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

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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. Nr2f 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-fold 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
Swertianlarin modulates liver detoxification 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

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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, norUDCA, 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.
Swertianlarin modulates liver detoxification

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), 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 salt concentrations

Figure 1A illustrates that the serum ALP level in rats treated with 100 mg/kg/day of swertianlarin...
Swertianlarin modulates liver detoxification

The Cyp8b1 mRNA expression in the rat liver was induced 1.8-fold by the swertianlarin treatment, compared with that 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

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Swertianlarin modulates liver detoxification

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-fold, 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...
Swertianlarin modulates liver detoxification

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, Oct1 and 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.

The mRNA expression of nuclear receptors Fxr, Shp, Pxr, Car, Vdr, Ppara, Hnf1α, Hnf4α, Rxrα, Rarα, Lxr, and Lrh-1 were not significantly altered in the rats treated with swertianlarin, compared 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, Ppara, 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,
Swertianlarin modulates liver detoxification

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, Ppara, Hnf1α, Hnf4α,
Swertianlarin modulates liver detoxification

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-
Swertianlarin modulates liver detoxification

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 Mdr1 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.

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Disclosure of conflict of interest

None.

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Swertianlarin modulates liver detoxification
