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RESEARCH PAPER

Protective Effects of Alisol B 23-Acetate Via Farnesoid X Receptor-Mediated Regulation of Transporters and Enzymes in Estrogen-Induced Cholestatic Liver Injury in Mice

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ABSTRACT

Purpose To investigate protective effects of alisol B 23-acetate (AB23A) against hepatotoxity and cholestasis induced by 17α-ethinylestradiol (EE) in association with farnesoid X receptor (FXR) activation *in vivo* and *in vitro*.

Methods The cholestatic liver injury model was established by subcutaneous injections of EE in C57BL/6 mice. Serum biomarkers, bile flow assay and H&E staining were used to identify the amelioration of cholestasis after AB23A treatment. Mice primary hepatocytes culture, gene silencing experiment, real-time PCR and Western blot assay were used to elucidate the mechanisms underlying AB23A hepatoprotection.

Results AB23A treatment protected against liver injury induced by EE through increasing hepatic efflux and reducing uptake of bile acid via an induction in efflux transporters (Bsep and Mrp2) and an inhibition in hepatic uptake transporter (Ntcp) expression. AB23A also reduced bile acid synthesis through repressing Cyp7a1 and Cyp8b1, and increased bile acid metabolism through an induction in gene expression of Sult2a1. We further demonstrated that the changes in transporters and enzymes, as well as ameliorative liver histology in AB23A-treated mice were abrogated by FXR antagonist guggulsterone *in vivo* and were abrogated after FXR was silenced *in vitro*.

Conclusions AB23A produces protective effects against EE-induced cholestasis, due to FXR-mediated gene regulation.

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KEY WORDS alisol B 23-acetate · cholestasis · EE · FXR · transporter

ABBREVIATIONS

AB23A	Alisol B 23-acetate
ALP	Alkaline phosphatase
Bsep	Bile salt export pump
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
Cyp7a1	Cholesterol 7α-hydroxylase
Cyp8b1	Sterol-12α-hydroxylase
EE	17α-ethinylestradiol
Fgf15	fibroblast growth factor 15
FXR	Farnesoid X receptor
GS	Guggulsterone
H&E	Haematoxylin & eosin
Mrp2	Multidrug resistance-related protein 2
Ntcp	Na+/taurocholate cotransporting polypeptide
PXR	Pregnane X receptor
Shp	Small heterodimer partner
Sult2a1	Hydroxysteroid sulfotransferase 2a1
Ugt1a1	UDP-glucuronosyltransferase 1a1

INTRODUCTION

Estrogen–induced cholestasis is one of the most common and devastating manifestations in many conditions of susceptible women, such as pregnancy, administration of oral contraceptives, or postmenopausal hormone replacement therapy (1–3). Since therapeutic drug for treating estrogen–induced cholestasis are limited, there is an urgent medical need to develop drugs that can protect against cholestatic liver injury. 17α-ethinylestradiol (EE) is a hepatotoxicant widely used in rodents to examine molecular mechanisms involved in estrogen-induced cholestasis.

Decreases in bile flow and bile acid synthesis induced by EE, have been demonstrated to be related with the changes in transporters and enzymes involved in bile acid homeostasis (4). Therefore, appropriate regulation of hepatobiliary transporters and enzymes has provided a novel strategy to treat cholestatic disorders.

Bile acid homeostasis has been shown to be tightly regulated by nuclear receptors and their target genes. Farnesoid X receptor (FXR), a member of the nuclear receptor superfamily of intracellular ligand-activated transcription factors, is highly expressed in the liver, intestine, kidney and adrenals (5, 6). FXR has been reported to play a critical role in bile acid homeostasis (7–9). FXR activation transiently induces the expression of small heterodimer partner (Shp) and fibroblast growth factor 15 (Fgf15) in mice, and elevated levels of Shp and Fgf15 in turn lead to transcriptional repression of genes involved in bile acid synthesis (10, 11). In addition, FXR can down-regulate Na^+/taurocholate cotransporting polypeptide (Ntcp), which prevents bile acids uptake into hepatocytes, and induce expression of bile salt export pump (Bsep), which increases bile acid efflux from the liver into bile. Therefore, FXR regulates synthesis and transport of bile acid and protects against bile acid accumulation-induced hepatic toxicity.

Alisol B 23-acetate (AB23A), is a triterpenoid that exists naturally in medicinal plants. Its chemical structure is shown in Fig. 1. Many pharmacological studies have revealed that AB23A has several pharmacological activities, such as anti-hepatitis virus, anti-proliferative activity of cancer cell lines and antibacterial effects (12–14). Recently, we have demonstrated that AB23A has promotive effect on liver regeneration and protective effect through FXR activation in mice (15, 16). Therefore, an intriguing and important question arises whether AB23A has hepatoprotective effect on cholestatic liver injury such as EE-induced hepatic toxicity and cholestasis. Another further question is that whether FXR and its target genes contribute to its hepatoprotection if AB23A possesses protective effect against EE-induced cholestatic liver injury.

In the present study, we aimed to investigate the hepatoprotective effects of AB23A on EE-induced hepatotoxicity and cholestasis in mice, and further to explore the potential mechanisms *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials

AB23A (purity > 98%) was purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China). EE, chenodeoxycholic acid (CDCA), pregnenolone 16α-carbonitrile (PCN), 1,4-Bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and guggulsterone (GS) were purchased from Sigma-Aldrich (St. Louis, MO). All biochemical indicators kits and other chemicals were commercially available.

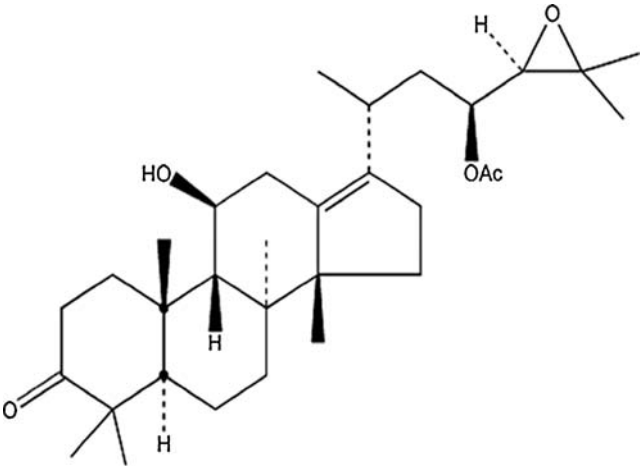

Fig. 1 The chemical structure of AB23A.

Animals and Treatments

All animal maintenance and treatment protocols were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China. Male C57BL/6 mice (8–9 weeks) were housed in laboratory animal facilities under a 12-h light/dark cycle with access to standard chow and water *ad libitum*. AB23A (7.5, 15 or 30 mg/kg) or vehicle (10% hydroxypropyl-beta-cyclodextrin in 500 mM phosphate pH 7.0) alone was treated to mice by oral gavage once daily for 7 days. Since the 3rd day, 4 h after AB23A or vehicle treatment, mice received subcutaneous injections of EE (10 mg/kg) or vehicle (80% 1, 2-propanediol with 0.15% NaCl) once daily for 5 successive days. GS was dissolved in 100 mM DMSO and diluted in methylcellulose 1% as previously described (17). The mice were injected intraperitoneally with 10 mg/kg of GS 4 h before vehicle or AB23A administration every time. GS administration time is 7 days, which is same to duration time of AB23A administration. On the 7th day, 4 h after EE or vehicle administration, mice were sacrificed. Blood, liver and intestine were collected.

Serum Biochemistry and Biliary Bile Acids Analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (γ-GTP), total bilirubin and biliary total bile acids were analyzed using commercial kits according to the manufacturer's protocols.

Histopathology

Liver fragments were fixed in 10% neutral buffered formalin, embedded in paraffin, sliced for 5 μm, stained with H&E using standard protocols and examined microscopically for structural changes.

Surgery for Bile Collection and Bile Flow Measurements

On the 7th day, surgery for bile collection was performed between 9:00 am and 11:00 am to minimize influence of circadian variations. Animals were anesthetized with a single dose of pentobarbital (50 mg/kg body weight) and maintained under this condition throughout the experiments. The gallbladder of mice was cannulated with PE-10 polyethylene tubing (Becton, DicKinson and Co., Franklin Lakes, NJ). The bile was collected every 20 min for 1 h when mice were kept under anesthesia at 37°C. Bile volume was determined gravimetrically with the density of 1.0 g/mL.

Mice Primary Hepatocytes Culture

Two-step collagenase digestion method was used to isolate hepatocytes from C57BL/6 mice as described previously (18). The isolated hepatocytes were cultured with the william's E medium containing 10% heat-inactivated fetal bovine serum, 0.1 μM dexamethasone, 1× insulin-transferrin-selenium-sodium pyruvate solution and 1× glutamine, and incubated for 4 h. Then hepatocytes were incubated with the fresh medium and were cultured for 10 h to a density of 6×10^5 cells per dish.

RNA Silencing Experiment

Twelve hours later, mice primary cultured hepatocytes were transiently transfected 200 nM siRNA targeting at mouse FXR (siGENOME SMARTpool, Dharmacon) or negative control siRNA using lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) and 5 μM AB23A was added to the culture medium for 48 h. After that, the cells were harvested for quantitative real-time PCR.

Quantitative Real-Time PCR

Total RNAs from mouse hepatic, intestinal tissue or mice primary hepatocytes were extracted by RNAiso Plus reagent (TaKaRa Biotech, Dalian, China) according to the manufacturer's instructions. Total RNAs (1 μg) was reverse-transcribed to cDNA using PrimeScript RT reagent kit (TaKaRa Biotech, Dalian, China). The levels of mRNA expression were quantified using SYBR Green PCR Master Mix and an ABI prim 7500 Sequence Detection System (Applied Biosystems, USA). The quantity of mRNA was normalized with an internal standard mouse β-actin. The sequence of the primers in mice is shown in Table I.

Protein Isolation and Western Blot

Liver tissues were homogenized in protein lysis buffer containing 1 mM PMSF. Fifty μg of total protein were resolved with 8–12% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline, membranes were incubated overnight with primary antibodies, including Bsep (H-180), Mrp2 (H-17) and Ntcp (M-130) (Santa Cruz Biotechnology, Santa Cruz, CA). Specific bands were detected by an enhanced chemiluminescence (ECL) method using Bio-Spectrum Gel Imaging System (UVP, USA).

Statistical Analysis

Data are expressed as means ± S.D. Statistical analysis between two groups were performed by a Student's *t* test and multiple comparisons were performed by a one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

AB23A Protects Against Hepatotoxicity and Cholestasis Induced by EE

Serum ALP and total bile acids, the biochemical indicators of hepatotoxicity and cholestasis, in mice orally administered vehicle, 7.5, 15 or 30 mg/kg of AB23A respectively, indicated significant difference. These two indicators were increased in vehicle-treated EE mice and were significantly reduced by AB23A treatment in a dose-dependent manner (Fig. 2a–b). CDCA is a known FXR agonist used as a positive control drug. Other biochemical indicators of liver function such as serum ALT, AST, γ-GTP and total bilirubin were not significantly changed by EE (data not shown), which is consistent with previous report (19). To further investigate the protective effect of AB23A, the effect of different doses of AB23A on bile flow and biliary bile acid output was determined. The bile flow rate was decreased in mice with EE administration, however, the EE-suppressed bile flow was significantly ameliorated in AB23A-treated mice (Fig. 2c). Besides, AB23A orally administration dose-dependently reversed the EE-induced the decrease in biliary bile acid output (Fig. 2d). Since the best protective effect was observed in the high-dose group, 30 mg/kg of AB23A was used for the subsequent studies. The histological assessments further indicated hepatotoxicity

Table I The Primer Sequences Used for Real-Time PCR Assay in Mice

Gene	GenBank accession	Forward primer (5'-3')	Reverse primer (5'-3')
Ntcp	U95132.1	GCATGATGCCACTCCTCTTATAC	TACATAGTGTGGCCTTTTGGACT
Bsep	NM_021022.3	AGCAGGCTCAGCTGCATGAC	AATGGCCCGAGCAATAGCAA
Mrp2	NM_013806.2	AACTGCCTCTTCAGAATCTTA	GCCAGCCACGGAACCAGCTGCT
Cyp7a1	NM_007824.2	CAAGAACCTGTACATGAGGGAC	CACTTCTTCAGAGGCTGCTTTC
Cyp8b1	NM_010012.3	CCCCTATCTCTCAGTACACATGG	GACCATAAGGAGGACAAAGGTCT
Shp	NM_011850.2	GTCTTTCTGGAGCCTTGAGCTG	GTAGAGGCCATGAGGAGGATTC
Fgf15	NM_008003.2	GAGGACCAAAACGAACGAAATT	ACGTCCTTGATGGCAATCG
Cyp3a11	NM_007818.3	CCACCAGTAGCACACTTTCC	TTCCATCTCCATCACAGTATCA
Cyp2b10	NM_009999.4	CAATGGGGAACGTTGGAAGA	TGATGCACTGGAAGAGGAAC
Sult2a1	NM_001111296.2	GGAAGGACCACGACTCATAAC	GATTCTTCACAAGGTTTGTGTTACC
Ugt1a1	NM_201645.2	TCTGAGCCCTGCATCTATCTG	CCCCAGAGGCGTTGACATA
β-actin	NM_007393.3	TATTGGCAACGAGCGGTTC	ATGCCACAGGATTCCATACCC

induced by EE. H&E stained liver sections showed a large number of inflammatory cells infiltration in vehicle-treated EE mice. In comparison, the scope of inflammatory cells infiltration was reduced by AB23A and CDCA treatment (Fig. 2e). Taken together, these results suggested that AB23A can provide remarkable protection against hepatotoxicity and cholestasis induced by EE in mice.

AB23A Alters Gene and Protein Expression of Hepatic Transporters Involved in Bile Acid Transport

To elucidate the mechanism underlying alleviated EE-induced hepatotoxicity and cholestasis in AB23A-treated mice, we examined the expression of hepatic key genes which are involved in bile acid homeostasis. Firstly, we determined expression of the canalicular efflux transporter Bsep and multidrug resistance-related protein 2 (Mrp2), both of which are responsible for transporting hepatic bile acid into bile and constitute the limiting step of bile acid efflux. Bsep and Mrp2 expression levels were reduced by 59 and 27% respectively by EE. The decreases in expression of Bsep and Mrp2 lead to the decreases in bile flow and biliary bile acid output in mice after EE administration. AB23A treatment increased Bsep and Mrp2 gene expression, which resulted in ameliorated cholestasis (Fig. 3a–b). Next we determined the expression of basolateral uptake transporter Ntcp which is involved in bile acid uptake into hepatocytes in mice. Fig. 3c illustrated that EE markedly decreased the expression of Ntcp and AB23A treatment further enhanced the down-regulation of Ntcp. To confirm the quantitative real-time PCR results regarding changes in transporter gene, we further determined the protein levels using Western blotting analysis. As shown in Fig. 3d, AB23A treatment caused increases in Bsep, Mrp2 and a decrease in Ntcp protein levels. Together, the above results suggested that hepatoprotection of AB23A against EE was due to up-regulation of Bsep, Mrp2 and down-regulation of Ntcp, resulting in an increase in efflux and a decrease in influx of bile acid in liver.

AB23A Decreases Expression of Bile Acid Synthetic Enzymes

Besides the above transporters, bile acid synthetic enzymes are also involved in bile acid homeostasis. To further elucidate the mechanism of hepatoprotective effect of AB23A, we determined expression levels of enzymes in bile acid synthesis. A decrease in the gene expression of cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting enzyme in bile acid synthesis, was observed in vehicle-treated EE mice. Sterol-12α-hydroxylase (Cyp8b1), another bile acid synthetic enzyme, was also decreased by EE (Fig. 4a). The alterations in bile acid synthetic enzymes may be adaptive regulation to limit hepatotoxicity induced by EE. Cyp7a1 and Cyp8b1 gene expression were further reduced by AB23A treatment. We further determined the expression of hepatic Shp and intestinal Fgf15, which are essential for regulating bile acid biosynthesis by repressing Cyp7a1 and Cyp8b1. Shp and Fgf15 expression were increased by EE and was further induced by AB23A treatment (Fig. 4b). Taken together, these results indicated that AB23A through Shp and Fgf15 induction repressed Cyp7a1 and Cyp8b1 expression, resulting in a decrease in bile acid synthesis.

AB23A Increases Expression of Enzyme Involved in Bile Acid Metabolism

Detoxification of bile acid in mice was mainly mediated through phase I enzymes such as Cyp3a11 and Cyp2b10, and phase II enzymes such as hydroxysteroid sulfotransferase 2a1 (Sult2a1) and UDP-glucuronosyltransferase 1a1

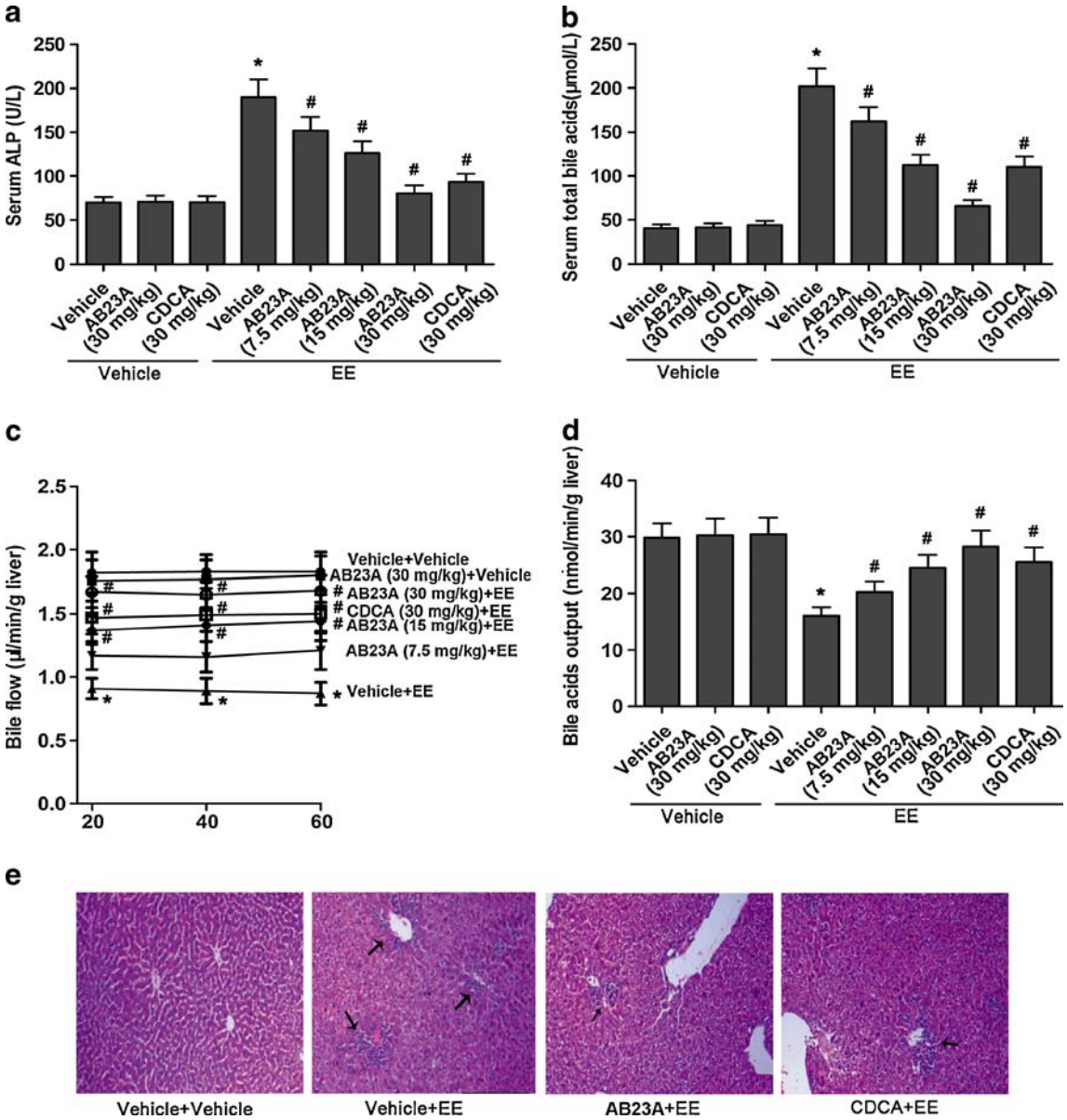

Fig. 2 Hepatoprotection of AB23A against EE-induced hepatotoxity and cholestasis. Serums ALP (**a**) and total bile acids (**b**) levels elevated by EE were significantly reduced by treatment with different doses of AB23A. The bile flow rate (**c**) was decreased in mice over 60 min by EE, and was significantly ameliorated in AB23A-treated mice. Besides, EE decreased biliary bile acid output (**d**) and AB23A orally administration significantly reversed the EE-induced the decrease in biliary bile acid output. (**e**) The images of representative H&E stained liver sections (100 ×magnification) were shown. Areas of inflammatory cells infiltration were marked by *arrows*. Data are the mean ± S.D. ($n = 10$). *$p < 0.05$ *versus* Vehicle + Vehicle; #$p < 0.05$ *versus* Vehicle + EE.

(Ugt1a1). PCN and TCPOBOP, a PXR or CAR agonist respectively, are used as positive control drugs. Sult2a1 has been shown to be a downstream target gene of FXR. As illustrated in Fig. 5, EE induced the mRNA expression of Cyp3a11 and Sult2a1 by 37 and 42% respectively, whereas the mRNA levels of Cyp2b10 and Ugt1a1 were not changed. In comparison, the Cyp3a11, Cyp2b10 and Ugt1a1 mRNA levels were not altered, while Sult2a1 was increased in AB23A-treated EE mice. These findings suggested that AB23A can promote bile acid metabolism through inducing Sult2a1 expression.

The Regulation of Gene Involved in Bile Acid Homeostasis is Abrogated by FXR Antagonist GS

Since Bsep, Mrp2, Ntcp and genes including Cyp7a1, Cyp8b1 involved in bile acid synthesis are downstream target genes of FXR, as well as AB23A has been shown to be an exogenous activator of FXR (15, 16), we hypothesized that AB23A may activate FXR to regulate gene expression in bile acid homeostasis. To verify this hypothesis, we blocked FXR by use of the FXR antagonist guggulsterone (GS) in mice. GS decreased the gene expression of Bsep and Shp, which are the

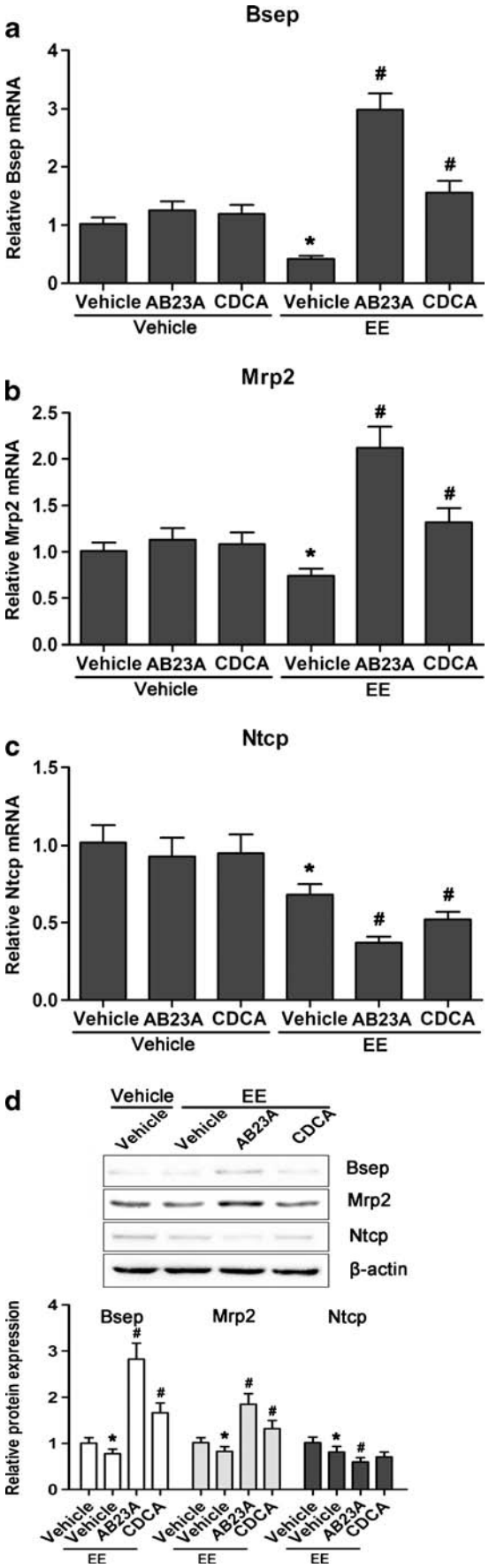

Fig. 3 AB23A alters gene and protein expression of hepatic transporters involved in bile acid transport in EE-induced cholestatic mice. Quantitative real-time PCR analysis was performed to measure the gene expression of (**a**) Bsep, (**b**) Mrp2 and (**c**) Ntcp. (**d**) Western blot analysis was used to measure Bsep, Mrp2 and Ntcp protein expression. Specific band intensity was quantified, normalized to β-actin. Data are the mean ± S.D. ($n = 10$). *$p < 0.05$ *versus* Vehicle + Vehicle; #$p < 0.05$ *versus* Vehicle + EE.

classical FXR direct target genes, in AB23A-treated EE mice (Fig. 6a). Under these conditions, the increase in Mrp2 mRNA and the decreases in Ntcp, Cyp7a1 and Cyp8b1 gene were abrogated by GS administration (Fig. 6a–b). And GS also reduced the hepatoprotective effect of AB23A (Fig. 6c). Taken together, these results clearly demonstrated that AB23A protected against EE-induced liver injury through activating FXR in mice.

The Regulation of FXR Target Gene Expression by AB23A is Abrogated by FXR Gene Silencing *In Vitro*

In *in vivo* experiments, the effects of AB23A on the expression of FXR target genes had been measured; however, the changes may not be enough to represent the effect of AB23A on FXR activation. Thus, the effect of AB23A on FXR activation was subsequently examined using mice primary cultured hepatocytes by FXR gene silencing experiment *in vitro*. As shown in Fig. 7a, the FXR expression was decreased after specific siRNA targeting FXR mRNA transfection, which was ensured by Western blot analysis. *In vitro* evidences demonstrated that the changes in Bsep, Mrp2 and Cyp7a1 induced by AB23A were abrogated by FXR silencing (Fig. 7b). These results further demonstrated the involvement of FXR activation in the hepatoprotective effect of AB23A.

DISCUSSION

Cholestasis results in systemic and hepatic retention of potentially toxic bile acid that causes liver injury, ultimately leading to biliary fibrosis and cirrhosis (20, 21). Estrogens are well known to cause cholestasis in susceptible women during pregnancy, administration of oral contraceptives and postmenopausal replacement therapy (22, 23). In the present study, we demonstrated that AB23A had at least three roles in protection against EE-induced cholestatic liver injury. The first role is to increase hepatic efflux and decrease uptake of bile acid through an induction in efflux transporters (Bsep and Mrp2) and an inhibition in hepatic uptake transporter (Ntcp) expression. The second role is to reduce hepatic bile acid synthesis through repressing bile acid synthetic enzymes Cyp7a1 and Cyp8b1. The third role is to increase bile acid metabolism through an induction in gene expression of Sult2a1. We also clarified that the hepatoprotective effect of AB23A against

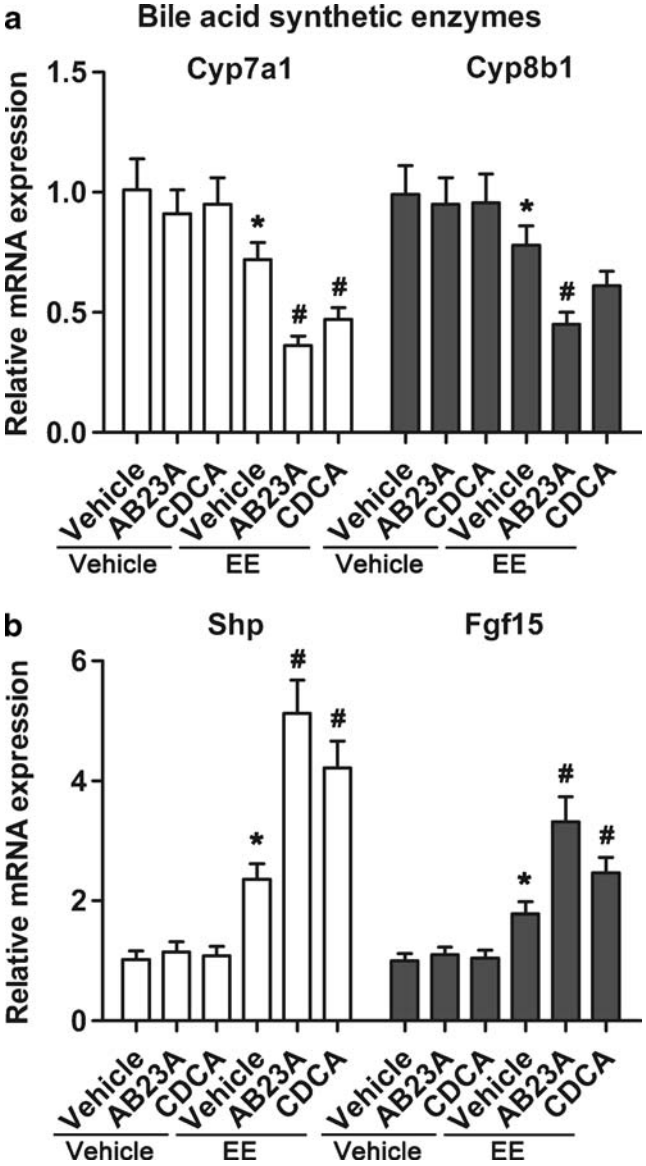

Fig. 4 Effects of AB23A on gene expression of hepatic enzymes involved in bile acid synthesis in cholestatic mice induced by EE. The gene expression levels of bile acid synthetic enzymes Cyp7a1, Cyp8b1 (**a**) and their upstream genes Shp, Fgf15 (**b**) were shown. Data are the mean ± S.D. ($n = 10$). *$p < 0.05$ *versus* Vehicle + Vehicle; #$p < 0.05$ *versus* Vehicle + EE.

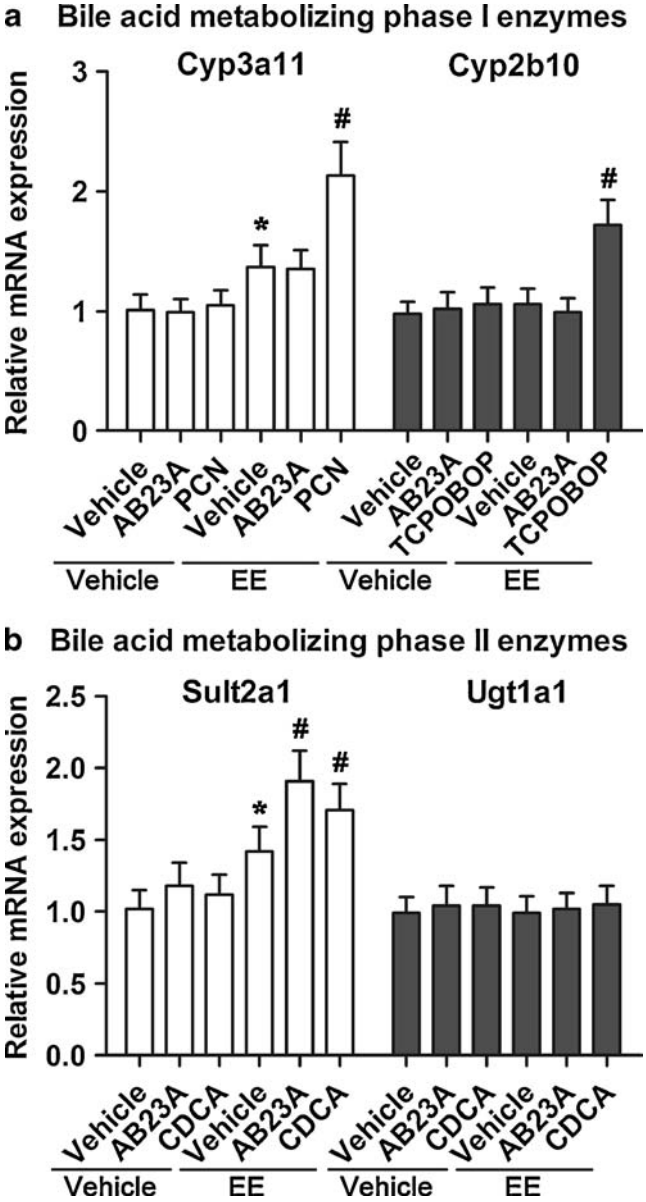

Fig. 5 Effects of AB23A on gene expression of hepatic enzymes involved in bile acid metabolism in cholestatic mice induced by EE. Quantitative real-time PCR analysis was performed to measure the gene expression of metabolic enzymes including phase I enzymes Cyp3a11 and Cyp2b10 (**a**), and phase II enzymes Sult2a1 and Ugt1a1 (**b**). Data are the mean ± S.D. ($n = 10$). *$p < 0.05$ *versus* Vehicle + Vehicle; #$p < 0.05$ *versus* Vehicle + EE.

EE-induced cholestatic liver injury was due to FXR-mediated regulation of above genes.

EE is widely used to cause experimental cholestasis in rodents to examine molecular mechanisms involved in estrogen-induced cholestasis. The present study clearly demonstrated that AB23A had the potential to protect against EE-induced cholestatic liver injury in a dose-dependent manner, as evidenced by the ameliorative liver histology and the significant decreases in serum ALP and total bile acids, as well as the increases in bile flow and biliary bile acid output (Fig. 2). Particularly worth mentioning is that EE-induced cholestatic liver injury has been shown to be related with the decreases in bile acid efflux in hepatocytes which leads to decreases in bile flow and biliary bile acid output (4, 24). Therefore, in the present study, we focused on the effects of AB23A on bile acid transport, as well as bile acid synthesis and metabolism (or detoxification).

A variety of transporters and enzymes have been demonstrated to play crucial roles in hepatic bile acid homeostasis (25–27). The ATP-binding cassette (ABC) transporters including Bsep and Mrp2 are responsible for transporting bile acid and other organ anions including bilirubin across the canalicular membrane of hepatocytes into bile in mice (28, 29). And

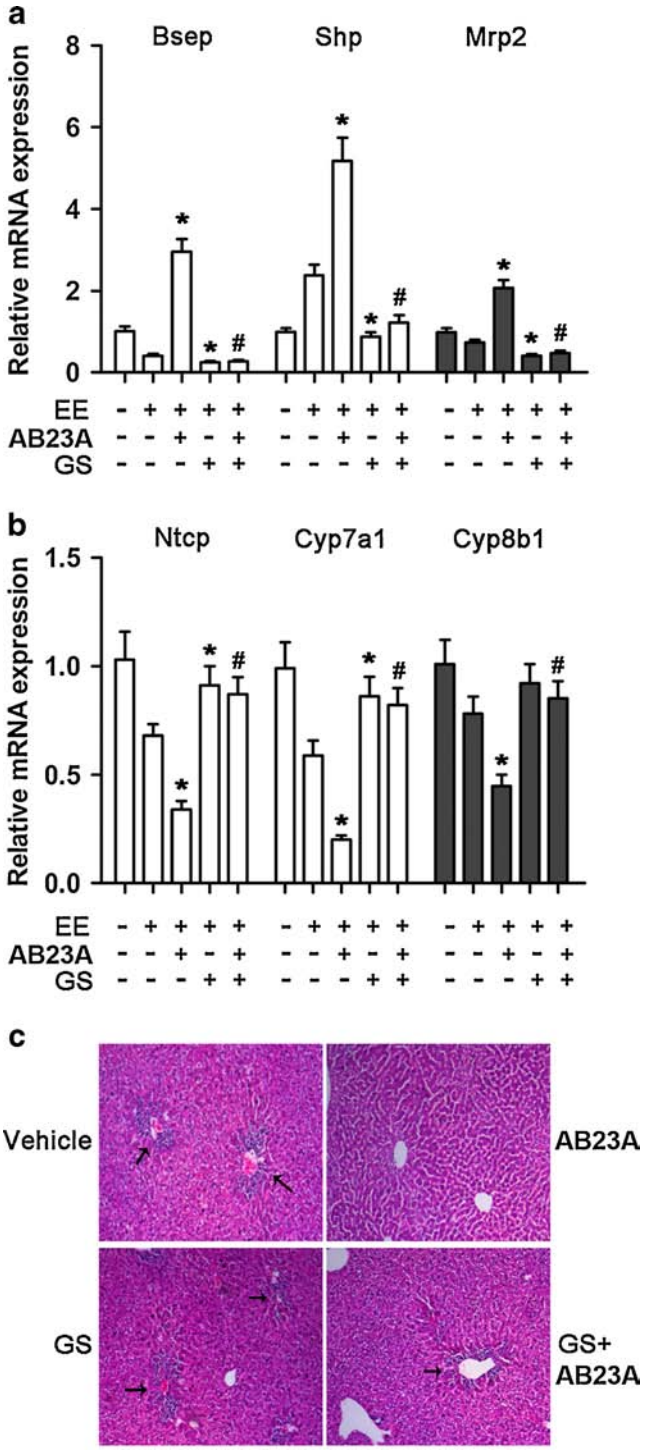

Fig. 6 Effects of AB23A on the regulation of genes involved in bile acid homeostasis is abrogated by FXR antagonist GS. (**a**) The hepatic expression of Bsep, Shp and Mrp2 in mice with vehicle, AB23A, FXR antagonist GS or GS + AB23A. The changes in expression of (**b**) Ntcp, Cyp7a1 and Cyp8b1 in AB23A-treated mice were abrogated by GS. Data are the mean ± S.D. ($n = 5$). *$p < 0.05$ *versus* EE only; #$p < 0.05$ *versus* EE + AB23A. (**c**) The images of representative H&E stained liver sections (100 ×magnification) after GS administration were shown. Areas of inflammatory cells infiltration were marked by *arrows*.

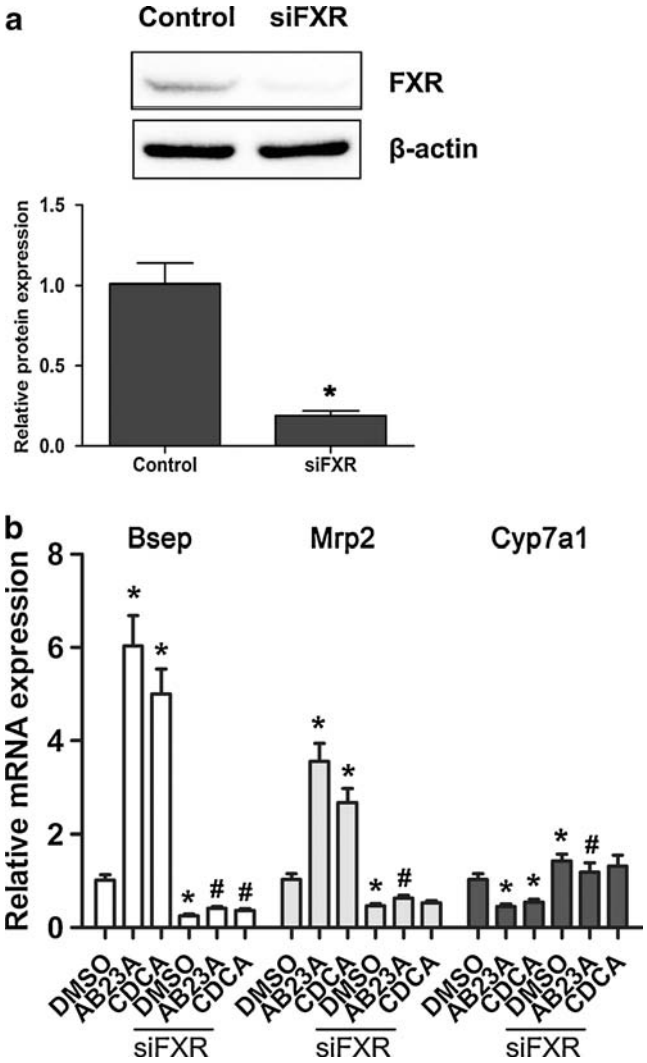

Fig. 7 *In vitro* evidences on FXR activation by AB23A. (**a**) FXR silencing efficiency was measured by Western blot. (**b**) FXR silencing abrogated the regulation of Bsep, Mrp2 and Cyp7a1 by AB23A in mice primary hepatocytes. Quantitative real-time PCR analysis was performed to measure the gene expression. Data are expressed as mean ± S.D. ($n = 5$). *$p < 0.05$ *versus* DMSO alone; #$p < 0.05$ *versus* AB23A alone.

this process constitutes the rate-limiting step in hepatic bile acid excretion. In the current study, EE reduced Bsep and Mrp2 expression, resulting in the decreases in bile flow and biliary bile acid output. In addition, hepatic uptake of bile acids takes place at the basolateral membrane of hepatocytes and is mediated through Ntcp in mice (30). Ntcp mediates Na^+-dependent uptake of all physiological bile acid in their conjugated form. In this study, Ntcp were greatly inhibited by EE to defense against excessive bile acids entering hepatocytes. Through activating FXR, AB23A increased expression of bile acid export transporter Bsep which is a FXR direct downstream gene and Mrp2, another FXR downstream gene, and decreased hepatic uptake transporter Ntcp expression (Fig. 3). Besides transporters, bile acid synthetic enzymes including Cyp7a1, Cyp8b1 and bile acid metabolizing enzymes including Cyp3a11, Cyp2b10, Sult2a1 and Ugt1a1 also play important roles in bile acid homeostasis. AB23A treatment

reduced FXR downstream target genes Cyp7a1 and Cyp8b1 expression via FXR-Shp and FXR-Fgf15 axis, leading to suppressing bile acid synthesis. AB23A treatment further increased Sult2a1 gene expression, while had no effects on gene expression of Cyp3a11, Cyp2b10 and Ugt1a1 (Fig. 4 and 5). In our previous study, AB23A treatment was found to reduce Ntcp, Cyp7a1 and Cyp8b1 expression, while increasing Bsep and Mrp2 expression in alpha-naphthylisothiocyanate (ANIT)-induced hepatotoxity model (16). The effects of AB23A on the expression of genes involved in bile acid homeostasis in EE-induced cholestatic liver injury are similar to the effects of AB23A in ANIT-induced hepatotoxity and cholestasis in mice. This may be due to the fact that the effects of AB23A on the regulation of gene expression are related with FXR activation.

FXR has been proved to be one of the most important upstream nuclear receptors. It has been demonstrated that Bsep and Shp are direct target genes of FXR and could be induced by FXR in rodent livers (31, 32). Shp has been proved to be the upstream gene of Ntcp, Cyp7a1 and Cyp8b1, and FXR through inducing Shp, suppresses expression of Ntcp, Cyp7a1 and Cyp8b1 (33). Fgf15 is an intestinal hormone that travels to liver where it interacts with its receptor fibroblast growth factor receptor 4 (Fgfr4) to suppress bile acid synthesis. Fgf15 is also a FXR target gene and FXR through inducing Fgf15, suppresses expression of Cyp7a1 and Cyp8b1. Most importantly, EE was proved to orchestrate adaptive response by activating FXR, indicating that as the key regulatory transcription factor for bile acid, FXR may become the focus of targeted therapies in cholestasis (34). We have shown that AB23A is an exogenous activator of FXR in the previous studies (15, 16). In the present study, we used FXR antagonist GS in mice to verify that AB23A activate FXR to regulate expression level of genes in bile acid homeostasis. The changes in hepatic gene expression of transporters and enzymes, as well as ameliorative liver histology in AB23A-treated mice were abrogated by GS (Fig. 6). To further demonstrate whether FXR activation is involved in the hepatoprotective effect of AB23A, FXR gene silencing experiment was performed using mice primary cultured hepatocytes *in vitro*. *In vitro* evidences demonstrated that the significant regulation of Bsep, Mrp2 and Cyp7a1 by AB23A were abrogated by FXR silencing (Fig. 7). The ovariectomy has some disadvantageous effects on somatic function including metabolic disturbance in bone, glucose and lipid, *etc*. Also, ovariectomy can lead to disorders in immune system and the decrease in bone mineral density. The metabolic disorders may influence the expression of transporters and enzymes involved in bile acid homeostasis. In addition, endogenous estrogens have been shown to affect the expression of transporters and enzymes *in vivo*. There is no effect of estrogens in male mice. Therefore, we chose male mice as the investigated subject in the present study.

The FXR-target gene expression levels were elevated by AB23A treatment in hepatocytes (Fig. 7), but not *in vivo* (Figs. 3 and 4). This may be due to the fact that the effect of AB23A on expression levels of FXR-target genes *in vivo* is influenced by a variety of factors in the body, such as first-pass effect. When AB23A with oral gavage firstly pass the intestine and liver, some portion of AB23A was metabolized by the intestinal or hepatic enzymes, leading to the decreased AB23A entering into the blood circulation. Therefore, the effect of AB23A on regulation of FXR-target gene expression was reduced *in vivo*. However, the effect of AB23A in mice primary cultured hepatocytes is not influenced by other organs in the body. Therefore, AB23A treatment can affect the FXR-target gene expression levels in mice primary cultured hepatocytes, but not *in vivo*.

In addition to FXR, pregnane X receptor (PXR) and constitutive androstane receptor (CAR) which are two other nuclear receptors, have been shown to also play important roles in regulating transporters and enzymes of bile acid (35). However, AB23A had no effects on gene expression of Cyp3a11 which is a PXR target gene, and Cyp2b10 which is a CAR target gene in mice, suggesting that the hepatoprotection of AB23A is not through PXR and CAR activation.

To preferably observe the hepatoprotective effect of AB23A, we selected three doses (7.5, 15 and 30 mg/kg) by repeated administration for 7 consecutive days before mice sacrifice. The dose of 30 mg/kg is the optimal dose of AB23A that can play roles in protecting against EE-induced liver injury in mice. This result may support the potential therapeutic use of AB23A. And 10 mg/kg of GS is the minimal dose we observed which can efficiently hold AB23A back from FXR activation in mice.

Despite a large number of basic studies searching for novel therapeutic agents to protect against cholestastic liver injury, few options are currently available for clinical use. Our study suggests the possibility that AB23A may be an effective pharmacological strategy to protect against cholestasis and improve the prognosis of many patients after cholestasis.

CONCLUSION

AB23A protects against EE-induced cholestatic liver injury via activating FXR signaling pathway, resulting in increases in bile flow through increasing hepatic efflux and metabolism of bile acids, and decreasing hepatic uptake and synthesis of bile acid.

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REFERENCES

1. Williamson C, Miragoli M, Sheikh Abdul Kadir S, Abu-Hayyeh S, Papacleovoulou G, Geenes V, *et al.* Bile acid signaling in fetal tissues: implications for intrahepatic cholestasis of pregnancy. Dig Dis. 2011;29:58–61.
2. Paulen ME, Folger SG, Curtis KM, Jamieson DJ. Contraceptive use among solid organ transplant patients: a systematic review. Contraception. 2010;82:102–12.
3. Vihma V, Ropponen A, Aittomaki K, Ylikorkala O, Tikkanen MJ. Postmenopausal estrogen therapy and serum estradiol fatty acid esters in women with and without previous intrahepatic cholestasis of pregnancy. Ann Med. 2004;36:393–9.
4. Henriquez-Hernandez LA, Flores-Morales A, Santana-Farre R, Axelson M, Nilsson P, Norstedt G, *et al.* Role of pituitary hormones on 17alpha-ethinylestradiol-induced cholestasis in rat. J Pharmacol Exp Ther. 2007;320:695–705.
5. Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, *et al.* Identification of a nuclear receptor that is activated by farnesol metabolites. Cell. 1995;81:687–93.
6. Wang YD, Chen WD, Moore DD, Huang W. FXR: a metabolic regulator and cell protector. Cell Res. 2008;18:1087–95.
7. Matsubara T, Li F, Gonzalez FJ. FXR signaling in the enterohepatic system. Mol Cell Endocrinol. 2013;368:17–29.
8. Modica S, Petruzzelli M, Bellafante E, Murzilli S, Salvatore L, Celli N, *et al.* Selective activation of nuclear bile acid receptor FXR in the intestine protects mice against cholestasis. Gastroenterology. 2012;142:355–65.
9. Fiorucci S, Mencarelli A, Distrutti E, Palladino G, Cipriani S. Targetting farnesoid-X-receptor: from medicinal chemistry to disease treatment. Curr Med Chem. 2010;17:139–59.
10. Kong B, Wang L, Chiang JY, Zhang Y, Klaassen CD, Guo GL. Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. Hepatology. 2012;56:1034–43.
11. Wooton-Kee CR, Coy DJ, Athippozhy AT, Zhao T, Jones BR, Vore M. Mechanisms for increased expression of cholesterol 7alpha-hydroxylase (Cyp7a1) in lactating rats. Hepatology. 2010;51:277–85.
12. Jiang ZY, Zhang XM, Zhang FX, Liu N, Zhao F, Zhou J, *et al.* A new triterpene and anti-hepatitis B virus active compounds from Alisma orientalis. Planta Med. 2006;72:951–4.
13. Xu YH, Zhao LJ, Li Y. Alisol B acetate induces apoptosis of SGC7901 cells via mitochondrial and phosphatidylinositol 3-kinases/Akt signaling pathways. World J Gastroenterol. 2009;15:2870–7.
14. Jin HG, Jin Q, Ryun Kim A, Choi H, Lee JH, Kim YS, *et al.* A new triterpenoid from Alisma orientale and their antibacterial effect. Arch Pharm Res. 2012;35:1919–26.
15. Meng Q, Chen X, Wang C, Liu Q, Sun H, Sun P, *et al.* Alisol B 23-acetate promotes liver regeneration in mice after partial hepatectomy via activating farnesoid X receptor. Biochem Pharmacol. 2014;92:289–98.
16. Meng Q, Chen X, Wang C, Liu Q, Sun H, Sun P, *et al.* Alisol B 23-acetate protects against ANIT-induced hepatotoxity and cholestasis, due to FXR-mediated regulation of transporters and enzymes involved in bile acids homeostasis. Toxicol Appl Pharmacol. 2015;283:178–86.
17. Mencarelli A, Renga B, Palladino G, Distrutti E, Fiorucci S. The plant sterol guggulsterone attenuates inflammation and immune dysfunction in murine models of inflammatory bowel disease. Biochem Pharmacol. 2009;78:1214–23.
18. Klaunig JE, Goldblatt PJ, Hinton DE, Lipsky MM, Chacko J, Trump BF. Mouse liver cell culture. I. Hepatocyte isolation. In Vitro. 1981;17:913–25.
19. Sanchez Pozzi EJ, Crocenzi FA, Pellegrino JM, Catania VA, Luquita MG, Roma MG, *et al.* Ursodeoxycholate reduces ethinylestradiol glucuronidation in the rat: role in prevention of estrogen-induced cholestasis. J Pharmacol Exp Ther. 2003;306:279–86.
20. Corpechot C. Primary biliary cirrhosis and bile acids. Clin Res Hepatol Gastroenterol. 2012;36:70016–5.
21. Wagner M, Zollner G, Trauner M. Nuclear receptors in liver disease. Hepatology. 2011;53:1023–34.
22. Chen J, Zhao KN, Liu GB. Estrogen-induced cholestasis: pathogenesis and therapeuticimplications. Hepatogastroenterology. 2013;60:1289–96.
23. Boaglio AC, Zucchetti AE, Sanchez Pozzi EJ, Pellegrino JM, Ochoa JE, Mottino AD, *et al.* Phosphoinositide 3-kinase/protein kinase B signaling pathway is involved in estradiol 17beta-D-glucuronide-induced cholestasis: complementarity with classical protein kinase C. Hepatology. 2010;52:1465–76.
24. Ruiz ML, Villanueva SS, Luquita MG, Ikushiro S, Mottino AD, Catania VA. Beneficial effect of spironolactone administration on ethynylestradiol-induced cholestasis in the rat: involvement of up-regulation of multidrug resistance-associated protein 2. Drug Metab Dispos. 2007;35:2060–6.
25. Alrefaiand WA, Gill RK. Bile acid transporters: structure, function, regulation and pathophysiological implications. Pharm Res. 2007;24:1803–23.
26. Huang W, Ma K, Zhang J, Qatanani M, Cuvillier J, Liu J, *et al.* Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. Science. 2006;312:233–6.
27. Yang H, Ramani K, Xia M, Ko KS, Li TW, Oh P, *et al.* Dysregulation of glutathione synthesis during cholestasis in mice: molecular mechanisms and therapeutic implications. Hepatology. 2009;49:1982–91.
28. Nicolaou M, Andress EJ, Zolnerciks JK, Dixon PH, Williamson C, Linton KJ. Canalicular ABC transporters and liver disease. J Pathol. 2012;226:300–15.
29. Blazquez AG, Briz O, Romero MR, Rosales R, Monte MJ, Vaquero J, *et al.* Characterization of the role of ABCG2 as a bile acid transporter in liver and placenta. Mol Pharmacol. 2012;81:273–83.
30. Gundala S, Wells LD, Milliano MT, Talkad V, Luxon BA, Neuschwander-Tetri BA. The hepatocellular bile acid transporter Ntcp facilitates uptake of the lethal mushroom toxin alpha-amanitin. Arch Toxicol. 2004;78:68–73.

31. Herraez E, Gonzalez-Sanchez E, Vaquero J, Romero MR, Serrano MA, Marin JJ, *et al.* Cisplatin-induced chemoresistance in colon cancer cells involves FXR-dependent and FXR-independent up-regulation of ABC proteins. Mol Pharm. 2012;9:2565–76.
32. Fiorucci S, Antonelli E, Rizzo G, Renga B, Mencarelli A, Riccardi L, *et al.* The nuclear receptor SHP mediates inhibition of hepatic stellate cells by FXR and protects against liver fibrosis. Gastroenterology. 2004;127:1497–512.
33. Kir S, Zhang Y, Gerard RD, Kliewer SA, Mangelsdorf DJ. Nuclear receptors HNF4alpha and LRH-1 cooperate in regulating Cyp7a1 in vivo. J Biol Chem. 2012;287:41334–41.
34. Fiorucci S, Clerici C, Antonelli E, Orlandi S, Goodwin B, Sadeghpour BM, *et al.* Protective effects of 6-ethyl chenodeoxycholic acid, a farnesoid X receptor ligand, in estrogen-induced cholestasis. J Pharmacol Exp Ther. 2005;313: 604–12.
35. Staudinger JL, Woody S, Sun M, Cui W. Nuclear-receptor-mediated regulation of drug- and bile-acid-transporter proteins in gut and liver. Drug Metab Rev. 2013;45:48–59.