Int J Clin Exp Pathol 2015;8(1):184-195
www.ijcep.com /ISSN:1936-2625/IJCEP0003923

Original Article

Swertianlarin, isolated from *Swertia mussotii Franch*, increases detoxification enzymes and efflux transporters expression in rats

Xin-Chan Feng[1*], Xiaohuang Du[2*], Sheng Chen[3], Dongmei Yue[4], Ying Cheng[1], Liangjun Zhang[1], Yu Gao[1], Shaoxue Li[1], Lei Chen[1], Zhihong Peng[1], Yong Yang[5], Weizao Luo[5], Rongquan Wang[1], Wensheng Chen[1], Jin Chai[1]

Departments of [1]Gastroenterology, [2]Traditional Chinese Medicine, [3]Pediatrics, Southwest Hospital, [4]Employee Health, Third Military Medical University, Chongqing 400038; [5]Chongqing Academy of Chinese Material Medical, Chongqing 400065, P. R. China. [*]Equal contributors.

Received November 17, 2014; Accepted December 24, 2014; Epub January 1, 2015; Published January 15, 2015

Abstract: Swertianlarin, isolated from *Swertia mussotii Franch* and *Enicostemma axillare*, has hepatoprotective effects against cholestasis in rat models of hepatotoxicity. However, the underlying molecular mechanism is not clear. We then treated rats with swertianlarin for 7 d and then measured serum liver injury markers, lipids, and bile salts, as well as the expression of bile acid synthesis and detoxification enzymes (e.g. Cyp7a1 and Cyp3a), membrane influx and efflux transporters (e.g. Ntcp and Mrp3), nuclear receptors (e.g. Pxr and Fxr/Shp) and transcriptional factors (e.g. Nrf2 and Hnf3β) in the liver. We found a significant induction of the expression of the basolateral efflux transporters Mrp3 and Mrp4 and canalicular transporter Mdr1 in rats treated with swertianlarin, compared with the controls (1.9-fold and 2.2-fold, $P < 0.005$, and 3.4-fold, $P < 0.05$, respectively). The expression of detoxification enzymes Cyp3a, Ugt2b, Sult2a1 and Gsta1 in rats treated with swertianlarin was significantly higher than that in controls (3.7-fold, 2.8-fold, 2.1-fold, and 1.7-fold, respectively, all $P < 0.05$). Expression of the synthetic enzyme, Cyp8b1, was higher in rats treated with swertianlarin than that in controls (1.8-fold at mRNA level and 3.4-flod at protein level, $P < 0.05$). Elevated serum levels of the conjugated bile acids, taurocholic acid and taurodeoxycholic acid, and a reduction in levels of serum ALP, unconjugated bile acid αMCA, and TG were observed (all $P < 0.05$). In conclusion, swertianlarin significantly up-regulates hepatic bile acid detoxification enzymes and efflux transporters in rats, which can increase the water solubility of hydrophobic bile acids and elimination of conjugated bile acids.

Keywords: Swertianlarin, synthetic and detoxification enzymes, hepatic efflux transporters, nuclear receptors and transcriptional factors

Introduction

Swertia mussotii Franch is a Chinese traditional medicinal plant that has long been used for the treatment of jaundice resulting from virus hepatitis and gallstone obstruction [1, 2]. Swertianlarin, isolated from *Swertia mussotii Franch*, is a typical iridoid [3, 4] and has hepatoprotective effects similar to that reported previously with swertianlarin isolated from *Enicostemma axillare* [5]. However, the underlying molecular mechanism for the hepatoprotective effects remains unclear.

The accumulation of toxic bile acids in the liver contributes to hepatocellular damage in cholestasis [6, 7]. Liver injury triggers a hepatic adaptive response to attenuate cholestasis, such as repressing hepatic basolateral bile acid uptake and *de novo* bile acid synthesis, increasing water solubility of bile acids, and enhancing basolateral bile acid excretion [6-15]. Down-regulation of influx transporters, such as Na^+/taurocholate cotransporter (Ntcp) and organic anion transporter (Oatp1b1), and up-regulation of bile acid efflux transporters, such as multidrug resistance-associated protein 3 (Mrp3), Mrp4 and organic solute transporter alpha and beta (Ostα/β), are crucial adaptive responses that reduce accumulation of toxic bile acids in cholestatic hepatocytes [6-8, 10-16]. The inhibition of the expression of bile acid synthesis

Table 1. Sense and antisense primers used for real-time qPCR (SYBR Green)

Gene	Accession No.	Sense Primer (5'→3')	Antisense Primer (5'→3')
Cyp7a1	NM_012942.2	GCAAAACCTCCAATCTGTCAT	GCTTCAAACATCACTCGGTAAC
Cyp7b1	NM_019138.1	GGTTCTGAGGTTGTGCTCCTAC	GACTTCTGGGTCATTGTGTATCA
Cyp8b1	NM_031241.1	AGGTTGGAAGCCGAGACAT	TGGCACAGAGACGAAGAGTC
Cyp27a1	NM_178847.2	CAGCAAGAAGGTGAGCCTAC	TGTCTATGGGAAGGGCAGAG
Cyp3a	AB084894.1	CACAAAGACCCAAAGTGCTG	CCATTACCAAAGGGCAGGT
Ugt2b	NM_031533.5	CCCACCACCGTAGATGAGAC	GCAAGGGTTTAGCAGGTTTG
Sult2a1	NM_131903.1	AGGAACGAACTGGCTGATTG	ATGGGAAGATGGGAGGTCAT
Gsta2	NM_017013.4	GCAGGAGTGGAGTTTGATGAG	TTTGGTGGCGATGTAGTTGA
Gsta3	NM_031509.2	GGCTAAGGAATGATGGGAGTT	TGGTGGCAATGTAGTTGAGAA
Gsta4	NM_001106840.1	GCTGGAGTGGAGTTTGAAGAA	GATGGCTCTGGTCTGTGTCA
Gstm1	NM_017014.1	CCCATCTCCTCAACCTCAC	GGGCAGACCTCAAATCACAG
Gstm2	NM_177426.1	TGCTCCCGACTATGACAGAA	CGTCCACACGAATCCTCTC
Gstm3	NM_020540.1	TGGACACTTTGGAGAACCAG	CTTGCCCAGGAACTCTGAAT
Gstm4	NM_001024304.1	TCACACTGGCTCTGGCTTCT	CCCTGCCTATCCAACTGAAAT
Mrp2	NM_012833.1	CCCAGTCTTCGCTATCATCA	GACAGAATCCAACCGTCTCAG
Bsep	NM_031760.1	CGTGCTTGTGGAAGAAGTTG	GGGAGTAGATGGGTGTGACTG
Abcg2	NM_181381.2	CTTACTGGCTTCTGGGAAACTC	AGGGTTGTTGTAGGGCTCAC
Abcg5	NM_053754.2	TAAGATGGCAGGCAGGAAAG	AGCAAAGGACGGTGAGTTCT
Abcg8	NM_130414.2	CTCATCGTCATTGGCATCA	GTGGAAGCAAGGCTGAACAT
Mdr1	NM_012623.2	GAGCCCATCCTGTTTGACTG	TGTCTCCCACTCTGGTGTTG
Mdr2	NM_012690.2	TCAGCAACCAGAGCAGAGAA	GCCCAGGAGCATAAACAAT
Mrp3	NM_080581.1	TTCCGATTCACCACTTTCTACA	GGCAAGGATTTGTGTCAAGATT
Mrp4	NM_133411.1	GAAGGAAAATGAGGAAGCAGAG	GGATGACTGTTGAGACCAAATC
Ostα	NM_001107087.1	AAGTCGGAAGGGTTGGGTAG	ATCCTCTGCTGTGCCATCTC
Ostβ	XM_001076555.1	TTTGGTATTTCCGTTCAGAGG	GCATTCCGTTGTCTTGTGG
Ntcp	NM_017047.1	CTGGCTACCTCCTCCCTGAT	ATGCTGATGGTGCGTCTG
Oatp1	NM_017111.1	TGTATGGAGAACCGAACACAGA	AAGGGCACAATAGGAGTTTCAC
Oct1	NM_012697.1	TGTGGCTTTGCCTGAGACTA	CTTGCCTGTTTGGACCTGAA
Fxr	NM_021745.1	TGAGCGTCTACAGCGAAAGTG	GGGATGGTGGTCTTCAAATAAG
Shp	NM_057133.1	ATCTCTTCTTCCGCCCTGTC	AGGTTTTGGGAGCCATCAAG
Pxr	NM_052980.2	ACATCATCCCTCACCCTTCA	TCAGGTCTCATCTCCAGGTTTA
Car	NM_022941.4	CCAAGGAACTGTGTGGTGTG	CTGGACAATGGCGTCTCTG
Vdr	NM_017058.1	GCCGCCTGTCTGTGTTATTC	GGTCATCTTGGCAGTGAGTG
Pparα	NM_013196.1	CGGTGTGTATGAAGCCATCTT	TCTTTAGGAACTCTCGGGTGAT
Hnf1α	NM_012669.1	AGAGGGAAGCAGGGTGAAG	CACAGAAATCCAGGCAGTCA
Hnf4α	NM_022180.2	TAGCAGAGATGAGCCGTGTG	GCTTTGAGGCAGGCGTATT
Rxrα	NM_012805.2	TTCTCCCACCGCTCCATAG	CGTTAGCACCCTGTCAAAGA
Rarα	NM_031528.2	CACCTGAGCAAGACACAATGA	GCGAAGGCAAAGACCAAGT
Lxr	NM_031626.1	CGCTACAACCACGAGACAGA	GGCAATGAGCAAGGCATACT
Lrh-1	NM_021742.1	ATGGGAAGGAAGGGACAATC	CAAACTGAAGGGAACGGAGTC
Nrf2	NM_031789.2	CCTTCCTCTGCTGCCATTAG	GTGCCTTCAGTGTGCTTCTG
Hnf3β	NM_012743.1	AACAAGATGCTGACGCTGAG	GAATGACGGATGGAGTTCTG
Ahr	NM_013149.2	CAGGACCAGTGTAGAGCACAAG	CTGCCGTGACAACCAGAAC

enzymes Cyp7a1 and Cyp7b1, and induction of the expression of detoxification enzymes Cyp3a, Ugt2b and Sult2a1 contribute to the repression of bile acid synthesis and reduction of bile salt toxicity [6-8, 10, 16, 17]. Inhibiting bile acids synthesis and enhancing the water

solubility and elimination of bile acids alleviate liver injury in cholestatic animal models and human cholestatic patients [17-20]. For example, ursodeoxycholic acid (UDCA), a modulator of pregnane X receptor (Pxr) and NF-E2-related factor (Nrf2), and the only drug approved for the treatment of cholestatic disorder, exerts its anti-cholestatic effect through stimulating canalicular membrane transporters MRP2, hepatic bile salt export pump (BSEP), and basolateral membrane transporters MRP3 and MRP4 [13, 16, 21-25]. In addition, *nor*UDCA, a side chain shortened UDCA derivate, also markedly induces the expression of Phase I and Phase II detoxification enzymes [26]. The agonists of nuclear receptor FXR, such as GW4064 and INT747, attenuate cholestasis through stimulating the expression of MRP2, BSEP, and MDR3 [7, 27, 28]. Thus, we hypothesized that swertianlarin exerts its protective effects on cholestasis through inhibiting the hepatic influx transporters and bile acid synthesis enzymes, and stimulating efflux transporters and detoxification enzymes in the liver.

To test this hypothesis, we determined the serum levels of bile salts and the expressions of genes associated with bile acids homeostasis in rats treated with swertianlarin. Our results may provide a basis for future investigations on the use of swertianlarin in the therapy of human cholestasis.

Materials and methods

Chemicals

Swertianlarin, isolated from *Swertia mussotii Franch*, was provided by Chongqing Academy of Chinese Material Medical, with a purity of 98% as analyzed by HPLC. All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co., St Louis, MO, USA).

Animals and treatments

The animal use and experimental protocols were reviewed and approved by the Ethics Committee of Third Military Medical University, Chongqing, China. Male Sprague-Dawley (SD) rats, weighing 200-250 g, were purchased from the Center of Laboratory Animals of Third Military Medical University, Chongqing, China. The rats were housed in plastic cages individually in temperature-controlled (20-23°C) rooms with a 12/12-h light/dark cycle, with free access to food and water. The animals were allowed one week to adapt to the new environment before experiments. The rats were randomized into two groups (5-7/group); one group was given swertianlarin dissolved in 1% Tween-20 saline (100 mg/kg/day) by gavage for 7 days while the other group was used as a control and given 1% Tween-20 saline for 7 days. After the 7-day treatment, animals were sacrificed in random order. For blood sample collection, the rat heart was opened and the blood sample was placed on ice for 1 h and centrifuged at 8,000 g for 10 min to prepare serum. The serum was immediately stored at -80°C for serum biochemistry and analysis of bile salts. The rat livers were removed, washed with cold PBS, and immediately cut into small pieces and rapidly frozen in liquid nitrogen and kept in liquid nitrogen until analysis.

Serum biochemistry, lipids and bile salt analyses

The following analyses were performed using the corresponding ELISA Kits (BlueGene Biotechnology, Shanghai, China) according to the manufacturer's instructions [29]: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bile salts (TBA), total bilirubin (TBIL), and direct bilirubin (DBIL), chenodeoxycholic acid (CDCA), taurochenodeoxycholic acid (TCDCA), cholic acid (CA), taurocholic acid (TCA), deoxycholic acid (DCA), taurodeoxycholic acid (TDCA), tauroursodeoxycholic acid (TUDCA), tauro-alpha/-beta-muricholic acid (Tα/βMCA), alpha-muricholic acid (αMCA), and beta-muricholic acid (βMCA); and serum lipid triglyceride hydrolase (Tgh), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).

RNA extraction and Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Frozen liver samples (100 mg each) were ground and total RNA was extracted with Trizol agent (Invitrogen, San Diego, CA, USA). The cDNA was prepared and Real-time qPCR was subsequently performed as described previously [10, 11]. The primers used in this study are given in **Table 1**.

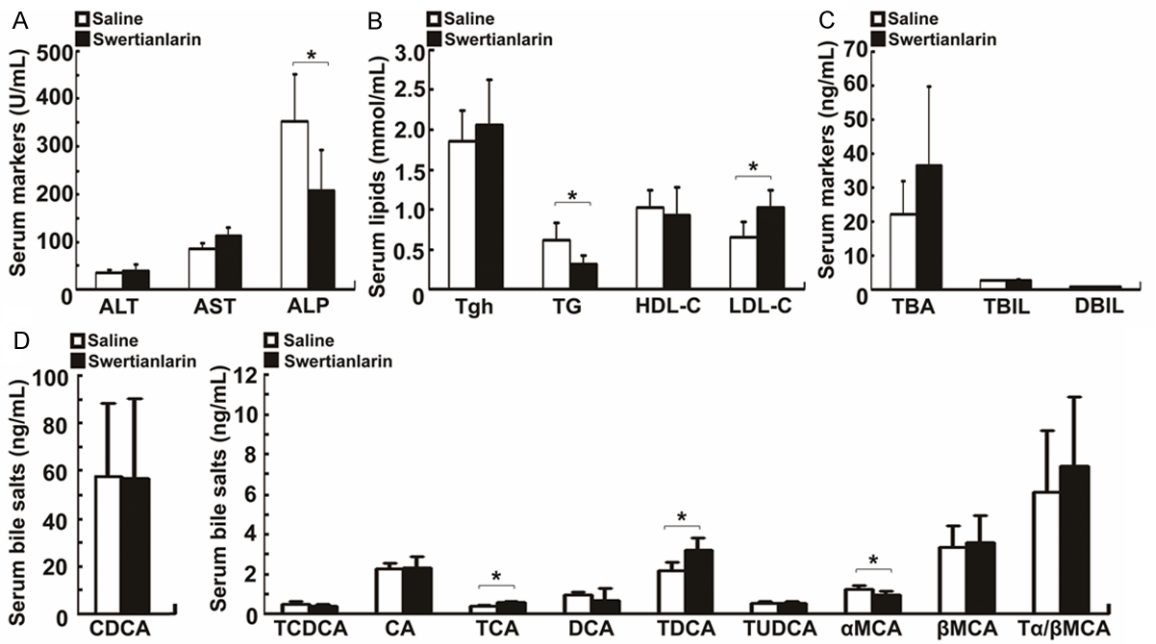

Figure 1. Changes in serum biochemistry, and lipids and bile salt levels in rats treated with swertianlarin compared with the controls. A: Changes in ALT, AST and ALP levels; B: Changes in lipids Tgh, TG, HDL-C and LDL-C; C: Changes in TBIL, DBIL and TBA levels; and D: Changes in serum bile salts CDCA, TCDCA, CA, TCA, DCA, TDCA, TUDCA, Tα/βMCA, αMCA, and βMCA levels. n = 5 per group; $^{*}P < 0.05$.

Western blot analysis

Western-blotting was performed as described previously [10, 11]. The dilution of primary antibodies were as follows: CYP7A1 (1:2000), CYP7B1 (1:2000), CYP8B1 (1:2000), CYP27A1 (1:2000), CYP3A4 (1:4000), UGT2B (1:4000), SULT2A1 (1:2000), GSTA1 (1:1000), GSTM2 (1:1000), MDR1 (multidrug resistance transporter 1) (1:2000), MDR2 (1:2000), OSTα (1:4000), ABCG5 (1:3000), ABCG8 (1:2000), BSEP (1:1000), NTCP (1:800), PXR (1:1000), CAR (1:1000), VDR (1:1000), RXRα (1:1600), RARα (1:1600), HNF1α (hepatocytes nuclear factor 1alpha) (1:2000), HNF4α (1:2000) and LXR (1:1000) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA); GSTA2 (1:4000) (GeneTex, Irvine, CA, USA), GSTM1 (1:1000), PPARα (1:1000), and AhR (1:1000) (Proteintech Group, Chicago, IL, USA), OSTβ (1:500) (Sigma-Aldrich, St Louis, MO, USA), MRP2 (1:2000), ABCG2 (1:2000), FXR (1:10,000), SHP (1:1000), LRH-1 (1:2,000), HNF3β (1:10,000), and NRF2 (1:10,000) (Abcam, Cambridge, MA, USA). GAPDH (1:40,000) (Abcam) was used as the loading control. The band intensities of Western blots were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence analysis

Rat liver tissues embedded in Tissue-Tek O.C.T.™ Compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) were snap-frozen in liquid nitrogen. The frozen block was then used to prepare 6-μm thick sections at -25°C. Immunofluorescence (IF) microscopy was performed with the antibodies of MDR1 (1:100), MRP3 (1:100), MRP4 (1:100), PXR (1:50), VDR (1:50), HNF1α (1:50), RARα (1:50) and Nrf2 (1:100) as previously described [10, 11].

Statistical analysis

All data are expressed as the means ± standard deviations (SDs) and analyzed using the independent-samples Student's t test (two-tailed) using the SPSS software (PASW Statistics 18, IBM; SPSS, Inc., Chicago, IL, USA). A P value of < 0.05 was considered statistically significant.

Results

Swertianlarin reduces serum ALP levels and alters bile salts concentrations

Figure 1A illustrates that the serum ALP level in rats treated with 100 mg/kg/day of swertianla-

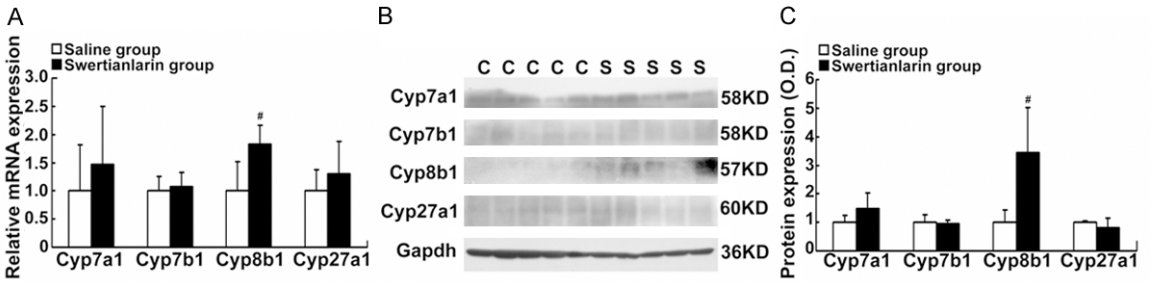

Figure 2. Alteration of bile acid synthetic enzymes Cyp7a1, Cyp7b1, Cyp8b1 and Cyp27a1 in rats treated with swertianlarin, compared with controls. A: The mRNA levels of Cyp8b1, Cyp7a1, Cyp7b1 and Cyp27a1; B: Representative Western blotting for protein levels of Cyp7a1, Cyp7b1, Cyp8b1 and Cyp27a1; and C: Western blot densitometry analyses (% of control group). *C*, the control group; *S*, the swertianlarin group. $^{\#}P < 0.05$. n = 5 per group.

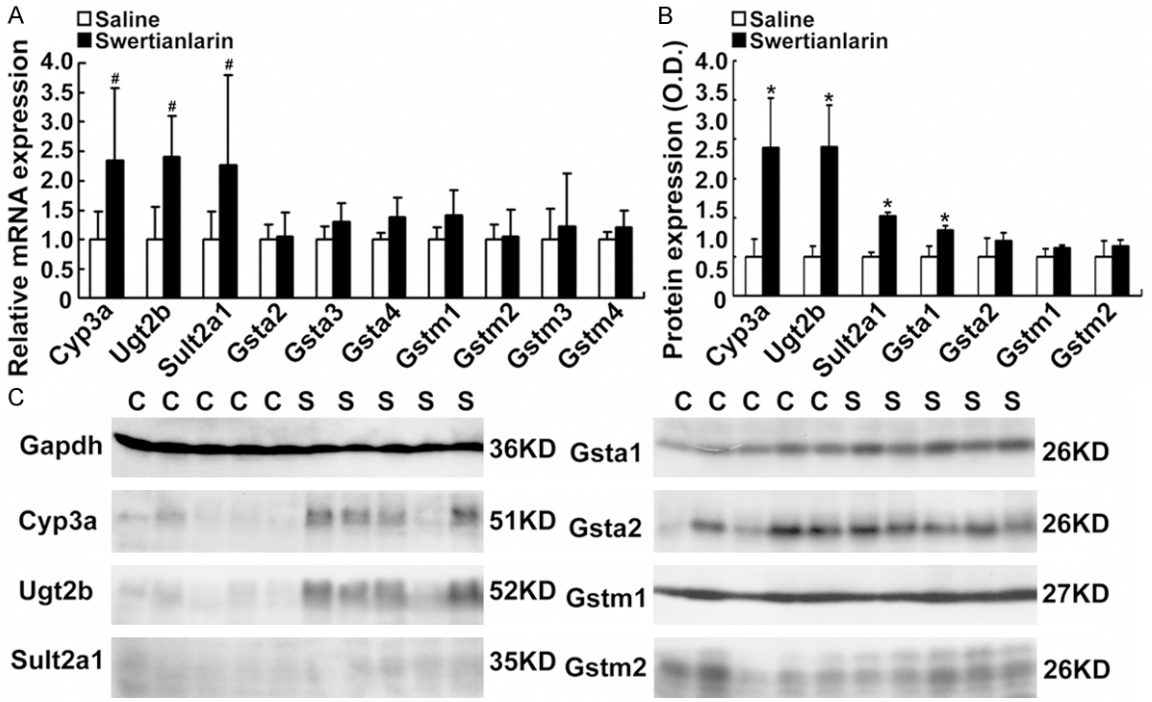

Figure 3. Changes in expression of bile acid detoxification enzymes Cyp3a, Ugt2b, Sult2a1, Gsta1-4 and Gstm1-4, Cyp8b1 and Cyp27a1 in rats treated with swertianlarin, compared with controls. A: The mRNA expression of Cyp3a, Ugt2b, Sult2a1, Gstm1-m4 and Gsta2-4; B: The protein levels of Cyp3a, Ugt2b, Sult2a1, Gsta1, Gsta2, Gstm1 and Gstm2, analyzed by densitometry of the Western blotting (% of control group); and C: Representative Western blots. $^{*}P < 0.01$; $^{\#}P < 0.05$; n = 5 per group. *C*, the control group; *S*, the swertianlarin group.

rin for 7 days was decreased to 59% of the control ($P < 0.05$). However, there were no significant changes in the serum ALT and AST (**Figure 1A**). The concentration of serum TG in the swertianlarin group was reduced to 52% of the control, LDL-C was increased 1.5-fold, and Tgh and HDL-C levels were not significantly altered (**Figure 1B**). The serum TBA tended to increase while TBIL and DBIL were not altered in the swertianlarin group (**Figure 1C**). The serum TCA and TDCA levels were significantly increased while the αMCA level was decreased in the swertianlarin group, compared with the control group (**Figure 1D**). However, CDCA, TCDCA, CA, DCA, TUDCA, βMCA and Tα/βMCA levels were not significantly changed.

Swertianlarin modulates bile acid synthetic enzymes in the rat liver

The Cyp8b1 mRNA expression in the rat liver was induced 1.8-fold by the swertianlarin treatment, compared with that of the control ($P <$

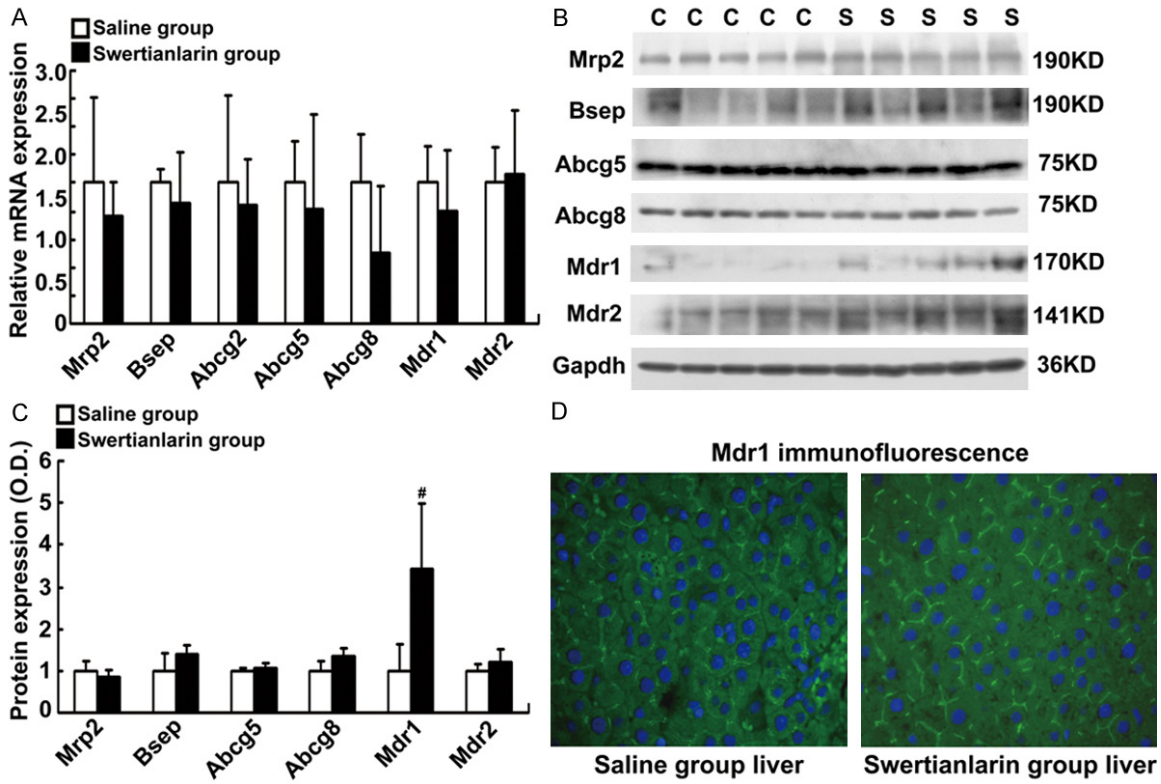

Figure 4. Changes in the expression of canalicular membrane transporters in rats treated with swertianlarin, compared with controls. A: The mRNA expression of Mrp2, Bsep, Abcg2, Abcg5/8, Mdr1, and Mdr2; B: Representative Western blots for Mrp2, Bsep, Abcg2, Abcg5/8, Mdr1, and Mdr2; and C: Densitometry analyses of Western blots (% of control group); D: Immunofluorescent labeling of Mdr1. $^{\#}P < 0.05$; n = 5 per group. *C*, the control group; *S*, the swertianlarin group.

0.05, **Figure 2A**), while the mRNA expressions of Cyp7a1, Cyp7b1, and Cyp27a1 were not significantly changed (**Figure 2A**). Western blotting results further confirmed that the protein level of Cyp8b1 was increased by 3.4-fold in the swertianlarin group, compared with the control group ($P < 0.05$, **Figure 2B**, **2C**), with no significant changes being seen in the protein levels of Cyp7a1, Cyp7b1 and Cyp27a1 (**Figure 2B**, **2C**).

Swertianlarin increases bile acid detoxification enzymes in the rat liver

Real time qPCR results displayed that the detoxification enzymes Cyp3a, Ugt2b, and Sult2a1 were markedly increased in the rats following administration of swertianlarin, compared to the controls (2.3-fold, 2.4-fold, and 2.2-fold, respectively, all $P < 0.05$, **Figure 3A**), while the mRNA levels of Gsta2, Gsta3, Gsta3, Gstm1, Gstm2, Gstm3, and Gstm4 remained unchanged (**Figure 3A**). Western blotting results demonstrated that the protein levels of Cyp3a, Ugt2b, and Sult2a1 in the swertianlarin group were also induced, compared with the controls (3.7-flod, 3.8-fold, and 2.1-fold, respectively, all $P < 0.01$, **Figure 3B**, **3C**). The Gsta1 protein level was also significantly increased, (1.7-fold, $P < 0.05$, **Figure 3B**, **3C**), with no significant changes being seen with the protein levels of Gsta2, Gstm1 and Gstm2.

Swertianlarin alters canalicular membrane transporters in rat liver

Figure 4A shows that the mRNA expression of canalicular membrane transporters Mrp2, Bsep, Abcg2, Abcg5/8, Mdr1, and Mdr2 were not significantly altered in the rat liver of the swertianlarin group, compared with that of the control. However, the protein level of Mdr1 was significantly increased in the swertianlarin group, compared with the control (3.4-fold, $P < 0.05$), with no significant changes being seen with the protein levels of Mrp2, Bsep, Abcg5/8 and Mdr2 (**Figure 4B**, **4C**). The induction of

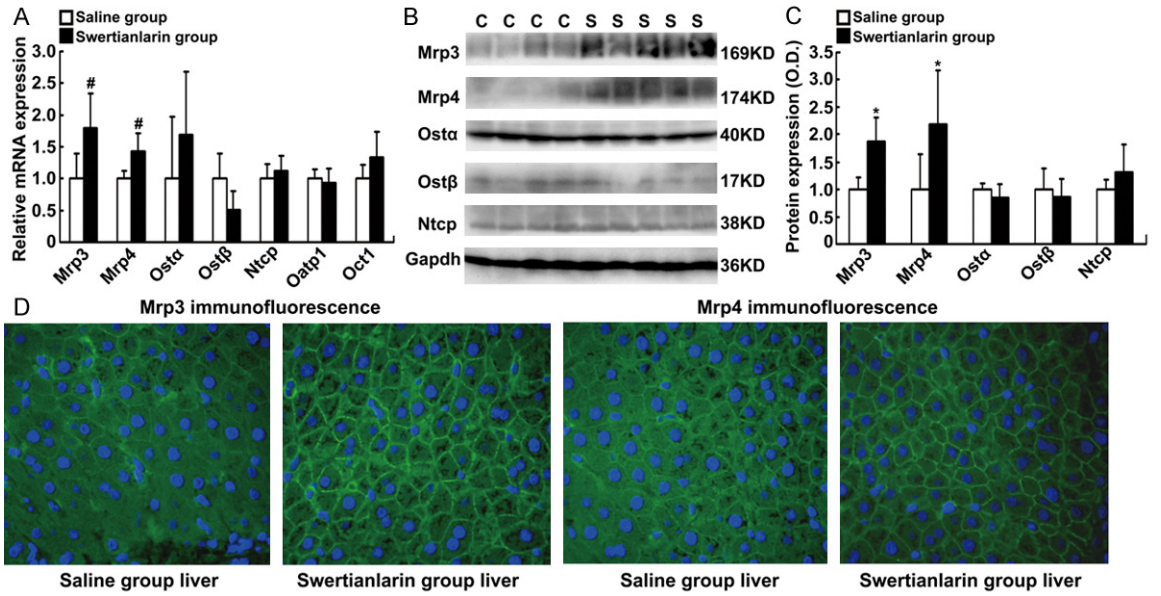

Figure 5. Expression level of basolateral membrane transporters Mpr3, Mrp4 Ostα/β, Ntcp, Oct1 and Oatp1b1 in rats treated with swertianlarin, compared with controls. A: The mRNA expressions of Mrp3, Mrp4, Ostα/β, Ntcp, Oct1 and Oatp1b1; B: Representative western blotting for Mrp3, Mrp4, Ostα/β and Ntcp; and C: Densitometry analysis of Western blots (% of control group); D: Immunofluorescent labeling of Mrp3 and Mrp4. $^{*}P < 0.01$; $^{\#}P < 0.05$; n = 5 per group; *C*, the control group; S, the swertianlarin group.

Mdr1 protein expression was further confirmed by immunofluorescence labeling with Mdr1 antibody (**Figure 4D**). The protein expression of Mdr1 at the canalicular membrane of hepatocytes in the rat liver of the swertianlarin group was more prominent than that in the control.

Swertianlarin increases the expression of basolateral membrane transporters, Mrp3 and Mrp4 in rat liver

As shown in **Figure 5A**, the mRNA expression of basolateral transporters Mrp3 and Mrp4 in the rat liver were induced by swertianlarin treatment, compared with the control (1.8-fold and 1.5-fold, respectively, all $P < 0.05$), while the mRNA expressions of Ostα, Ostβ, Ntcp, Oatp1b1, and Oct1 were not significantly altered. The protein levels of Mrp3 and Mrp4 were also elevated in the swertianlarin group, compared with the control (1.9-fold and 2.2-fold, respectively, all $P < 0.005$), whereas the protein levels of Ostα, Ostβ and Ntcp were not affected (**Figure 5B**, **5C**). The induction of Mrp3 and Mrp4 expression at the basolateral membrane of hepatocytes were further confirmed by immunofluorescence labeling with Mrp3 and Mrp4 antibodies (**Figure 5D**). The basolateral membrane of Mrp3 and Mrp4 expressions in the rat liver of the swertianlarin group was more prominent than that in the control.

Swertianlarin alters the expressions of nuclear receptors and transcriptional factors in rat liver

The mRNA expression of nuclear receptors Fxr, Shp, Pxr, Car, Vdr, Pparα, Hnf1α, Hnf4α, Rxrα, Rarα, Lxr, and Lrh-1 were not significantly altered in the rats treated with swertianlarin, compare to the control group (**Figure 6A**). The expression of transcriptional factors Nrf2, Hnf3β and Ahr were also unchanged (**Figure 6A**). However, the protein expressions of Pxr, Vdr, Hnf1α and Rarα were significantly induced in the swertianlarin group, compared with the control (3.9-fold, 2.5-fold, 2.2-fold, and 2.6-fold, respectively, all $P < 0.01$, **Figure 6B**, **6C**), while Shp was also increased 1.3-fold ($P < 0.05$, **Figure 6B**, **6C**). However, the protein levels of nuclear receptors Fxr, Car, Pparα, Hnf4α, Rxrα, Rarα, Lxr, and Lrh-1 were not significantly changed (**Figure 6B**, **6C**). The protein levels of Nrf2 and Ahr were also elevated in the swertianlarin group, compared with the control (2.5-fold, and 1.5-fold, respectively, all $P < 0.05$, **Figure 6B**, **6C**). Immunofluorescence results further confirmed that the presence of Pxr, Vdr,

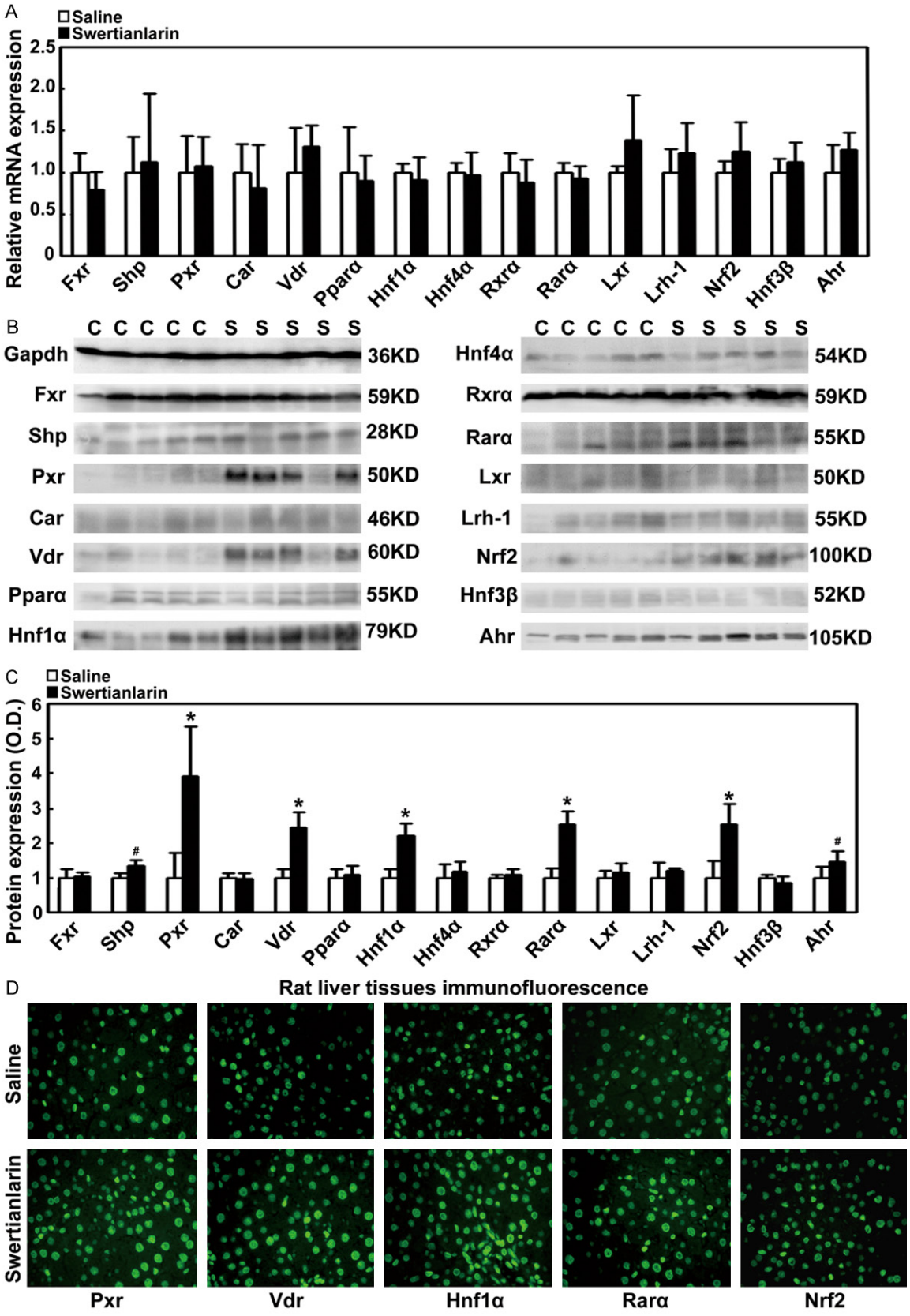

Figure 6. Changes in nuclear receptor and transcriptional factor expression in rats treated with swertianlarin, compared with the controls. A: The mRNA expression of nuclear receptors Fxr, Shp, Pxr, Car, Vdr, Pparα, Hnf1α, Hnf4α,

Rxrα, Rarα, Lxr, and Lrh-1, and transcriptional factors Nrf2, Hnf3β, and Ahr; B: Representative Western blots for Fxr, Shp, Pxr, Car, Vdr, Pparα, Hnf1α, Hnf4α, Rxrα, Rarα, Lxr, Lrh-1factors, Nrf2, Hnf3β, and Ahr; C: Densitometry analysis of Western blots (% of control group); and D: Immunofluorescent labeling of Pxr, Vdr, Hnf1α Rarα, and Nrf2 $^{*}P < 0.01$; $^{\#}P < 0.05$; n = 5 per group; *C*, the control group; *S*, the swertianlarin group.

Hnf1α, Rarα, and Nrf2 in the nuclei of hepatocytes was more prominent in the swertianlarin group than that in the control (**Figure 6D**).

Discussion

Cholestasis caused by bile duct obstruction (e.g., gallstone and pancreas tumors), hepatitis, and overdose of some drugs, may lead to liver failure, fibrosis and cirrhosis [6-10]. The accumulation of bile acids in hepatocytes exerts a key role in cholestatic liver injury [6, 7]. Drugs that are capable of inhibiting bile acids synthesis, and enhancing water solubility and elimination of hydrophobic bile acids, such as UDCA and INT747, have been shown to have anti-cholestasis effects in cholestatic animal models and patients [25-28]. In the present study, we made at least four major discoveries, including that swertianlarin: (1) increased the serum conjugated bile acids TCA and TDCA, and reduced unconjugated bile acid αMCA and ALP levels, (2) induced the levels of synthetic enzyme Cyp8b1, (3) stimulated expression of detoxification enzymes Cyp3a, Ugt2b, Sult2a1 and Gsta1, and (4) increased canalicular transporter Mdr1 and basolateral transporters Mrp3 and Mrp4. These results indicate that swertianlarin alters bile acid transporters and bile acids synthetic and detoxification enzymes which reduces liver injury by enhancing the water solubility and elimination of toxic bile acids in hepatocytes.

Nuclear receptors and transcriptional factors play a crucial role in regulating the bile acids synthetic and detoxification enzymes and the canalicular and basolateral membrane transporters in cholestasis [19-21, 24]. We speculated that the alteration of bile acid synthetic and detoxification enzymes and membrane transporters in rats treated with swertianlarin may be also mediated by these nuclear receptors and transcriptional factors. The observations of the significant induction of nuclear receptors Pxr, Vdr, Hnf1α, Rarα, and Shp, and transcriptional factors Nrf2 and Ahr at the protein level supported our hypothesis, although the mRNA of these genes were not always significantly changed, indicating that the post-transcriptional regulation or nuclear translocation may be more functionally relevant. The significant induction of Vdr, Pxr and Hnf1α following swertianlarin treatment may contribute to the increase in the levels of detoxification enzymes Cyp3a, Ugt2b, Sult2a1, and Gsta1 in rats because their activation has been shown to induce expression of these detoxification enzymes in rodent liver and hepatoma cells [6-8, 16-18]. These induced detoxification enzymes can increase the water solubility of hydrophobic bile acids through hydroxylation (Cyp3a), glucuronidation (Ugt2b), and sulphation (Sult2a1) [6-8]. Moreover, the induced Pxr and Vdr may also up-regulate the expression of Mrp3, and the induction of Nrf2 and Ahr may contribute to the increase in Mrp3 and Mrp4 expression [6-8, 11, 20, 30-31]. The basolateral transporters Mrp3 and Mrp4, are bile acids efflux transporters that eliminate conjugated bile acids from hepatocytes [6-8], which can explain why the increased serum levels of conjugated bile acids, TCA and TDCA were observed in rats treated with swertianlarin in the present study. These results imply that swertianlarin can stimulate detoxification enzymes and basolateral efflux transporter expression to reduce the toxicity and increase elimination of hydrophobic bile acids from hepatocytes highlighting the protective role for swertianlarin in cholestasis.

The up-regulation of Cyp8b1 gene at mRNA and protein levels in rats treated with swertianlarin may help direct bile acid synthesis pathways towards CA but not CDCA, considering that the loss of CYP8B1 activity leads to the production of CDCA over CA in rodents and that CDCA is more toxic than CA to hepatocytes [32, 33]. Furthermore, Cyp8b1 also exerts an important role in maintaining cholesterol and triglyceride (TG) balance [34, 35]. The significant reduction of serum TG in rats treated with swertianlarin may be associated with the up-regulation of hepatic Cyp8b1, considering that the deficiency of Cyp8b1 and the product of 12α-hydroxylated BAs hinder the TG-lowering effects of the BA receptor, Fxr [34, 35]. However, the mechanism for the swertianlarin-induced up-regulation of Cyp8b1 remains elusive, because the modula-

tors Hnf4α and Lrh-1 were not significantly changed in rats treated with swertianlarin. In addition, the induced Mdr1 expression observed in rats treated with swertianlarin may partially contribute to the changes in cholesterol and bile acids homeostasis, considering that Mrd1 may function as an intracellular transport of cholesterol and secondary bile salt export pump [7, 36]. However, the increased Mdr1 expression in the rats treated with swertianlarin was not at the mRNA level but at the protein level, implying that post-transcriptional regulation is involved. Further study to elucidate the underlying mechanisms is needed.

In summary, the present study provided the first evidence that swertianlarin significantly increased the expression of bile acids detoxification enzymes and bile acids efflux transporters, which may increase the water solubility of hydrophobic bile acids and elimination of conjugated bile acids. Our results may help better understand the protective role of swertianlarin against liver injury in cholestasis and drug-induced acute liver damage. We also suggest that swertianlarin may be a potential drug for the treatment of cholestatic patients. Future studies are needed to demonstrate its therapeutic effects in the clinic.

Acknowledgements

This study was supported by National Natural Science Foundation of China (81070320, 81100280, and 81470880), Scholarship Foundation of China Scholarship Council (CSC No. 201307610015), and Scholarship Foundation of Third Military Medical University (2013).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jin Chai, Department of Gastroenterology, Southwest Hospital, Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel: +86-23-68765183; Fax: 86-23-65410853; E-mail: chaijinsw@yahoo.com

References

[1] Fan G, Luo WZ, Luo SH, Li Y, Meng XL, Zhou XD, Zhang Y. Metabolic discrimination of Swertia mussotii and Swertia chirayita known as "Zangyinchen" in traditional Tibetan medicine by H NMR-based metabolomics. J Pharm Biomed Anal 2014; 98: 364-370.

[2] Yang H, Ding C, Duan Y, Liu J. Variation of active constituents of an important Tibet folk medicine Swertia mussotii Franch (Gentianaceae) between artificially cultivated and naturally distributed. J Ethnopharmacol 2005; 98: 31-35.

[3] Tian C, Zhang T, Wang L, Shan Q, Jiang L. The hepatoprotective effect and chemical constituents of total iridoids and xanthones extracted from Swertia mussotii Franch. J Ethnopharmacol 2014; 154: 259-266.

[4] Gao R, Wang L, Yang Y, Ni J, Zhao L, Dong S, Guo M. Simultaneous determination of oleanolic acid, ursolic acid, quercetin and apigenin in Swertia mussotii Franch by capillary zone electrophoresis with running buffer modifier. Biomed Chromatogr 2014; 29: 402-9.

[5] Jaishree V, Badami S. Antioxidant and hepatoprotective effect of swertianlarin from Enicostemma axillare against D-galactosamine induced acute liver damage in rats. J Ethnopharmacol 2010; 130: 103-106.

[6] Trauner M, Meier PJ, Boyer JL. Molecular pathogenesis of cholestasis. N Engl J Med 1998; 339: 1217-27.

[7] Wagner M, Zollner G, Trauner M. New molecular insights into the mechanisms of cholestasis. J Hepatol 2009; 51: 565-580.

[8] Zollner G, Marschall HU, Wagner M, Trauner M. Role of nuclear receptors in the adaptive response to bile acids and cholestasis: pathogenetic and therapeutic considerations. Mol Pharm 2006; 3: 231-51.

[9] Trauner M, Wagner M, Fickert P, Zollner G. Molecular regulation of hepatobiliary transport systems: clinical implications for understanding and treating cholestasis. J Clin Gastroenterol 2005; 39: S111-S124.

[10] Chai J, He Y, Cai SY, Jiang Z, Wang H, Li Q, Chen L, Peng Z, He X, Wu X, Xiao T, Wang R, Boyer JL, Chen W. Elevated hepatic multidrug resistance-associated protein 3/ATP-binding cassette Subfamily C 3 expression in human obstructive cholestasis is mediated through tumor necrosis factor alpha and c-Jun NH2-terminal kinase/stress-activated protein kinase-signaling pathway. Hepatology 2012; 55: 1485-94.

[11] Chai J, Luo D, Wu X, Wang H, He Y, Li Q, Zhang Y, Peng ZH, Xiao T, Wang R, Chen W. Changes of organic anion transporter MRP4 and related nuclear receptors in human obstructive cholestasis. J GastrointestSurg 2011; 15: 996-1004.

[12] Bohan A, Chen WS, Denson LA, Held MA, Boyer JL. Tumor necrosis factor alpha-dependent up-

regulation of Lrh-1 and Mrp3 (Abcc3) reduces liver injury in obstructive cholestasis. J Biol Chem 2003; 278: 36688-36698.
[13] Zollner G, Fickert P, Silbert D, Fuchsbichler A, Marschall HU, Zatloukal K, Denk H, Trauner M. Adaptive changes in hepatobiliary transporter expression in primary biliary cirrhosis. J Hepatol 2003; 38: 717-727.
[14] Mennone A, Soroka CJ, Cai SY, Harry K, Adachi M, Hagey L, Schuetz JD, Boyer JL. Mrp4-/- mice have an impaired cytoprotective response in obstructive cholestasis. Hepatology 2006; 43: 1013-21.
[15] Boyer JL, Trauner M, Mennone A, Soroka CJ, Cai SY, Moustafa T, Zollner G, Lee JY, Ballatori N. Upregulation of a basolateral FXR-dependent bile acid efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents. Am J Physiol Gastrointest Liver Physiol 2006; 290: G1124-1130.
[16] Zollner G, Wagner M, Fickert P, Silbert D, Gumhold J, Zatloukal K, Denk H, Trauner M. Expression of bile acid synthesis and detoxification enzymes and the alternative bile acid efflux pump MRP4 in patients with primary biliary cirrhosis. Liver Int 2007; 27: 920-929.
[17] Wagner M, Halilbasic E, Marschall HU, Zollner G, Fickert P, Langner C, Zatloukal K, Denk H, Trauner M. CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. Hepatology 2005; 42: 420-430.
[18] Aleksunes LM, Klaassen CD. Coordinated regulation of hepatic phase I and II drug-metabolizing genes and transporters using AhR-, CAR-, PXR-, PPARα-, and Nrf2-null mice. Drug Metab Dispos 2012; 40: 1366-79.
[19] Boyer JL. Nuclear receptor ligands: rational and effective therapy for chronic cholestatic liver disease? Gastroenterology 2005; 129: 735-740.
[20] Jonker JW, Liddle C, Downes M. FXR and PXR: potential therapeutic targets in cholestasis. J Steroid Biochem Mol Biol 2012; 130: 147-158.
[21] Wagner M, Zollner G, Trauner M. Nuclear receptor regulation of the adaptive response of bile acid transporters in cholestasis. Semin Liver Dis 2010; 30: 160-177.
[22] Paumgartner G, Pusl T. Medical treatment of cholestatic liver disease. Clin Liver Dis 2008; 12: 53-80.
[23] Marschall HU, Wagner M, Zollner G, Fickert P, Diczfalusy U, Gumhold J, Silbert D, Fuchsbichler A, Benthin L, Grundström R, Gustafsson U, Sahlin S, Einarsson C, Trauner M. Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. Gastroenterology 2005; 129: 476-485.
[24] Zollner G, Fickert P, Fuchsbichler A, Silbert D, Wagner M, Arbeiter S, Gonzalez FJ, Marschall HU, Zatloukal K, Denk H, Trauner M. Role of nuclear bile acid receptor, FXR, in adaptive ABC transporter regulation by cholic and ursodeoxycholic acid in mouse liver, kidney and intestine. J Hepatol 2003; 39: 480-488.
[25] Beuers U. Drug insight: mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nat Clin Pract Gastroenterol Hepatol 2006; 3: 318-328.
[26] Fickert P, Wagner M, Marschall HU, Fuchsbichler A, Zollner G, Zatloukal K, Liu J, Waalkes MP, Cover C, Denk H, Hofmann AF, Jaeschke H, Trauner M. 24-norUrsodeoxycholic acid is superior to ursodeoxycholic acid in the treatment of sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 2006; 130: 465-481.
[27] Liu Y, Binz J, Numerick MJ, Dennis S, Luo G, Desai B, MacKenzie KI, Mansfield TA, Kliewer SA, Goodwin B, Jones SA. Hepatoprotection by the farnesoid X receptor agonist GW4064 in rat models. J Clin Invest 2003; 112: 1678-1687.
[28] Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid Signalling for metabolic diseases. Nature Reviews Drug Discovery 2008; 7: 678-693.
[29] Wang Y, Jing L, Zhao XM, Han JJ, Xia ZL, Qin SC, Wu YP, Sun XJ. Protective effects of hydrogen-rich saline on monocrotaline-induced pulmonary hypertension in a rat model. Respir Res 2011; 12: 26.
[30] Xu S, Weerachayaphorn J, Cai SY, Soroka CJ, Boyer JL. Aryl hydrocarbon receptor and NF-E2-related factor 2 are key regulators of human MRP4 expression. Am J PhysiolGastrointest Liver Physiol 2010; 299: G126-135.
[31] Aleksunes LM, Slitt AL, Maher JM, Augustine LM, Goedken MJ, Chan JY, Cherrington NJ, Klaassen CD, Manautou JE. Induction of Mrp3 and Mrp4 transporters during acetaminophen hepatotoxicity is dependent on Nrf2. Toxicol Appl Pharmacol 2008; 226: 74-83.
[32] Li-Hawkins J, Gafvels M, Olin M, Lund EG, Andersson U, Schuster G, Björkhem I, Russell DW, Eggertsen G. Cholic acid mediates negative feedback regulation of bile acid synthesiSs in mice. J Clin Invest 2002; 110: 1191-1200.
[33] Monte MJ, Marin JJ, Antelo A, Vazquez-Tato J. Bile acids: chemistry, physiology, and pathophysiology. World J Gastroenterol 2009; 15: 804-816.
[34] Haeusler RA, Pratt-Hyatt M, Welch CL, Klaassen CD, Accili D. Impaired generation of 12-hydroxylated bile acids links hepatic insulin sig-

naling with dyslipidemia. Cell Metab 2012; 15: 65-74.

[35] Lee SD, Thornton SJ, Sachs-Barrable K, Kim JH, Wasan KM. Evaluation of the contribution of the ATP binding cassette transporter, P-glycoprotein, to in vivo cholesterol homeostasis. Mol Pharm 2013; 10: 3203-12.

[36] Connelly-Smith L, Pattinson J, Grundy M, Shang S, Seedhouse C, Russell N, Pallis M. P-glycoprotein is downregulated in KG1a-primitive leukemia cells by LDL cholesterol deprivation and by HMG-CoA reductase inhibitors. Exp Hematol 2007; 35: 1793-800.