

DMD # 66969

Hepato-protective Effects of *Schisandra sphenanthera* Extract against Lithocholic Acid-induced Cholestasis in Male Mice are Associated with the Activation of PXR Pathway and Promotion of Liver Regeneration

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DMD # 66969

Running title page

Running title: Schisandra extract protects against LCA-induced cholestasis

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ABBREVIATIONS

ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; CCND1, cyclin D1; CYP, cytochrome P450; UGT, urineglucorotransferase; LCA, lithocholic acid; PCN, pregnenolone-16 α -carbonitrile; PCNA, proliferating cell nuclear antigen; SinA, schisandrin A; SinB, schisandrin B, SinC, schisandrin C; SolA, schisandrol A; SolB, schisandrol B, StnA, schisantherin A; TBA, total bile acids; Tbili, total bilirubin; WZ, Wuzhi tablet.

DMD # 66969

ABSTRACT

We previously reported the ethanol extract of *Schisandra sphenanthera* (Wuzhi tablet, WZ) significantly protects against acetaminophen-induced hepatotoxicity. However, whether it exerts protective effect against cholestasis remains unclear. In this study, the protection effect of WZ on lithocholic acid (LCA)-induced intrahepatic cholestasis in mice was characterized and the involved mechanisms were investigated. WZ pre-treatment (350 mg/kg) with LCA significantly reversed liver necrosis and decreased activity of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). More importantly, serum total bile acids (TBA) and total bilirubin (Tbili) were also remarkably reduced. RT-qPCR and Western blot analysis showed that hepatic expression of PXR target genes such as CYP3A11 and UGT1A1 were significantly increased by WZ treatment. Luciferase assays performed in LS174T cells illustrated that WZ extract and its six bioactive lignans could all activate hPXR. Additionally, WZ treatment significantly promoted liver regeneration via inhibition of p53/p21 to induce cell proliferation-associated proteins such as CCND1 and PCNA. In conclusion, WZ performs protective effect against LCA-induced intrahepatic cholestasis, partially due to the activation of PXR pathway and promotion of liver regeneration.

DMD # 66969

INTRODUCTION

Cholestasis is a clinical syndrome with toxic bile acids retention which is caused by the obstacle of bile formation or secretion. Varieties of liver diseases are developed from cholestasis, including biliary atresia, primary sclerosing cholangitis and primary biliary cirrhosis (Trauner et al., 1998). Lithocholic acid (LCA) is a toxic hydrophobic secondary bile acid primarily formed in the intestine by bacterial 7α -dehydroxylation of chenodeoxycholic acid (CDCA) (Ridlon et al., 2006). In humans, elevated levels of LCA are found in cholestasis patients (Carey et al., 1966). Rodents treating with LCA can lead to development of cholestasis and liver damage, which has been used as an applied model of intrahepatic cholestasis (Ceryak et al., 1998; Lucangioli et al., 2009; Wang et al., 2012). The therapeutic strategy against cholestasis is mainly focused on promotion of elimination for excess toxic bile acids. However, until now, ursodeoxycholic acid (UDCA) is the only FDA-approved drug to treat cholestasis whereas its efficacy is limited to early stages of primary biliary cirrhosis (Ishizaki et al., 2005).

A number of metabolizing enzymes and transporters participates in bile acid elimination, such as CYP3A, UGT1A1, SULT2A1, BSEP, MRP2, MRP3, MRP4, NTCP, OATP2, OST β . Nuclear receptors and transcription factors, such as pregnane X receptor (PXR), farnesoid X receptor (FXR), constitutive androstane receptor (CAR), and nuclear factor-E2-related factor (Nrf2), which can regulate these genes are thought as potential therapeutic targets of cholestasis (Yu et al., 2002; Rizzo et al., 2005; Tan et al., 2010; Zollner et al., 2010). Agonist of these factors may have

DMD # 66969

potential prevention effect against cholestasis (Zollner et al., 2010; Chen et al., 2014). PXR and its human homolog steroid and xenobiotic receptor (SXR) are known to induce CYP3A, UGT1A1 expression (Goodwin et al., 1999; Chen et al., 2003). It has been reported that PCN (pregnenolone-16 α -carbonitrile) as a rodent PXR agonist can prevent the LCA-induced hepatotoxicity in rodents (Staudinger et al., 2001; Xie et al., 2001). In PXR null mice, PCN failed to prevent LCA-induced cholestasis and liver damage. In addition, glycyrrhizin as a PXR agonist, has been demonstrated to protect against LCA-induced cholestasis (Wang et al., 2012). These findings suggest PXR plays an important role in bile acids elimination and its agonists may have potential prevention efficacy on cholestasis. In addition, p53/p21 pathway plays an important role in liver regeneration after liver injury (Wu et al., 1996). Several cell cycle-related proteins such as Cyclin D1 (CCND1), proliferating cell nuclear antigen (PCNA) are regulated by p21, which may explain for the therapeutic effect of treating cholestatic liver damage (Yang et al., 2011).

Schisandra sphenanthera, the dried ripe fruit of *Schisandra sphenanthera* Rehd. et Wils, is widely used as a tonic and nutrition in many countries. Wuzhi tablet (WZ), containing 7.5 mg Schisantherin A per tablet, is a preparation of the ethanol extract of *Schisandra sphenanthera*, is a prescribed drug in China used to protect liver function in chronic hepatitis and liver dysfunction patients (registration number: Z20025766). Chemical fingerprint of WZ was determined by HPLC-DAD analysis in our previous study and the relative amounts of its major active lignans including Schisandrin A (SinA), Schisandrin B (SinB) , Schisandrin C (SinC), Schisandrol A (SolA),

DMD # 66969

Schisandrol B (SolB), and Schisantherin A (StnA) were determined (Qin et al., 2014). Previous studies have demonstrated that WZ protects a number of hepatotoxins induced acute liver damage including carbon tetrachloride and acetaminophen (Zhu et al., 2000; Xie et al., 2010; Bi et al., 2013). Based on these facts, we assumed that WZ may have potential hepatoprotective effect against cholestasis. Therefore, this study aimed to investigate the protective effect of WZ against LCA-induced cholestasis in mice, and to explore the potential mechanisms.

DMD # 66969

Materials and Methods

Chemicals and reagents

Wuzhi tablets were manufactured by Fanglue Pharmaceutical Company (Guangxi, China), which has been quantified to 7.5 mg Schisantherin A per tablet. WZ extract prepared by ethanol extraction was performed as described previously and quantified by the concentration of Schisandrin A in it. Schisandrin A (Sin A), schisandrin B (Sin B), schisandrin C (Sin C), schisandrol A (Sol A), schisandrol B (Sol B), and schisantherin A (Stn A) were all produced by Shanghai Winherb Medical Science and Technology Development Co., Ltd. (Shanghai, China, <http://www.winherb.cn/>). LCA (purity >98%) was purchased from Aladdin Company (Shanghai, China). PCN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pSG5-hPXR expression vector was generously provided by Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX, USA) (Lehmann et al., 1998). The pGL3-CYP3A4-XREM luciferase reporter construct containing the basal promoter (−362/+53) with the proximal PXR response element (ER6) and the distal xenobiotic responsive enhancer module (XREM, −7836/−7208) of the CYP3A4 gene 5'-flanking region inserted to pGL3-Basic reporter vector was generously provided by Dr. Jeff Staudinger (University of Kansas, Lawrence, KS, USA) (Goodwin et al., 1999). pRL-TK *Rotylenchulus reniformis* control vector was obtained from Promega (Madison, WI, USA). All other chemicals and solvents were commercially available and of analytical grade.

DMD # 66969

Animals and Treatment

Adult male C57BL/6 mice, weighting 21-25 g, were purchased from Guangdong Animal Experimental Centre (Guangzhou, China). Mice were maintained on standard laboratory chow and were allowed food and water ad libitum. The animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Sun Yat-sen University, Guangzhou, China. WZ (350 mg/kg) was dispersed in saline and administered orally twice a day for 7 days. The dose was calculated from the dose used in clinical practice as described previously (Bi et al., 2013; Qin et al., 2013; Fan et al., 2014). PCN and LCA were dissolved in corn oil and injected intraperitoneally either once (PCN, 50 mg/kg) a day for 7 days or twice (LCA, 250 mg/kg) a day for 4 days as described previously (Staudinger et al., 2001). For WZ and PCN protection, WZ and PCN were administered for 3 days previously. Subsequently, WZ and PCN treatment were continued for another 4 days, during which time animals were also injected with LCA (250 mg/kg/d) (Staudinger et al., 2001). Mice were sacrificed 12 h following the final LCA injection. Liver photos were taken firstly, followed by collection of serum and liver samples which were snap-frozen in liquid nitrogen and stored at -80°C until use. The whole procedure of animal study is illustrated in Supplemental Figure 1.

Histological and Biochemical Evaluation

Liver tissues fixed in neutral buffered formalin were embedded in paraffin, cut into sections, and stained with hematoxylin and eosin (H&E) according to a standard protocol. H&E-stained liver

DMD # 66969

sections were examined using an LEICA DM5000B microscope (Leica, Heidelberg, Germany). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) were determined using commercial kits (Kefang biotech, Guangzhou, China) on a Beckman Synchron CX5 Clinical System. Serum total bile acids (TBA) and total bilirubin (Tbili) levels were analyzed by commercial kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions. For histology evaluation, tissues were immediately fixed in formaldehyde, embedded in paraffin wax, sectioned and stained for hematoxylin and eosin (H&E).

RT-qPCR analysis

Total RNA from mice liver tissues or cultured HepG2 cells was isolated using Trizol reagent according to the manufacturer's instruction (Invitrogen, Grand Island, NY). 1 μ g RNA was purified and randomly reverse-transcribed to cDNA by using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotech, Kyoto, Japan). RT-qPCR analysis was performed using SYBR Premix Ex Taq II (Tli RnaseH Plus) kit (TaKaRa Biotech, Kyoto, Japan) in Applied Biosystems 7500 Real-Time PCR System. *Gapdh* for mice was run for each sample to normalize expression. The gene-specific primers were obtained from a primerbank (Spandidos et al., 2010; Wang et al., 2012), and the sequences are listed in Supplemental Table 1.

Western blot analysis

DMD # 66969

Proteins extracted from mice liver tissue were prepared using RIPA lysis buffer (Bicolor BioScience and Technology, China) and were determined by BCA protein assay (Thermo Scientific, Rockford, IL). 40 μ g of protein extracts was separated in 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrophoretically transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline, membranes were incubated overnight with primary antibodies, including CYP3A11 (L-14) , p21 (F-8) (Santa Cruz Biotechnology, Santa Cruz, CA); UGT1A1 (ab62600), p53 (pab 240, ab26) (Abcam, Cambridge, MA); GAPDH(14c10) (Cell Signaling Technologies, Danvers, MA, USA); CCDN1 (AB20509b), PCNA (AB20014) (Sangon, China). Subsequently, a secondary horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied, and then specific bands were visualized using ECL detection kit (Engreen Biosystem, China).

Transient transfection assays

LS174T cells (ATCC) were maintained in DMEM containing 10% FBS and 100 U penicillin/streptomycin. Cells were seeded to 96 well plate at a density of 1.5×10^4 cells per well without antibiotics. For PXR transactivation assay, each well contains 100 ng pGL3-CYP3A4-XREM-Luc, 50 ng pSG5-hPXR and 5 ng pRL-TK. The transfection procedure was followed by instruction of Lipofectamine 2000 (Invitrogen, Grand Island, NY). 6 h later, the transfection mixtures were removed and replaced with phenol red free DMEM containing 10% charcoal-stripped delipidated FBS.

DMD # 66969

Transfected cells were then treated with SinA, SinB, SinC, SolA, SolB, StnA, WZ extract (2.5, 10, 40 μ M), or respective positive agonist Rifampicin (10 μ M) for 24 h. The concentration of these lignans and WZ extract were chosen as described previously (Qin et al., 2010). Luciferase activity was assayed in an Amersham Pharmacia Biotech luminometer using Dual Reporter Assay System (Promega, Madison, WI) according to the manufacturer's directions. Firefly luciferase activity was normalized to Renilla activity for each well.

Statistical Analysis

All values were expressed as mean \pm S.E.M. One-way ANOVA followed by Dunnett's multiple comparison post hoc test or unpaired Student's *t* test was used for statistical analysis of data using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Only the comparisons indicated above the bars are being made and difference was considered as significant if the probability (*P* value) was less than 0.05 ($P < 0.05$).

DMD # 66969

RESULTS

WZ protects LCA-induced cholestasis and liver injury

The morphological and histological evaluations illustrate that 4-day LCA treatment resulted in severe hepatic necrosis, diffuse vacuolization, infiltrating neutrophils, as well as gall bladder enlargement (Fig. 1, A and B). Mice pretreated with 700 mg/kg WZ or 50 mg/kg PCN abrogated the LCA-induced liver damage, with similar livers appearance to the vehicle group (Fig. 1, A and B).

Consistent with liver histopathology, LCA treatment caused serum ALT, AST, ALP levels huge elevation to 4731, 4536 and 410 U/L, respectively (Fig. 2, A-C). Pre-administration of WZ reversed LCA-induced increase of ALT to 15 %; AST to 45 %; and ALP to 38 %, respectively (Fig. 2, A-C). Similarly, serum TBA and serum Tbili levels were strikingly increased to 947 and 27 $\mu\text{mol/L}$ by LCA treatment, which was 8.2- and 3.8- fold higher than that of the vehicle group, respectively (Fig. 2, D and E). WZ pre-treatment reversed serum TBA and Tbili levels to 23 %, 56 %, respectively (Fig. 2, D and E). Moreover, similar protective effects were also observed in 50 mg/kg PCN pre-treatment group. Taken together, these data evidently demonstrate that WZ can protect against LCA-induced intrahepatic cholestasis and hepatotoxicity.

WZ up-regulates PXR targeted gene expression involved in bile acid homeostasis

Figure 3 (A-B) illustrates that WZ alone significantly up-regulated *Cyp3a11*, *Ugt1a1* and *Oatp2* mRNA levels, while *Cyp7a1* mRNA level was not affected. LCA also

DMD # 66969

increased *Cyp3a11* mRNA, whereas had inhibition effect on *Ugt1a1* and *Cyp7a1* expression, as well as no significant effect on *Oatp2*. Interestingly WZ pre-treatment only significantly up-regulated *Ugt1a1*, *Oatp2* and *Cyp7a1* expression, while no further induction effect was found for *Cyp3a11*, compared with LCA group. In addition, PCN as a PXR agonist performed similar induction pattern in these genes.

To confirm the above RT-qPCR results, we further determined their protein levels using western blot analysis. As shown in Figure 3 (D-E), the protein expression patterns of CYP3A11, UGT1A1 were consistent with the mRNA levels.

Taken together, these data indicate that the protection effect of WZ on LCA-induced cholestasis may be due to its induction effect on hepatic *Cyp3a11* and *Ugt1a1*, which results in accelerating detoxification of toxic bile acids.

WZ and its lignans activate hPXR on human cell lines

To further confirm whether WZ and its active ingredients can activate hPXR, dual-luciferase reporter gene assay was performed in LS174T cells via transiently transfection with reporter plasmids to determine WZ and its bioactive lignans' activation effect on hPXR. As shown in Fig. 4, Rifampicin, a classic hPXR agonist, significantly enhanced the luciferase activity of the hPXR reporter gene to a level 4.8-fold than that in the control group, whereas WZ enhanced the hPXR reporter gene activity, with a notably 3.8-fold elevation at 10 μ M. Meanwhile, all the six bioactive ligands including SinA, SinB, SinC, SolA, SolB and StnA showed similar activation effect on hPXR at 10 and 40 μ M. These results demonstrate that WZ and its main

DMD # 66969

active lignans can activate hPXR, which may play a role in its hepato-protective effect against LCA-induced cholestasis.

WZ suppresses p53/p21 signaling pathway to promote liver regeneration

p53 and p21 levels were then investigated to explore whether WZ can block p53/p21 signaling to promote liver repair in mice after LCA-induced toxicity. The expression of p53 and p21 were slightly affected by WZ treatment, but were dramatically elevated by LCA administration, compared with vehicle group. Additionally, WZ pre-treatment strikingly reversed the elevation of p53 and p21 expression by LCA treatment. Furthermore, levels of cell cycle regulatory proteins CCND1 and PCNA expression were enhanced by WZ administration, compared to that of vehicle group. CCND1 and PCNA expression were also slightly induced by LCA treatment (Fig. 5, A and B), whereas WZ pre-administration further induced the expression levels, compared with LCA group. Interestingly, PCN showed similar impact like WZ on these proteins expression. Taken together, these findings suggest that WZ can inhibit p53/p21 pathway and induce CCND1/PCNA-mediated liver regeneration during cholestatic liver injury.

DMD # 66969

DISCUSSION

The present study clearly revealed WZ's protection effect on LCA-induced intrahepatic cholestasis and hepatotoxicity, as evidenced by ameliorative liver morphology and histology, as well as significant decrease in serum ALT/AST/ALP, serum total bile acids, total bilirubin, and hepatic total bile acids. As a matter of fact, the estrogen hormone is proved to induce intra-hepatic cholestasis during pregnancy, which suggests that the dynamic change of estrogen in female mice would affect the effectiveness and stability of cholestasis modeling. Therefore, male mice are more suitable for cholestasis study (Accatino et al., 1995). Further mechanism studies illustrated that the hepatoprotective effect of WZ against LCA-induced cholestasis was possibly due to PXR-mediated up-regulation of CYP3A11 and UGT1A1, as well as promotion on p53/p21-mediated liver regeneration.

In the current study, the dose of WZ was 700 mg/kg/d, which is converted from the clinical dose. Previously, we also observed obvious hepatoprotective effect of WZ at this dose against acetaminophen-induced liver damage (Bi et al., 2013; Fan et al., 2014). The LCA-induced liver damage, as well as serum ALT, AST, ALT, TBA and Tbili levels were significantly attenuated by WZ pre-treatment. However, WZ displayed weaker efficacy compared with PCN, as PCN reversed most of the above index back to normal level. This may be explained by the fact that the induction effect of WZ on CYP3A11, UGT1A1, CCND1 and PCNA's mRNA transcription and/or protein expression is significantly weaker than PCN.

Bile acids and bilirubin detoxification is mainly mediated via phase I

DMD # 66969

hydroxylation and phase II conjugation by metabolizing enzymes, which produces more hydrophilic, less toxic metabolites that are more apt to be excreted by urine. On the other hand, bile acids are eliminated by transporters either on canalicular membrane into bile or on sinusoidal membrane into blood (Ghonem et al., 2015). PXR has been demonstrated as a bile acid sensor, which is due to its induction net effect on phase I enzyme CYP3A, phase II enzyme UGT1A1 to avoid the accumulation of bile acids and bilirubin to toxic levels. LCA was demonstrated to be a substrate of CYP3A4, which is the human homolog of mouse CYP3A11 (Xie et al., 2001). The metabolites are more hydrophilic and easier to be transported out of hepatocytes. In addition, PXR is the main activator of CYP3A. PCN, a classic mPXR activator, has been demonstrated to protect against LCA-induced cholestasis, with the mechanism that PCN could induce CYP3A11 (Staudinger et al., 2001). In the present study, WZ induces hepatic CYP3A11 expression, which indicates a possible mechanism to illustrate the anti-cholestatic effect of WZ.

Besides bile acids, bilirubin is another important hepatotoxic material during cholestasis. UGT1A1, a phase II metabolizing enzyme, mediates the glucuronidation of bilirubin, which makes the metabolite be more hydrophilic and prone to be excreted (Levesque et al., 2007; Bock, 2012). The study revealed that WZ treatment could significantly up-regulate liver UGT1A1, which may due to the observed reversal effect on serum total bilirubin by WZ pre-treatment. Considering PXR is an modulator of UGT1A1 (Chen et al., 2003), we assume that the induction effect of WZ on UGT1A1 is also via the activation of PXR.

DMD # 66969

Therefore, we further studied whether WZ and its active components can activate PXR. *In vitro* luciferase reporter gene assay revealed that WZ together with its main six components including Sin A, Sin B, Sin C, Sol A, Sol B, and Stn A could activate hPXR, which was consistent with previous report (Mu et al 2006). Therefore, we assume that WZ produces hepato-protective effect against LCA-induced cholestasis by activating PXR and subsequently inducing CYP3A11 and UGT1A1, resulting the acceleration of LCA detoxification.

CYP7A1 is the rate-limiting enzyme during bile acids synthesis and plays a crucial role in bile acids homeostasis. CYP7A1 could be negatively regulated by PXR agonist (Staudinger et al., 2001; Li and Chiang, 2005). Interestingly, no significant regulation of WZ on hepatic *CYP7A1* was found without LCA treatment. On the contrary, WZ pre-treatment elevated *CYP7A1*'s transcription compared with LCA group. This may be due to the net regulation of CYP7A1, WZ may affect other CYP7A1's modulators that counteract the regulation effect of PXR. This result was crucial and indicates that WZ would not affect normal bile acids synthesis, which suggests the safety of WZ treatment on normal status.

Besides the genes mentioned above, a variety of metabolism or transporter genes such as *Mrp2*, *Mrp3*, *Mrp4*, *Bsep*, *Ntcp*, *Mdr2*, *Ost α* , *Ost β* etc. are involved in bile acids homeostasis. We observed several genes hepatic mRNA levels were significantly altered by LCA treatment, while WZ pre-treatment reversed these changes (Supplemental Figure 2). However, WZ alone treatment could not affect any of these genes expression, which suggests that the reversal effect on these gene

DMD # 66969

expression by WZ pre-treatment with LCA was due to the reduced toxic bile acids retention which can cause the alteration of these genes expression.

Furthermore, we previously found significant induction effect of WZ on several proteins related to liver regeneration after APAP-induced hepatotoxicity (Fan et al., 2014), thus p53/p21 related liver regeneration pathways was also investigated. Firstly, LCA treatment caused remarkably elevation of p53/p21 expression, as well as slightly up-regulation of PCNA and CCND1. As expected, WZ pre-treatment displayed significant inhibition on p53/p21 expression, along with induction on PCNA and CCND1. Moreover, PCN pre-treatment exhibited similar regulation pattern, which suggests potential role of PXR on liver regeneration. This is further supported by the report that liver regeneration process was delayed in PXR deficient mice after partial hepatectomy (Dai et al., 2008). Interestingly, p53 pathway could also negatively regulate PXR activity (Elias et al., 2013), indicating the possibility that WZ activates PXR via inhibition effect on p53/p21 pathway.

In a conclusion, the current study clearly demonstrated that *Schisandra Sphenanthera* extract WZ could prevent LCA-induced intrahepatic cholestasis and hepatotoxicity. And this hepatoprotective effect is possibly due to activation of PXR pathway and promotion of liver regeneration.

DMD # 66969

Authorship Contributions

Participated in research design: Bi, Huang, H. Zeng.

Conducted experiments: H. Zeng, D.S. Li, Qin, P. Chen, Tan, X.Z. Zeng, X. Li, Fan, Jiang, Zhou, Y.X. Chen, Wang.

Performed data analysis: H. Zeng, D. S. Li, Bi.

Wrote or contributed to the writing of the manuscript: H. Zeng, Bi, Huang.

DMD # 66969

References:

- Accatino L, Figueroa C, Pizarro M and Solis N (1995) Enhanced biliary excretion of canalicular membrane enzymes in estrogen-induced and obstructive cholestasis, and effects of different bile acids in the isolated perfused rat liver. *J Hepatol* **22**:658-670.
- Bi H, Li F, Krausz KW, Qu A, Johnson CH and Gonzalez FJ (2013) Targeted Metabolomics of Serum Acylcarnitines Evaluates Hepatoprotective Effect of Wuzhi Tablet (Schisandra sphenanthera Extract) against Acute Acetaminophen Toxicity. *Evid Based Complement Alternat Med* **2013**:985257.
- Bock KW (2012) Human UDP-glucuronosyltransferases: Feedback loops between substrates and ligands of their transcription factors. *Biochemical Pharmacology* **84**:1000-1006.
- Carey JJ, Wilson ID, Zaki FG and Hanson RF (1966) The metabolism of bile acids with special reference to liver injury. *Medicine (Baltimore)* **45**:461-470.
- Ceryak S, Bouscarel B, Malavolti M and Fromm H (1998) Extrahepatic deposition and cytotoxicity of lithocholic acid: studies in two hamster models of hepatic failure and in cultured human fibroblasts. *Hepatology* **27**:546-556.
- Chen C, Staudinger JL and Klaassen CD (2003) Nuclear receptor, pregnane X receptor, is required for induction of UDP-glucuronosyltransferases in mouse liver by pregnenolone-16 alpha-carbonitrile. *Drug Metab Dispos* **31**:908-915.
- Chen P, Zeng H, Wang Y, Fan X, Xu C, Deng R, Zhou X, Bi H and Huang M (2014)

DMD # 66969

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.

Dai G, He L, Bu P and Wan YJ (2008) Pregnane X receptor is essential for normal progression of liver regeneration. *Hepatology* **47**:1277-1287.

Elias A Wu J and Chen T (2013) Tumor suppressor protein p53 negatively regulates human pregnane X receptor activity. *Mol Pharmacol* **83**:1229-1236.

Fan X, Jiang Y, Wang Y, Tan H, Zeng H, Wang Y, Chen P, Qu A, Gonzalez FJ, Huang M and Bi H (2014) Wuzhi tablet (Schisandra Sphenanthera extract) protects against acetaminophen-induced hepatotoxicity by inhibition of CYP-mediated bioactivation and regulation of NRF2-ARE and p53/p21 pathways. *Drug Metab Dispos* **42**:1982-1990.

Ghonem NS Assis DN and Boyer JL (2015) On fibrates and cholestasis: A review. *Hepatology*.

Goodwin B Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* **56**:1329-1339.

Ishizaki K Imada T and Tsurufuji M (2005) Hepatoprotective bile acid 'ursodeoxycholic acid (UDCA)' Property and difference as bile acids. *Hepatol Res* **33**:174-177.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT and Kliewer SA

DMD # 66969

(1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**:1016-1023.

Levesque E, Girard H, Journault K, Lepine J and Guillemette C (2007) Regulation of the UGT1A1 bilirubin-conjugating pathway: role of a new splicing event at the UGT1A locus. *Hepatology* **45**:128-138.

Li T and Chiang JY (2005) Mechanism of rifampicin and pregnane X receptor inhibition of human cholesterol 7 alpha-hydroxylase gene transcription. *Am J Physiol Gastrointest Liver Physiol* **288**:G74-G84.

Lucangioli SE, Castano G, Contin MD and Tripodi VP (2009) Lithocholic acid as a biomarker of intrahepatic cholestasis of pregnancy during ursodeoxycholic acid treatment. *Ann Clin Biochem* **46**:44-49.

Qin XL, Bi HC, Wang XD, Li JL, Wang Y, Xue XP, Chen X, Wang CX, Xu LJ, Wang YT and Huang M (2010) Mechanistic understanding of the different effects of Wuzhi Tablet (Schisandra sphenanthera extract) on the absorption and first-pass intestinal and hepatic metabolism of Tacrolimus (FK506). *Int J Pharm* **389**:114-121.

Qin XL, Chen X, Zhong GP, Fan XM, Wang Y, Xue XP, Wang Y, Huang M and Bi HC (2014) Effect of Tacrolimus on the pharmacokinetics of bioactive lignans of Wuzhi tablet (Schisandra sphenanthera extract) and the potential roles of CYP3A and P-gp. *Phytomedicine* **21**:766-772.

Qin XL, Yu T, Li LJ, Wang Y, Gu HM, Wang YT, Huang M and Bi HC (2013)

DMD # 66969

Effect of long-term co-administration of Wuzhi tablet (Schisandra sphenanthera extract) and prednisone on the pharmacokinetics of tacrolimus.

Phytomedicine **20**:375-379.

Ridlon JM Kang DJ and Hylemon PB (2006) Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* **47**:241-259.

Rizzo G, Renga B, Mencarelli A, Pellicciari R and Fiorucci S (2005) Role of FXR in regulating bile acid homeostasis and relevance for human diseases. *Curr Drug Targets Immune Endocr Metabol Disord* **5**:289-303.

Spandidos A, Wang X, Wang H and Seed B (2010) PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res* **38**:D792-D799.

Staudinger J, Liu Y, Madan A, Habeebu S and Klaassen CD (2001) Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* **29**:1467-1472.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98**:3369-3374.

Tan KP, Wood GA, Yang M and Ito S (2010) Participation of nuclear factor (erythroid 2-related), factor 2 in ameliorating lithocholic acid-induced cholestatic liver injury in mice. *Br J Pharmacol* **161**:1111-1121.

Trauner M Meier PJ and Boyer JL (1998) Molecular pathogenesis of cholestasis. *N*

DMD # 66969

Engl J Med **339**:1217-1227.

Wang X, Spandidos A, Wang H and Seed B (2012) PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Res* **40**:D1144-D1149.

Wang YG, Zhou JM, Ma ZC, Li H, Liang QD, Tan HL, Xiao CR, Zhang BL and Gao Y (2012) Pregnane X receptor mediated-transcription regulation of CYP3A by glycyrrhizin: a possible mechanism for its hepatoprotective property against lithocholic acid-induced injury. *Chem Biol Interact* **200**:11-20.

Wu H, Wade M, Krall L, Grisham J, Xiong Y and Van Dyke T (1996) Targeted in vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development and regeneration. *Genes Dev* **10**:245-260.

Xie W, Radomska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* **98**:3375-3380.

Xie Y, Hao H, Kang A, Liang Y, Xie T, Sun S, Dai C, Zheng X, Xie L, Li J and Wang G (2010) Integral pharmacokinetics of multiple lignan components in normal, CCl₄-induced hepatic injury and hepatoprotective agents pretreated rats and correlations with hepatic injury biomarkers. *J Ethnopharmacol* **131**:290-299.

Yang H, Li TW, Peng J, Tang X, Ko KS, Xia M and Aller MA (2011) A mouse

DMD # 66969

model of cholestasis-associated cholangiocarcinoma and transcription factors involved in progression. *Gastroenterology* **141**:378-388, 381-388.

Yu J, Lo JL, Huang L, Zhao A, Metzger E, Adams A, Meinke PT, Wright SD and Cui

J (2002) Lithocholic acid decreases expression of bile salt export pump through farnesoid X receptor antagonist activity. *J Biol Chem* **277**:31441-31447.

Zhu M, Yeung RY, Lin KF and Li RC (2000) Improvement of phase I drug metabolism with *Schisandra chinensis* against CCl₄ hepatotoxicity in a rat model. *Planta Med* **66**:521-525.

Zollner G, Wagner M and Trauner M (2010) Nuclear receptors as drug targets in cholestasis and drug-induced hepatotoxicity. *Pharmacol Ther* **126**:228-243.

DMD # 66969

Footnotes

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DMD # 66969

Figure captions

Figure 1. Hepatoprotective effects of WZ against LCA-induced cholestatic liver damage. (A) In situ photos of representative livers. Gall bladders were marked by arrows and the apparent subcapsular necrotic foci in the liver could be seen in LCA group. (B) Representative H&E stained liver sections (10×10). Areas of severe liver necrosis were indicated with arrows.

Figure 2. Hepatoprotective effects of WZ against LCA-induced cholestatic liver damage. (A-E) Serum ALT, AST and ALP activity, as well as serum total bile acids and total bilirubin levels elevated by LCA were significantly reduced by pre-treatment with 350 mg/kg WZ or 50 mg/kg PCN. Data are the mean \pm S.E.M (n = 6-8). *P < 0.05 versus vehicle; #P < 0.05 versus LCA alone.

Figure 3. WZ altered hepatic gene expression profile involved in bile acid synthesis, metabolism and transport. RT-qPCR analysis was used to determine the gene expression of (A) *Cyp3a11*, (B) *Ugt1a1*, (C) *Oatp2*, (D) *Cyp7a1* in liver tissues of mice relative to house-keeping gene *Gapdh*. Data are the mean \pm S.E.M. (n=6). (E) Protein levels of CYP3A11 and UGT1A1 in mouse livers were detected by Western Blot analysis. (F) Specific band intensity was quantified, normalized to GAPDH, and expressed as mean \pm S.E.M. (n=3). *P < 0.05 versus vehicle, #P < 0.05 versus LCA alone.

DMD # 66969

Figure 4. Effects of WZ and its six bioactive lignans on hPXR activation. LS174T

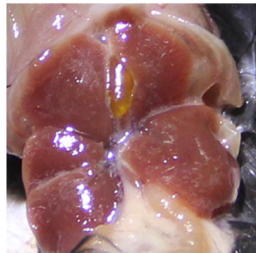
cells were transiently transfected with expression vectors encoding human PXR (pSG5-hPXR), reporter plasmid (pGL3-CYP3A4- XREM-Luc) and control plasmid (pRL-TK). Cells were treated with different concentrations of WZ ethanol extraction, SinA, SinB, SinC, SolA, SolB, StnA or positive agonist Rifampicin 24 h prior to harvest. Data are the mean \pm S.E.M. (n = 4). *P < 0.05 versus Control.

Figure 5. WZ altered p53/p21-related liver regeneration pathway in

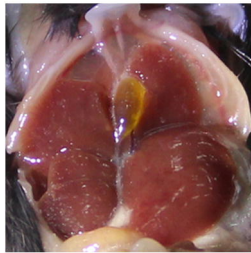
LCA-induced cholestasis model. (A) Western blot was used to measure p53, p21, PCNA and CCND1 protein expression. (B) Specific band intensity was quantified, normalized to GAPDH, and expressed as mean \pm S.E.M. (n=3). *P<0.05 versus vehicle, #P<0.05 versus LCA alone.

Figure 1

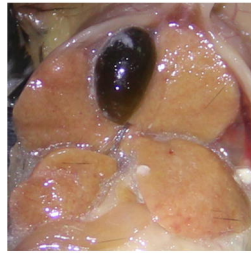
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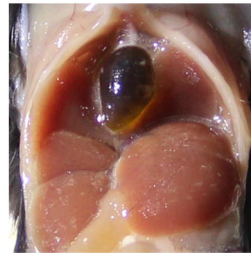
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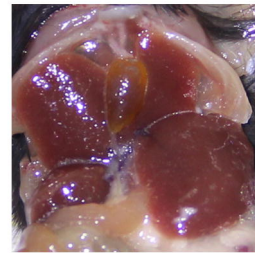
WZ



LCA

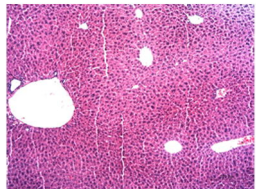


LCA+WZ

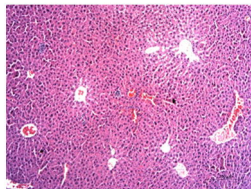


LCA+PCN

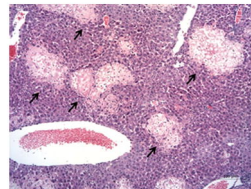
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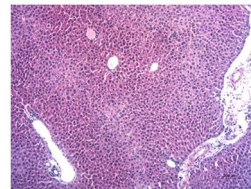
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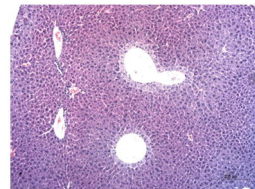
WZ



LCA



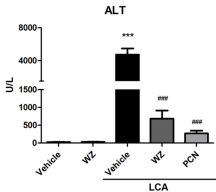
LCA+WZ



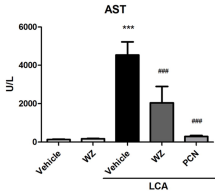
LCA+PCN

Figure 2

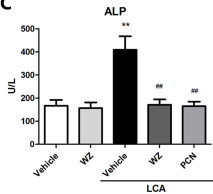
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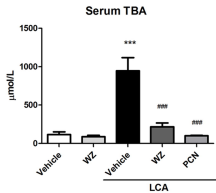
B



C



D



E

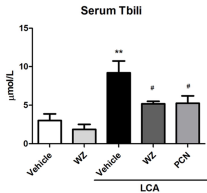


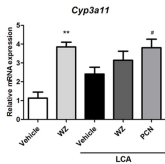
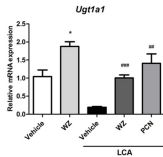
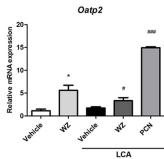
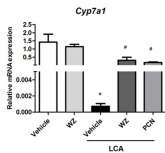
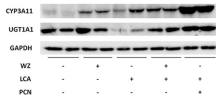
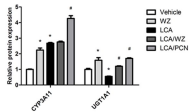
Figure 3**A****B****C****D****E****F**

Figure 4

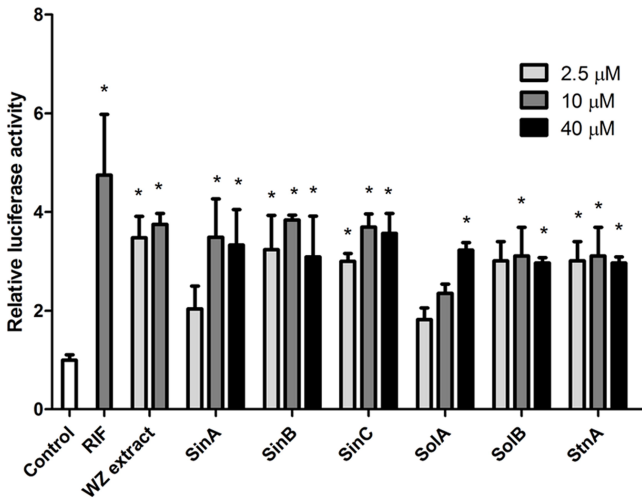
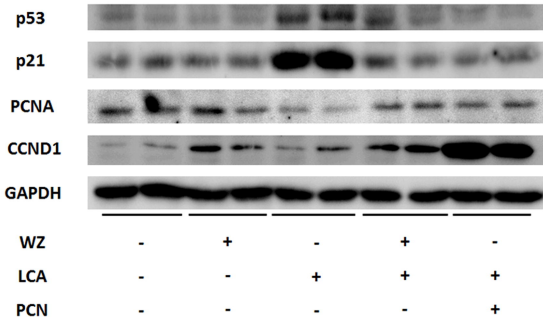


Figure 5

A



B

