Effects of corilagin on alleviating cholestasis via FXR-associated pathways in vitro and in vivo

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BACKGROUND AND PURPOSE

This aim of this study was to investigate the effects of corilagin on alleviating intrahepatic cholestasis by regulating liver FXR (farnesoid X receptor)-associated pathways in vitro and in vivo.

EXPERIMENTAL APPROACH

Cellular and animal models were treated with different concentrations of corilagin. In the cellular experiments, FXR expression was up-regulated by either lentiviral transduction or GW4064 treatment and down-regulated by either siRNA technology or treatment with guggulsterones. Real-time PCR and western blotting were employed to detect the mRNA and protein levels of FXR, SHP1, SHP2, UGT2B4, BSEP, CYP7A1, CYP7B1, Ntcp, MRP2 and SULT2A1. Immunohistochemistry (IHC) was used to examine the expression of BSEP in liver tissues. Rat liver function and pathological changes in hepatic tissue were assessed using biochemical tests and HE staining.

RESULTS

Upon corilagin intervention, the mRNA and protein levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 were remarkably increased, and those of CYP7A1, CYP7B1 and Ntcp were decreased. After either up- or down-regulating FXR using different methods, corilagin could still increase the mRNA and protein levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 and decrease the protein levels of CYP7A1, CYP7B1 and Ntcp, especially when administered at a high concentration. Corilagin also exerted a notable effect on the pathological manifestation of intrahepatic cholestasis, BSEP staining in liver tissues, and liver function.

CONCLUSIONS AND IMPLICATIONS

Corilagin exerts a protective effect in hepatocytes and can rescue the deleterious activities of intrahepatic cholestasis by stimulating FXR-associated pathways.
KEYWORDS

Corilagin; intrahepatic cholestasis; farnesoid X receptor; small heterodimer partner; uridine diphosphate glucuronosyltransferase 2 family polypeptide B4; bile salt export pump

Abbreviations

farnesoid X receptor, FXR; small interfering RNA, siRNA; small heterodimer partner 1, SHP1; small heterodimer partner 2, SHP2; uridine diphosphate glucuronosyltransferase 2 family polypeptide B4, UGT2B4; bile salt export pump, BSEP; cytochrome P450 family 7 subfamily A polypeptide 1, CYP7A1; cytochrome P450 family 7 subfamily B polypeptide 1, CYP7B1; sodium taurocholate cotransporting polypeptide, Ntcp; multidrug resistance-associated protein 2, MRP2; sulfotransferase family 2A member 1, SULT2A1; immunohistochemistry, IHC; haematoxylin and eosin, HE; bile acids, BAs; total bilirubin, TBIL; direct bilirubin, DBIL; alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP; γ-glutamyl transpeptidase, γ-GGT; total bile acids, TBA; ursodeoxycholic acid, UDCA; Cell Counting Kit 8, CCK8; dexamethasone, DEX; foetal bovine serum, FBS; horseradish peroxidase, HRP; transducing units, TU; bicinchoninic acid, BCA; sodium dodecyl sulphate, SDS; polyvinylidene difluoride, PVDF; enhanced chemiluminescence, ECL; diaminobenzidine, DAB; image-pro plus, IPP; one-way analysis of variance, ANOVA; green fluorescent protein, GFP; enhanced green fluorescent protein, EGFP; cytochromes P450s, CYP; cholesterol 7α-hydroxylase, CYP7A1; Food and Drug Administration, FDA; S-adenosyl-L-methionine, SAMe; peroxisome proliferator-activated receptor γ, PPARγ; 6α-ethyl-chenodeoxycholic acid, 6-ECDCA; primary biliary cirrhosis, PBC; alpha-naphthyl isothiocyanates, ANIT.
Introduction

Intrahepatic cholestasis is an impairment of hepatocytes and cholangiocytes that can lead to bile formation and flow blockage, especially bile acid (BA) retention. Accordingly, biochemical indicators of this condition include elevated levels of total bilirubin (TBIL), direct bilirubin (DBIL), γ-glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), and total bile acids (TBA) (Beuers et al., 2015). If patients do not undergo effective treatment, they will develop liver fibrosis, cirrhosis and even liver failure, ultimately requiring liver transplantation (Ding et al., 2006).

Ursodeoxycholic acid (UDCA) and glucocorticoids are recognized as effective drugs that are frequently used to treat cholestatic hepatitis (Parés et al., 2015). However, UDCA is ineffective in approximately 1/3 of patients with cholestasis, and the treatment is tedious (McKiernan et al., 2002), while glucocorticoids cause obvious side effects (Purohit et al., 2015). Therefore, identifying drugs with fewer side effects with fast efficacy for treating cholestatic hepatitis is critical. During the progression of cholestasis, the nuclear receptor FXR plays a central role in bile composition metabolism, which controls the balance of BAs by regulating bile acid synthesis, detoxification and transport (Ding et al., 2015). Thus, FXR is considered as a key target for the treatment of cholestasis (Sepe et al., 2015).

Corilagin (beta-1-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-D-glucose), which is found in many medicinal herbaceous plants such as Phyllanthus urinaria, is a member of the tannin family (Shen et al., 2003). Its molecular formula is C\textsubscript{27}H\textsubscript{22}O\textsubscript{18} (Duan et al., 2005). It has been reported that corilagin has strong anti-oxidant (Zhao et al., 2008b; Jin et al., 2013), anti-inflammatory (Zhao et al., 2008b), hepatoprotective (Shiota et al., 2004), thrombolytic and antihypertensive (Cheng et al., 1995), antiatherogenic (Duan et al., 2005) and anti-tumoural (Jia et al., 2013) properties. Corilagin can also suppress schistosomiasis liver
fibrosis by regulating the IL-13/JAK/STAT6 and miR-21/smad7/ERK signalling pathways (Li et al., 2016; Yang et al., 2016; Du et al., 2016). Moreover, corilagin inhibits the expression of TNF-α and NF-κB (Gambari et al., 2012) and protects against HSV1 encephalitis by inhibiting the TLR2 signalling pathways (Guo et al., 2015; Guo et al., 2010).

In our previous study, we demonstrated that corilagin exerts anti-inflammatory and anti-oxidative effects against acute cholestasis (Jin et al., 2013). However, the molecular mechanism of how corilagin alleviates intrahepatic cholestasis is still unknown. As the pathological and biochemical changes in the liver due to alpha-naphthylisothiocyanate (ANIT) treatment mimic cholestatic hepatitis (Chen et al., 2016; Ding et al., 2008), this study used the normal liver tissue LO2 cell line and an ANIT-induced rat model to determine whether corilagin can alleviate cholestasis via the FXR-associated signalling pathway and to find a new strategy to prevent and treat intrahepatic cholestasis.

Methods

Chemicals and reagents

Corilagin (purity >99%) for cell experiments was purchased from the China National Institutes for Food and Drug Control. Corilagin for animal experiments (purity >80%), which was used in our previous study (Yang et al., 2016), was provided by Chengdu PureChem-Standard Co., Ltd. (Chengdu, China). A Cell Counting Kit 8 (CCK8) kit was purchased from Dojindo Laboratories (Kumamoto, Japan). ANIT was purchased from Sigma (St Louis, MO, USA). UDCA capsules were obtained from Dr Falk at Pharma GmbH (Freiburg, Germany). Dexamethasone (DEX) was purchased from Xinxiang Changle Pharmaceutical Company Ltd. (Xinxiang, China). Guggulsterones and GW4064 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Foetal bovine serum
(FBS) and RPMI 1640 medium were obtained from Gibco (Grand Island, NY, USA). Protein extraction kits were purchased from Wuhan Aspen Biological Technology co., Ltd. (Wuhan, China). Affinity-purified rabbit anti-rat antibodies targeting BSEP, UGT2B4, SHP1, SHP2, CYP7A1, CYP8B1, Ntcp, MRP2, SULT2A1 and cytokeratin-18 (CK-18) were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-rat intercellular FXR was obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Biotin-conjugated goat anti-rabbit IgG and streptavidin-horseradish peroxidase (HRP) conjugate were obtained from Wuhan Boster Biotechnology Co., Ltd. (Wuhan, China). RNAiso, a PrimeScript RT Reagent kit and a SYBR Premix Ex Taq kit were obtained from Takara Biotechnology Dalian Co., Ltd. (Dalian, China).

Cell culture and cytotoxic effects of corilagin

The LO2 human embryo liver cell line was purchased from Chinese Academy of Sciences. RPMI 1640 medium supplemented with 10% FBS was used to culture the LO2 cells in an incubator at 37 °C and maintained in a humidified atmosphere containing 5% CO₂. The cytotoxic effect of corilagin was evaluated using a CCK8 assay, which was conducted as previously described (Wang et al., 2016).

FXR in cells up- or down-regulated with GW4064 or guggulsterones

The intervention groups were divided into a normal group, a DEX group, a UDCA group and corilagin 25 μg/ml, 50 μg/ml and 100 μg/ml groups. LO2 cells were maintained in RPMI 1640 media supplemented with 10% FBS. As previously described (Ding et al., 2016), cells were passed into either 6-well plates for real-time PCR and western blotting or 96-well plates.
for CCK8 experiments and preliminary lentiviral transfection experiments for 24 h. When the cell cultures reached 70% density, they were treated with corilagin, UDCA or DEX. After 24 h, the cells were harvested for real-time PCR and western blotting. For the GW4064 or guggulsterones intervention experiments, LO2 cells were maintained in RPMI 1640 media supplemented with 10% FBS. After the cells were grown on 6-well plates for 24 h and cultured to 70% density, GW4064 (1 μmol/L, diluted in 1640 medium) or guggulsterones (1 μmol/L, diluted in 1640 medium) were added to the wells of all the groups (except the normal group) for 24 h. Then, the cells were treated with corilagin, UDCA or DEX, as indicated above. After 24 h, the cells were harvested for real-time PCR and western blotting.

**Small interfering RNA (siRNA) transfection of LO2 cells**

Human FXR siRNA (sense 5′-CAAGTGACCTCGACAACAA-3′) was synthesized by RiboBio Co., Ltd. GenePharma Co. Ltd. (Guangzhou, China). LO2 cells were seeded onto 6-well plates and transfected with 50 μmol/ml siRNA constructs using 20 pmol/ml Lipofectamine 2000 (Invitrogen, San Diego, USA) according to the manufacturer's instructions. The medium was replaced 6 h later, and then the cells were cultured for up to 48 h. In addition, the siRNA-treated cells were treated with corilagin, UDCA or DEX 24 h before harvesting.

**Over-expression of FXR in LO2 cells via lentiviral transduction**

The FXR and control lentiviral vectors were constructed by GeneChem Co., Ltd. (Shanghai, China). GV367-FXR/NC-enhanced green fluorescent protein (eGFP) was transfected into LO2 cells, and the viral supernatant was harvested after 48 h (2×10^8 transducing units
[TU/ml]. LO2 cells were seeded onto 96-well or 6-well plates and transduced with lentivirus with a multiplicity of infection of 50 according to the manufacturer's instructions. The medium was replaced 6 h later, and then the cells were grown for an additional 72 h. In addition, the lentivirus-transfected cells were treated with corilagin, UDCA or DEX for another 24 h.

**Animals**

Neonatal Sprague-Dawley male rats (3 weeks old) weighing 40 g-50 g and rats for isolating primary hepatocytes were born and raised in specific pathogen-free conditions and were purchased from Hubei Provincial Centre for Disease Control and Prevention (Wuhan, China). Animal studies were reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). Rats were housed in stainless polypropylene cages (size: 465 * 290 * 150 mm) with wood shavings as bedding materials. A maximum of five rats was kept in a single cage. The rats were maintained as described previously (Du *et al.*, 2016) under standard laboratory conditions at 25 ± 2 °C, 50 ± 15% relative humidity, and a normal circadian period (12-h dark/12-h light cycle). The animals were provided a normal diet and water ad libitum. All study procedures were approved by internationally accepted principles and the Guidelines for the Care and Use of Laboratory Animals of Huazhong University of Science and Technology.

**Rat model establishment and drug administration**

Thirty-five rats were equally divided into 7 groups: corilagin (40 mg/kg), corilagin (20 mg/kg), corilagin (10 mg/kg), UDCA, model and normal (rats without ANIT intervention).
The reported median lethal dose (LD50) of corilagin is 1.78 g/kg (Zhang et al., 2013), which is much larger than our experimental dose. Corilagin was prepared as a 0.4% suspension in sodium carboxymethylcellulose. UDCA was prepared as a 0.6% suspension in water. Dexamethasone was dissolved in water at the concentration of 0.045%. Our previous studies confirmed that liver damage and pathological changes began to increase at 24 h after ANIT treatment, peaked at 48 h and trended towards restoration at 72 h (Ding et al., 2008). ANIT was dissolved in sesame oil at a concentration of 1% and administered for 48 h. Before establishing the cholestasis model, corilagin (40 mg/kg), corilagin (20 mg/kg), corilagin (10 mg/kg), UDCA (60 mg/kg/d) and DEX (1.8 mg/kg/d) were intragastrically administered to the rats in their respective groups for 4 days. Model and normal groups were administered normal saline. On the 5th day, we induced the rat model. In addition, after 12 h, all the groups (except the normal group) were intragastrically administered ANIT (50 mg/kg). After 8-h intervals of ANIT administration, the rats were still administered the respective drug or control agents. After 48 h of continuous ANIT treatment, the rats in each group were euthanized to collect specimens (Table 1 and Figure 9A). Rats were assigned to groups randomly, and no rats were excluded from the statistical analysis.

**Specimen collection**

The procedure was conducted as previously described (Yang et al., 2016). Following anaesthetization with 4% chloral hydrate by intraperitoneal injection (1 ml/100 g), the rat abdomen was opened, and the abdominal aorta was separated. Then, 2-3 ml of arterial blood was collected in a test tube containing anticoagulant. After centrifugation at 3250 g, blood serum was obtained and stored at -20 °C until testing. Subsequently, the rat liver was dissected using an aseptic, RNase-free device. After washing with normal saline, the entire
hepatic tissue was divided into two parts—one part was sheared and stored at -80 °C, and the other was fixed in 10% formalin for 48 h, dehydrated, embedded in paraffin and sliced.

**Isolation, culture and treatment of rat primary hepatocytes**

Hepatocytes from male SD rats (8-9 weeks) weighing 200 g-220 g were isolated by a two-step collagenase digestion method and cultured according to the published procedures (Klaunig et al., 1981; Chen et al., 2014). CK-18 protein in the cells was detected by immunofluorescence to identify whether the isolated cells were rat primary hepatocytes (Banaudha et al., 2010). Then, the hepatocytes were seeded in 6-well plates. When the cell cultures reached 70% density, they were treated with corilagin, UDCA or DEX. After 24 h, the cells were harvested for real-time PCR and western blotting.

**Biochemical tests**

The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), TBIL, DBIL, ALP, γ-GGT and TBA levels were assayed using a fully automated Aeroset Chemistry Analyser provided by Abbott Co., Ltd. (Ding et al., 2008).

**Real-time quantitative polymerase chain reaction**

Following our previous protocols (Huang et al., 2013; Zhou et al., 2016), total RNA from liver tissues and cells was isolated using RNAiso Plus following the manufacturer’s instructions. cDNAs were produced using a PrimeScript RT reagent kit and incubated at 37 °C for 15 min and 85 °C for 5 s. Real-time PCR reactions were performed using a
StepOne Plus device (Applied Biosystems) at 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s according to the instructions provided by the SYBR Premix Ex Taq kit. Data were analysed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen et al., 2008). All primers were synthesized by TSINGKE (Wuhan, China). The sequences of all primers are listed in Table 1.

**Western blot analysis**

Western blotting was performed as previously described (Zhao et al., 2008b). To detect FXR, SHP1, SHP2, BSEP and UGT2B4 expression, total protein was extracted from the liver tissues and hepatocytes. Protein concentration was determined using the bicinchoninic acid (BCA) method. In each sample, an equivalent volume of 2× sodium dodecyl sulphate (SDS) loading buffer (100 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerine; 10% β-mercaptoethanol; and 0.2% bromophenol blue) was added and mixed again. The mixtures were then denatured at 95 °C for 10 min, and approximately 30 mg of protein was loaded into each well and separated on 10% SDS-polyacrylamide electrophoresis gels. After separation for approximately 80 min, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes, and the membranes were saturated and blocked with 5% fat-free milk at 37 °C for 1 h. The membranes were probed with rabbit polyclonal antibodies targeting the rat homologs of FXR (1:1000), BSEP (1:1,000), UGT2B4 (1:500), SHP 1 (1:1,000), SHP2 (1:1,000), CYP7A1 (1:1000), CYP8B1 (1:1000), Ntcp (1:1000), MRP2 (1:1000), SULT2A1 (1:2000) and β-actin (1:5,000) followed by treatment with horseradish peroxidase-conjugated secondary immunoglobulin IgG (1:1000). The membranes were then treated with an enhanced chemiluminescence (ECL) reagent (Merck Millipore, MA, USA), and the signals were detected by exposing the membranes to X-ray films (Kodak, Rochester, NY, USA). The relative signal intensity was quantified using densitometry with Gel pro3.0 image software.
Immunohistochemistry (IHC) for detecting BSEP expression in liver tissue

IHC was conducted as previously described (Jin et al., 2015). The liver tissue specimens were sliced into 10-μm sections after dewaxing and rehydrating. The sections were incubated in 3% H₂O₂/methanol to eliminate endogenous peroxidase activity. Then, the sections were incubated with normal goat serum for 10 min followed by BSEP antibody (1:400) overnight at 4 °C and biotin-conjugated goat anti-rabbit IgG (1:500) at 37 °C for 45 min. The sections were rinsed again with PBS and incubated with horseradish peroxidase-conjugated streptavidin at 37 °C. The samples were developed with diaminobenzidine (DAB) and stained with haematoxylin. After being rinsed with distilled water and dehydrated, the sections were made transparent and mounted for examination under a microscope. After immunohistochemical analysis, Image-Pro plus (IPP) software version 6.0 was used to analyse the optical density of the images as described previously.

Histomorphology

After the tissues were fixed in 4% formaldehyde, they were embedded in paraffin and cut into 4-mm-thick serial sections for haematoxylin and eosin (HE) staining as previously described (Huang et al., 2013).

Statistical analysis

The statistical analyses were conducted using SPSS 12.0 software. Data were expressed as
the mean ± SD. The significance of differences between two groups was determined by Student's paired two-tailed t-test. For all other statistical analyses and comparisons of multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was defined as $P < 0.05$ (Ding et al., 2015; Dang et al., 2016). The data and statistical analysis complied with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

**Nomenclature of targets and ligands**

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015a, b).

**Results**

**Cytotoxic effect of corilagin and cell morphology observation**

Cell viability (%) was calculated as follows: 
\[ \frac{[A \text{ (experimental well)} - A \text{ (blank well)}]}{[A \text{ (control well)} - A \text{ (blank well)}]} \times 100 \]. Moreover, we assessed the viability of cells treated with different concentrations (0, 6.25, 12.5, 25, 50, 100, 200 and 400 $\mu$g/ml) of corilagin after 24 h. Based on the CCK8 assay, the concentration-effect curve was represented by the equation \( y = \frac{100}{(1+10^{((2.228-x)*(-2.210))})} \) (Figure 1A), and the specific parameters $IC_{50}$ (169.0 $\mu$g/ml), Hillslope (-2.210) and $R^2$ (0.9777) were also assessed. Then, using this equation, the concentration for 75% viability was calculated as 102.8272 $\mu$g/ml. For convenience, we approximated high concentration as 100 $\mu$g/ml, middle concentration as 50...
μg/ml and low concentration as 25 μg/ml. Thus, the concentrations of corilagin for pretreatment of unstimulated LO2 cells were 25 μg/ml, 50 μg/ml and 100 μg/ml for 24 h, none of which significantly affected cell viability (Figure 1B). Cell morphology observations showed the same result (Figure 1C-1D). Therefore, we chose to treat cells with corilagin at 25 μg/ml, 50 μg/ml and 100 μg/ml for 24 h. We suggest that the IC50 of corilagin for LO2 cells is 169.0 μg/ml.

Effects of corilagin on FXR and downstream effectors in LO2 cells

BAAs are synthesized from cholesterol in hepatocytes, and hepatocytes play an important role in the entire process of BA metabolism. Therefore, we administered the selected three concentrations of corilagin to normal LO2 cells and observed whether corilagin could activate the related FXR pathway. Compared with the untreated cells, the corilagin-treated cells showed significantly elevated mRNA expression of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05). The mRNA expression of FXR in the DEX group (n = 5; P < 0.05) and the mRNA expression of SHP, UGT2B4 and BSEP in the UDCA group (n = 5; P < 0.05) were also increased. More importantly, the increases in the mRNA levels of FXR, SHP, UGT2B4 and BSEP in the 100 μg/ml corilagin group were more significant than those in the DEX and UDCA groups (n = 5; P < 0.05) (Figure 2A). The protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 were also significantly increased. Furthermore, CYP7A1, CYP8B1 and Ntcp were decreased in the corilagin groups compared with the normal group (n = 5; P < 0.05), and there were no obvious increases in protein expression in the DEX and UDCA groups (Figure 2B-2C).
**Effects of corilagin on FXR and downstream effectors in LO2 cells stimulated with guggulsterones**

To identify the role of FXR in cholestasis, various researchers have sought to use chemicals or genetic tools to inhibit hepatic FXR. Some reports showed that Fxr-knockout (Fxr−/−) mice had excessive levels of BAs, cholesterol and triglycerides (Anakk *et al.*, 2011). Guggulsterones, which are FXR antagonists, have been proven to exacerbate cholestasis in liver cells (Zhao *et al.*, 2014). Therefore, in our experiment, LO2 cells were treated with guggulsterones to down-regulate FXR. Compared with the normal group, the guggulsterones group showed significantly decreased mRNA expression of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05), while compared with the guggulsterones group, the corilagin and UDCA groups showed significantly elevated mRNA expression of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05). The FXR and SHP mRNA levels in the 50 μg/ml corilagin group and the FXR, SHP, UGT2B4 and BSEP mRNA levels in the 100 μg/ml corilagin group were greater than those in the UDCA group (n = 5; P < 0.05) (Figure 3A). The protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 were also significantly reduced, while CYP7A1, CYP8B1 and Ntcp were elevated by guggulsterones (n = 5; P < 0.05). The corilagin groups exhibited significantly higher protein expression of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 and decreased protein expression of CYP7A1, CYP8B1 and Ntcp (n = 5; P < 0.05) than the guggulsterones group. It was worth noting that the activating effect of FXR protein increased as the concentration of corilagin increased (n = 5; P < 0.05) (Figure 3B-3C).
Effects of corilagin on FXR and downstream molecules in LO2 cells stimulated with GW4064

Either the FXR agonist 6α-ethyl-chenodeoxycholic acid (6-ECDCA, obeticholic acid) or GW4064 can reduce oestradiol-induced cholestasis in liver cells (Seok et al., 2015) to increase the uptake of bile salts, restore the flow of bile and reduce the levels of serum bile salt. These compounds have been approved for the treatment of primary biliary cirrhosis (PBC) and NASH (Neuschwander-Tetri et al., 2015). More importantly, to observe whether corilagin could continue to promote the FXR signal pathway in the condition of high FXR levels, we used GW4064 to up-regulate FXR. Compared with the normal group, the GW4064 group showed significantly increased mRNA expression of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05), while compared with the GW4064 group, the 50 µg/ml and 100 µg/ml corilagin groups showed significantly increased mRNA expression of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05) (Figure 4A). FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 protein expression was also significantly elevated and CYP7A1, CYP8B1 and Ntcp protein expression was reduced in the GW4064 group (n = 5; P < 0.05). Compared with the GW4064 group, the corilagin and UDCA groups exhibited significantly elevated protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1, together with reduced protein expression levels of CYP7A1, CYP8B1 and Ntcp (n = 5; P < 0.05). The BSEP protein levels in the 100 µg/ml corilagin group were significantly higher than those in the UDCA group (n = 5; P < 0.05) (Figure 4B-4C).
**Effect of corilagin on FXR and downstream effectors in LO2 cells after siRNA down-regulation of FXR**

To precisely down-regulate the expression of FXR, we used siRNA to interfere with FXR in LO2 cells. In addition, to verify whether the FXR-siRNA was transfected into the cells, the Cy3 molecule in LO2 cells was conjugated to the siRNA and observed under a fluorescence microscope after siRNA transfection for 24 h (Figure 5A-5C). The cells were then treated with corilagin (25, 50 or 100 μg/ml), UDCA or DEX for 24 h. Compared with the normal group, the Si-FXR group showed significantly decreased mRNA expression levels of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05) (Figure 5D), and the protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 were also significantly lower, while CYP7A1, CYP8B1 and Ntcp were higher in the Si-FXR group (n = 5; P < 0.05) (Figure 5E-5G). Compared with the Si-FXR group, the corilagin and UDCA groups exhibited significantly elevated mRNA expression levels of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05). In addition, the FXR mRNA levels in the 50 μg/ml and 100 μg/ml corilagin groups and the SHP, UGT2B4 and BSEP mRNA levels in 100 μg/ml corilagin group were notably more elevated than those in the UDCA group (n = 5; P < 0.05). The activating effect of FXR mRNA increased as the concentration of corilagin increased (n = 5; P < 0.05) (Figure 5H). Compared with the Si-FXR group, the corilagin and UDCA groups showed significantly elevated protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1, as well as significantly reduced protein expression levels of CYP7A1, CYP8B1 and Ntcp (n = 5; P < 0.05). In addition, the SHP1 protein levels in the 50 μg/ml and 100 μg/ml corilagin groups and the FXR, SHP2, UGT2B4, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp protein levels in the 100 μg/ml corilagin group were notably more elevated than those in the UDCA group (n = 5; P < 0.05) (Figure 5I-5L).
Effect of corilagin on downstream effectors in LO2 cells after up-regulation of FXR using a lentiviral vector

To precisely down-regulate the expression of FXR, we constructed the FXR lentiviral vector GV273 and transduced LO2 cells in vitro. GFP (green fluorescent protein) was observed under a fluorescence microscope at 48 h and 72 h after transduction (Figure 6A-6B). Then, the cells were treated with corilagin (25, 50 or 100 μg/ml), UDCA or DEX for 24 h. Compared with the normal group, the lentivirus-treated group showed significantly increased mRNA expression levels of FXR, SHP, UGT2B4 and BSEP (n = 5; P < 0.05) (Figure 6C), and the protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 were also significantly elevated, while CYP7A1, CYP8B1 and Ntcp were reduced in the lentivirus-treated group (n = 5; P < 0.05) (Figure 6D-6F). The corilagin and UDCA groups exhibited significantly higher mRNA expression levels of FXR, SHP, UGT2B4 and BSEP than the lentivirus-treated group (n = 5; P < 0.05). In addition, the FXR and BSEP mRNA levels in the 100 μg/ml corilagin groups were more pronounced than those in the UDCA group (n = 5; P < 0.05) (Figure 6G). Compared with the lentivirus-treated group, the corilagin and UDCA groups showed significantly elevated protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 and reduced protein expression levels of CYP7A1, CYP8B1 and Ntcp (n = 5; P < 0.05). In addition, the FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp protein levels in the 100 μg/ml corilagin groups were significantly different (enhanced or reduced) than those in the UDCA group (n = 5; P < 0.05). Additionally, the activating effect of the FXR protein increased as the concentration of corilagin increased (n = 5; P < 0.05) (Figure 6H-6K).
Effects of corilagin on FXR and downstream molecules in rat primary hepatocytes

We used rat primary hepatocytes to investigate the effect of corilagin on cholestasis. To verify whether the isolated cells were rat primary hepatocytes, an immunofluorescence method was used to detect CK-18 protein in hepatocytes. The green fluorescence molecule Dylight 488 in primary hepatocytes was observed under a fluorescence microscope (Figure 7A-7C). After corilagin treatment, compared with the untreated cells, the corilagin group showed significantly elevated mRNA expression of FXR, SHP, UGT2B4 and BSEP ($n = 5$; $P < 0.05$), while the mRNA expression of SHP and BSEP in the UDCA group was also increased ($n = 5$; $P < 0.05$). More importantly, the increases in the mRNA levels of SHP and BSEP in the 100 $\mu$g/ml corilagin group were more significant than those in the UDCA groups ($n = 5$; $P < 0.05$) (Figure 7D). The protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 were also significantly increased. Furthermore, CYP7A1, CYP8B1 and Ntcp were decreased in the corilagin groups compared with the normal group ($n = 5$; $P < 0.05$), and there were no obvious increases protein expression in the DEX and UDCA groups (Figure 7E-7F). The primary hepatocyte experimental results were consistent with those of the LO2 liver cell line, as the FXR-associated pathways were markedly upregulated in the 50 $\mu$g/ml and 100 $\mu$g/ml corilagin groups in vitro.

Effect of corilagin on serum biochemical indicator

As shown in Table 2, compared with the model group, the corilagin-treated groups showed significant decreases in ALT, AST, TBIL, DBIL, ALP, GGT and TBA levels ($n = 5$; $P < 0.05$). Corilagin (20 mg/kg) and corilagin (40 mg/kg) induced similar effects as UDCA on ALT. Corilagin (40 mg/kg) exhibited the greatest effects on AST, ALP and DBIL ($n = 5$; $P <
0.05), but they were inferior to those induced by UDCA. Corilagin (40 mg/kg, 20 mg/kg) had notable effects on TBIL ($n = 5; P < 0.05$), and UDCA induced a similar effect on TBIL levels as corilagin (10 mg/kg). Corilagin (10 mg/kg, 20 mg/kg, 40 mg/kg) significantly influenced GGT ($n = 5; P < 0.05$), and corilagin (40 mg/kg) was more effective on GGT ($n = 5; P < 0.05$) than UDCA.

**Effects of corilagin on liver morphology as assessed using HE staining**

As shown in Figure 8, the hepatic tissue in the normal group showed a regular arrangement of hepatic lobules and cells, and intact epithelial cells from the bile duct could also be observed. Compared with the normal group, the model group showed typical pathological changes, including significant swelling of hepatic cells, swelling of cytoplasm, disorganized nuclei and strongly stained nucleoli. Moreover, in the model group, many punctiform or focused necrotic zones were observed in the hepatic tissue, and proliferation of bile duct epithelial cells and Kupffer's cells could also be observed. In addition, the bile duct showed a narrower canal, a bile thrombus and necrotic cells. In the corilagin groups, the pathological changes were less pronounced than those in the model group, and the manifestations in the UDCA group were similar to those observed in the corilagin (20 mg/kg) group. However, the pathological impairment in the DEX group appeared to be more severe in hepatic tissue.

**Effect of corilagin on BSEP protein expression in liver tissues as examined by immunohistochemistry**

As shown in Figure 8, the rate of BSEP-positive staining at the cytoplasm in specimens from the model group significantly decreased ($n = 5; P < 0.05$) compared with that in the normal
The staining rate in the UDCA group was higher than that in the model group \((n = 5; P < 0.05)\). Compared with the UDCA group, the corilagin (20 mg/kg) and corilagin (40 mg/kg) groups had markedly higher positive staining rates \((n = 5; P < 0.05)\), while the positive staining rate in the DEX group showed no remarkable differences compared with the model group.

**Effects of corilagin on FXR pathways in a rat model of cholestatic hepatitis**

Compared with the normal group, the model group showed significantly decreased mRNA levels of FXR, SHP, BSEP and UGT2B4 \((n = 5; P < 0.05)\). The mRNA expression of FXR, SHP, BSEP and UGT2B4 in the corilagin and UDCA groups and the mRNA expression of SHP in the DEX group were significantly altered compared with the model group \((n = 5; P < 0.05)\). In addition, the SHP mRNA level in the 20 mg/kg and 40 mg/kg corilagin groups and the UDCA group were significantly higher than that in the DEX group \((n = 5; P < 0.05)\). The FXR, SHP, BSEP and UGT2B4 mRNA levels in the 40 mg/kg corilagin group were greater than those in the UDCA group \((n = 5; P < 0.05)\). Furthermore, the activating effect of FXR mRNA increased as the concentration of corilagin increased \((n = 5; P < 0.05)\) (Figure 9B).

Compared with the normal group, the model group exhibited significantly decreased protein expression levels of FXR, SHP1, SHP2, UGT2B4 and BSEP \((n = 5; P < 0.05)\). Compared with the model group, the corilagin and UDCA groups showed significantly elevated protein expression levels of FXR, SHP1, SHP2, UGT2B4, BSEP, MRP2 and SULT2A1 as well as reduced protein expression levels of CYP7A1, CYP8B1 and Ntcp. The protein expression levels of SHP2, UGT2B4, BSEP, CYP8B1 and SULT2A1 in the DEX group were also significantly elevated or reduced \((n = 5; P < 0.05)\) (Figure 9C-9D). Although UDCA exerted
a positive effect on the protein expression of FXR, SHP1, SHP2, BSEP, UGT2B4, DEX, CYP8A1 and CYP8B1, and although DEX induced a positive effect on the protein expression of SHP2, BSEP, UGT2B4, CYP8B1 and SULT2A1, the corilagin (40 mg/kg) group presented better improvement than the UDCA and DEX groups ($n = 5; P < 0.05$).

Discussion

Bile acids (BAs) are produced in the liver by the oxidation of cholesterol through a series of reactions carried out by various cytochromes P450 enzymes (CYP) and CYP7A1, the latter of which is generally considered the rate-limiting enzyme that initiates bile acid synthesis (Gonzalez et al., 2016). The physiological concentration of BAs can regulate liver regeneration (Huang et al., 2006), energy expenditure (Watanabe et al., 2006), triglyceride levels (Watanabe et al., 2004) and glucose homeostasis (Thomas et al., 2009). However, cholestasis patients who have excessive BAs can suffer from oxidative stress, hepatocyte death and mitochondrial abnormality, all of which can ultimately induce liver damage.

Therefore, early effective interventions to control cholestasis development are particularly important. UDCA is currently the only Food and Drug Administration (FDA)-approved drug to treat primary biliary cirrhosis, but its efficacy is limited to early stages of the disease (Corpechot et al., 2011). UDCA has shown the ability to promote the excretion of endogenous cholic acid, change the composition of BAs, increase the proportion of hydrophilic BAs, protect liver cells and bile duct cells from toxic BAs and inhibit liver cell apoptosis (Beuers et al., 2006). However, depending on age and sex, nearly 30–50% of patients who are biochemical non-responders are still at risk of disease progression and poor survival (Kuiper et al., 2009; Corpechot et al., 2016). Other medications such as S-adenosyl-L-methionine (SAMe) suffer from a lack of randomized controlled trials that

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have validated their efficacy (Lu et al., 2012). Although glucocorticoids have a limited effect on cholestasis, long-term use of glucocorticoids can cause many adverse effects, including weight gain, hyperglycaemia, osteoporosis, cataracts, increased risk of opportunistic infections, and so on (Purohit et al., 2015).

Thereby, it is critical to find new drugs with fewer side effects and reliable curative effects for treating cholestasis. Although the mechanisms of aberrant BA retention via synthesis, detoxification and transport are complicated, the multistep feedback loop that regulates BA involves the nuclear receptors FXR and SHP and culminates in the repression of CYP7A1 expression (Anakk et al., 2011). FXR is a member of the nuclear receptor superfamily and is largely expressed in the liver (Fiorucci et al., 2015). Additionally, FXR has been recognized as a therapeutic target in cholestasis and has been confirmed as a key node to maintain BAs by regulating target genes related to the metabolism of BAs—particularly its synthesis, detoxification, and transportation (Sepe et al., 2015; Mazuy et al., 2015). The role of FXR in cholestasis is as follows (Figure 10). In the bile acid detoxification pathway, UGT2B4 and SULT2A1 are influenced by FXR and transduce a detoxification signal that overrides other signals (Zollner et al., 2009). In the bile acid transportation process, BSEP, Ntcp and MRP2 play roles in the transfer of bile components after they are activated by FXR. BSEP, Ntcp and MRP2 are abundantly expressed at the capillary bile duct membrane of hepatocytes and function as rate-limiting enzymes, making them key proteins in bile transportation and excretion. FXR up-regulates the expression of BSEP by binding to its DNA response element and assisting BA transport from hepatocytes to the biliary duct (Gonzalez et al., 2012). During the process of bile acid synthesis, FXR either directly or indirectly represses transcription of CYP7A1 and CYP8B1, which is required for the synthesis of BAs, by either mediating the expression of SHP or suppressing the degradation of SHP. This results in inhibition of BA synthesis, the FXR can increase the
content of bile phospholipids via small heterodimer partner 1/2 (SHP1/2) and inhibit liver fibrosis via SHP1/2 and peroxisome proliferator activated receptor γ (PPARγ) (Wagner et al., 2009).

For the *in vitro* experiment, the mRNA and protein expression levels of FXR, SHP, UGT2B4, BSEP, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp were significantly elevated or reduced in the corilagin group compared with those in the normal group. Then, LO2 cells were treated with guggulsterones or si-FXR to inhibit FXR. Compared with the guggulsterones or si-FXR group, the corilagin groups showed significantly elevated mRNA and protein expression levels of FXR, SHP, UGT2B4, BSEP, MRP2 and SULT2A1 and reduced protein expression levels of CYP7A1, CYP8B1 and Ntcp. Furthermore, to confirm the efficacy of corilagin, we treated LO2 cells with either GW4064 or lentivirus to enhance FXR activity. Compared with the GW4064 or lentivirus-treated group, the corilagin groups showed significantly elevated mRNA and protein expression of FXR, SHP, UGT2B4, BSEP, MRP2 and SULT2A1, together with reduced protein expression levels of CYP7A1, CYP8B1 and Ntcp. In the *in vivo* experiments, FXR signalling pathways were activated in ANIT-induced cholestasis rats. After establishing the model and providing treatments, the serum TBIL, DBIL, TBA, ALP, GGT, ALT and AST levels as well as pathological changes in the liver were monitored. Corilagin at a dose of 20 mg/kg in rats exert remarkable effects on TBA and can also improve liver functions, related enzyme dysregulation and the jaundice index. Based on biochemical and pathological observations, ANIT-induced intrahepatic cholestasis and liver damage were observed in the model group, thus proving successful establishment of the animal model. Regarding the hepatic pathology, the livers from rats subjected to ANIT administration exhibited typical damage, such as infiltration of neutrophils, necrosis of hepatocytes, proliferation of inflammatory cells and epithelial cells in the bile duct and formation of bile thrombus. Upon corilagin intervention, improvement in acute
hepatic impairment was achieved. The expression levels of FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp were significantly decreased or increased in the model group. After treatment with corilagin, the FXR pathways were markedly activated, and the expression levels of FXR, SHP, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp were promoted or inhibited to varying degrees.

In our experiments, several details deserve further attention. First, one concentration cannot reflect a dose-dependent effect, and therefore, we chose three corilagin concentrations. Cells treated with siRNA-FXR + corilagin 100 μg/ml had higher levels of FXR than normal cells (seen in Figure 5), and siRNA-FXR + UDCA treatment increased FXR expression, while UDCA alone did not affect FXR in the normal group based on the literature and the data shown in Figure 2. In LO2 cells treated with siRNA with lower levels of FXR, the effect of the drug on promoting FXR was notable, and corilagin 100 μg/ml promoted FXR in normal LO2 cells. UDCA had no effects on normal LO2 cells, but whether it exerts effects on distressed cells remains unknown. However, from the subsequent results, regardless of treatment with guggulsterones, GW4064, siRNA or lentivirus, UDCA has notable effects. Furthermore, although guggulsterones and siRNA as well as GW4064 and the lentivirus vector exerted similar effects on FXR gene expression to either inhibit or promote FXR by 50% percent, respectively, the mechanisms of action are completely different. siRNA and lentiviral vectors target the specific gene, whereas the use of a chemical antagonist (guggulsterones) or agonist (GW4064) can affect FXR-associated pathways in multiple manners, the exact mechanisms of which are unknown. Therefore, we choose both chemical and biological approaches to interfere with the expression of FXR. Moreover, Fxr-knockout (Fxr−/−) animals have excessive levels of BAs, cholesterol and triglycerides. In addition, the FXR-associated pathways in human cholestasis have been proven to be suppressed, although there are few studies that have knocked out FXR to observe the effect of the FXR gene in
humans. Therefore, the LO2 cells were treated with guggulsterones or siRNA to down-regulate FXR expression to simulate low FXR expression. In contrast, the FXR agonist GW4064 reduced cholestasis and has been approved for the treatment of PBC and NASH. However, to observe whether corilagin continues to promote the FXR signalling pathway under conditions of high FXR expression, we choose GW4064 and a lentiviral vector to up-regulate FXR in cells. Additionally, to maintain stable blood drug concentrations, we pretreated cells with the respective compounds. Cholestasis in an animal model is a process with rapid onset, and the most serious damage occurs at 48 h. Drugs administered after model induction would miss the opportunity to exert effects during the acute stage of disease. However, in clinical practice, cholestasis normally requires an extended treatment regimen, and the efficacy would be obvious after the medication arrived at a stable blood drug concentration. Therefore, we administered medication prior to inducing the model in order to achieve a stable blood drug concentration and to reflect the efficacy of corilagin for treating cholestasis. As a result, corilagin was shown to be an effective therapy. However, further work is necessary before progressing to clinical trials.

In summary, we investigated the efficacy of corilagin in activating the FXR signalling pathway to alleviate cholestasis *in vitro* and *in vivo*, which could specifically affect the synthesis, detoxification and transport of Bas. More importantly, we demonstrated that corilagin could regulate the key target molecule FXR, which has been verified as a viable therapeutic target for the development of drugs to treat intrahepatic cholestasis. This experiment not only focuses on the reliable curative effects but also opens up a new area of gene targeting therapy, providing a new avenue for clinical research and treatment and forming the foundation for future precision medicine for intrahepatic cholestasis. Further studies on the mechanism of corilagin regarding anti-liver cholestasis may help identify new methods to prevent and treat intrahepatic cholestasis.
Ethics Approval

The study was reviewed and approved by the Research Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology ([2015] IACUC Number: 513).

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81371840 and 81600373), the Hubei Province Health and Family Planning Scientific Research Project (WJ2017Q021), the Hubei Provincial Natural Science Foundation of China (2017CFB471), the Fundamental Research Funds for the Central Universities (2017KFYXJJ238), Shandong Provincial Natural Science Foundation of China (2016ZRBB14450).

Author contributions

Lei Zhao takes responsibility for the integrity of the work as a whole, from inception to publication. Fan Yang conceived and designed the experiments. Yao Wang, Zhi-Lin Chen, Juan Xue and Lei Luo performed the experiments. Gang Li analysed the data. Zhi-Lin Chen, Feng Jin, Lei Luo, Xuan Zhou, Qian Ma, Xin Cai and Hua-Rong Li contributed reagents, materials, or analysis tools. Yao Wang, Li Gang, Zhi-Lin Chen wrote the paper. Lei Zhao edited the article. All authors approved the final version of the manuscript.

Conflict of interest

No potential conflicts of interest are disclosed.
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D1054-D1068.


compounds from the ethanol extracts of Geranium wilfordii. J Ethnopharmacol. 147: 204-207.


Figure 1 (A) The concentration-effect curve was $y = \frac{100}{(1+10^{((2.228-x)*(-2.210))})}$. IC50: 169.0 µg/ml, HillSlope: -2.210, $R^2$: 0.9777. (B) CCK8 assay of LO2 viability after corilagin treatment. (C-D) Morphologies of LO2 after corilagin treatment for 24 h. Data were shown as the mean ± SD. n = 5.
Figure 2 Effect of corilagin on the expression of FXR, SHP, BSEP and UGT2B4 in LO2 normal cells. (A) The mRNA levels of FXR SHP, BSEP and UGT2B4 were detected by RT-PCR. (B-C) The protein levels of FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp were detected by western blotting. Data were shown as the mean ± SD. n = 5. (*P<0.05 compared to the Normal group.)
Figure 3 Effect of corilagin on the expression of FXR, SHP, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1 and CYP8B1 in the Gugg treated LO2 cells. (A) The mRNA levels of FXR, SHP, BSEP and UGT2B4 were detected by RT-PCR. (B-C) The protein levels of FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp were detected by western blotting. Data were shown as the mean ± SD. n = 5. (*P<0.0 compared to the Gugg group; #P<0.05 compared to the Normal group; ΔP<0.05 compared to the UDCA group) Gugg: Guggulsterones, the biochemical inhibitor of FXR.
Figure 4 Effect of corilagin on the expression of FXR, SHP, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1 and CYP8B1 in the GW4064-treated LO2 cells. (A) The mRNA levels of FXR, SHP, BSEP and UGT2B4 were detected by RT-PCR. (B-C) The protein levels of FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1 and CYP8B1 were detected by western blotting. Data were shown as the mean ± SD. n = 5. (*P<0.05 compared to the GW group; #P<0.05 compared to the Normal group; △P<0.05 compared to the UDCA group) GW: GW4064, the biochemical agonist of FXR.
Figure 5 Down-regulation of FXR in LO2 cells via siRNA-FXR. SiRNA interfered with LO2 cells for 24 h to mediate FXR down-expression, and then the cells were treated with different drug intervention for 24 h. (A-C) The fluorescent molecules Cy3 were observed with a
fluorescence microscope after siRNA was introduced into LO2 cells for 24 h compared with the same perspective of ordinary light. (D, H) The mRNA levels of FXR, SHP, BSEP and UGT2B4 were detected by RT-PCR. (E-G, I-L) The protein levels of FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1 and CYP8B1 were detected by western blotting. Data were shown as the mean ± SD. n = 5. (*P<0.05 compared to the Si-FXR group; △P<0.05 compared to the UDCA group) SiRNA-FXR: FXR was knocked down in LO2 cells by siRNA. SiRNA-NC: SiRNA negative control group.
Figure 6 Up-regulation of FXR in LO2 cells via the lentiviral vector. The lentiviral vector interfered with LO2 cells for 72 h to mediate FXR overexpression. Then, the cells were treated with different drug interventions for 24 h. (A-B) The expression of GFP was observed with a fluorescence microscope after the lentivirus was introduced into LO2 cells for 48 and
72 h. (C, G) The mRNA levels of FXR SHP, BSEP and UGT2B4 were detected by RT-PCR.

(D-F, H-K) The protein levels of FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1 and CYP8B1 were detected by western blotting. Data were shown as the mean ± SD. n = 5. (*P<0.05 compared to the Normal group/Lentivirus-treated group; △P<0.05 compared to the UDCA group) Lentivirus-treated: FXR was overexpressed in LO2 cells by the lentiviral vector. Lentivirus-NC: Lentivirus negative control group. GFP: Green fluorescent protein.
Figure 7 Effects of corilagin on FXR and downstream effectors in rat primary hepatocytes. Immunofluorescence was used to detect CK-18 protein in hepatocytes and the green fluorescence molecule Dylight 488 in primary hepatocytes was observed under a fluorescence microscope (Figure 7A-7C). The mRNA levels of FXR, SHP, BSEP and UGT2B4 were detected by RT-PCR. (D) The protein levels of FXR, SHP1, SHP2, BSEP,

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UGT2B4, MRP2, SULT2A1, CYP7A1, CYP8B1 and Ntcp were detected by western blotting. Data were shown as the mean ± SD. n = 5. (*P<0.05 compared to the Normal group.)
Figure 8 Effect of corilagin on cholestasis rat model pathological manifestation of hepatic tissue by HE staining in 400 × magnification and effect of corilagin on BSEP expression was examined with IHC.
Figure 9 Effect of corilagin on the levels of FXR, SHP, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1 and CYP8B1 in ANIT-treated rats. (A) The administration time point: Drugs were administered to the rats in the respective groups for 4 days. On the 5th day, we administered drugs to the rats. After 12 h, all groups except the normal group were intragastrically administered ANIT (50 mg/kg). After an 8-hr interval of ANIT treatment, the rats were

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administered the respective drug or control agent. At the time of ANIT treated for modelling with 48 h, rats in each group were killed for taking specimens. (B) The mRNA levels of FXR, SHP, BSEP and UGT2B4 were measured by real-time PCR. (C-D) The protein levels of FXR, SHP1, SHP2, BSEP, UGT2B4, MRP2, SULT2A1, CYP7A1 and CYP8B1 were detected by western blotting. Data were expressed as the mean ± SD. n = 5. (*p<0.05 compared to the Model group; #p<0.05 compared to the Normal group; △P<0.05 compared to the UDCA group; ※P<0.05 compared to the DEX group)
Figure 10 FXR played an important role in bile acid synthesis, detoxification and transport.

Corilagin was effective in activating the FXR signalling pathways to alleviate cholestasis.
### Tables

**Table 1** Sequences of primers for real-time quantitative

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<td>β-actin</td>
<td>forward primer</td>
<td>5'-GTGGCATGCCGTAAAGACCT-3'</td>
<td>5'-GTCCACCGCAATGCTTTA-3'</td>
</tr>
<tr>
<td></td>
<td>reverse primer</td>
<td>5'-TAGGAGCCAGGGAATCTC-3'</td>
<td>5'-TGCTGTACCTTTACCGTTC-3'</td>
</tr>
</tbody>
</table>
Table 2 Effect of Corilagin on liver function tests. Data were shown as mean±SD. n=5. (*P<0.01 vs Normal group; #P<0.05 vs Model group; &P<0.05 vs UDCA group; ▲P<0.05 vs Dexamethasone group.)

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT(U/L)</th>
<th>AST(U/L)</th>
<th>TBIL(μmol/L)</th>
<th>DBIL(μmol/L)</th>
<th>ALP(U/L)</th>
<th>γ-GGT(U/L)</th>
<th>TBA(μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corilagin(10mg/kg)</td>
<td>785.8±56.4*</td>
<td>1243.8±120.6*</td>
<td>70.7±12.9*</td>
<td>55.2±12.1*</td>
<td>802.0±97.6*</td>
<td>5.4±1.1*</td>
<td>156.6±18.3*</td>
</tr>
<tr>
<td>Corilagin (20mg/kg)</td>
<td>739.6±104.1*</td>
<td>996.6±216.6*</td>
<td>57.4±6.8*</td>
<td>47.4±9.3*</td>
<td>507.8±94.9*</td>
<td>4.8±0.8*</td>
<td>149.0±32.3*</td>
</tr>
<tr>
<td>Corilagin (40mg/kg)</td>
<td>749.6±172.1*</td>
<td>764.2±119.8*</td>
<td>55.7±7.6*</td>
<td>37.4±10.1*</td>
<td>353.0±58.3*</td>
<td>3.4±0.5*</td>
<td>117.2±27.2*</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1372.4±226.3*</td>
<td>1572.2±227.0*</td>
<td>72.5±14.5*</td>
<td>60.3±9.6*</td>
<td>833.0±49.7*</td>
<td>6.6±1.5*</td>
<td>235.6±18.8*</td>
</tr>
<tr>
<td>UDCA</td>
<td>527.2±217.1*</td>
<td>1076.±231.9*</td>
<td>69.2±16.0*</td>
<td>58.8±10.3*</td>
<td>596.4±99.7*</td>
<td>4.0±1.2*</td>
<td>136.6±43.8*</td>
</tr>
<tr>
<td>Model</td>
<td>888.6±41.5*</td>
<td>1471.2±254.2*</td>
<td>92.3±13.4*</td>
<td>76.4±13.6*</td>
<td>949.4±70.8*</td>
<td>10.8±2.3*</td>
<td>199.8±37.6*</td>
</tr>
<tr>
<td>Normal</td>
<td>51.6±22.7</td>
<td>140.4±30.3</td>
<td>1.6±0.5</td>
<td>0.9±0.2</td>
<td>272.6±45.8</td>
<td>3.4±0.5</td>
<td>15.8±6.1</td>
</tr>
</tbody>
</table>
Table 3 The dosage of medication and time administration in the animal experiments

<table>
<thead>
<tr>
<th>Dosage of medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>high dose of corilagin: 40 mg/kg/d</td>
</tr>
<tr>
<td>middle dose of corilagin: 20 mg/kg/d</td>
</tr>
<tr>
<td>low dose of corilagin: 10 mg/kg/d</td>
</tr>
<tr>
<td>DEX: 1.8 mg/kg/d</td>
</tr>
<tr>
<td>UDCA: 60 mg/kg/d</td>
</tr>
<tr>
<td>ANIT: 50 mg/kg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day1-Day5: corilagin, DEX and UDCA administration for 4 days</td>
</tr>
<tr>
<td>Day5: after 12h with corilagin, DEX and UDCA administration, modeled for ANIT (we defined this modeling time point as time point A.)</td>
</tr>
<tr>
<td>8 hours after time point A: corilagin, DEX and UDCA administration</td>
</tr>
<tr>
<td>24 hours after time point A: specimen collected</td>
</tr>
</tbody>
</table>