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18 β -Glycyrrhetic acid protects against alpha-naphthylisothiocyanate-induced cholestasis through activation of the Sirt1/FXR signaling pathway

Shou-yan Wu^{1,2}, Shi-chao Cui^{2,3}, Le Wang^{1,2}, Yi-ting Zhang^{1,2}, Xiao-xia Yan^{1,2}, Heng-lei Lu^{1,2}, Guo-zhen Xing^{1,2}, Jin Ren^{1,2} and Li-kun Gong^{1,2}

Cholestasis is a common feature of liver injury, which manifests as bile acid excretion and/or enterohepatic circulation disorders. However, very few effective therapies exist for cholestasis. Recently, 18 β -Glycyrrhetic acid (18b-GA), a major metabolic component of glycyrrhizin, which is the main ingredient of licorice, was reported to protect against alpha-naphthylisothiocyanate (ANIT)-induced cholestasis. However, its protective mechanism remains unclear. We hypothesized that 18b-GA may stimulate the signaling pathway of bile acid (BA) transportation in hepatocytes, resulting its hepatoprotective effect. According to the results, 18b-GA markedly attenuated ANIT-induced liver injury as indicated the hepatic plasma chemistry index and histopathology examination. In addition, the expression levels of nuclear factors, including Sirt1, FXR and Nrf2, and their target efflux transporters in the liver, which mainly mediate bile acid homeostasis in hepatocytes, significantly increased. Furthermore, we first revealed that 18b-GA treatment significantly activated FXR, and which can be significantly reduced by EX-527 (a potent and selective Sirt1 inhibitor), indicating that 18b-GA activates FXR through Sirt1. Taken together, 18b-GA confers hepatoprotection against ANIT-induced cholestasis by activating FXR through Sirt1, which promotes gene expression of the efflux transporter, and consequently attenuates dysregulation of bile acid homeostasis in hepatocyte compartments.

Keywords: hepatoprotective effect; cholestatic liver injury; 18 β -glycyrrhetic acid (18b-GA); FXR; Sirt1; bile acid transporters

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INTRODUCTION

Cholestasis is clinically associated with a variety of liver diseases, has a very high incidence, and mainly manifests as a bile secretion disorder and excessive bile acid (BA) accumulation in the liver. Without effective intervention, cholestasis will eventually evolve into hepatic fibrosis and cirrhosis [1, 2]. The etiology of cholestasis is more complex, but cholestatic disease due to any cause can lead to the retention of toxic substances, such as BA, leading to liver damage and cholestatic liver disease [3–6]. Currently, obeticholic acid and ursodeoxycholic acid (UDCA) are two FDA-approved therapeutic drugs used as single agents or in combination for primary biliary cholangitis (PBC) treatment in adults, while there are no approved drugs for other cholestatic diseases that are found throughout the world [7, 8]. Therefore, novel therapeutic strategies for the treatment of cholestasis are needed.

Many traditional Chinese medicines have been shown to protect against liver diseases, such as hepatitis, cholestasis and liver fibrosis [9]. Glycyrrhizin, which is the principal triterpene component of licorice root, has been used to treat patients with chronic hepatitis B in China and Japan for many years [10]. 18 β -Glycyrrhetic acid (18b-GA) is a product of glycyrrhizin

metabolism through the intestinal flora [11]. 18b-GA has recently been reported to have anti-inflammatory [12], anticancer [13, 14], and hepatoprotective [15] effects. In addition, recent evidence indicates that 18b-GA exerts a powerful protective effect in multiple liver injury models, including models of exposure to free fatty acids [16], BA [9], a choline-deficient L-amino acid-defined diet [17], and carbon tetrachloride [18]. Although 18b-GA has been reported to protect against alpha-naphthylisothiocyanate (ANIT)-induced hepatotoxicity [10, 19], the mechanism remains unclear.

Interrupting BA metabolism and transportation in hepatocyte compartments is widely believed to play a role in cholestasis. BAs are steroid acids that are mainly synthesized in the liver via cholesterol oxidation and are subsequently secreted to help digest fats [20]. When BA metabolism is impaired, BAs accumulate in the liver at high concentrations, which may cause hepatocyte apoptosis and necrosis [6]. Additionally, abnormal BA transporter and enzyme expression and function may cause BA retention in the liver, ultimately leading to cholestasis [21, 22]. The expression and function of BA transporters and enzymes can be regulated by nuclear receptors and transcription factors. Farnesoid X receptor (FXR) plays important regulatory roles in repressing BA synthetic

¹Center for Drug Safety Evaluation and Research, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 201203 Shanghai, China; ²University of Chinese Academy of Sciences, 100049 Beijing, China and ³State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 201203 Shanghai, China

Correspondence: L.-k. Gong (lkgong@cdser.simm.ac.cn)

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enzymes, inhibiting the hepatic uptake transporter, inducing bile efflux transporters and increasing BA metabolism in the liver [23]. Nuclear factor-E2-related factor-2 (Nrf2), which is known to be mainly involved in the body's antioxidant response [24], also participates in mediating phase II drug metabolism enzymes and the efflux transporters [25]. Recent studies have reported that Sirt1, a nuclear factor family receptor, can regulate the activities of FXR and Nrf2, indicating that Sirt1 is a transcription factor and plays an important role in BA homeostasis [8, 26, 27]. Therefore, we hypothesized that the therapeutic effect of 18b-GA on hepatic cholestasis may be related to its regulatory effect on Sirt1 to affect downstream nuclear receptors, consequently attenuating the imbalance of metabolism and transport of BA.

In this paper, based on an assessment of the protective effect of 18b-GA on ANIT-induced intrahepatic cholestasis, we further systematically studied the effect of 18b-GA on BA transporter expression and the ability of this compound to reverse ANIT-induced toxicity. Therefore, we investigated key nuclear receptors, which are crucial in regulating BA homeostasis. Subsequent studies using luciferase assays on transfected cells were performed to explore the effect of 18b-GA on signaling pathways of Sirt1-FXR.

MATERIALS AND METHODS

Animals and experimental design

All animal experiments were performed in accordance with the Institutional Ethics Committee of Shanghai Institute of Materia Medica. Eighteen 6- to 8-week-old male Sprague-Dawley specific pathogen-free (SPF)-grade rats were purchased from Shanghai Laboratory Animal Co. (Shanghai, China). They were housed under conditions of 55% humidity at 20–25 °C, provided a standard diet with water available *ad libitum* and were kept on a 12-h light/dark cycle. The rats were fed for one week and allowed to acclimate to the environment before the experiment. The rats were then randomly divided into three groups. Control group rats were injected intraperitoneally with 5% dimethyl sulfoxide (DMSO) in olive oil for 7 days, and olive oil was orally administered on the 5th day. ANIT-treated rats were injected intraperitoneally with 5% DMSO in olive oil for 7 days and, on the 5th day, were orally administered ANIT (dissolved in olive oil) at 100 mg/kg body weight, which was selected according to the supplemental data (Supplementary Figure S1). ANIT and 18b-GA cotreated rats were injected intraperitoneally with 18b-GA (60 mg/kg, which was selected according to preliminary data (Supplementary Figure S2) for 7 days and given ANIT (100 mg/kg) via oral administration 4 h after the 5th 18b-GA injection. On the 7th day, 48 h after ANIT (100 mg/kg) treatment, the rats were sacrificed to collect the livers

and blood. Blood samples were centrifuged at 3000 rpm for 10 min and stored at –80 °C. Liver samples were immediately frozen and stored at –80 °C. ANIT, olive oil, and 18b-GA were purchased from Sigma-Aldrich (St Louis, MO, USA).

Plasma biochemistry

Reagents for measuring plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL), and γ -glutamyl transpeptidase (GGT) were obtained from Roche, Germany and were tested using a biochemical analyzer (Roche, Germany).

Histopathological analysis

After rats were euthanized, the livers were fixed in 10% paraformaldehyde. Tissue samples were then dehydrated and embedded in paraffin, sectioned (4 μ m thickness), and stained with hematoxylin and eosin (H&E). Histological lesions were scored by a pathologist. We evaluated tissue damage by a semi-quantitative method according to the literature [28]. For semiquantitative analyses, the area of necrosis or lesions in the detected area was scored as +1 for <25%, +2 for 25–50%, +3 for 50–75%, and +4 for >75%.

RNA isolation and qRT-PCR analysis of mRNA expression

Total RNA was extracted from 30 mg liver samples using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized using a PrimeScript RT kit (Takara, Japan), and quantitative real-time polymerase chain reactions (qRT-PCR) were analyzed on a Rotor-Gene Q 2plex HRM system (Qiagen, USA) using a SYBR[®] premix Ex Taq[™] II kit (Takara, Japan). The primer sequences are listed in Table 1. All results were normalized to GAPDH expression and calculated using the $\Delta\Delta C_t$ method.

Western blotting

Rat livers (30 mg) were lysed using radio immunoprecipitation assay buffer (Beyotime, China). Protein lysates were separated on 10% SDS-PAGE gels and then transferred to PVDF membranes (Millipore, USA). The membranes were incubated overnight at 4 °C with antibodies against FXR (sc-1204, Santa Cruz Biotechnology, USA), sirtuin 1 (Sirt1) (8469, Cell Signaling Technology, USA), p-glycoprotein (P-gp; ab170904, Abcam, USA), bile salt export pump (Bsep; ab112494, Abcam, USA), multidrug resistance-associated protein 3 (Mrp3; sc-5774, Santa Cruz Biotechnology, USA) and GAPDH (ab9485, Abcam, USA). Membranes were then washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., USA). Protein band chemiluminescence was detected using an ECL Plus immunoblot detection system

Table 1. Primer sequences for quantitative real-time PCR analysis

Gene	Forward	Reverse
r-Ahr	GGATGAAGAAGGACGCGAAC	TCCTTACTCGGGTTGACTG
r-Bsep	CAACGCATTGCTATTGCTCG	GTTCTGGATGGTGGACAAACG
r-FXR	CTCCCATTTACAAGCCACGG	GACGAGGAGAGATCTGTGG
r-GAPDH	TTCCAGGAGCGAGATCCCCTAAC	CATGAGCCCTCCACGATGCCAAAG
r-Hnf4 α	GAGCCATCACCACCATCGTC	TGAGCCAGCAGAAGCCTCAC
r-Mrp2	GGAGCTGGTTGGAACTTGG	TTGGTCTCTGCTTCTGACGT
r-Mrp3	GAGAACCTTCACCTCCAGCT	TGGAGCTCACGAACATCAGT
r-Mrp4	AATTGAGGAGACCACCCGAG	TAGTATGGCCAGGATGAGCG
r-NF- κ B	AAGCAGGAAGATGTGGTGGGA	GATAAGGAGTGTGCCTTGC
r-Nrf2	CTTCTAGCAGAGCCCAGT	AAATGCCGGAGTCAGAGTCA
r-Shp	CCTTGGATGCTTAGGCAAG	CACCACTGTTGGGTTCTCT
r-Sirt1	TGCCATCATGAAGCCAGAGA	CATCGAGTCTCCAAGAAGC

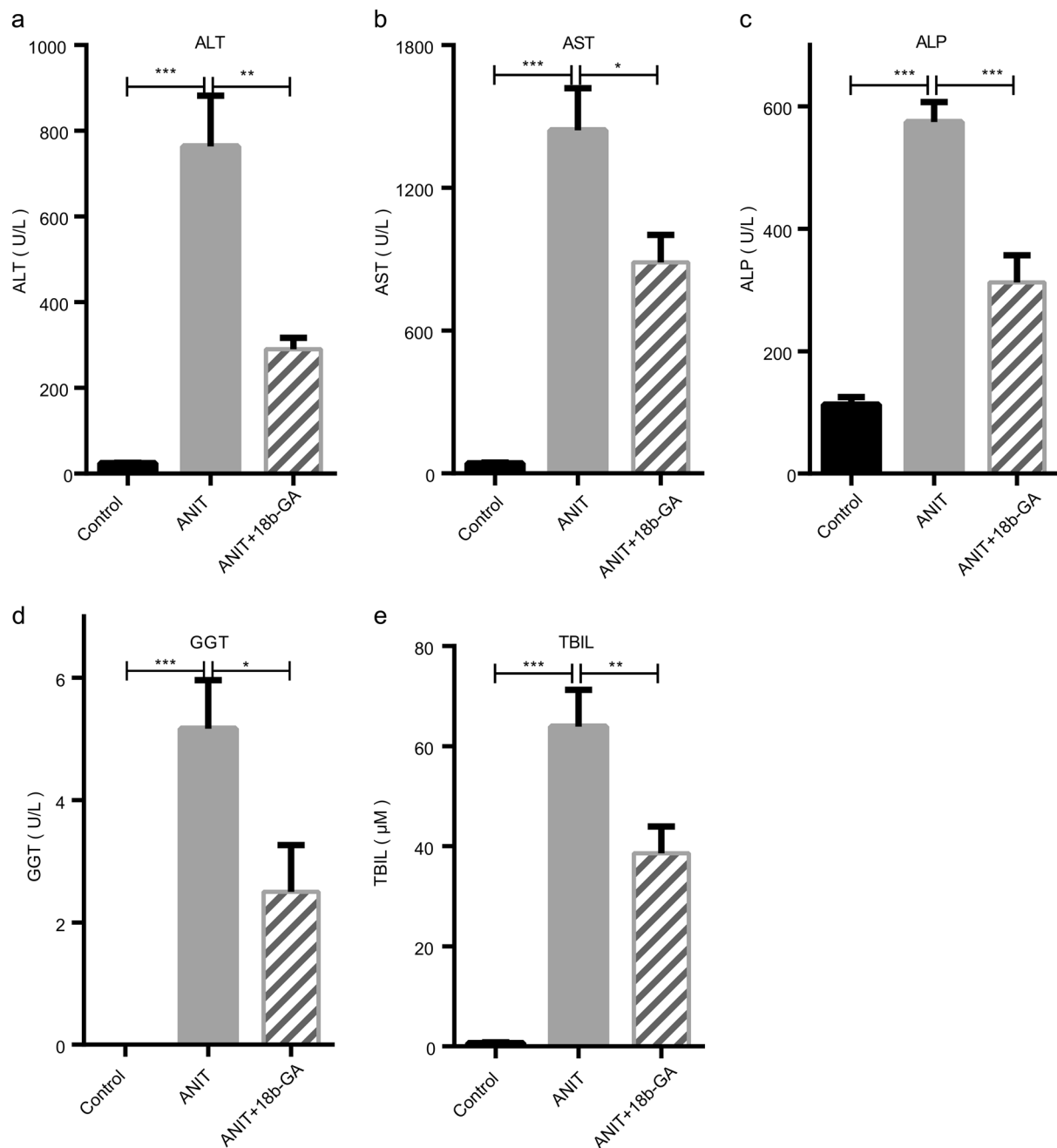


Fig. 1 Protective effects of 18b-GA against ANIT-induced cholestasis in rats. Rats were intraperitoneally administered 18b-GA (60 mg/kg) or vehicle for 7 days and were orally provided ANIT (100 mg/kg, ig) or vehicle after the 5th 18b-GA dose. The levels of biochemical indicators (a) ALT, (b) AST, (c) ALP, (d) GGT, and (e) TBIL were measured. Blood samples were collected and analyzed, as described in the Materials and Methods. Data are shown as the mean \pm SEM. ($n = 6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the ANIT-treated group

(Millipore, Billerica, MA, USA), and protein band density was quantified by ImageQuant software (GE Healthcare, UK).

Primary rat hepatocyte isolation and sandwich culture
Primary hepatocytes were isolated from adult male Sprague-Dawley rats by a two-step collagenase perfusion method and purified by 45% isotonic Percoll [29]. The cell treatments and culture methods have been described previously [30].

Under Mrp2 transport in cells, 5-(and 6)-carboxy-2',7'-dichloro-fluorescein (CDF), which is a probe substrate for Mrp2, is hydrolyzed from a fluorescent CDF diacetate promoiety (CDF-DA). CDF fluorescence intensity is indicative of Mrp2 transport

function. CDF-DA was obtained from Sigma-Aldrich (St Louis, MO, USA).

FXR luciferase assay

The luciferase reporter expression plasmids pcDNA3.1-FXR α , pcDNA3.1-RXR α , and pGL3-FXRE-Luc and the Renilla luciferase gene-containing plasmid pRL-SV40 were kind gifts from Prof. Xu Shen (School of Medicine and Life Sciences, Nanjing University of Chinese Medicine, Nanjing, China). We seeded Huh7 cells (ATCC, USA) in 48-well plates for luciferase assay experiments. The experimental method was consistent with that of Prof. Shen's laboratory [31]. Cell extracts were collected at 24 h after

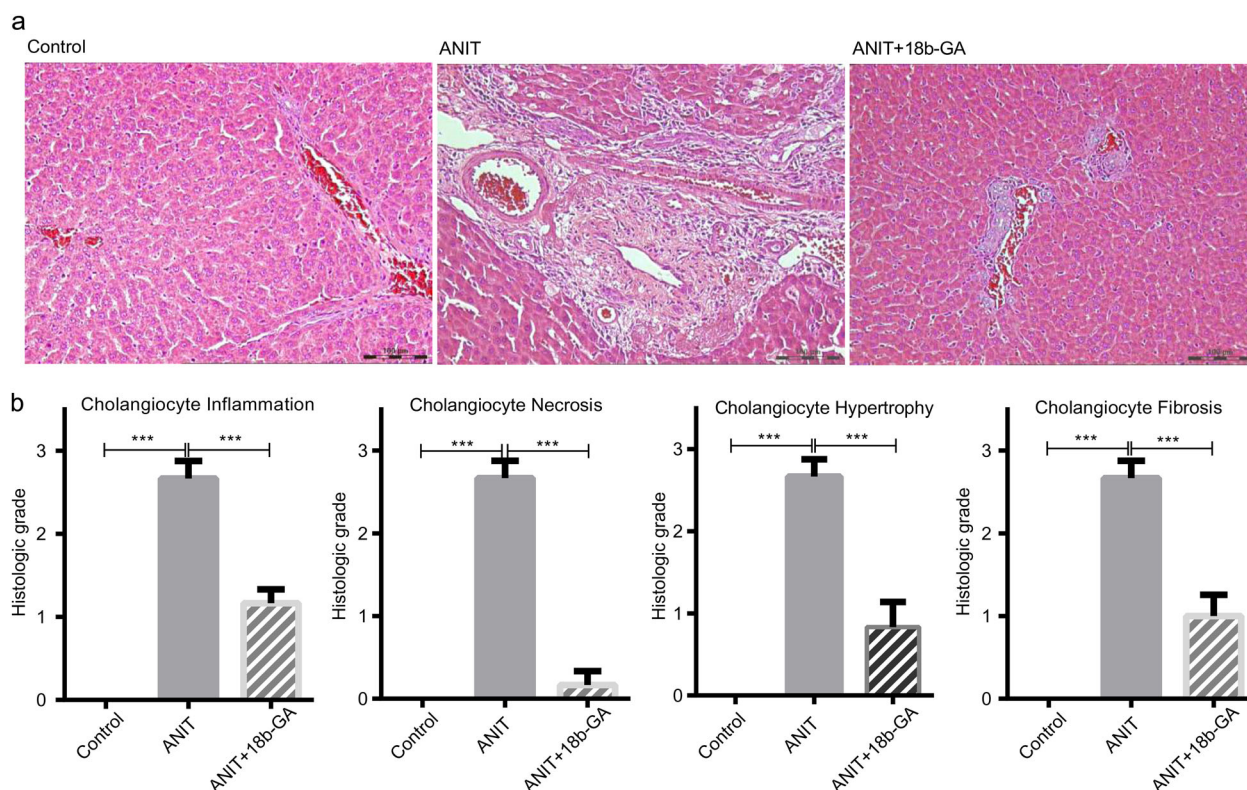


Fig. 2 Liver histology of ANIT-treated rats and 18b-GA-attenuated liver injury induced by ANIT. **a** Liver tissues were fixed, followed by H&E staining ($\times 200$). No histological change was observed in the control group, large areas of necrosis and inflammation were observed in the ANIT-treated group, and a few spotty areas of necrosis and inflammation were observed in the ANIT and 18b-GA cotreated group. **b** The graph shows the semi-quantitative analysis of cholangiocyte inflammation, necrosis, hypertrophy, and fibrosis. Data are shown as the mean \pm SEM. ($n = 6$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the ANIT-treated group

transfection, and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA). Renilla luciferase was used for normalization.

Statistical analysis

Data were statistically analyzed and graphed using GraphPad Prism software (version 5.03; GraphPad Software, Inc., CA, USA). All cell experiments were performed independently at least three times. The data are expressed as the mean \pm SEM, and Student's *t*-tests were used to analyze the significance of differences. $P < 0.05$ was considered statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

RESULTS

18b-GA has anti-cholestatic and hepatoprotective effects in vivo

To estimate the protective effect of 18b-GA on ANIT-induced hepatotoxicity and cholestasis, rats were intraperitoneally injected with 18b-GA (60 mg/kg) for 7 days, and ANIT was administered orally (100 mg/kg) 48 h before euthanasia. Then, plasma biochemistry was analyzed. Plasma ALT, AST, ALP, GGT, and TBIL levels were increased by 31.2-, 33.4-, 5.1-, 5.0-, and 91.3-fold, respectively, in ANIT-induced rats ($P < 0.0001$), and all levels were significantly decreased by 62.0%, 38.5%, 45.7%, 51.6%, and 39.7%, respectively, after 18b-GA treatment ($P < 0.05$) (Fig. 1a–e).

H&E staining of liver sections showed that compared with the control group, the ANIT-induced group displayed multifocal portal vein degeneration/coagulation necrosis, fibrosis, neutrophil and monocyte accumulation, and bile duct epithelial cell apoptosis and hypertrophy. 18b-GA and ANIT coadministration significantly

improved the above symptoms in bile duct epithelial cells, as shown in Fig. 2a. Histological scores revealed a significant improvement in the animals treated with 18b-GA (Fig. 2b).

18b-GA altered receptor factors involved in BA homeostasis

To elucidate the protective effects of 18b-GA in ANIT-induced cholestasis, we measured the gene expression of several nuclear receptors and transcription factors associated with BA homeostasis. As shown in Fig. 3a, 18b-GA and ANIT cotreatment significantly increased FXR, Nrf2, and Sirt1 mRNA levels and had little effect on NF- κ B, Ahr, and Hnf4a levels. These data demonstrated that FXR, Nrf2, and Sirt1, but not NF- κ B, Ahr, and Hnf4a, are key factors in the anti-cholestatic effect of 18b-GA. According to the literature, 18b-GA may protect the liver through Nrf2-induced Mrp3/4 upregulation (Fig. 3b). Next, FXR target genes in rats were studied by real-time PCR, which revealed involvement of Shp, Bsep, and Mrp2 (Fig. 3c). These data revealed that 18b-GA regulates the BA balance through FXR.

To verify the accuracy of the qRT-PCR results on the induction of transporters and nuclear factors by 18b-GA, we measured protein levels using western blot analysis. The results revealed that ANIT treatment marginally changed Bsep and FXR protein expression and slightly increased P-gp, Mrp3 and Sirt1 protein expression. 18b-GA cotreatment dramatically increased Bsep, P-gp, Mrp3, FXR, and Sirt1 protein levels (Fig. 3d). Therefore, 18b-GA and ANIT coadministration can increase Bsep, Mrp3, P-gp, FXR, and Sirt1 mRNA and protein expression. This finding suggested that the protective effect of 18b-GA against cholestasis may be a result of enhanced BA output, which is possibly mediated via FXR or Sirt1 activation.

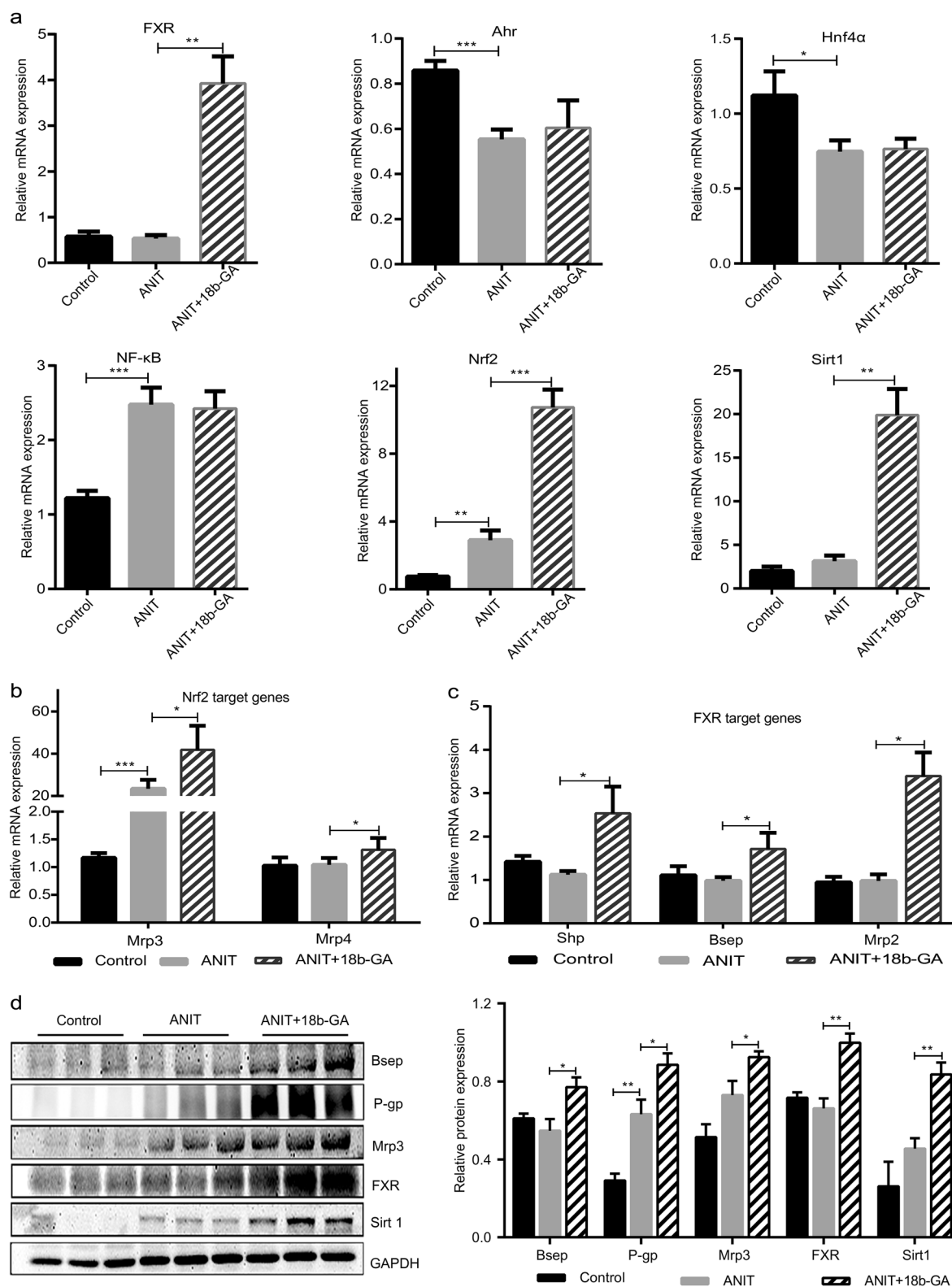


Fig. 3 Effects of 18b-GA on bile acid nuclear receptor and transcription factor homeostasis in vivo. Quantitative real-time PCR analysis was performed to measure (a) the BA transcription factor; (b) the expression levels of Nrf2 target genes, including Mrp3 and Mrp4; and (c) the expression levels of FXR target genes, including Shp, Bsep, and Mrp2. **d** Western blot was used to measure transcription factor and transporter levels. Data are reported as the mean \pm SEM. ($n = 6$). * $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$ compared with the ANIT-treated group

18b-GA activated FXR through Sirt1 in vitro. As FXR plays an important role in regulating BA homeostasis, we tested whether 18b-GA exerted protective effects against

cholestasis through modulating FXR activities. Interestingly, 18b-GA exposure resulted in dose-dependent FXR activation when the FXR ligand chenodeoxycholic acid (CDCA) was present, and

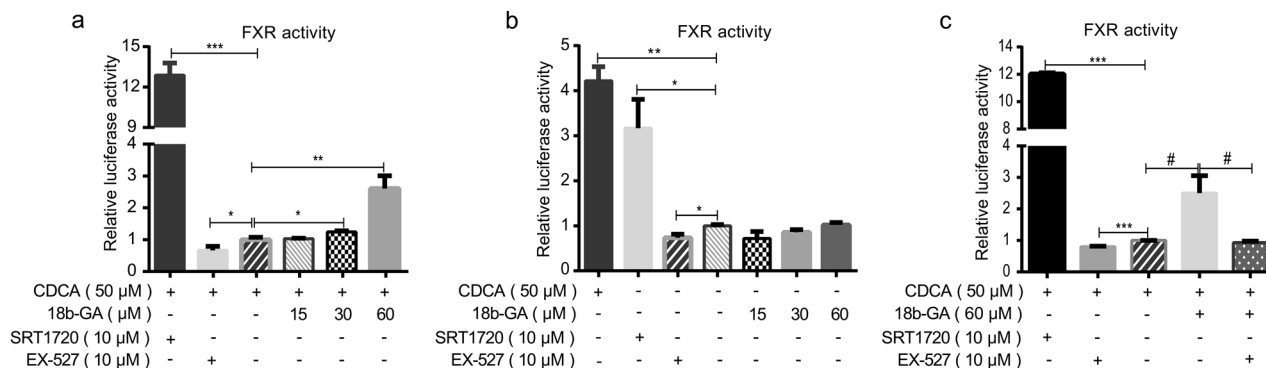


Fig. 4 18b-GA activates FXR in vitro. After transfection with FXR plasmid DNA for 6 h, the dose-dependent response of Huh7 cells to 18b-GA exposure for 24 h with (a) and without (b) CDCA (50 μM), an effective FXR endogenous ligand, was measured. (c) Luciferase reporter assays confirmed that FXR is regulated by Sirt1. 18b-GA treatment significantly activated FXR, and FXR activity was significantly reduced by EX-527. SRT1720, a selective activator of human Sirt1, and EX-527, a potent and selective Sirt1 inhibitor, were used. Data are reported as the mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with DMSO, #P < 0.05 compared with 18b-GA

18b-GA exposure did not activate FXR when CDCA was absent. (Fig. 4a, b), as indicated by the FXR luciferase assay in Huh7 cells (SRT1720, a selective activator of human Sirt1, as a positive control). Furthermore, as Sirt1 is a mediator of FXR, we investigated whether 18b-GA activated FXR through Sirt1. The results revealed that 18b-GA treatment significantly activated FXR, which was significantly reduced by EX-527 (a potent and selective Sirt1 inhibitor) (Fig. 4c), indicating that 18b-GA-mediated FXR activation is associated with Sirt1. Similar results were obtained when another tool, HEK293T cells, were used in the FXR luciferase assay (Supplementary Figure S3).

18b-GA regulated FXR activity and FXR target gene expression via Sirt1

Since 18b-GA activated FXR through Sirt1 in vitro, we further evaluated the protective effect of 18b-GA against EX-527 by transporters and nuclear factors in sandwich-cultured rat hepatocytes (SCRHs). As illustrated in Fig. 5a, 18b-GA can significantly increase Sirt1 and FXR mRNA levels, which are significantly reduced by EX-527. Then, we examined the mRNA levels of downstream FXR genes, such as Shp, Bsep and Mrp2. The results revealed that 18b-GA significantly increased Shp, Bsep and Mrp2 mRNA expression, which was inhibited by EX-527 (Fig. 5b). In addition, cells were pretreated with 18b-GA for 1 day and then re-treated with ANIT for 24 h, as shown in Fig. 5c. After treatment with ANIT, the fluorescence substrate of Mrp2, CDF, was decreased. Compared with ANIT treatment, 18b-GA and SRT1720 (a selective activator of human Sirt1) cotreatment increased CDF fluorescence intensity. ANIT treatment reduced the fluorescence intensity of CDF, which was reversed when the cells were cotreated with 18b-GA and ANIT, thus increasing the intensity of the fluorescent substrate of Mrp2. These results indicated that 18b-GA can protect against ANIT-induced impairment of the efflux function of Mrp2. Taken together, these observations indicated that 18b-GA ameliorated ANIT-induced cholestasis through the Sirt1/FXR pathway.

DISCUSSION

Our data highlight the protective effect of 18b-GA against ANIT-induced liver injury by stimulating the expression of Sirt1, FXR and Nrf2 nuclear factors. Nrf2 activation stimulated the expression of its targeted efflux transporter gene (*Mrp3*, *Mrp4*) and regulated bile acid homeostasis. In addition, 18b-GA treatment significantly activated FXR, which promotes the expression of its targeted efflux transporter gene (*Mrp2*, *Bsep*), and this effect was prevented by the selective Sirt1 inhibitor EX-527. Therefore, 18b-GA activated FXR through activation of Sirt1 and exerted a hepatoprotective

effect against ANIT-induced cholestasis, which promotes the expression of efflux transporter genes, thus impairing bile acid homeostasis disorders in hepatocytes.

ANIT, a hepatotoxic agent that is widely used to induce intrahepatic cholestasis in rodents, leads to cholestasis by impairing parenchymal hepatic cell polarization, directly injuring biliary epithelial cells and inhibiting BA transporter function and expression [32]. According to the literature, treating rats with ANIT significantly decreases bile flow, and cotreatment with ANIT and 18b-GA significantly increases bile flow [10]. We speculated that a close relationship exists between increased bile flow and BA efflux. Since BA content is regulated by various nuclear factors, we investigated whether the expression of these genes was changed in the liver by 18b-GA cotreatment. The results revealed that compared with the ANIT treatment group, 18b-GA cotreatment significantly increased FXR, Nrf2 and Sirt1 gene expression levels. However, changes in BA-related nuclear receptors induced by ANIT are inconsistent across studies [21, 23, 33–39]. Our results show that compared with the control rats, the levels of FXR, Sirt1, and Nrf2 mRNA in the rats treated with ANIT did not change. The diversity of this effect may be due to the different experimental conditions and the complex regulation of nuclear receptors, i.e., the negative feedback of BA balance, the body's stress response [35, 36], cross-regulation of BA in the body [39], and even intestinal flora [40, 41].

Activating Nrf2 may ameliorate bile duct ligation (BDL)- or ANIT-induced cholestasis [7, 42, 43]. Recent studies have reported that *Mrp2*, *Mrp3*, and *Mrp4* are direct Nrf2 target genes whose expression was increased by prototypical Nrf2 chemical activators in rodent livers and decreased to basal expression levels in Nrf2-knockout mice [44–46]. Our results revealed that 18b-GA significantly increased *Mrp2*, *Mrp3*, and *Mrp4* mRNA expression (Fig. 3c). The protective effect of 18b-GA in ANIT-induced cholestasis has been indicated to be due to increased bile efflux, which is closely related to Nrf2-mediated *Mrp2*, *Mrp3*, and *Mrp4* upregulation. Under baseline conditions, FXR-knockout (*FXR*^{-/-}) mice develop plasma BA, and when fed cholic acid, these mice develop severe liver toxicity [47, 48]. Previous studies have demonstrated that the synthetic FXR agonist GW4064 improves cholestatic symptoms induced by ANIT [49] and BDL [50]. To further confirm the effect of 18b-GA on the FXR pathway, the expression of FXR and FXR target genes, including *Bsep*, *Mrp2*, and *Shp*, were quantified in rats treated with 18b-GA. The results demonstrated that 18b-GA improved FXR, *Shp*, *Bsep* and *Mrp2* expression, as shown in Fig. 3b, d, indicating that 18b-GA alleviates liver injury via activation of the FXR/*Shp* signaling pathway.

Sirt1, a class III NAD-dependent histone deacetylase, plays a key role in lipid, glucose, and BA metabolism. Sirt1 and FXR can form

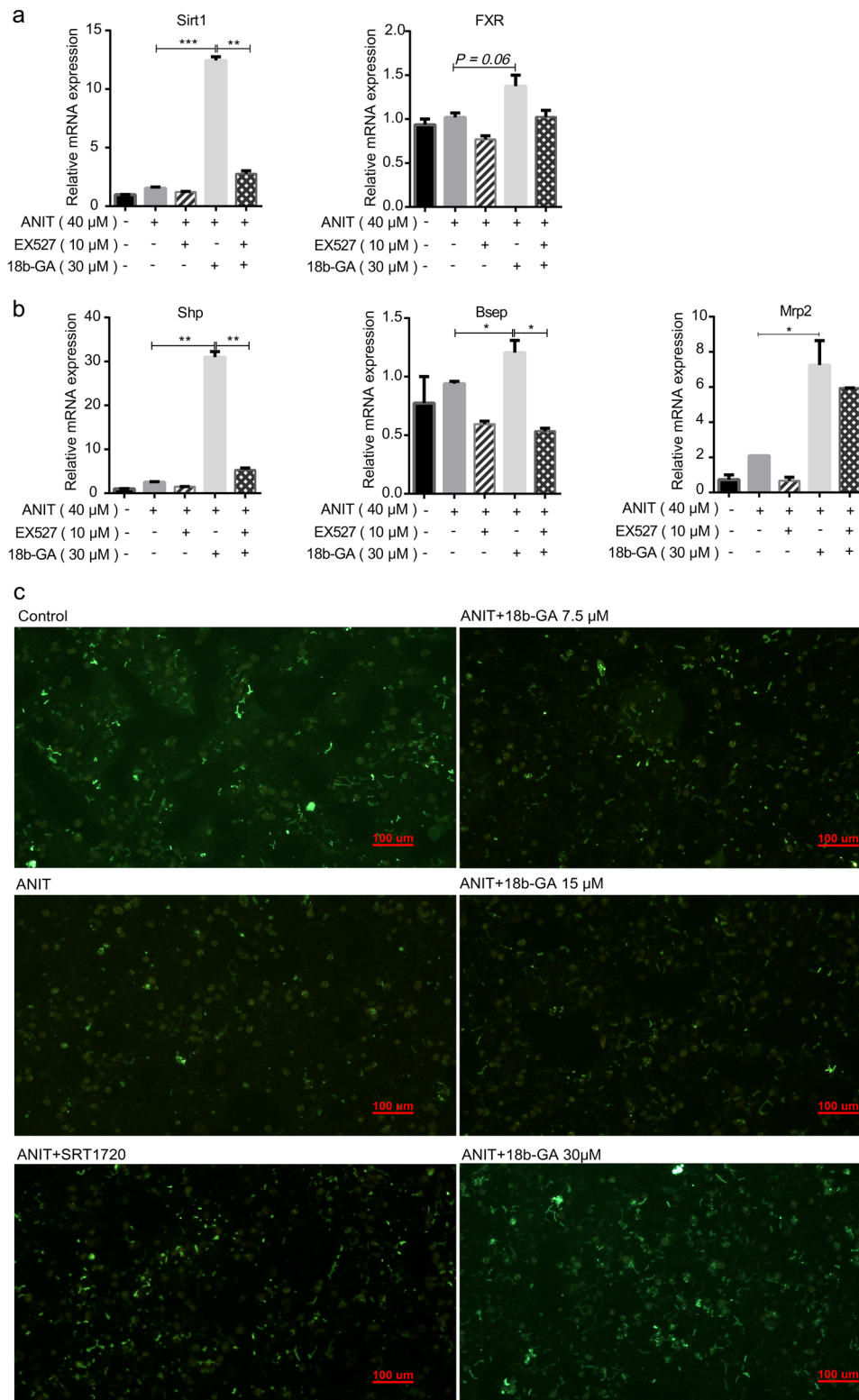


Fig. 5 18b-GA upregulates the expression of FXR and FXR target genes, and 18b-GA promotes Mrp2 function in SCRHS. Rat primary hepatocytes were treated with 18b-GA (30 μM) and/or EX-527 (10 μM) for 3 days and with ANIT (40 μM) on the 4th day. RNA isolation and quantitative real-time PCR analysis were performed to measure the levels of the transcription factors Sirt1 and FXR (a) and FXR target genes (b). Data are reported as the mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with ANIT and 18b-GA cotreatment). c Intracellular amounts of CDF were determined by spectrofluorimetry. Rat primary hepatocytes were treated with ANIT (40 μM) and 18b-GA (7.5, 15, 30 μM); 18b-GA pretreatment lasted for 1 day, and cells were treated with ANIT the following day. Then, a fluorescent substrate of Mrp2, CDF, was added. The fluorescence represents the function of the Mrp2-related canalicular efflux pump. Scale bar = 100 μm

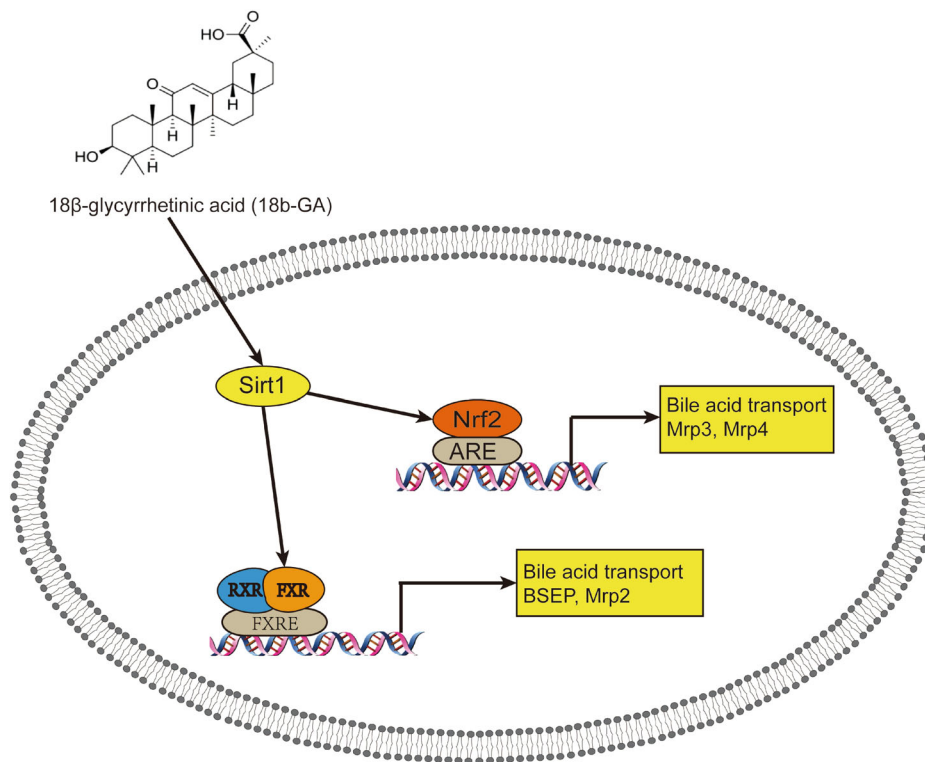


Fig. 6 The possible mechanism of Sirt1/FXR activation by 18b-GA in an ANIT-induced cholestasis rat model. 18b-GA can activate Sirt1 and promote the binding of FXR and RXR, which promotes upregulation of canalicular efflux transporters and entry into the bile. On the other hand, activation of Sirt1 promotes the expression of Nrf2, leading to marked upregulation of the basolateral efflux transporters, thereby promoting bile into the blood. ARE: antioxidant response element, RXR: retinoid X receptor, FXRE: FXR-responsive element

an interactive regulatory network: acetylation stabilizes FXR but reduces its activity by decreasing FXR/RXR heterodimerization and the ability of FXR to bind FXRE and transactivate other genes [51, 52]. Liver-specific Sirt1 deletion can lead to BA metabolic dysfunction by downregulating FXR signaling and affects cholesterol gallstone development in lithogenic diets, which can be reversed by Sirt1 overexpression [53]. To analyze the influence of 18b-GA on Sirt1/FXR signaling, we first tested whether 18b-GA can activate FXR in Huh7 cells. In the presence of CDCA, an endogenous FXR ligand, 18b-GA activated FXR in a dose-dependent manner; this effect did not occur in the absence of CDCA (Fig. 4b). Reports in the literature have also demonstrated that when no ligand is present, FXR remains in a silent state [54]. Subsequently, we found that 18b-GA activated FXR, and this effect was prevented by EX-527, a potent and selective Sirt1 inhibitor, indicating that 18b-GA-mediated FXR activation is associated with Sirt1.

To further analyze the effect of 18b-GA on the Sirt1/FXR pathway in vitro, Sirt1 and FXR target gene expression levels were quantified in SCRHS. The results demonstrated that 18b-GA increased Sirt1, FXR, Shp, Mrp2, and Bsep expression levels, which were significantly inhibited by EX-527 (Fig. 5a, b). In addition, we found that CDF fluorescence intensity was markedly increased in 18b-GA-treated SCRHS compared with that in ANIT-treated SCRHS (Fig. 5c), indicating that 18b-GA can improve BA efflux into bile. As CDF is a substrate of efflux transporter Mrp2, its fluorescence in bile pockets of SCRHS may reflect the function of efflux transporters at the bile canalicular side of hepatocytes.

In conclusion, the current study indicates that 18b-GA protects against ANIT-induced cholestasis through activation of the Sirt1/FXR signaling pathway and the Sirt1/Nrf2 signaling pathway (Fig. 6). However, how 18b-GA activates FXR through Sirt1 is still unclear. Further studies focusing on molecular structure may help determine their interactive mechanism.

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AUTHOR CONTRIBUTIONS

SW, LG, GX, and JR designed the research project; SW, SC, LW, YZ, and XY performed the experiments; SW, SC, and LG analyzed the data; HL, as a pathologist, scored the histological lesions; and SW and LG wrote the manuscript.

ADDITIONAL INFORMATION

The online version of this article (<https://doi.org/10.1038/s41401-018-0110-y>) contains supplementary material, which is available to authorized users.

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