

## ORIGINAL ARTICLE

# Comparative potency of obeticholic acid and natural bile acids on FXR in hepatic and intestinal in vitro cell models

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## Abstract

Obeticholic acid (OCA) is a semisynthetic farnesoid X receptor (FXR) agonist, an analogue of chenodeoxycholic acid (CDCA) which is indicated for the treatment of primary biliary cholangitis (PBC) in combination with ursodeoxycholic acid (UDCA). OCA efficiently inhibits bile acid synthesis and promotes bile acid efflux via activating FXR-mediated mechanisms in a physiologically relevant in vitro cell system, Sandwich-cultured Transporter Certified™ human primary hepatocytes (SCHH). The study herein evaluated the effects of UDCA alone or in combination with OCA in SCHH. UDCA ( $\leq 100 \mu\text{mol/L}$ ) alone did not inhibit CYP7A1 mRNA, and thus, no reduction in the endogenous bile acid pool observed. UDCA  $\leq 100 \mu\text{mol/L}$  concomitantly administered with  $0.1 \mu\text{mol/L}$  OCA had no effect on bile acid synthesis beyond what was observed with OCA alone. Furthermore, this study evaluated human Caco-2 cells (clone C2BBE1) as in vitro intestinal models. Glycine conjugate of OCA increased mRNA levels of FXR target genes in Caco-2 cells, FGF-19, SHP, OST $\alpha/\beta$ , and IBABP, but not ASBT, in a concentration-dependent manner, while glycine conjugate of UDCA had no effect on the expression of these genes. The results suggested that UDCA  $\leq 100 \mu\text{mol/L}$  did not activate FXR in human primary hepatocytes or intestinal cell line Caco-2. Thus, co-administration of UDCA with OCA did not affect OCA-dependent pharmacological effects.

## KEYWORDS

bile acids, caco-2 cells, FXR, hepatocytes, obeticholic acid, ursodeoxycholic acid

## 1 | INTRODUCTION

Natural bile acids are derived from cholesterol in the liver through a series of enzymatic reactions.<sup>1</sup> There are two bile acid synthesis

**Abbreviation:** C4, 7 $\alpha$ -hydroxy-4-cholesten-3-one; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7 $\alpha$ -hydroxylase; CA, cholic acid; DCA, deoxycholic acid; FXR, farnesoid X receptor; FGF-19, fibroblast growth factor 19; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IBABP, intestine bile acid binding protein; LCA, lithocholic acid; NAFLD, nonalcoholic fatty liver disease; OCA, obeticholic acid; PBC, primary biliary cholangitis; qRT-PCR, quantitative real-time polymerase chain reaction; SCHH, sandwich-cultured human hepatocytes; UDCA, ursodeoxycholic acid; VDR, vitamin D receptor.

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pathways: classic and alternative pathways. Cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) is the first and rate-limiting enzyme<sup>2,3</sup> in the classic pathway. Cholic acid (CA) and chenodeoxycholic acid (CDCA), the primary bile acids in humans, are extensively conjugated with glycine or taurine in hepatocytes, effluxed into bile, stored in the gallbladder, and eventually released into the intestine upon ingestion of a meal. Intestinal bacteria de-conjugate a portion of bile acids and further convert them to secondary bile acids, deoxycholic acid (DCA) from CA, and lithocholic acid (LCA) from CDCA.<sup>4</sup> In humans, epimerization of a small quantity of CDCA to form ursodeoxycholic acid (UDCA) occurs at the C-7 hydroxyl group.<sup>5,6</sup>

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Endogenous bile acids are also signaling molecules for bile acid homeostasis.<sup>7</sup> Bile acids directly activate nuclear receptors including the farnesoid X receptor (FXR; NR1H4),<sup>8-10</sup> and the pregnane X receptor (PXR; NR1I2).<sup>11,12</sup> Although bile acids share the same C<sub>24</sub> steroid backbone, they differentiate from each other in potency and selectivity of nuclear receptors. For example, CDCA is the most potent activator of FXR among natural bile acids<sup>8,9,13</sup> with an EC<sub>50</sub> value of approximately 10  $\mu\text{mol/L}$ .<sup>1</sup>

Farnesoid X receptor is a key regulator of bile acid homeostasis.<sup>7</sup> Obeticholic acid (OCA) is an analog of CDCA that differs in structure by a single ethyl group at C<sub>6</sub>.<sup>14</sup> FXR activation by OCA has an EC<sub>50</sub> value of approximately 100  $\text{nmol/L}$ .<sup>14</sup> Previous studies by the authors utilizing a physiologically relevant cellular model of sandwich-cultured human hepatocytes (SCHH) demonstrated a significant reduction in endogenous bile acids following 72-hour incubation of OCA.<sup>15</sup> In the same study, a head-to-head comparison confirmed that OCA was 100-fold more potent than CDCA on FXR. In a Phase 3 clinical trial<sup>16</sup>, patients with primary biliary cholangitis (PBC), treated with OCA once daily, consistently had significant reductions in plasma bile acid levels compared to placebo controls.

Ursodeoxycholic acid is an epimer of CDCA, but UDCA was reported to be a weak FXR agonist<sup>17</sup> or did not activate FXR in *in vitro* models.<sup>13,18,19</sup> An *in vivo* study<sup>20</sup> reported that UDCA exerted FXR-antagonistic effects in nonalcoholic fatty liver disease (NAFLD) patients by demonstrating induction of hepatic CYP7A1, elevation of circulating 7 $\alpha$ -hydroxy-4-cholesten-3-one (C4) and reduction in fibroblast growth factor 19 (FGF-19). Controversially, there are no published studies showing UDCA is a direct antagonist<sup>21</sup> with the exception of one *in silico* FXR binding study showing an inhibitory effect of UDCA.<sup>22</sup>

Ursodeoxycholic acid is the frontline treatment for many cholestatic liver diseases including PBC.<sup>23</sup> Its beneficial effects in cholestasis have been studied over decades. Multiple mechanisms of action have been proposed for its effects in the liver and intestine.<sup>24,25</sup> Beneficial effects of UDCA could be attributed to its hydrophilic properties. Recently, OCA was approved for the treatment of PBC in patients who were intolerant to UDCA or who had an inadequate response to UDCA. The concomitant use of UDCA and OCA in PBC therapy demands an understanding of the pharmacological interactions between UDCA and OCA.

Bile acid synthesis occurs and is regulated in the liver. The hepatic regulation pathway mediated by OCA has been clarified previously in SCHH.<sup>15</sup> However, *in vivo* bile acid homeostasis is regulated through FGF-15/FGF-19-mediated cross-talk between the liver and small intestine where FXR is highly expressed and activated by bile acids.<sup>26,27</sup> FGF-15 is an orthologue of FGF-19 in rodents.<sup>28</sup> When intestinal FXR is activated by bile acids, high levels of FGF-19/FGF-15 are released from ileum to activate FGFR4 in the liver, resulting in repression of CYP7A1 and bile acid synthesis.<sup>29</sup> In intestinal specific FXR null mice, the bile acid pool increased,<sup>26</sup> due to the lack of intestinal FXR activation and thus reduced circulating FGF-15. In this study, we evaluated human Caco-2 cells (clone C2BBE1) as *in vitro*

intestinal models to characterize FXR potency of OCA and natural bile acids (UDCA, CDCA, and CA).

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

Obeticholic acid was provided by Intercept Pharmaceuticals, Inc. (San Diego, CA). UDCA was procured from Sigma Aldrich (St. Louis, MO), CDCA and CA from Steraloids, Inc. (Newport, RI), and d<sub>5</sub>-CDCA from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). Proprietary cell culture media formulations, QualGro™ Seeding Medium and QualGro™ Hepatocyte Culture Induction Medium, were developed at Qualyst Transporter Solutions, LLC (Durham, NC). Cell culture base medium and supplements were purchased from Thermo Fisher Scientific (Waltham, MA). Matrigel™ and Bio-Coat culture plates were acquired from BD Biosciences (San Jose, CA). CellTiter-Glo® Luminescent Cell Viability Assays were purchased from Promega (Madison, WI). All quantitative real-time polymerase chain reaction (qRT-PCR) reagents were purchased Thermo Fisher Scientific (Carlsbad, CA). Pierce™ BCA™ Protein Assays were purchased from Thermo Fisher Scientific (Waltham, MA). Human gene-specific TaqMan® primers and probes were purchased from Thermo Fisher Scientific.

### 2.2 | Methods

#### 2.2.1 | Sandwich-cultured human hepatocyte (SCHH) preparation and treatment

Cryopreserved human hepatocytes were purchased from Triangle Research Laboratories, LLC (Durham, NC) and Xenotech, LLC (Lenexa, KS), and Thermo Fisher Scientific. Liver donors' demographic information (ethnicity, gender, and age) was listed in Table S1. Hepatocytes were thawed following manufacturer's instructions. SCHH were prepared by plating Transporter Certified™, cryopreserved human hepatocytes suspended in QualGro™ Seeding Medium at a density of  $0.8\text{--}1.2 \times 10^6$  cells/mL onto BioCoat® 24-well cell culture plates. Following plating, cells were allowed to attach for 2-4 hours, rinsed, and fed with 37°C QualGro™ Seeding Medium. Eighteen to 24 hours later, cells were fed and overlaid with QualGro™ Hepatocyte Culture Induction Medium supplemented with 0.25 mg/mL Matrigel®. Cells were maintained in QualGro™ Hepatocyte Culture Induction Medium at 37°C in a humidified incubator with 95% air/5% CO<sub>2</sub>.

Stock solutions of OCA, UDCA, d<sub>5</sub>-CDCA, and CA were prepared in DMSO and diluted 1000 times directly into QualGro™ Hepatocyte Culture Induction Medium. The final DMSO concentration in the cell culture medium was  $\leq 0.1\%$  in mono-treatment, and  $\leq 0.2\%$  in combination treatment. In mono-treatment assays, SCHH were treated for 72 hours with increasing concentrations of OCA (0.00316-3.16  $\mu\text{mol/L}$ ), UDCA (0.316-316  $\mu\text{mol/L}$ ), CDCA (0.1-100  $\mu\text{mol/L}$ ), or CA (0.1-100  $\mu\text{mol/L}$ ). In assessing co-administration of OCA with UDCA, SCHH were treated for 72 hours with increasing

concentrations of UDCA (1.0–316  $\mu\text{mol/L}$ ) in the presence of a fixed concentration of OCA of 0.1  $\mu\text{mol/L}$ . Cell culture medium was changed on daily basis during treatment. In parallel, SCHH treated with DMSO served as controls. Each treatment was performed in triplicate wells.

### 2.2.2 | Human intestinal Caco-2 cell culture and treatment

Caco-2 cells (clone C2BBe1) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were seeded at a density of 60 000 cells/cm<sup>2</sup> on 12-well collagen-coated Transwell plates. Cells were housed in a humidified incubator at 37°C with 5% CO<sub>2</sub> and grown in a maintenance medium comprised of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 100  $\mu\text{mol/L}$  nonessential amino acids, 4 mmol/L L-glutamine, 100 IU/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin. The culture medium was changed three times weekly, and cell growth was observed by light microscopy. Cell monolayers were grown for 28 days to confluence. Both apical and basolateral compartments were dosed for 24 hours with increasing concentrations of glyco-OCA (0.1–30  $\mu\text{mol/L}$ ), glyco-UDCA (1–100  $\mu\text{mol/L}$ ), glyco-CDCA (1–100  $\mu\text{mol/L}$ ), or glyco-CA (1–100  $\mu\text{mol/L}$ ). At the end of treatment, cells were collected for mRNA expression analysis. Each treatment was performed in triplicate wells.

### 2.2.3 | RT-PCR quantitation of mRNA expression

Total RNA was isolated from human hepatocytes and Caco-2 cells after treatment using Qiagen RNeasy kit following manufacturer's instructions. Isolated RNA was quantified using Quant-iT<sup>TM</sup> RiboGreen<sup>®</sup> RNA Assay Kit (Thermo Fisher Scientific, Carlsbad, CA). Total RNA was pooled together from triplicate wells, and a total amount of 500 ng was converted to cDNA following the manufacturer's procedure of the High Capacity cDNA Archive Kit (Thermo Fisher Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. mRNA content was determined for each treatment group relative to the endogenous control gene expression and the calibrator, 0.1% vehicle control (DMSO) using the ViiA<sup>TM</sup> 7 system software (Thermo Fisher Scientific, Carlsbad, CA).

### 2.2.4 | Bioanalysis of endogenous bile acid pool (EBAP)

EBAP in SCHH was analyzed using B-CLEAR<sup>®</sup> technology and calculated as the sum of endogenous primary bile acids (CA and CDCA) and their glycine and taurine conjugates (glyco-CA, tauro-CA, glyco-CDCA, and tauro-CDCA) in cell culture medium, and hepatocyte lysate (cell + bile). Endogenous bile acids were extracted from cell culture medium samples and hepatocyte lysate using the same procedure described in the previous paper.<sup>15</sup> Prepared samples were filtered and analyzed by LC-MS/MS using a Shimadzu binary HPLC

system (Columbia, MD) and tandem mass spectrometry using Thermo Electron TSQ<sup>®</sup> Quantum Discovery MAX<sup>TM</sup> (Waltham, MA) with an Ion Max ESI source operating in negative ion electrospray ionization mode using multiple reaction monitoring. Protein content was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific) following manufacturer's instructions. All mass values were normalized to the mean protein (mg) per well.

### 2.2.5 | Data analysis

Data were analyzed with GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA). All data are normalized to the vehicle control (DMSO). For hepatocyte studies, mean and standard deviation (SD) were calculated from hepatocyte preparations of three liver donors. For the gene expression from Caco-2 cell study, mean and 95% confidence intervals (CI) were calculated from technical triplicate.

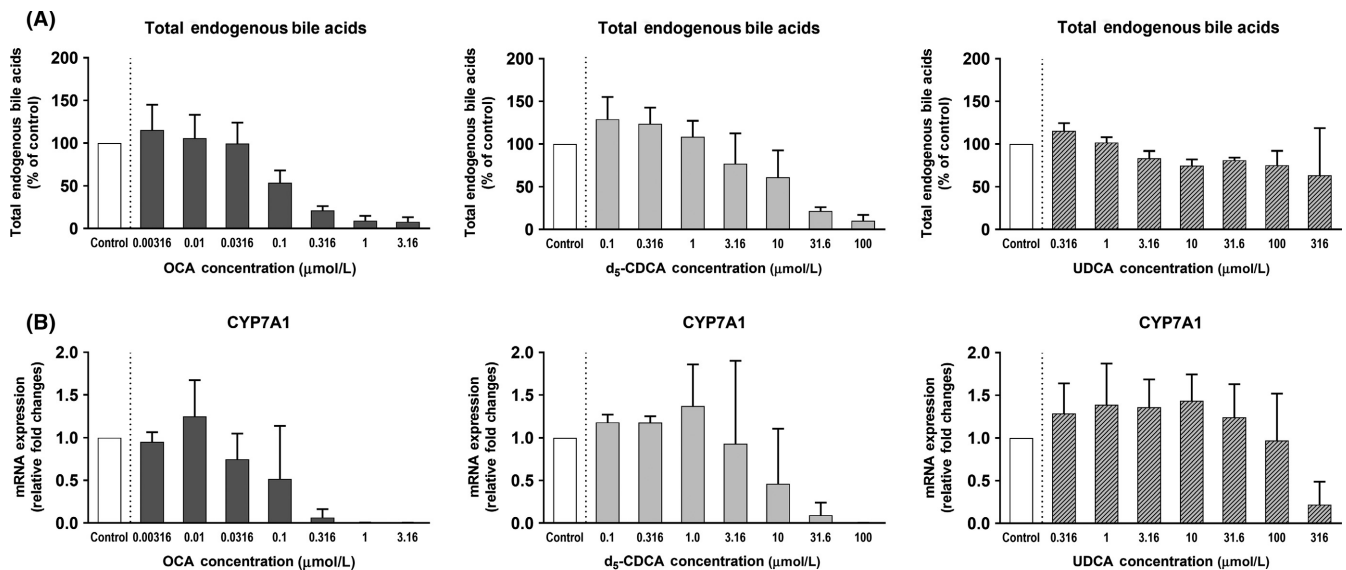
## 3 | RESULTS

### 3.1 | Effects of OCA, UDCA, CDCA and CA on bile acid synthesis in SCHH

Previous studies in SCHH have demonstrated that OCA and CDCA up to 100  $\mu\text{mol/L}$  showed no overt cytotoxicity.<sup>15</sup> To optimize treatment concentration of UDCA, cytotoxicity of increasing concentrations of UDCA (1.0–1000  $\mu\text{mol/L}$ ) was evaluated by morphological and ATP assessments (Figure S1). Following 72-hour treatment with UDCA  $\leq 316$   $\mu\text{mol/L}$ , no marked changes were observed in hepatocyte morphology (data not shown) with a reduction in ATP content <21%. These results demonstrate that UDCA was well tolerated in SCHH up to 316  $\mu\text{mol/L}$ . In contrast, 1000  $\mu\text{mol/L}$  UDCA reduced ATP content by 32.4%, which was in line with observed morphological changes including weakening of cuboidal cell shape and increased cell detachment. Therefore, concentrations of UDCA  $\leq 316$   $\mu\text{mol/L}$  were used in the subsequent studies.

The effect of UDCA on bile acid synthesis was compared to OCA and natural bile acids (CDCA and CA) in the SCHH. The expression of CYP7A1 and total EBAP are indicators of bile acid synthesis. Following 72 hours of exposure to OCA and d<sub>5</sub>-CDCA, total EBAPs were decreased in a concentration-dependent manner (Figure 1A). Maximal suppression of total EBAPs was observed starting at 1  $\mu\text{mol/L}$  OCA (9.2  $\pm$  5.6% relative to control) and 100  $\mu\text{mol/L}$  d<sub>5</sub>-CDCA (10.2  $\pm$  6.9% relative to control). CYP7A1 mRNA was consistently suppressed by OCA or d<sub>5</sub>-CDCA in a dose-dependent manner (Figure 1B). Estimated IC<sub>50</sub> values, the concentrations of OCA and d<sub>5</sub>-CDCA to suppress CYP7A1 mRNA by 50%, were consistent with IC<sub>50</sub> values for reducing EBAP (Table 1).

In contrast, following 72 hours of exposure to UDCA from 0.316  $\mu\text{mol/L}$  to 100  $\mu\text{mol/L}$ , there was no concentration-dependent reduction in total EBAP observed. The maximal reduction in total EBAP observed at 100  $\mu\text{mol/L}$  UDCA was <25% relative to the vehicle control (Figure 1A). Consistently, there was no dose-dependent suppression of CYP7A1 mRNA observed with the treatment of



**FIGURE 1** Effects of bile acids on bile acid synthesis. Sandwich-cultured human hepatocytes (SCHH) were treated for 72 hours with increasing concentrations of OCA, d<sub>5</sub>-CDCA, and UDCA. (A) Endogenous bile acids, CA, glyco-CA, tauro-CA, CDCA, glyco-CDCA, and tauro-CDCA, were determined in the cell culture media (CCM) and total cell lysate (cell + bile). Total endogenous bile acid pool was calculated as the sum of these six bile acids in CCM + cell + bile. The data were normalized to the vehicle control treatment (DMSO 0.1%) and shown as percent (%) of control. (B) The mRNA expression of CYP7A1 was evaluated by quantitative RT-PCR and shown as fold change relative to control. The data represented means with SD error bars of individual SCHH preparation of three liver donors (n = 3)

**TABLE 1** EC<sub>50</sub> values of OCA and CDCA to inhibit bile acid synthesis and CYP7A1 in human hepatocytes

EC <sub>50</sub> (μmol/L)	Bile acid pool	CYP7A1
OCA	0.088	0.079
CDCA	5.772	7.066

OCA, obeticholic acid; CDCA, chenodeoxycholic acid.

UDCA ≤100 μmol/L. Although CYP7A1 mRNA in the treatment of UDCA at the highest concentration of 316 μmol/L was 0.2 ± 0.3-fold of the vehicle control, total EBAP was 63.4 ± 55.3% of the vehicle control – <40% decrease.

The effect of CA (0.1–100 μmol/L) on CYP7A1 mRNA expression was also examined in SCHH following 72-hour exposure (Figure S2A). CA concentrations ≤1.0 μmol/L increased CYP7A1 mRNA by five-fold to six-fold, but not in a dose-dependent manner. CA at concentrations ≥3.16 μmol/L had no effect on CYP7A1 mRNA.

These results suggest CA or UDCA ≤100 μmol/L had no suppressive effect on CYP7A1 expression and no potential to reduce bile acid synthesis and thus is unlikely to activate FXR in the human hepatocytes.

### 3.2 | CYP7A1-negative regulators (SHP and FGF-19) in SCHH

SHP and FGF-19 genes contain FXR response DNA elements. FXR directly regulates the transcription of SHP and FGF-19 that subsequently suppress CYP7A1 expression.<sup>30,31</sup> SHP and FGF-19 mRNA were dose-dependently increased in SCHH treated with OCA or

d<sub>5</sub>-CDCA. Compared to controls, SHP mRNA increased 5.6 ± 1.7-fold with 1 μmol/L OCA and 5.4 ± 2.1-fold with 100 μmol/L d<sub>5</sub>-CDCA (Figure 2A). FGF-19 mRNA increased by 397 ± 295-fold with treatment of 1 μmol/L OCA and a 1046 ± 911-fold increase with 100 μmol/L d<sub>5</sub>-CDCA (Figure 2B). These data are consistent with CYP7A1 mRNA suppression and the reduction in bile acid synthesis, suggesting OCA and CDCA both are effective at activating FXR in the human primary hepatocytes, but with 100-fold difference in potency.

In the parallel treatment of UDCA ≤316 μmol/L, no changes in SHP were observed at any concentrations of UDCA (Figure 2A). FGF-19 mRNA was not changed by UDCA ≤100 μmol/L, which was consistent with no observed reduction of CYP7A1 mRNA with UDCA ≤100 μmol/L (Figure 2B). UDCA at the maximal tested concentration of 316 μmol/L modestly increased FGF-19 levels 5.5 ± 2.9-fold. Additionally, no dose-dependent changes in SHP or FGF-19 were observed with CA ≤100 μmol/L treatment (Figure S2B, C). These results suggest CA or UDCA ≤100 μmol/L did not activate FXR in the human hepatocytes.

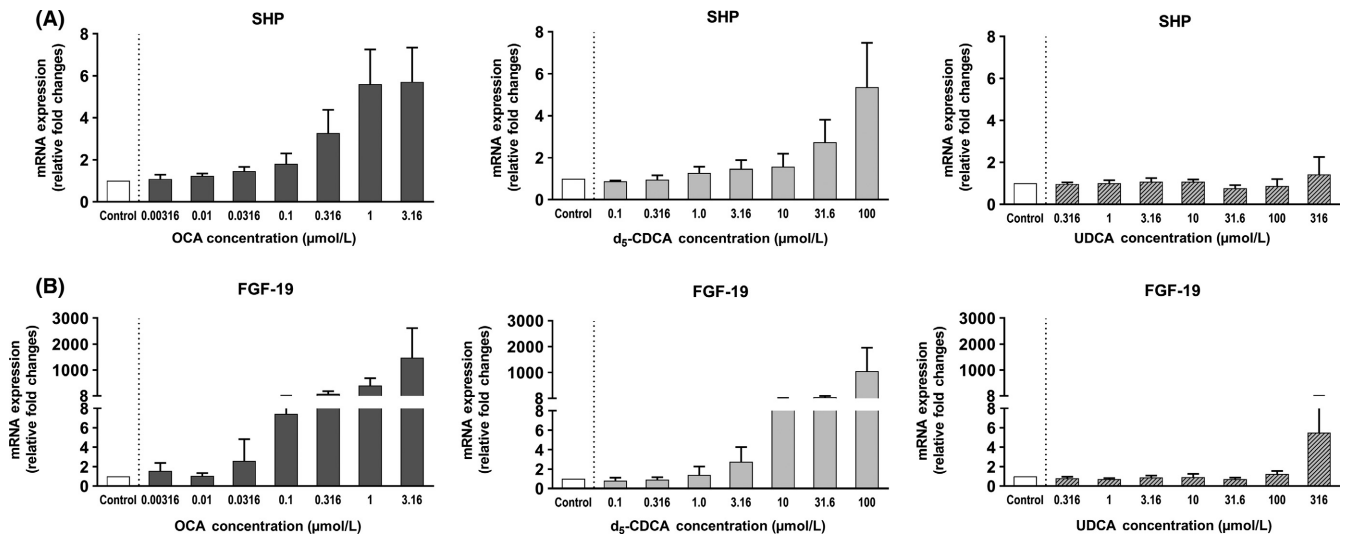
### 3.3 | Effects on bile acid export transporters (OST<sub>α</sub>, OST<sub>β</sub>, and BSEP) in SCHH

FXR also directly regulates the transcription of OST<sub>α</sub>, OST<sub>β</sub>, and BSEP that are, respectively, located on the basolateral and canalicular membranes of hepatocytes and efflux bile acids out of hepatocytes.

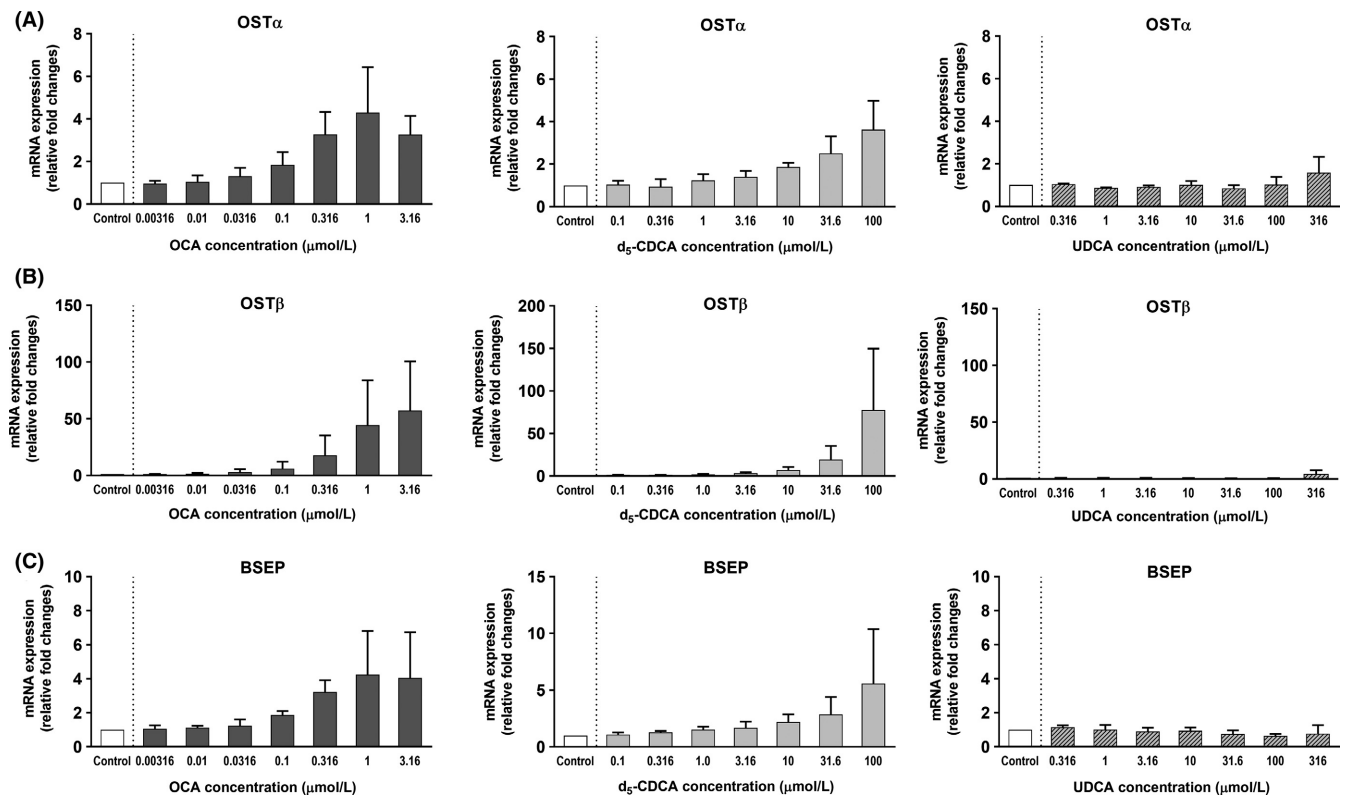
Dose-dependent increases of bile acid transporters OST<sub>α</sub>/OST<sub>β</sub> (basolateral) and BSEP (canalicular) mRNA were observed in SCHH

treated with OCA or d<sub>5</sub>-CDCA. OST<sub>α</sub> increased 4.3 ± 2.1-fold with 1 μmol/L OCA and 3.6 ± 1.4-fold with 100 μmol/L d<sub>5</sub>-CDCA. OST<sub>β</sub> mRNA increased 44.3 ± 39.5-fold with 1 μmol/L OCA and

77.5 ± 72.3-fold with 100 μmol/L d<sub>5</sub>-CDCA. In contrast, UDCA did not alter OST<sub>α</sub> expression (Figure 3A). No induction or suppression of OST<sub>β</sub> was observed with treatment of UDCA ≤100 μmol/L. But



**FIGURE 2** Effects of bile acids on SHP and FGF-19 in SCHH. Sandwich-cultured human hepatocytes (SCHH) were treated for 72 hours with increasing concentrations of OCA, d<sub>5</sub>-CDCA, and UDCA. SHP (A) and FGF-19 (B) are FXR direct target genes and CYP7A1-negative regulators. The mRNA expression was evaluated by quantitative RT-PCR and shown as fold change relative to control (DMSO 0.1%). The data represented means with SD error bars of individual SCHH preparation of three liver donors (n = 3)



**FIGURE 3** Effects of bile acids on bile acid transporters in SCHH. Sandwich-cultured human hepatocytes (SCHH) were treated for 72 hours with increasing concentrations of OCA, d<sub>5</sub>-CDCA, and UDCA. OST<sub>α/β</sub> are heterodimer transporters on the basolateral membrane of hepatocytes, and BSEP is on the canalicular membrane. OST<sub>α/β</sub> (A and B) and BSEP (C) are FXR direct target genes. The mRNA expression was evaluated by quantitative RT-PCR and shown as fold change relative to control (DMSO 0.1%). The data represented means with SD error bars of individual SCHH preparation of three liver donors (n = 3)



UDCA at 316  $\mu\text{mol/L}$  showed a modest increase in  $\text{OST}_{\beta}$  mRNA expression of  $4.3 \pm 3.3$ -fold (Figure 3B).

A dose-dependent increase in BSEP mRNA was observed following 72-hour exposure of OCA and  $d_5$ -CDCA. The increases were  $4.2 \pm 2.6$ -fold with 1  $\mu\text{mol/L}$  OCA and  $5.6 \pm 4.8$ -fold with 100  $\mu\text{mol/L}$   $d_5$ -CDCA. UDCA had no observed effect on BSEP expression (Figure 3C). CA had no observed effect on  $\text{OST}_{\alpha}$  and BSEP mRNA content; however, a mild induction of  $\text{OST}_{\beta}$  mRNA at 100  $\mu\text{mol/L}$  ( $4.2 \pm 5.1$ -fold) was observed (Figure S2D-F).

### 3.4 | Effect of cotreatment of UDCA with OCA in SCHH

In parallel to 0.1  $\mu\text{mol/L}$  OCA mono-treatment, UDCA (1.00–316  $\mu\text{mol/L}$ ) were co-administered with 0.1  $\mu\text{mol/L}$  OCA. Bile acid synthesis and CYP7A1 mRNA were examined. In the mono-treatment of 0.1  $\mu\text{mol/L}$  OCA, total cholic acid levels, the sum of CA, glyco-CA, and tauro-CA, were decreased to  $24.3 \pm 12.7\%$  of the control (Figure 4A). Co-administration of UDCA ( $\leq 100$   $\mu\text{mol/L}$ ) with 0.1  $\mu\text{mol/L}$  OCA did not impact suppression of total cholic acid levels by OCA, which was consistent with the fact that cotreatment of UDCA did not change CYP7A1 mRNA compared to the mono-treatment of OCA (Figure 4B). UDCA of 316  $\mu\text{mol/L}$  further decreased total cholic acid levels to  $7.9 \pm 4.2\%$  of the vehicle control, secondary to further decreased CYP7A1 mRNA levels ( $0.015 \pm 0.01$ -fold relative to the control). The further reduction in total EBAP content and CYP7A1 mRNA following cotreatment of UDCA at 316  $\mu\text{mol/L}$  could be explained by increased FGF-19 mRNA ( $16.3 \pm 11.8$ -fold higher relative to the control) compared to 0.1  $\mu\text{mol/L}$  OCA mono-treatment ( $6.2 \pm 8.1$ -fold higher relative to the control) (Figure 4C).

Ursodeoxycholic acid has been previously reported to activate PXR and induce CYP3A4 mRNA expression which is a prototypical target gene of PXR.<sup>19</sup> CYP3A4 expression was examined in the mono-treatment of OCA, UDCA and cotreatment of UDCA with OCA. OCA mono-treatment dose-dependently inhibited CYP3A4 mRNA (Figure 5A). UDCA ( $\leq 100$   $\mu\text{mol/L}$ ) mono-treatment showed no effect on CYP3A4 mRNA levels, but UDCA at 316  $\mu\text{mol/L}$

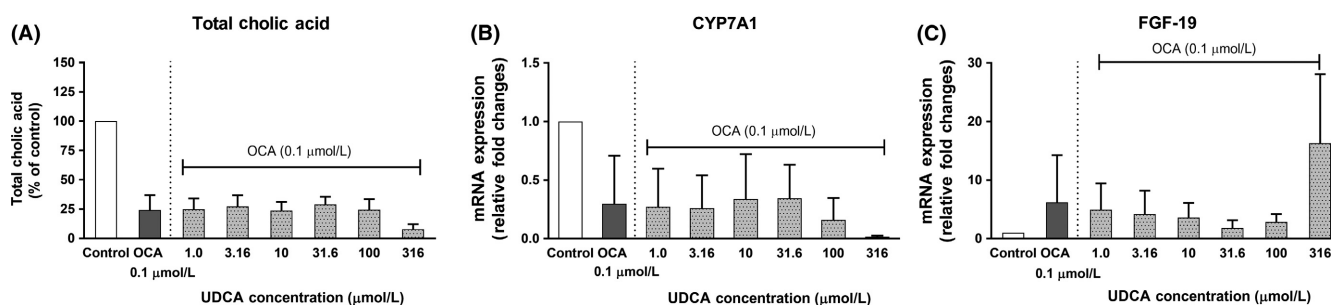
increased CYP3A4 mRNA  $15.4 \pm 9.8$ -fold relative to the control (Figure 5B). In the cotreatment assays, UDCA at 316  $\mu\text{mol/L}$  abolished the suppression of CYP3A4 mRNA levels by OCA; instead, a  $10.5 \pm 2.7$ -fold increase in CYP3A4 mRNA was observed relative to the control (Figure 5C). This result confirmed that in SCHH, UDCA at 316  $\mu\text{mol/L}$  is capable to activate PXR.

### 3.5 | FXR activation in human Caco-2 cells

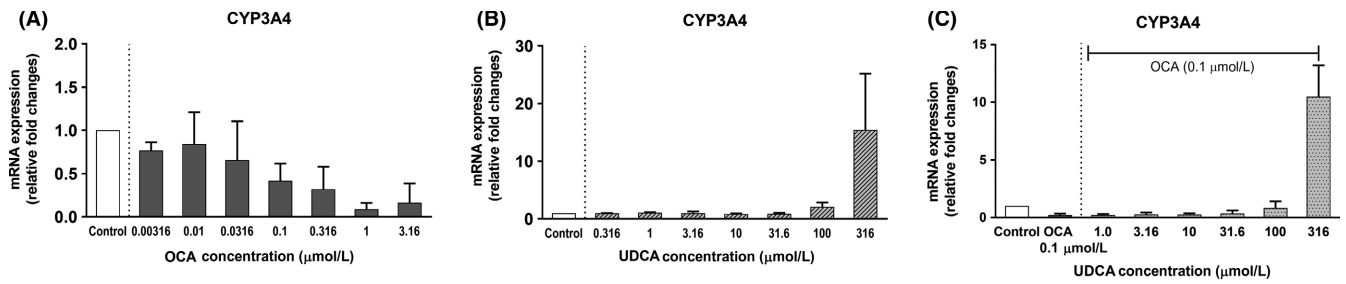
In Caco-2 cells, intestinal FXR target genes were evaluated following 24-hour incubation with increasing concentrations of glyco-OCA, glyco-CDCA, glyco-UDCA, and glyco-CA. Glyco-OCA (0.1–30  $\mu\text{mol/L}$ ) induced concentration-dependent increases in mRNA expression of FGF-19, SHP, intestine bile acid binding protein (IBABP), and basolateral membrane transporters  $\text{OST}_{\alpha/\beta}$ . The expression of these genes reached maximal levels at 10  $\mu\text{mol/L}$  of glyco-OCA. Relative to the vehicle control, FGF-19 increased by 6.99-fold, SHP by 7.76-fold, IBABP by 335-fold,  $\text{OST}_{\alpha}$  by 9.85-fold, and  $\text{OST}_{\beta}$  by 11.6-fold (Figures 6 and 7). Although there was a trend of dose-dependent increase following glyco-CDCA treatment, 100  $\mu\text{mol/L}$  glyco-CDCA mildly increased FGF-19 (3.13-fold), SHP (2.52-fold), IBABP (30.9-fold),  $\text{OST}_{\alpha}$  (3.55-fold), and  $\text{OST}_{\beta}$  (3.83-fold). These responses were less than the increases in these genes observed following 10  $\mu\text{mol/L}$  glyco-OCA treatment. In contrast, UDCA as well as CA (Figure S3) exerted no changes on the expression of these target genes. Interestingly, ASBT was not changed by any compounds. Other bile acid transporters on apical and basolateral membranes, OATP2B1, OATP1A2, MRP4, MRP2, P-gp, BCRP, and MRP3, were not affected by 10  $\mu\text{mol/L}$  of glyco-OCA or glyco-CDCA treatment (Figure S4).

## 4 | DISCUSSION

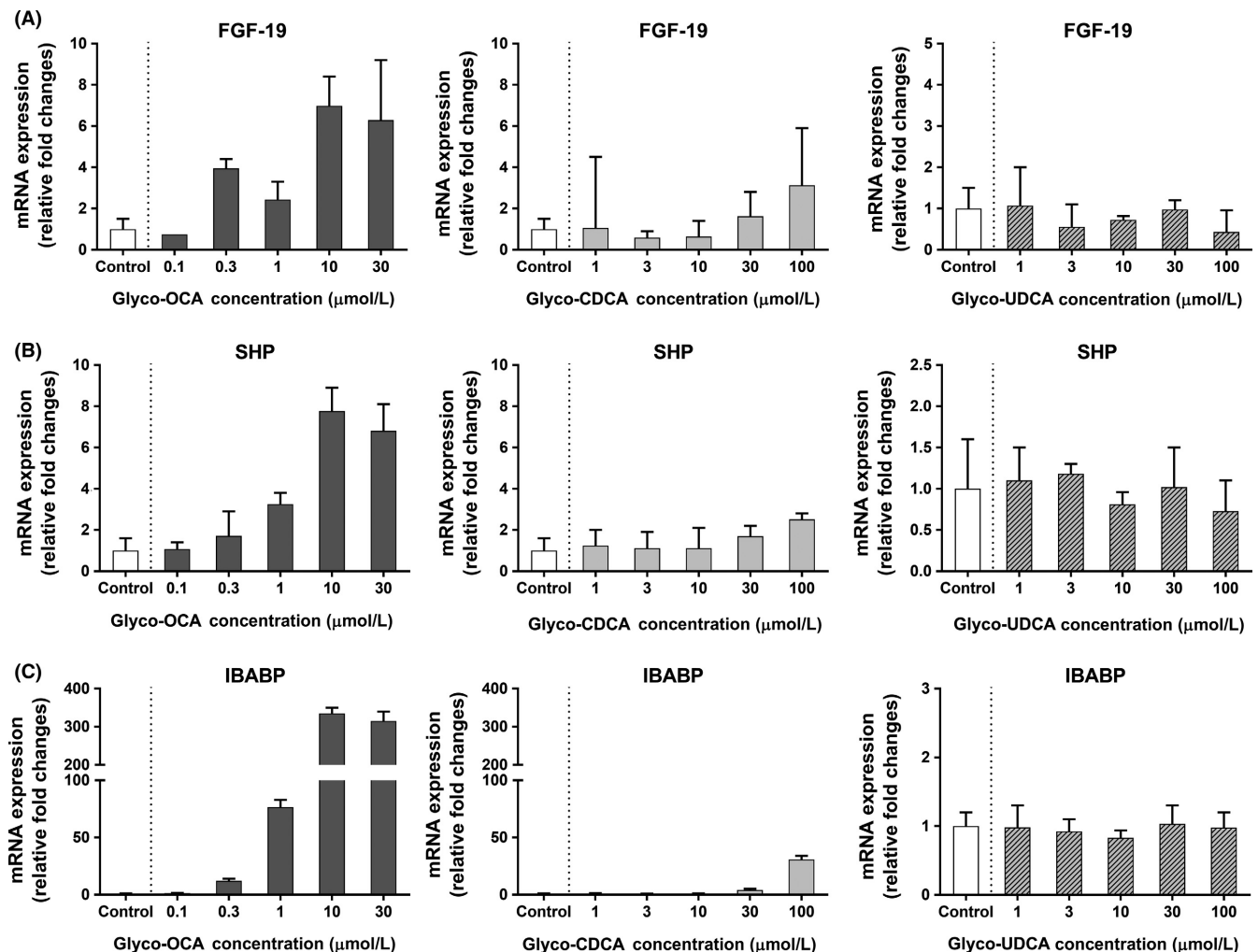
This study characterized FXR activation potentials of UDCA in comparison with OCA and natural bile acids, CDCA and CA, in hepatic and intestinal in vitro cell models. The results demonstrated that UDCA and CA are devoid of FXR activity at clinically relevant



**FIGURE 4** Effects of concomitant UDCA on bile acid synthesis in the presence of OCA in SCHH. Sandwich-cultured human hepatocytes were treated for 72 hours with increasing concentrations of UDCA in the presence of OCA at a fixed concentration of 0.1  $\mu\text{mol/L}$ . (A) Total endogenous cholic acid pool was calculated as the sum of CA, glyco-CA, and tauro-CA in CCM + cell + bile, representing the bile acid synthesis ability in the hepatocytes. The data were normalized to the vehicle control treatment (DMSO 0.2%) and shown as percent (%) of control. The mRNA expression of CYP7A1 (B) and FGF-19 (C) was evaluated by quantitative RT-PCR and shown as fold change relative to control. The data represented means with SD error bars of individual SCHH preparation of three liver donors ( $n = 3$ )



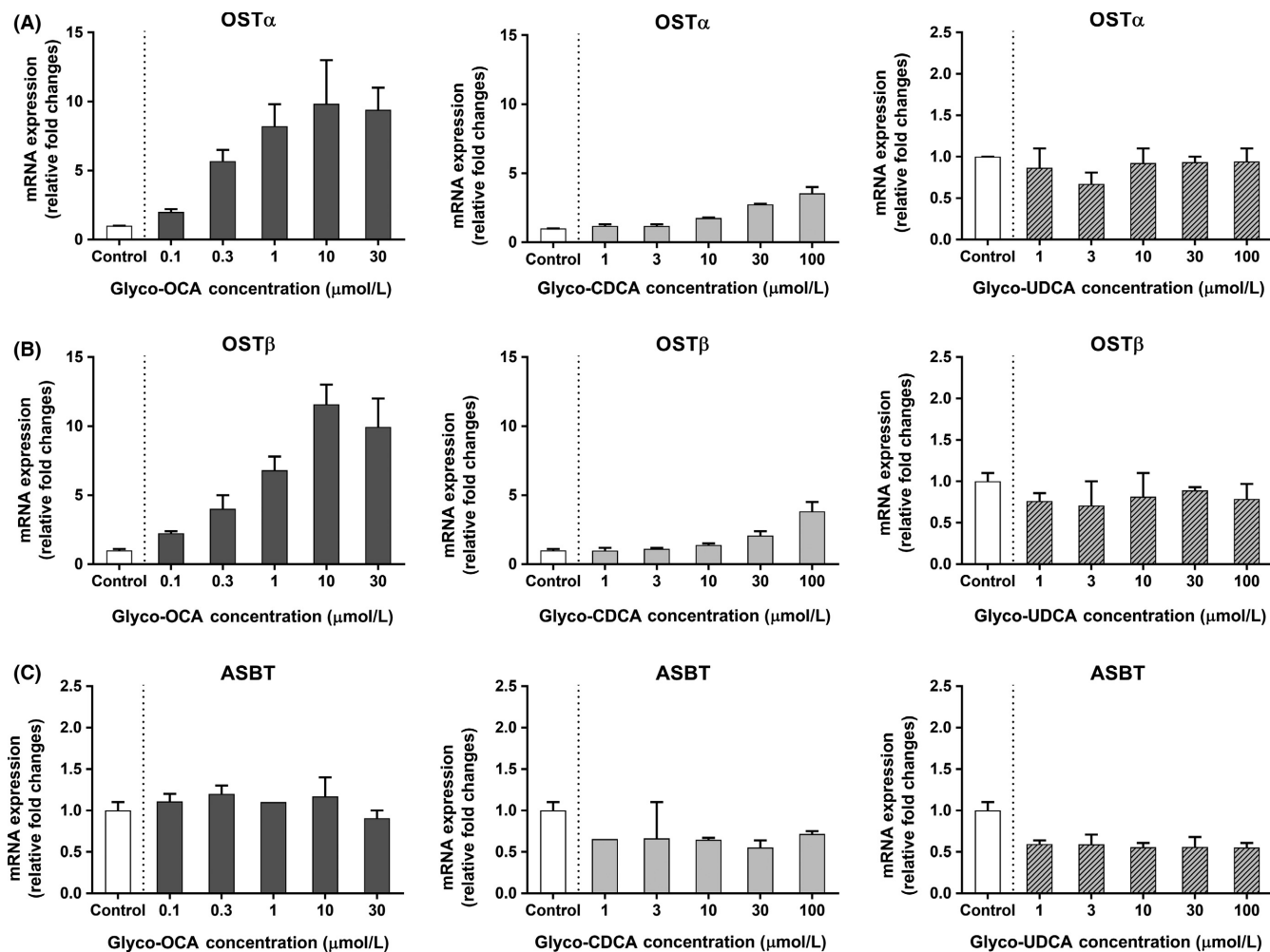
**FIGURE 5** Different effects of OCA and UDCA on CYP3A4 mRNA expression in SCHH. CYP3A4 is a prototypical PXR target genes. CYP3A4 mRNA expression was evaluated following 72 hours of the mono-treatments of increasing concentrations of OCA (A), UDCA (B), or concomitant treatment of increasing concentrations of UDCA with a fixed concentration of OCA at 0.1 μmol/L (C). The mRNA expression was evaluated by quantitative RT-PCR and shown as fold change relative to control. The data represented means with SD error bars of individual SCHH preparation of three liver donors (n = 3)



**FIGURE 6** Farnesoid X receptor (FXR) activation in Caco-2 cells. Caco-2 cells were incubated with increasing concentrations of glycine conjugates of OCA, and natural bile acids (CDCA and UDCA) for 24 hours. The mRNA expression of FXR intestinal target genes, FGF-19 (A), SHP (B), and IBABP (C), was evaluated by quantitative RT-PCR and shown as fold change relative to control (DMSO 0.1%). The data represented means with upper 95% CI error bars of technical triplicate (n = 3). The shaded area was between 0.5 and twofold changes relative to the vehicle control

concentrations. OCA is the most potent FXR agonist with about 100-fold greater potency than CDCA shown in human hepatocytes and enterocytes.

The concentrations evaluated in these studies covered the clinically relevant range of concentrations. In a Phase 3 study investigating daily OCA in patients with PBC,<sup>16</sup> the plasma trough



**FIGURE 7** Effects on bile acid transporters in Caco-2 cells. Caco-2 cells were incubated with increasing concentrations of glycine conjugates of OCA, and natural bile acids (CDCA and UDCA) for 24 hours. The mRNA expression of bile acid transporters, OST $\alpha$  (A) and OST $\beta$  (B) on the basolateral membrane, and ASBT (C), was evaluated by quantitative RT-PCR and shown as fold change relative to control (DMSO 0.1%). The data represented means with upper 95% CI error bars of technical triplicate ( $n = 3$ ).

concentration of total OCA (unconjugated and conjugated OCA) was 0.290  $\mu\text{mol/L}$  for the OCA 10 mg treatment group. The plasma trough concentrations of total UDCA (unconjugated and conjugated UDCA) in the same study was 13  $\mu\text{mol/L}$  (average dose = 16  $\text{mg kg}^{-1} \text{day}^{-1}$ ). The ranges of concentrations tested in SCHH, 0.00316–3.16  $\mu\text{mol/L}$  of OCA, 0.316–316  $\mu\text{mol/L}$  of UDCA cover therapeutic and supratherapeutic concentrations of OCA and UDCA. Intestinal concentrations of total OCA in daily OCA 10 mg group is approximately 40  $\mu\text{mol/L}$  estimated by simulation (internal data). Molino et al.<sup>32</sup> estimated the physiological concentration of glyco-CDCA in the intestine is approximately 35  $\mu\text{mol/L}$ . The concentration ranges of glyco-OCA and glyco-CDCA tested in Caco-2 cells are 0.1–30  $\mu\text{mol/L}$  and 1–100  $\mu\text{mol/L}$ , respectively. Therefore, the conclusions from these studies in human hepatocytes and intestinal Caco-2 cells are clinically relevant.

These studies suggest that UDCA and OCA do not share the same mechanisms of action – UDCA is not an agonist/antagonist of FXR, while OCA is a potent and selective FXR agonist. UDCA was

ineffective in activating hepatic or intestinal FXR. In SCHH or Caco-2 cells, UDCA did not alter FXR target genes (SHP, FGF-19, BSEP, OST $\alpha$ , and OST $\beta$ ). UDCA did not change bile acid synthesis in SCHH at therapeutic or supratherapeutic concentrations. Furthermore, UDCA was unable to antagonize the potential of OCA to activate FXR in SCHH. Cotreatment of UDCA at therapeutic and supratherapeutic concentrations did not alter OCA suppression of bile acid synthesis (Figure 4). The co-administration data suggested that OCA pharmacological effects should not be altered in the presence of UDCA.

Obeticholic acid does activate hepatic and intestinal FXR-FGF-19/SHP cascades, thereby substantially reducing bile acid synthesis. We have previously reported and discussed the hepatic mechanisms of action of OCA.<sup>15</sup> Herein, we further characterized glyco-OCA in Caco-2 cells to understand its intestinal mechanisms of action. As expected, glyco-OCA induced FXR target genes in Caco-2 cells. Increased intestinal FGF-19 by glyco-OCA may contribute to the suppression of bile acid synthesis in the liver. OST $\alpha/\beta$  are located on



the basolateral membrane of enterocytes and mediate bile acid transport into the portal vein. IBABP is an intestinal bile acid binding transporter protein. Increases in  $OST_{\alpha/\beta}$  and IBABP by glyco-OCA may reduce intracellular levels of free bile acids and thus prevent free bile acid cytotoxicity. A previous study in freshly isolated ileum biopsies reported similar FXR activation response to OCA and CDCA treatments,<sup>33</sup> leading to FGF-19 and  $OST_{\alpha/\beta}$  increases. Caco-2 cells are human colon adenocarcinoma cells and express different FXR isoforms from hepatocytes<sup>34</sup> and different levels of FXR from freshly isolated biopsies too,<sup>35</sup> which could explain the discrepancy of the response magnitude to FXR agonists between hepatocytes and Caco-2 cells, and between Caco-2 and isolated ileum biopsies.

Surprisingly, neither glyco-OCA nor glyco-CDCA suppressed ASBT mRNA in human Caco-2 cells. This result is consistent with a study in freshly isolated ileum biopsies.<sup>33</sup> However, Neimark<sup>36</sup> reported ASBT mRNA in Caco-2 cells was suppressed by 40-hour treatment with 100  $\mu\text{mol/L}$  CDCA and showed that SHP was a negative regulator. These differences may be due to the inherent variability of Caco-2 cells, or the incubation time, 24 hours in this study and 40 hours in Neimark's study. Mouse ASBT is suppressed by FXR-SHP axis in the intestine,<sup>37</sup> while rat ASBT is not.<sup>38</sup> There is significant species difference in regulation of the ASBT gene by bile acids.<sup>27,37,38</sup> The mechanisms of ASBT regulation in human enterocytes by bile acids require further investigation.

Our studies also demonstrated that UDCA may be a weak PXR agonist. UDCA at 316  $\mu\text{mol/L}$  induced the expression of CYP3A4, a prototypical PXR target gene.<sup>38</sup> Previous reports show that PXR activation induces FGF-19 expression.<sup>39</sup> We observed that high concentration of UDCA (316  $\mu\text{mol/L}$ ) increased FGF-19 mRNA, which may be why high concentrations of UDCA were found to decrease the expression of CYP7A1 and the total endogenous bile acid pool. Therefore, effects of UDCA at 316  $\mu\text{mol/L}$  on bile acid homeostasis are most likely through PXR signaling pathway and not mediated by FXR mechanisms. These effects of UDCA at supratherapeutic concentrations, however, are unlikely to occur in clinical use (even taking into account potential higher portal vein concentrations following oral administration) as 316  $\mu\text{mol/L}$  is ~24-fold greater than therapeutic concentrations of UDCA. Furthermore, previous clinical studies<sup>20,40</sup> demonstrated that UDCA treatment (15-20 mg  $\text{kg}^{-1}$   $\text{day}^{-1}$ ) did not suppress bile acid synthesis.

A limitation of this study was that only endogenous cholic acid was used for efficacy measurement when UDCA was administered with OCA. For mono-treatments, the level of the total endogenous bile acids represents the activity of bile acid synthesis. Total endogenous bile acids were calculated as the sum of CA, glyco-CA, tauro-CA, CDCA, glyco-CDCA, and tauro-CDCA. But we observed that, in the cotreatment of UDCA and OCA assays, total CDCA (the sum of CDCA, glyco-CDCA, and tauro-CDCA), marker for OCA effects was increased with the increasing concentrations of UDCA. This may be due to the epimerization of UDCA to CDCA. Instead of total endogenous bile acids, only total cholic acid was calculated as the sum of CA, glyco-CA, and tauro-CA, and used as the representative of the activity of bile acid synthesis in this SCHH system. This is

reasonable because cholic acid is synthesized from the classic pathway in which CYP7A1 is the rate-limiting enzyme.<sup>7</sup>

In summary, this study demonstrated that UDCA is not a FXR agonist. Co-administration of UDCA with OCA did not affect the ability of OCA to activate FXR and thus suppress bile acid synthesis.

## AUTHOR CONTRIBUTIONS

J. E. E., Y. Z., J. P. J., K. B., C. L., and S. K. participated in research design; J. P. J. and colleagues conducted experiments. None of the authors contributed to new reagents or analytical tools; Y. Z., J. E. E., and J. P. J. performed data analysis; J. E. E., Y. Z., and J. P. J. wrote or contributed to the writing of the manuscript.

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## SUPPORTING INFORMATION

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