Toxicology and Applied Pharmacology 305 (2016) 127–135

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Dioscin protects against ANIT–induced cholestasis via regulating Oatps, Mrp2 and Bsep expression in rats

Aijie zhang [a,c], Yongming Jia [a], Qinghan Xu [a], Changyuan Wang [a,b], Qi Liu [a,b], Qiang Meng [a,b], Jinyong Peng [a,b], Huijun Sun [a,b], Pengyuan Sun [a], Xiaokui Huo [a,b], Kexin Liu [a,b,*]

[a] Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, China
[b] Provincial Key Laboratory for Pharmacokinetics and Transport, Liaoning, Dalian Medical University, China
[c] State Key Laboratory of Drug Delivery Technology and Pharmacokinetics, Tianjin Institute of Pharmaceutical Research, Tianjin, China

ARTICLE INFO

Article history:
Received 12 March 2016
Revised 4 June 2016
Accepted 13 June 2016
Available online 15 June 2016

Keywords:
Dioscin
ANIT-induced cholestasis
Oatps
Mrp2
Bsep

ABSTRACT

Alpha-naphthylisothiocyanate (ANIT) is a toxicant that is widely used in rodents to model human intrahepatic cholestasis. The aim of the study is to investigate whether effects of dioscin on ANIT-induced cholestasis are related to changes in expression of hepatic transporters in rats. Effects of dioscin on cholestasis were examined by histology and biochemical marker levels. The functional changes of hepatic transporters were determined by *in vitro*, *in situ* and *in vivo*. qRT-PCR and western blot were used to assess the expression of hepatic transporters in cholestatic rats. Dioscin administration could ameliorate cholestasis, as evidenced by reduced biochemical markers as well as improved liver pathology. The uptakes of organic anion transporting polypeptide (Oatp) substrates were altered in liver uptake index *in vivo*, perfused rat liver *in situ* and isolated rat hepatocytes *in vitro* in cholestasis rats. qRT-PCR and western blot analysis indicated co-treatment of ANIT with dioscin prevented the adaptive down-regulation of Oatp1a1, 1b2, and prompted the up-regulation of Oatp1a4, multidrug resistance-associated protein (Mrp) 2 and bile salt export pump (Bsep). In addition, concerted effects on Mrp2 and Bsep occurred through up-regulation of small heterodimer partner by activating farnesoid X receptor. Dioscin might prevent impairment of hepatic function by restoring hepatic transporter expression.

1. Introduction

Liver disease is a common disorder caused by viral hepatitis, alcoholism and liver-toxic chemicals (Papay et al., 2009). Recently, herbal medicines have gained more attention and popularity for the treatment of liver diseases because of their safety and efficacy, such as silymarin and its derivates, quercetin, and curcumin (Del et al., 2012; Kobori et al., 2011). Di-ao-xin-xue-kang capsules prepared from *Dioscorea nipponica* Makino have been used for >10 years in China to treat coronary heart disease (Liu et al., 2004). Diosgenyl 2,4-di-*O*-a-L-rhamnopyranosyl-*p*-D-glucopyranoside (dioscin, Fig. 1D), a major component of di-ao-xin-xue-kang capsules, has been shown to protect against hepatotoxicity produced by carbon tetrachloride and acetaminophen (Lu et al., 2012; Zhao et al., 2012). Xu et al. (Xu et al., 2012) reported the no-observed-adverse-effect level (NOAEL) and the lowest-observed-adverse-effect level (LOAEL) of dioscin were estimated to be 300 mg/kg/day for female and male rats, respectively. Our previous study indicated that dioscin is a substrate of multiple organic anion transporting polypeptides in rats (Zhang et al., 2013).

Cholestasis is accompanied by retention of bile salts, which by themselves can cause hepatic injury and cholestasis. Alpha-naphthylisothiocyanate (ANIT) causes an intrahepatic cholestasis in rodents, which is widely used to model human intrahepatic cholestasis (Tanaka et al., 2009). Expression of hepatic transporters is altered during various forms of liver injury. In general, the expression of uptake transporters is reduced, and levels of efflux transporters are increased in damaged livers (Cui et al., 2009; Dietrich et al., 2001; Slitt et al.,

Abbreviations: ALT, alanine amiotransferase; ANIT, alpha-naphthylisothiocyanate; AST, aspartate transaminase; BIL, bilirubin; Bsep, bile salt export pump; CMC-Na, carboxymethylcellulose sodium; E3S, estrone-3-sulfate; FXR, Farnesoid X receptor; IS, hydrochlorothiazide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Mrp, multidrug resistance-associated protein; Ntcp, Na^+-taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; qRT-PCR, Quantitive Real Time-polymerase chain reaction; SHP, small heterodimer partner.

* Corresponding author at: Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, 9 West Section, Lvshun South Road, Lvshunkou District, Dalian 116044, China.
E-mail addresses: zhangaijie1986@163.com (A. zhang), yongmingjiahlj@126.com (Y. Jia), xulianglinyao@126.com (Q. Xu), wangcyuan@163.com (C. Wang), llaqii@yahoo.com.cn (Q. Liu), mengq531@yahoo.cn (Q. Meng), jinyongpeng2005@163.com (J. Peng), sunhuijun@hotmail.com (H. Sun), spfar1004@gmail.com (P. Sun), huoxiaokui@163.com (X. Huo), kexinliu@dlmedu.edu.cn (K. Liu).

http://dx.doi.org/10.1016/j.taap.2016.06.019
0041-008X/

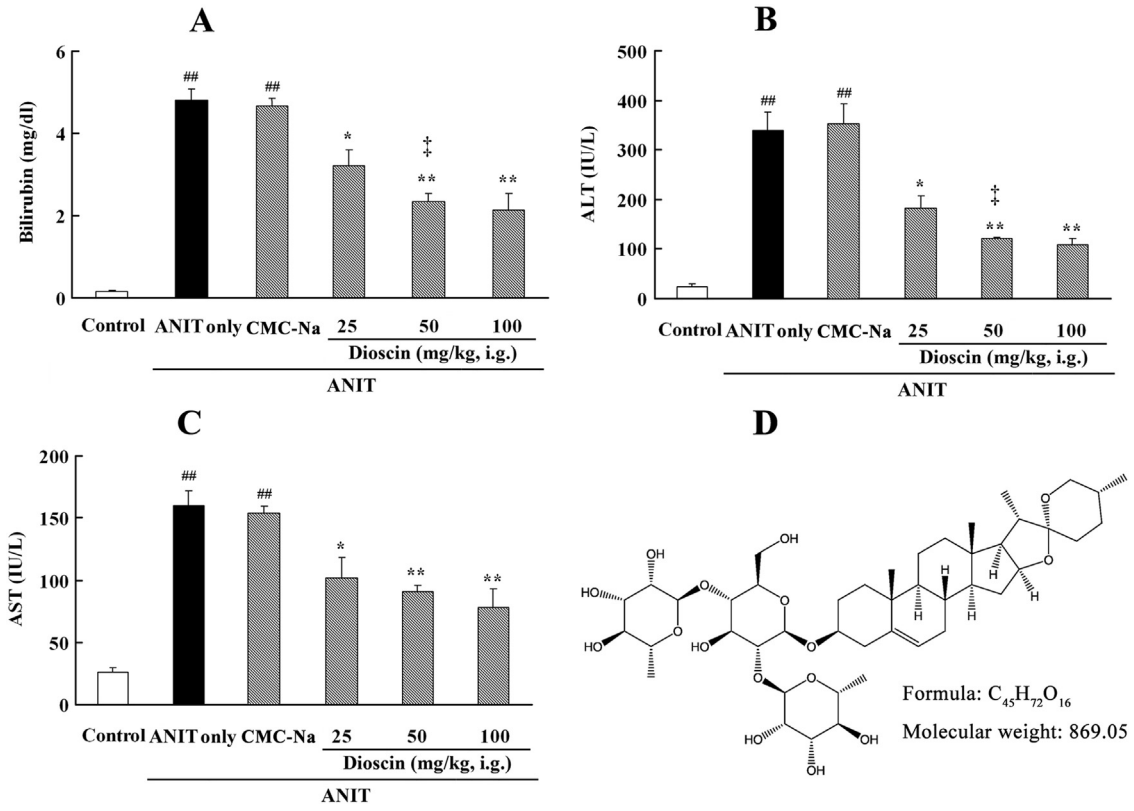

Fig. 1. Effect of dioscin on concentration of BIL (A), ALT (B) and AST (C) in serum of rat induced by ANIT and the chemical structure of dioscin (D). Dioscin was administered i.g. (25, 50 and 100 mg/kg) 24 h, 12 h before and 0.5, 20 and 40 h after ANIT treatment (50 mg/kg). Serum concentration of BIL, ALT and AST were measured 48 h after ANIT treatment. $^{\#\#}p < 0.01$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. ANIT only group; $^{‡}p < 0.05$ vs. 25 mg/kg group (n = 4 rats per group).

2007). The basolateral uptake transporters, sodium taurocholate–cotransporting polypeptide (Ntcp, *Slc10a1*), as well as organic anion transporting polypeptide (Oatp, *Slco*) 1a1, 1a4, and 1b2, transport bile acids, and other substrates (such as thyroid hormones (Hagenbuch, 2007), fexofenadine (Giacomini et al., 2010)) from portal blood into hepatocytes. The multidrug resistance-associated protein 2 (Mrp2, *Abcc2*) is a canalicular efflux transporter highly expressed in hepatocytes and is critical for the biliary excretion of glutathione, glutathione conjugates, and glucuronidated bile acids (Dietrich et al., 2001; Hayashi et al., 2012; Kong et al., 2012). Bile salt export pump (Bsep, *Abcb11*) is an ATP-binding cassette transmembrane transporter located on the canalicular membrane. Bsep and Mrp2 play an indispensable role in transporting bile acids into bile. Farnesoid X receptor (FXR), a hepatic nuclear receptor, could up-regulate efflux transporters through small heterodimer partner (SHP), while it was activated by bile acids (Chen et al., 2010; Cui et al., 2009).

Recently, the effect of expression and function of transporters on the improvement of disease has been reported. Naoi et al. (Naoi et al., 2014) reported 4-phenylbutyrate would have a therapeutic effect on a progressive familial intrahepatic cholestasis type 2 patient through partial restoration of decreased Bsep expression at the canalicular membrane. Chen et al. (Chen et al., 2014) reported oleanolic acid exert a protective effect against lithocholic acid (LCA)-induced cholestasis, possibly due to up-regulating expression of Mrps.

Other groups have examined the expression changes of hepatic transporters after ANIT treatment (Cui et al., 2009; Kong et al., 2012). However, little is known about the relationship between the changes in expression of transporters and cholestasis therapy. Therefore, the objectives of this work were to examine the relationships of changes in expression of Oatps, Mrp2, Bsep with ANIT-induced cholestasis, and to clarify whether the mechanism for the effect of dioscin on ANIT-induced cholestasis is related to improvement of expression of these transporters.

2. Material and methods

2.1. Materials

Dioscin (with purity of >99%) was kindly provided by Professor Jinyong Peng (Dalian Medical University, Dalian, China). Hydrochlorothiazide [internal standard (IS)], ANIT, inulin and estrone-3-sulfate (E3S) were purchased from Sigma (ST. Louis, Mo, USA). Quantitive real-time polymerase chain reaction (qRT-PCR) reagents were purchased from TaKaRa Biotechnology (Dalian, China) Co. Ltd.

2.2. Animals

Male Wistar rats of 220–250 g body weight obtained from the Experimental Animal Center of Dalian Medical University (permit number SCXK 2008–0002) were allowed free access to water and chow diet but were fasted for 12 h (with water ad libitum) before the experiments. All protocols involving the animal were approved by the Animal Care and Use Committee of Dalian Medical University and performed in strict accordance with the People's Republic of China Legislation Regarding the Use and Care of Laboratory Animals.

2.3. Evaluation of ANIT-induced Cholestatic Hepatitis

Rats were injected intraperitoneally (i.p) with olive oil (Control group, n = 4) or ANIT (ANIT only group, n = 4) at a dose of 50 mg/kg body weight (Liu et al., 2012a). 48 h after ANIT administration serum were collected for determining bilirubin (BIL), alanine amiotransferase (ALT) and aspartate transaminase (AST). All serum biochemistry was analyzed using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A portion of the liver was fixed in 10% (v/v) neutral formalin, processed by standard histological techniques,

stained with haematoxylin and eosin (H&E), and examined for morphological evidence of liver injury.

2.4. The effect of dioscin on ANIT-induced Cholestatic Hepatitis

ANIT was injected i.p. on the single dose of 50 mg/kg in all dosed groups and the ANIT + CMC-Na group. Dioscin suspended in 0.5% carboxymethylcellulose sodium (CMC-Na) was administered intragastrically (i.g.) at 25, 50, 100 mg/kg 24 h, 12 h before and 0.5, 20 and 40 h after ANIT treatment. The ANIT + CMC-Na group (n = 4) received equivalent volumes of 0.5% CMC-Na. 48 h after ANIT treatment, blood samples were collected to measure the serum concentration of BIL, ALT and AST, and livers were removed for Histological, PCR analysis and Western Blot analysis.

2.5. Pharmacokinetic studies

In vivo and *in situ* pharmacokinetic studies were performed in the Control group and ANIT only groups, while *in vitro* study included the Control, ANIT and ANIT + Dioscin (50 mg/kg) groups. Each group of the following experiments included four male rats.

2.5.1. In vivo liver uptake index (LUI) studies. Under light ether anesthesia, the portal vein was cannulated with polyethylene tubing (PE-50; BD Biosciences) in male Wistar rat. Dioscin (0.2 mg/kg) and inulin (1 mg/kg) dissolved in rat plasma, was injected rapidly into the portal vein immediately after ligation of the hepatic artery. After 18 s of bolus administration of compounds, which is enough time for the bolus to pass completely through the liver but brief enough to prevent recirculation of the compounds (Zhang et al., 2013), the portal vein was cut, the liver was excised, weighed and stored at −20 °C.

2.5.2. In situ perfused rat liver. Livers were prepared by standard techniques (Zhang et al., 2013). Following anesthesia, the bile duct was cannulated and the liver was perfused in a single pass via the portal vein with oxygenated Krebs–Henseleit buffer (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5.0 mM glucose, 2.5 mM $CaCl_2$ adjusted to pH 7.4). The liver was allowed to equilibrate for approximately 10 min before addition of the dose. Perfusion was continued with oxygenated Krebs–Henseleit buffer containing 20% (v/v) washed bovine erythrocytes at a flow rate of 12 mL/min. The superior vena cava was cannulated and used to collect the effluent perfusate. Liver viability was determined on the basis of the initial bile flow (>2 μL/min). Following equilibration, the liver was perfused for 45 min after addition of dioscin 2.5 μM in control or ANIT-treated rats. The effluent perfusate (~500 μL) was collected at timed intervals, and the samples were stored at −20 °C.

2.5.3. Uptake by using isolated rat hepatocytes. Rat hepatocytes were prepared by the collagenase perfusion method in rat as described (Maeda and Sugiyama, 2010; Zhang et al., 2013). Isolated hepatocytes (viability > 80%) were suspended in Krebs–Henseleit buffer, adjusted to 2.0×10^6 cells/mL and kept on ice. Before the uptake study, the cell suspension and Krebs–Henseleit buffer containing the substrate were incubated separately at 37 °C for 3 min and then transport was initiated by adding an equal volume of buffer (120–200 μL) to the cell suspension. After incubation at 37 °C for 0.5, 1, 2 or 5 min, the reaction was terminated by separating the cells from the substrate solution by centrifugal filtration. For this purpose, a 100 μL sample of incubation mixture was collected and placed into a 450 μL centrifuge tube (Hepatocyte Transporter Suspension Assay Kit, BD Gentest, Woburn, MA) containing 5 M sodium acetate under a 100 μL layer of an oil mixture (density 1.015, a mixture of silicone oil and mineral oil; BD Gentest), which was centrifuged at 10,000*g* for 10 s (ST-16R, Thermo Fisher, Germany) and the hepatocytes passed through the oil layer into the aqueous solution. The tube contents were frozen in liquid nitrogen immediately after centrifugation and stored at −20 °C. Before analysis, the sampling tube is cut at the middle of the oil layer and the upper and lower phases are transferred to separate tubes. The lower phase containing the lysed cells is prepared by biological sample preparation method.

2.6. Quantitive Real-Time PCR

Total tissue RNA was extracted using TRIzol® Reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol in all groups (n = 4). The assay was processed as described previously (Miao et al., 2011). The sequences of the primers are shown in (Table 1). The cDNA was amplified using SYBR® Premix Ex Taq™ kit (Takara, Dalian, China) and ABI PRISM® 7500 system (Applied Biosystems, Foster City, CA). The conditions for PCR were: 1 cycle of denaturation at 95 °C for 30 s, and 40 cycles of denaturation at 95 °C for 5 s, and combined annealing/extension (60 °C, 20 s). Gene expression changes were calculated by the comparative Ct method and the values were normalized to endogenous reference β-actin (Miao et al., 2011).

2.7. Western blot analysis

Western blot analysis was done as described previously (Guo et al., 2013). Briefly, Protein extracted from tissue samples was prepared using Radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentration was determined by the procedure of bicinchoninic acid (Solarbio, Beijing, China). Proteins (50 μg) were resuspended in electrophoresis sample buffer and separated by electrophoresis on a pre-cast 8–12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA), followed by electrotransfer to a PVDF membrane (Millipore, Bedford, MA). Membranes were blocked using 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at 37 °C. Membranes were incubated overnight at 4 °C with polyclonal antibody for Mrp2 and Bsep (Abcam, San Francisco, USA), and monoclonal antibody for Oatp1a1, Oatp1a4, Oatp1b2 and β-actin (Santa Cruz Biotechnology, Santa Cruz, USA). Then, membranes were rinsed three times with TBST and incubated for 2 h at 37 °C with secondary antibody. Emitted light was documented with a BioSpectrum–410 multispectral imaging system with a Chemi HR camera 410.

2.8. Biological sample preparation

Each liver obtained from the LUI study was weighed, added to a 3-fold volume of normal saline and homogenized in the ice-bath. Inulin preparation: the assay was processed as the reference (Zhang et al., 2013). Dioscin preparation: the LUI and uptake of hepatocytes samples were prepared as described previously (Zhang et al., 2013). The sample of E3S for rat hepatocytes, which was used as the positive control, was handled as described for dioscin. The samples were subjected to LC-MS/MS analysis.

2.9. LC-MS/MS analysis

An LC system (Agilent HP1200, CA) was used for LC-MS/MS analysis. Isocratic chromatographic separation was done by passage through a Hypersil BDS-C18 column (150 mm × 4.6 mm i.d., 5 μm; Dalian Elite Analytical Instruments Co. Ltd., China). The mobile phase consisted of 70% (v/v) acetonitrile, 10% (v/v) methyl alcohol and 20% (v/v) water with 10 mmol/L aqueous ammonium acetate for E3S at a flow rate of 0.5 mL/min. An API 3200 triple-quadruple mass spectrometer (Applied Biosystems, Canada) was operated with a TurboIonSpray interface in negative ion mode for E3S. The optimized truncated MRM fragmentation transitions were m/z 348.9 → 268.9 with CE of −45 eV for E3S and m/z 295.6 → 204.8 with CE of −50 eV for IS. The analysis of dioscin

Table 1
Sequences of primers used for real-time RT-PCR analysis.

Gene	GenBank#	Forward primer (5′-3′)	Reverse primer (5′-3′)
Ntcp	NM_017047	TGCACCATAGGGATCGTCCTC	GATGCTGTTGCCCACATTGA
Oatp1a1	NM_017111	AGACCTGTGGTGATTGGTATTGGA	AGTTGCCTGTAGGTGAGATCGTTG
Oatp1a4	U88036	TGCGGAGATGAAGCTTACC	TCCTCCGTCACTTTCGACCTT
Oatp1b2	AJ 271682	AGACGTTCCCATCACAACCAC	GCCTCTGCAGCTTTCCTTGA
Mrp2	NM_012833	ATCGCTCACAGGCTGCACAC	TCAGGACTGCCATACTCGACAATC
Bsep	NM_031760	GCCATTGTGCGAGATCCTAAA	TGCAGGTCCGACCCTCTCT
SHP	D86580	CTCGGTTTGCATACAGTGTTTGAC	GCATATTGGCCTGGAGGTTTT
beta-actin	NM_031144	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

and inulin by LC-MS/MS was done as described previously (Lin et al., 2010; Zhang et al., 2013).

2.10. Data analysis

Data obtained from LUI studies were expressed as percentage of LUI, which represents the ratio of the hepatic extraction of dioscin to that of inulin (Zhang et al., 2013). The hepatic extraction ratio (E_h) of dioscin in perfused rat liver was calculated as[6]:

$$E_h = 1 - F_h \quad (1)$$

$$F_h = C_{out}/C_{in} \quad (2)$$

where C_{out} and C_{in} are the concentration of dioscin in the effluent perfusate and the influent perfusate, respectively. F_h represents the bioavailability of liver.

Kinetic parameters for dioscin uptake in rat hepatocytes were calculated by specific uptake values of each concentration using non-linear, least-squares regression analysis via the Michaelis–Menten equation as described previously (Zhang et al., 2013).

Each experimental point represents the mean ± S.D. of 3–6 measurements. All statistical analysis was done with the SPSS11.5 package (SPSS Inc., Chicago, IL). To test for statistically significant differences among multiple treatments for a given parameter, one-way analysis of variance (ANOVA) with Dunnett's multiple comparison tests was used for comparison among various groups. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Protective effect of dioscin on ANIT-induced hepatitis

To understand whether dioscin could improve ANIT-induced hepatitis, we determined the change in the levels of BIL, ALT and AST in rat

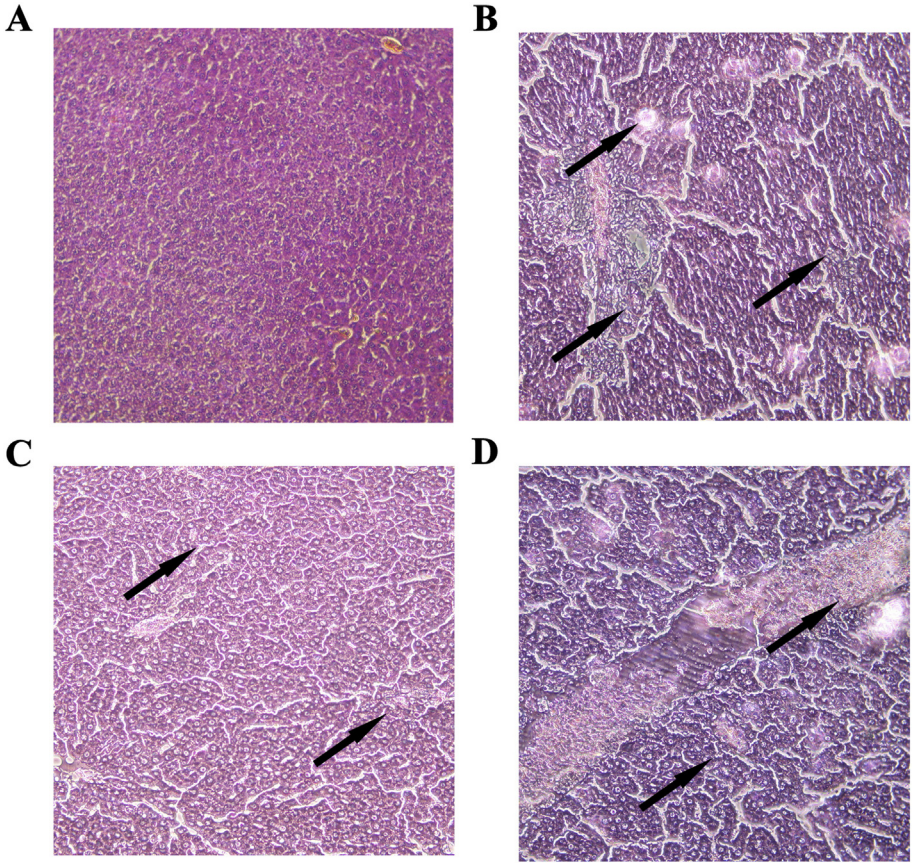

Fig. 2. Effect of dioscin on liver histochemistry 48 h after ANIT treatment in rats. (A) Control group; (B) ANIT only group (50 mg/kg); (C) Dioscin-treated group after ANIT treatment; (D) ANIT plus CMC-Na group. Dioscin was administrated i.g. (50 mg/kg) 24 h, 12 h before and 0.5, 20 and 40 h after ANIT treatment (50 mg/kg). (n = 4 rats per group). H&E, magnification ×200.

serum. When dioscin was administered at 25–100 mg/kg, the ANIT-induced elevation of BIL, ALT and AST in serum was decreased in a dose-dependent manner, and significant effects were observed in all the three Dioscin-treated groups although 50 mg/kg could function better than the 25 mg/kg (Fig. 1). Therefore, we select the dose of 50 mg/kg to elucidate the effect of dioscin on ANIT-induced hepatitis.

3.2. Effect of dioscin on the changes of histochemistry in ANIT-treated rats

To further confirm the effect of dioscin on ANIT-induced hepatitis, we examined the histological changes in rat liver. The portal areas and hepatic lobuli were shrunk with mononuclear and polymorphonuclear cells and massive necrosis with cytoplasmic swelling of most hepatocytes was observed in ANIT-treated rats by microscopic examination (Fig. 2B). Consistent with serology, ANIT-induced hepatocellular edema and necrosis were reduced in animals receiving dioscin administration (Fig. 2C). Arrows indicate necrosis in ANIT-treated animals, while in ANIT plus Dioscin-treated animals areas of cell loss were also reduced (Fig. 2).

3.3. Effect of ANIT on the pharmacokinetic characterization of Oatp substrates in rat

To understand whether ANIT-induced hepatitis could result in functional alteration in sodium-independent transport of the substrate for Oatps, we determined the pharmacokinetics of dioscin *in vivo*, *in situ* and *in vitro*.

A LUI study *in vivo* was performed with or without treatment of ANIT to evaluate the hepatic extraction of dioscin. The LUI value (Fig. 3A) was decreased significantly in ANIT-treated group (7.3 ± 1.2%) in comparison of control (14.1 ± 1.4%).

After perfusion of dioscin (2.5 μM) into control or ANIT-treated liver *in situ*, the effluent perfusate samples were collected at designate time. The hepatic extraction ratio in ANIT-treated liver was lower than control (15.3 ± 1.1%) with the value of 8.8 ± 0.9% (Fig. 3B).

To avoid the impact of physiological conditions, we used isolated rat hepatocytes *in vitro* to further investigate the mechanism for the changes in the hepatic uptake of dioscin in ANIT-treated rats. The V_{max} value of dioscin in ANIT-treated rats was significantly reduced compared with that of control group (Fig. 3C) (407 vs. 592 pmol/min/10^6 cells). The difference of K_m between control (3.84 μM) and ANIT-treated group (3.63 μM) was not observed.

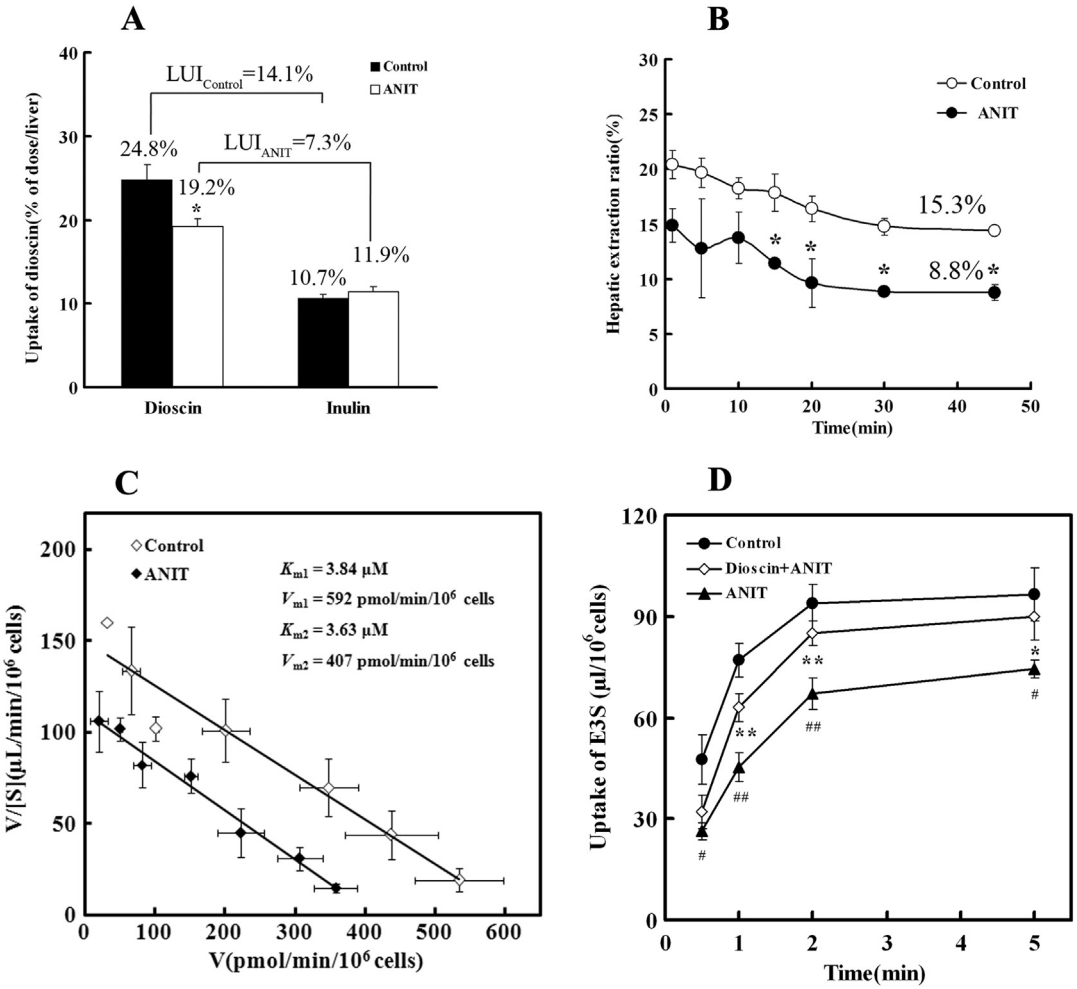

Fig. 3. The hepatic extraction ratio of dioscin in liver uptake index (LUI), perfused rat liver experiment and the uptake of dioscin, E3S by isolated rat hepatocytes. A: Dioscin or inulin was injected rapidly into the portal vein in control or ANIT-treated group, liver was excised, and concentration of dioscin or inulin was measured by LC-MS/MS as described in the text. Each value was normalized by the total injected dose. B: Rat livers were perfused with 2.5 μM dioscin in control, ANIT-treated group and the hepatic extraction ratio was calculated from the concentration of dioscin in the effluent and the influent perfusate. *$p < 0.05$ vs. control group ($n = 4$ rats per group). C: The uptake of dioscin for Eadie–Hofstee plot analysis by isolated rat hepatocytes was measured at a concentration between 0.2 μM and 25 μM (0.2, 0.5, 1, 2, 5, 10, 25 μM) in control and ANIT-treated group. D: Uptake of E3S (1 μM) was measured by isolated rat hepatocytes in control, ANIT-treated group and Dioscin + ANIT group. #$p < 0.05$, ##$p < 0.01$ vs. control group; *$p < 0.05$, **$p < 0.01$ vs. ANIT only group ($n = 4$ rats per group).

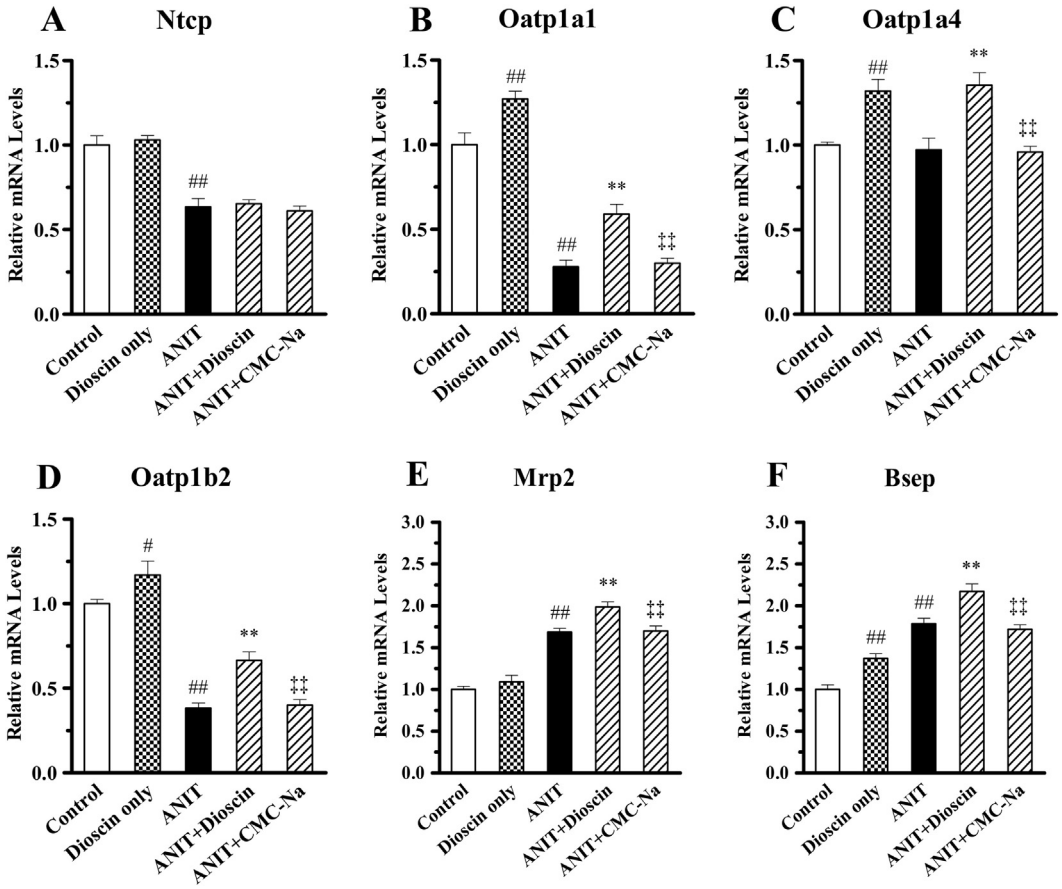

Fig. 4. The effect of dioscin on the mRNA expression of uptake (A–D) and efflux (E–F) transporters 48 h after ANIT treatment. The bar graph shows the relative expression ratio of each mRNA calculated after normalization by β-actin, a housekeeping gene. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. ANIT only group; $^{‡}p < 0.05$, $^{‡‡}p < 0.01$vs. ANIT + Dioscin group (n = 4 rats per group).

In addition, we also investigated the uptake of typical Oatps substrate (E3S) in control and ANIT-treated rats. The uptake of E3S in ANIT-treated rats was reduced significantly in comparison of control group (Fig. 3D). Moreover, the uptake of E3S was increased in ANIT + Dioscin group in contrast to ANIT-treated group but not completely recovered to control (Fig. 3D).

3.4. Effect of dioscin on the mRNA and protein levels of hepatic tansporters after ANIT treatment

To examine whether the changes of pharmacokinetics for dioscin and E3S in ANIT-treated rats were related to the expression of hepatic transporters, we investigated the changes in mRNA of hepatic uptake and efflux transporters by qRT-PCR. For uptake transporters, ANIT treatment reduced mRNA expression of Oatp1a1, Oatp1b2 and Ntcp (Fig. 4). In ANIT + Dioscin group, the mRNA levels increased in comparison of ANIT only group (Fig. 4). Compared with control group, dioscin alone markedly up-regulated the expression of Oatp1a1, Oatp1a4 and Oatp1b2 (Fig. 4). For efflux transporters, ANIT treatment increased mRNA levels of Mrp2 and Bsep (Fig. 4E and F). In ANIT + Dioscin group, the expression of Mrp2 and Bsep was increased further in contrast to ANIT only group.

As shown in Fig. 5, ANIT-induced cholestasis led to a decrease of Oatp1a1 and Oatp1b2, but not Oatp1a4. Dioscin treatment in ANIT-induced cholestasis resulted in a partial restoration of Oatp1a1 and Oatp1b2 protein levels, and an increase of Oatp1a4 protein level was also observed in ANIT plus Dioscin group. For efflux transporters, we found that the protein levels of Mrp2 and Bsep were markedly increased in ANIT treatment group, and further increases were observed in ANIT + Dioscin group (Fig. 6).

3.5. Effect of dioscin on the mRNA of SHP in ANIT-induced cholestasis rats

To elucidate the mechanism of up-regulation of Mrp2 and Bsep by dioscin in ANIT-induced cholestasis rats, the expression of SHP was examined (Fig. 7). After ANIT treatment, the expression of SHP was increased compared to control. In ANIT + Dioscin group, further increase was observed compared to ANIT only group. In dioscin only group, SHP expression was also up-regulated by dioscin.

4. Discussion

The expression and function of transporters play an important role in treatment of some disease through restoring the function of excreting toxicants, such as acute renal failure and cholestasis (Liu et al., 2012b; Rost et al., 2003). Previous publications showed that the regulation of hepatic transporters might be a new therapeutic target which was closely linked to cholestasis treatment (Cui et al., 2009; Kong et al., 2012; Tanaka et al., 2009). In present study, we demonstrated that dioscin held the potential to protect against ANIT-induced cholestasis, as evidenced by improved liver pathology, as well as a significant reduction of ALT/AST/bilirubin in serum. Further *in vivo* and *in vitro* studies indicated that the hepatic protective effect of dioscin against ANIT-induced cholestasis was possibly due to regulating the expression and function of hepatic transporters.

Many publications reported expression of hepatic uptake and efflux transporters would be changed after cholestasis (Cui et al., 2009; Mennone et al., 2010). However, there are limit data about the pharmacokinetics change of drugs *in situ* or *in vivo* in association with cholestasis. Therefore, this challenge drives us to examine the relationships of

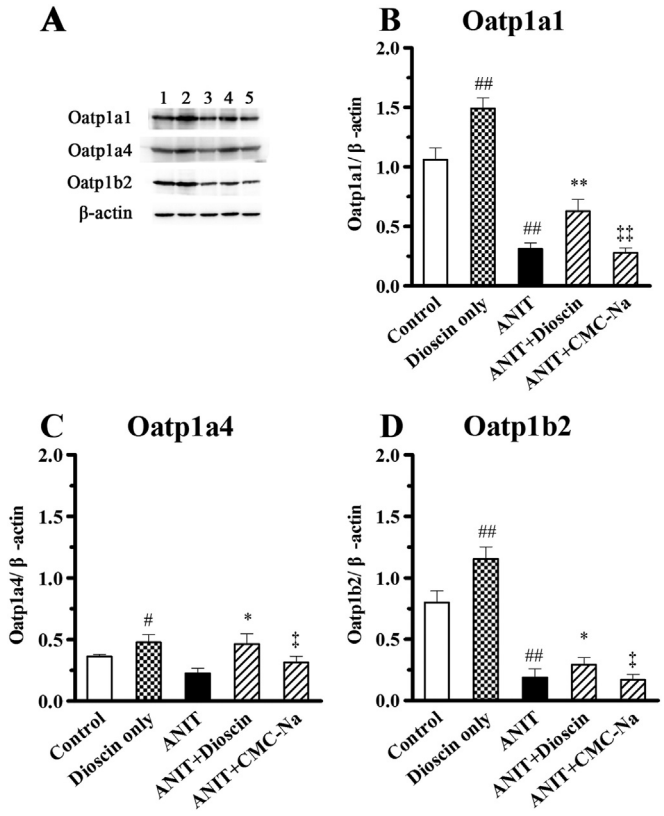

Fig. 5. The effect of dioscin on the protein expression of Oatp1a1, Oatp1a4 and Oatp1b2 in rats. For A: Lane 1: control group; lane 2: dioscin alone group (50 mg/kg); lane 3: ANIT alone group; lane 4: ANIT + Dioscin; lane 5: ANIT + CMC-Na. The bar graph shows the relative expression ratio of each protein calculated after normalization by β-actin protein. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. ANIT only group; $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$ vs. ANIT + Dioscin group (n = 3 rats per group).

changes in pharmacokinetics of drugs and expression of Oatps with

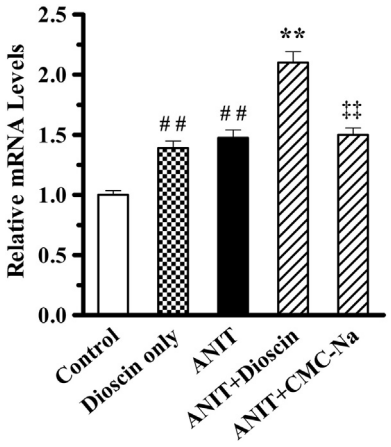

Fig. 7. The effect of dioscin on the mRNA expression of SHP in rats. The bar graph shows the relative expression ratio of each mRNA calculated after normalization by β-actin, a housekeeping gene. $^{\#\#}p < 0.01$ vs. control group; $^{**}p < 0.01$ vs. ANIT only group; $^{\ddagger\ddagger}p < 0.01$ vs. ANIT + Dioscin group (n = 4 rats per group).

ANIT-induced cholestasis. In present study, dioscin was selected as a model substrate of multiple Oatps (including Oatp1a1, Oatp1a4 and Oatp1b2) (Zhang et al., 2013). The decreases of LUI value and hepatic extraction ratio (Fig. 3A and B) might result from the decline of expression of hepatic Oatps. Then, dioscin uptake study using isolated rat hepatocytes indicated that compared to control group, the V_{max} value was decreased, without affecting K_m in ANIT-induced cholestasis (Fig. 3C). As we know, K_m value represents the transport function of transporter, while V_{max} value means the expression of transporter (Ashida et al., 2004; Miao et al., 2011). In other words, the reduction of V_{max} value for dioscin indicated the expression of Oatps might be decreased in ANIT-induced cholestasis. Moreover, the decrease of E3S (a prototypical substrate of Oatp1a1, Oatp1a4 and Oatp1b2) uptake in hepatocytes of cholestasis rats demonstrated the expression of Oatps might be decreased (Fig. 3D). These phenomena were consistent with that the expression of Ntcp, Oatp1a1, and Oatp1b2 in cholestasis rats was

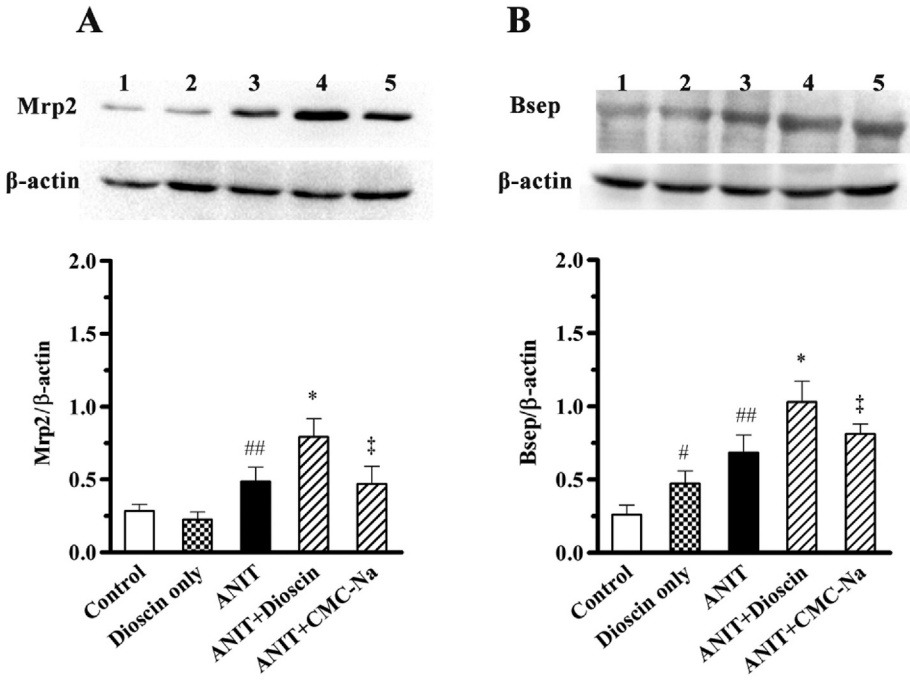

Fig. 6. The effect of dioscin on the protein expression of Mrp2 and Bsep in rats. For A and B: Lane 1: control group; lane 2: dioscin alone group (50 mg/kg); lane 3: ANIT alone group; lane 4: ANIT + Dioscin; lane 5: ANIT + CMC-Na. The bar graph shows the relative expression ratio of each protein calculated after normalization by β-actin protein. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control group; $^{*}p < 0.05$ vs. ANIT only group; $^{\ddagger}p < 0.05$ vs. ANIT + Dioscin group (n = 3 rats per group).

reduced in comparison of control (Figs. 4, 5). The reduction of expression of uptake transporters in basolateral membrane might result in the impairment of hepatic secretory function (Rost et al., 2003).

Then, dioscin was proved to protect against ANIT-induced cholestasis, as evidenced by improved liver pathology and decreased serum ALT/AST/bilirubin significantly (Figs. 1, 2). Our results of dioscin treatment for cholestasis rats indicated the expression of Oatp1a1 and Oatp1b2 was partially restored (Figs. 4, 5). Therefore, administration of dioscin might lead to a partial recovery of secretory function. Rost et al. (Rost et al., 2003) reported ursodeoxycholic acid treatment led to complete recovery of ^{14}C-taurocholic acid secretory rate in cholic acid induced cholestasis rats which might through the restoration of hepatic uptake transporters. A recent study also reports the danning tablets showed similar effect on the expression of Ntcp and Oatp1a1 in ANIT-induced cholestasis rats (Ding et al., 2014). It was reported that the substrate of transporter might up-regulate the expression of its transporter (Guo et al., 2013; Liu et al., 2012b). For example, a previous study found that the signal for human peptide transporter 1 (PEPT1) upregulation might be the dipeptide (the substrate of PEPT1) itself, or changes in levels of the constituent amino acids in the culture medium (Walker et al., 1998). Thamotharan et al. (Thamotharan et al., 1998) showed that dipeptides were able to stimulate their own transport by increasing the membrane population of Pept1. Guo et al. (Guo et al., 2013) reported organic anion transporter 1 (Oat1) and Oat3 were regulated by JBP485 (a substrate of Oat1 and Oat3) via substrate inducing. In addition, Geier et al. (Geier et al., 2007) reported TNF-α-dependent signals mediated the down-regulation of Oatp1a1 at both protein and mRNA levels during obstructive cholestasis. Therefore, the specific mechanism for upregulation of Oatps by dioscin needs to be investigated in future.

Cholestasis was closely linked to not only hepatic uptake transporters but also the canalicular efflux transporters (Cui et al., 2009; Kong et al., 2012; Tanaka et al., 2009). Publications showed that Mrp2 and Besp played an important role in excretion of ANIT-GSH and other biliary constituents (Tanaka et al., 2009). In comparison of down-regulation of uptake transporters, the expression of Mrp2 and Bsep was induced by ANIT treatment. Moreover, further up-regulation of Mrp2, Bsep and SHP occurred in the ANIT + Dioscin group in contrast to cholestasis model (Figs. 4, 6, 7). Taken together, these data indicated that the hepatoprotective effect of dioscin against ANIT-induced cholestasis may be due to enhancement of bile acid export, probably via upregulation of Mrp2 and Bsep. This phenomenon is of great interest, while Mrp2 and Bsep have been shown to be regulated by the FXR (Chen et al., 2013; Cui et al., 2009). Similarity to previous studies (Cui et al., 2009; Liu et al., 2003), we proved that FXR was activated by dioscin, as evidenced by increased mRNA expression of SHP. And this activation of FXR by dioscin might contribute in part to the up-regulation of Mrp2 and Bsep (Figs. 4, 6), which was likely to protect against liver injury through clearing toxic bile constituents from the liver.

The ANIT rat model of cholestasis has been used extensively to study cholestatic liver disease. In agreement with previous studies (Cui et al., 2009; Tanaka et al., 2009), we presented evidence that the expression of Bsep and Mrp2 were increased by ANIT treatment (Figs. 4 and 6). Interestingly, the results were contradictory to ANIT-reduced Mrp2 and Bsep expression reported in a recent study (Ou et al., 2016) as well as unchanged Bsep, Mrp2 and SHP expression reported in a previous study on FXR agonist GW4064 (Liu et al., 2003). In addition, bile duct ligation or lipopolysaccharide (LPS) treatment resulted in an IL-1β–mediated retinoic acid receptor alpha (RARα)/retinoid X receptor alpha (RXRα) downregulation, which in turn decreased Mrp2 transcription in rats (Denson et al., 2002; Takikawa, 2002). Wagner et al. (Wagner et al., 2010) reported inflammatory cholestasis, sepsis, and obstructive cholestasis could decrease Mrp2 expression by a cytokine-induced repression of transcriptional networks *in vitro* and in rodents. Indeed, the activity of ABC transporters, regulated by nuclear receptors and various posttranscriptional modifications, could either protect or damage cells of the hepatobiliary system (Halilbasic et al., 2013).

It is worth noting that the spectrum of diseases caused by defects of ABC transporter proteins in clinical is diverse and includes the liver diseases: progressive familial intrahepatic cholestasis (PFIC, defect of BSEP or P-glycoprotein 3) (Thompson and Jansen, 2000), Dubin–Johnson syndrome (defect of MRP2) (Devgun et al., 2012; Kartenbeck et al., 1996). Therefore, the induced expression of Mrp2 and Bsep in present or previous studies (Chen et al., 2014; Liu et al., 2003) may contribute to the hepatoprotection by enhancing bile acid output.

In summary, dioscin could relieve liver injury by restoring hepatic transporters expression and function partially. This therapeutic effect might be related to the up-regulated expression and function of Oatps, Mrp2 and Bsep. In addition, the up-regulation of Mrp2 and Bsep might be through activating FXR. Therefore, dioscin may be a candidate drug for the prevention of intrahepatic cholestasis.

Conflict of interest statement

All authors declare that there are no conflicts of interest in this study.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

This work was supported partly by a grant from the National Natural Science Foundation of China [no. 81072694, 81273580] and Dalian Government [no. 2010E12SF060].

References

Ashida, K., Katsura, T., Saito, H., Inui, K., 2004. Decreased activity and expression of intestinal oligopeptide transporter PEPT1 in rats with hyperthyroidism in vivo. Pharm. Res. 21, 969–975.

Chen, W.D., Wang, Y.D., Zhang, L., Shiah, S., Wang, M., Yang, F., Yu, D., Forman, B.M., Huang, W., 2010. Farnesoid X receptor alleviates age-related proliferation defects in regenerating mouse livers by activating forkhead box m1b transcription. Hepatology 51, 953–962.

Chen, Y., Song, X., Valanejad, L., Vasilenko, A., More, V., Qiu, X., Chen, W., Lai, Y., Slitt, A., Stoner, M., Yan, B., Deng, R., 2013. Bile salt export pump is dysregulated with altered farnesoid X receptor isoform expression in patients with hepatocellular carcinoma. Hepatology 57, 1530–1541.

Chen, P., Zeng, H., Wang, Y., Fan, X., Xu, C., Deng, R., Zhou, X., Bi, H., Huang, M., 2014. Low dose of oleanolic acid protects against lithocholic acid-induced cholestasis in mice: potential involvement of nuclear factor-E2-related factor 2-mediated upregulation of multidrug resistance-associated proteins. Drug Metab. Dispos. 42, 844–852.

Cui, Y.J., Aleksunes, L.M., Tanaka, Y., Goedken, M.J., Klaassen, C.D., 2009. Compensatory induction of liver efflux transporters in response to ANIT-induced liver injury is impaired in FXR-null mice. Toxicol. Sci. 110, 47–60.

Del, P.A., Scalera, A., Iadevaia, M.D., Miranda, A., Zulli, C., Gaeta, L., Tuccillo, C., Federico, A., Loguercio, C., 2012. Herbal products: benefits, limits, and applications in chronic liver disease. Evid. Based Complement. Alternat. Med. 2012, 837939.

Denson, L.A., Bohan, A., Held, M.A., Boyer, J.L., 2002. Organ-specific alterations in RAR alpha:RXR alpha abundance regulate rat Mrp2 (Abcc2) expression in obstructive cholestasis. Gastroenterology 123, 599–607.

Devgun, M.S., El-Nujumi, A.M., O'Dowd, G.J., Barbu, V., Poupon, R., 2012. Novel mutations in the Dubin-Johnson syndrome gene ABCC2/MRP2 and associated biochemical changes. Ann. Clin. Biochem. 49, 609–612.

Dietrich, C.G., Ottenhoff, R., de, W.D.R., Oude, E.R.P., 2001. Role of MRP2 and GSH in intrahepatic cycling of toxins. Toxicology 167, 73–81.

Ding, L., Zhang, B., Zhan, C., Yang, L., Wang, Z., 2014. Danning tablets attenuates α-naphthylisothiocyanate-induced cholestasis by modulating the expression of transporters and metabolic enzymes. BMC Complement. Altern. Med. 14, 249.

Geier, A., Dietrich, C.G., Trauner, M., Gartung, C., 2007. Extrahepatic cholestasis downregulates Oatp1 by TNF-alpha signalling without affecting Oatp2 and Oatp4 expression and sodium-independent bile salt uptake in rat liver. Liver Int. 27, 1056–1065.

Giacomini, K.M., Huang, S.M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L., Chu, X., Dahlin, A., Evers, R., Fischer, V., Hillgren, K.M., Hoffmaster, K.A., Ishikawa, T., Keppler, D., Kim, R.B., Lee, C.A., Niemi, M., Polli, J.W., Sugiyama, Y., Swaan, P.W., Ware, J.A., Wright, S.H., Yee, S.W., Zamek-Gliszczynski, M.J., Zhang, L., International Transporter Consortium, 2010. Membrane transporters in drug development. Nat. Rev. Drug Discov. 9, 215–236.

Guo, X., Meng, Q., Liu, Q., Wang, C., Sun, H., Peng, J., Ma, X., Kaku, T., Liu, K., 2013. JBP485 improves gentamicin-induced acute renal failure by regulating the expression and function of Oat1 and Oat3 in rats. Toxicol. Appl. Pharmacol. 271, 285–295.
Hagenbuch, B., 2007. Cellular entry of thyroid hormones by organic anion transporting polypeptides. Best Pract. Res. Clin. Endocrinol. Metab. 21, 209–221.
Halilbasic, E., Baghdasaryan, A., Trauner, M., 2013. Nuclear receptors as drug targets in cholestatic liver diseases. Clin. Liver Dis. 17, 161–189.
Hayashi, H., Mizuno, T., Horikawa, R., Nagasaka, H., Yabuki, T., Takikawa, H., Sugiyama, Y., 2012. 4-Phenylbutyrate modulates ubiquitination of hepatocanalicular MRP2 and reduces serum total bilirubin concentration. J. Hepatol. 56, 1136–1144.
Kartenbeck, J., Leuschner, U., Mayer, R., Keppler, D., 1996. Absence of the canalicular isoform of the MRP gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. Hepatology 23, 1061–1066.
Kobori, M., Masumoto, S., Akimoto, Y., Oike, H., 2011. Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice. Mol. Nutr. Food Res. 55, 530–540.
Kong, B., Csanaky, I.L., Aleksunes, L.M., Patni, M., Chen, Q., Ma, X., Jaeschke, H., Weir, S., Broward, M., Klaassen, C.D., Guo, G.L., 2012. Gender-specific reduction of hepatic Mrp2 expression by high-fat diet protects female mice from ANIT toxicity. Toxicol. Appl. Pharmacol. 261, 189–195.
Lin, C.C., Kuo, C.W., Pao, L.H., 2010. Development and validation of a liquid chromatography-tandem mass spectrometry method for simultaneous quantification of p-aminohippuric acid and inulin in rat plasma for renal function study. Anal. Bioanal. Chem. 398, 857–865.
Liu, Y., Binz, J., Numerick, M.J., Dennis, S., Luo, G., Desai, B., MacKenzie, K.I., Mansfield, T.A., Kliewer, S.A., Goodwin, B., Jones, S.A., 2003. Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models of intra- and extrahepatic cholestasis. J. Clin. Invest. 112, 1678–1687.
Liu, M.J., Wang, Z., Ju, Y., Zhou, J.B., Wang, Y., Wong, R.N., 2004. The mitotic-arresting and apoptosis-inducing effects of diosgenyl saponins on human leukemia cell lines. Biol. Pharm. Bull. 27, 1059–1065.
Liu, T., Guo, X., Meng, Q., Wang, C., Liu, Q., Sun, H., Ma, X., Kaku, T., Liu, K., 2012a. Effect of JBP485 on obstructive jaundice is related to regulation of renal Oat1, Oat3 and Mrp2 expression in ANIT-treated rats. Peptides 36, 78–85.
Liu, T., Meng, Q., Wang, C., Liu, Q., Guo, X., Sun, H., Peng, J., Ma, X., Kaku, T., Liu, K., 2012b. Changes in expression of renal Oat1, Oat3 and Mrp2 in cisplatin-induced acute renal failure after treatment of JBP485 in rats. Toxicol. Appl. Pharmacol. 264, 423–430.
Lu, B., Xu, Y., Xu, L., Cong, X., Yin, L., Li, H., Peng, J., 2012. Mechanism investigation of dioscin against CCl4-induced acute liver damage in mice. Environ. Toxicol. Pharmacol. 34, 127–135.
Maeda, K., Sugiyama, Y., 2010. The use of hepatocytes to investigate drug uptake transporters. Methods Mol. Biol. 640, 327–353.
Mennone, A., Soroka, C.J., Harry, K.M., Boyer, J.L., 2010. Role of breast cancer resistance protein in the adaptive response to cholestasis. Drug Metab. Dispos. 38, 1673–1678.
Miao, Q., Liu, Q., Wang, C., Meng, Q., Guo, X., Peng, J., Kaku, T., Liu, K., 2011. Inhibitory effect of zinc on the absorption of JBP485 via the gastrointestinal oligopeptide transporter (PEPT1) in rats. Drug Metab. Pharmacokinet. 26, 494–502.
Naoi, S., Hayashi, H., Inoue, T., Tanikawa, K., Igarashi, K., Nagasaka, H., Kage, M., Takikawa, H., Sugiyama, Y., Inui, A., Nagai, T., Kusuhara, H., 2014. Improved liver function and relieved pruritus after 4-phenylbutyrate therapy in a patient with progressive familial intrahepatic cholestasis type 2. J. Pediatr. 164, 1219–1227.
Ou, Q.Q., Qian, X.H., Li, D.Y., Zhang, Y.X., Pei, X.N., Chen, J.W., Yu, L., 2016. Yinzhihuang attenuates ANIT-induced intrahepatic cholestasis in rats through upregulation of Mrp2 and Bsep expressions. Pediatr. Res. 79, 589–595.
Papay, J.I., Clines, D., Rafi, R., Yuen, N., Britt, S.D., Walsh, J.S., Hunt, C.M., 2009. Drug-induced liver injury following positive drug rechallenge. Regul. Toxicol. Pharmacol. 54, 84–90.
Rost, D., Herrmann, T., Sauer, P., Schmidts, H.L., Stieger, B., Meier, P.J., Stremmel, W., Stiehl, A., 2003. Regulation of rat organic anion transporters in bile salt-induced cholestatic hepatitis: effect of ursodeoxycholate. Hepatology 38, 187–195.
Slitt, A.L., Allen, K., Morrone, J., Aleksunes, L.M., Chen, C., Maher, J.M., Manautou, J.E., Cherrington, N.J., Klaassen, C.D., 2007. Regulation of transporter expression in mouse liver, kidney, and intestine during extrahepatic cholestasis. Biochim. Biophys. Acta 1768, 637–647.
Takikawa, H., 2002. Hepatobiliary transport of bile acids and organic anions. J. Hepato-Biliary-Pancreat. Surg. 9, 443–447.
Tanaka, Y., Aleksunes, L.M., Cui, Y.J., Klaassen, C.D., 2009. ANIT-induced intrahepatic cholestasis alters hepatobiliary transporter expression via Nrf2-dependent and independent signaling. Toxicol. Sci. 108, 247–257.
Thamotharan, M., Bawani, S.Z., Zhou, X., Adibi, S.A., 1998. Mechanism of dipeptide stimulation of its own transport in a human intestinal cell line. Proc. Assoc. Am. Physicians 110, 361–368.
Thompson, R., Jansen, P.L., 2000. Genetic defects in hepatocanalicular transport. Semin. Liver Dis. 20, 365–372.
Wagner, M., Zollner, G., Trauner, M., 2010. Nuclear receptor regulation of the adaptive response of bile acid transporters in cholestasis. Semin. Liver Dis. 30, 160–177.
Walker, D., Thwaites, D.T., Simmons, N.L., Gilbert, H.J., Hirst, B.H., 1998. Substrate upregulation of the human small intestinal peptide transporter, hPepT1. J. Physiol. 507, 697–706.
Xu, T., Zhang, S., Zheng, L., Yin, L., Xu, L., Peng, J., 2012. A 90-day subchronic toxicological assessment of dioscin, a natural steroid saponin, in Sprague-Dawley rats. Food Chem. Toxicol. 50, 1279–1287.
Zhang, A., Wang, C., Liu, Q., Meng, Q., Peng, J., Sun, H., Ma, X., Huo, X., Liu, K., 2013. Involvement of organic anion-transporting polypeptides in the hepatic uptake of dioscin in rats and humans. Drug Metab. Dispos. 41, 994–1003.
Zhao, X., Cong, X., Zheng, L., Xu, L., Yin, L., Peng, J., 2012. Dioscin, a natural steroid saponin, shows remarkable protective effect against acetaminophen-induced liver damage in vitro and in vivo. Toxicol. Lett. 214, 69–80.