Paradoxical Effects of Emodin on ANIT-Induced Intrahepatic Cholestasis and Herb-Induced Hepatotoxicity in Mice

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ABSTRACT

Emodin is an active ingredient in many herbal medicines and has a broad spectrum of pharmacological activities. The current data indicate that emodin exerts its beneficial effect on alpha-naphthylisothiocyanate (ANIT)-induced intrahepatic cholestasis through its anti-oxidant and anti-inflammatory activities. Emodin has little effect on the concentrations of bile acids (BAs) in livers of ANIT-treated mice. Instead, emodin shows a potential pro-cholestatic effect by interfering with the crosstalk between AMP-activated protein kinase (AMPK) and farnesoid X receptor (Fxr) in the liver, which leads to a suppression of bile salt export pump (Bsep). Two emodin-containing herbs, namely Polygonum multiflorum (PM) and Semen cassiae (SC), markedly aggravate the intrahepatic cholestasis in ANIT-treated mice. SC interferes with the AMPK-Fxr crosstalk and suppresses Bsep in livers of mice. ANIT markedly increases the hepatic retention of emodin in SC-treated mice. The major SC constituents, in particular three anthraquinones, are able to activate AMPK in HepG2 cells and inhibit Bsep in primary mouse hepatocytes, with emodin showing the strongest activities. Together, the present study identifies a potential pro-cholestatic role of emodin in the hepatotoxicity of herbs.

Key words: ANIT-induced cholestasis; emodin; AMPK.

Cholestasis, characterized as the reduction of bile flow, is one of the most common features in many human liver diseases (Bohan and Boyer, 2002; Trauner et al., 1998). Currently, very few effective therapies are available for treating cholestasis, with ursodeoxycholic acid (UDCA) and obeticholic acid (OCA) being the only drugs approved by the Food and Drug Administration (Chascsa et al., 2017). Therefore, there is an urgent need for developing novel therapies to treat cholestasis.

Alpha-naphthylisothiocyanate (ANIT)-induced cholestatic animal models have been widely used for the identification of effective therapies for cholestatic liver diseases. Emodin has a broad spectrum of pharmacological effects, and has been suggested to be a valuable therapeutic option for the treatment of various diseases, including constipation, dermatitis, osteoarthritis, diabetes, neurodegenerative diseases, cancers, and liver diseases (Dong et al., 2016). Emodin was shown to inhibit the NF-κB-mediated inflammatory pathway in livers of ANIT-induced cholestatic rats (Ding et al., 2008). The hepatic retention of bile acids (BAs) has been suggested to be the main cause of cell death during cholestasis (Perez and Briz, 2009). However, very little is known about the effect of emodin on the BA homeostasis during cholestasis.

Many emodin-containing herbs, such as Polygonum multiflorum (PM) and Semen cassiae (SC), have been reported to possess...
hepatoprotective activities in various liver injury models (Lee et al., 2012; Paudel et al., 2018). Paradoxically, both PM and SC have been reported to produce hepatotoxicity in the clinical practice (Li et al., 2017a; Teo et al., 2016). Nonetheless, it remains unknown whether emodin-containing PM and SC protect against ANIT-induced cholestasis. In this study, mice were treated with ANIT to induce intrahepatic cholestasis. The effects of emodin on inflammation, oxidative stress, and BA homeostasis were systematically investigated. Furthermore, the effects of two emodin-containing herbs (PM and SC) on ANIT-induced cholestasis were also evaluated.

MATERIALS AND METHODS

Chemicals and reagents. BA standards were purchased from either Sigma-Aldrich (St. Louis, Missouri) or Steraloids, Inc. (Newport, Rhode Island). The tetra-OH-BA standards were originally a gift from Dr. Alan Hofmann (University of California, San Diego), and obtained from Dr. Curtis Klaassen (University of Kansas Medical Center, Kansas). ANIT and dorsomorphin (compound C) were purchased from Sigma-Aldrich (St. Louis, Missouri). Emodin was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Physcion, aurantio-obtusin, obtusifolin, obtusin, and norrubrofusarin were purchased from Yuanye Bio-technology (Shanghai, China). The SC and PM extracts were prepared according to our previous methods (Wang et al., 2017, 2018). The major compounds in PM extract were evaluated and identified as shown in Supplementary Figure 1. All other reagents were purchased from commercial vendors and were of the highest purity grade available.

Animal treatment. Male C57BL/6J mice (8-week-old) and SD rats (180–200 g body weights) were purchased from Vital River Laboratory Animal Technology (Beijing, China). Animals were kept in an environmentally controlled breeding room (temperature: 22 ± 2 °C, humidity: 60 ± 5%, 12 h dark/light cycle) at the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences (CAMS, Tianjin, China). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals at the CAMS. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the CAMS.

Dry SC (4 g) was crushed and extracted with 40 l of 70% ethanol. Similarly, dry PM (20 g) was extracted by ethanol and the obtained extract was about 3.4 kg. Chinese Pharmacopoeia recommends the dosages of SC at 9–15 g/day and PM at 6–12 g/day, respectively, for the therapeutic uses in humans. To study the pharmacological effects of herbs, rodents are usually treated with very high doses. For instance, rats were treated with 15 g/kg SC (equivalent to 9 times human consumption) to investigate the effects of SC on activity of hepatic microsomal CYP450s (Xu et al., 2016). Previous studies demonstrated that chronic treatments with SC or PM at a dosage more than 10 times of that of human dose did not produce obvious hepatotoxicity in rats (Li et al., 2017a; Pei et al., 2017). In the present study, we set the high doses for SC at 30 g/kg (3.27 g/kg of SC extract) and PM at 20 g/kg (3.4 g/kg of PM extract), respectively, which were equivalent to about 9 times the human consumption. The dose conversions were based on the normalization of doses to body surface area. SC and PM extracts were dissolved in normal saline.

The vehicle control was corn oil for ANIT, 10% PEG400 in 0.5% sodium carboxymethyl cellulose for emodin, as well as saline for PM and SC extracts. To evaluate the effect of emodin on ANIT-induced cholestasis, mice (n = 6–8/group) were divided into 4 groups (Control, Emodin, ANIT, and ANIT + Emodin). Emodin and ANIT + Emodin groups were treated with 150 mg/kg emodin (p.o.) for 7 days (Days 1–7), whereas control and ANIT groups were given 10% PEG400 in 0.5% sodium carboxymethyl cellulose. Two hours after emodin treatment on the fifth day (Day 5), a single dose of ANIT (50 mg/kg, p.o. in corn oil) was administered to ANIT and ANIT + Emodin groups. The other two groups were given corn oil (p.o.). Forty-eight hours after ANIT treatment, mice were anesthetized with 10% chloral hydrate (5 ml/kg, i.p.) and sacrificed. Blood was collected by orbital bleeding and serum was obtained by centrifuging blood at 3000 × g for 15 min. Livers were washed with saline, snap-frozen in liquid nitrogen, and stored at −80 °C.

To determine the effect of SC on ANIT-induced cholestasis, mice (n = 6–8/group) were randomly assigned into 6 groups (Control, SC-L, SC-H, ANIT, ANIT + SC-L, and ANIT + SC-H). SC-L and ANIT + SC-L were treated with 10 g/kg SC (p.o., about 3 times the clinical dose in humans) for 7 days (Days 1–7), whereas SC-H and ANIT + SC-H were treated with 30 g/kg SC (p.o., about 9 times the clinical dose in humans). Control and ANIT were given 10 ml/kg normal saline (p.o.). Two hours after saline or SC treatment on the fifth day (Day 5), a single dose of ANIT (50 mg/kg, p.o. in corn oil) was administered to ANIT, ANIT + SC-L, and ANIT + SC-H groups. The other three groups were given corn oil (p.o.). Forty-eight hours after ANIT treatment, mice were anesthetized 10% chloral hydrate (5 ml/kg, i.p.) and sacrificed. To determine the effect of PM on ANIT toxicity, mice (n = 6–8/group) were divided into 4 groups (Control, PM, ANIT, and ANIT + PM). The PM treatment procedure was the same as the SC treatment, except that mice were gavaged with 20 g/kg PM (3.4 g/kg PM extract).

Four-day and 14-day repeated dosing are frequently used to investigate the short-term and subchronic effects of drugs, respectively (Hayes et al., 1986; Xie et al., 2001; Zhang et al., 2004). To further evaluate the effect of emodin on BA homeostasis, mice (n = 6–9/group) were gavaged with emodin at 30 (Emodin-I), 90 (Emodin-M), and 300 mg/kg (Emodin-H) for either 4 or 14 days. For the study on the 14-day repeated-dose effect of SC, mice (n = 6–8/group) were orally administered with SC at 10 (SC-L) and 30 g/kg (SC-H), corresponding to 3 and 9 times the clinical dose in humans, respectively. To analyze the effect of emodin on bile flow, rats (n = 5/group) were randomly assigned into control or emodin groups. Rats in emodin group were treated with 150 mg/kg emodin (p.o.) for 7 consecutive days. Two hours after the last dose on the seventh day, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and the bile ducts were cannulated with PE-10 tubing after laparotomy. Bile samples were collected for a total of 30 min.

Blood biochemistry and histopathology. The serum ALT, ALP, total bilirubin, and total BAs were determined using standard enzymatic colorimetric assays in accordance with the manufacturer’s protocols (Jiancheng Biological Technology, Nanjing, China). Tissues were fixed in 10% formalin prior to routine processing and paraffin embedding. The paraffin tissues were cut into slices (5 μm). Tissue sections were stained with hematoxylin and eosin. The sections were analyzed blindly by a pathologist.

BA quantification by LC-MS. BA extraction was performed according to a method described previously (Zhang and Klaassen, 2010). BAs were determined by an Agilent 1260 HPLC system (Agilent Technologies, Palo Alto, California) coupled with an
Agilent 6420 Triple Quadrupole mass spectrometer equipped with an ESI source, according to a previous method (Zhou et al., 2014). The separation process was accomplished on an Eclipse Plus C18 (2.1 mm x 150 mm, 3.5 μm) column (Agilent Technologies, Palo Alto, California).

GSH quantification. Glutathione (GSH) was extracted from liver tissues according to a previous method (Yilmaz et al., 2009). GSH was determined by an Agilent 1260 HPLC system controlled by Agilent Lab Advisor software (Agilent Technologies, Palo Alto, California). The samples were separated on an Agilent ZORBAX SB-C18 column (5 μm, 4.6 x 150 mm) and detected by UV absorption at 210 nm. The mobile phase contained 10 mM NaClO₄ with 0.1% H₃PO₄ (pH 3.0) and 5% methanol. Flow rate was 1 ml/min and column temperature was set at room temperature.

Cell culture and luciferase assay. HepG2 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin. Cells were seeded in 12-well plates at a density of 2 x 10⁶ cells per well. The next day the adherent cells were cultured in DMEM without FBS supplement for 2 h. Subsequently, the cells were exposed with 5 μM of the indicated compounds for 30 min or 1 mM metformin for 2 h Cells were washed with 1 x PBS and sonicated with RIPA buffer. Protein concentration was quantified using bovine serum albumin (BSA) as the standard.

Primary hepatocytes were isolated from mice using a two-step in situ collagenase perfusion method (Tao et al., 2014). Primary hepatocytes were seeded in DMEM containing 10% FBS overnight, and were then exposed to 10 μM of the indicated compounds for 6 h. To determine the role of AMPK in emodin-induced inhibition of Bsep, primary mouse hepatocytes were pretreated with 5 μM of compound C for 30 min before starting emodin treatment (10 μM) for 6 h. At the end of treatment, cells were washed with 1 x PBS, and lysed for RNA and protein extraction.

For the luciferase assay, the plasmids (pGL4-SHP-TK, pCMV-ICIS-hFXR, and pCMV-renilla) were kindly provided by Dr Grace L. Guo (Rutgers University). HepG2 cells were transfected with the plasmids according to a previous method (Li et al., 2010). After transfection for 12 h, cells were treated with 0.1% DMSO (negative control), 500 nM GW4064 (positive control), or 10 μM of the testing compounds for 24 h. After treatment, cells were collected to quantify firefly and renilla luciferase activities using the Dual-Glo Luciferase Kit (Promega, Madison, Wisconsin) in a Tecan Infinite M200 Microplate Reader (Grödig, Austria). The luciferase activity of renilla was standardized to firefly luciferase.

RNA extraction and qPCR analysis. Total RNA was isolated using RNAiso Plus (Takara, Dalian, China) according to the manufacturer’s protocol. Total RNA was reverse-transcribed to synthesize cDNA using HiScript QRT SuperMix for qPCR (Vazyme Biotech, Nanjing, China). The amplification reactions were run on an ABI QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems, Foster City, California) using Ultra SYBR Mixture (Cwbio Bio Inc., Beijing, China). The comparative threshold cycle (Ct) method was used to quantify the fold change (2^ΔΔCt) for various mRNAs. The mRNA data were normalized to 36b4 mRNA and presented as relative fold change to the control group. Primer sequences for BA-related genes are shown in Supplementary Table 1.

Western blot and fluorescence immunostaining. Liver cytosol and plasma membrane preparations were made as described previously (Aleksunes et al., 2006). Nuclear proteins were prepared as follows. Tissues were homogenized in the hypotonic lysis buffer, and centrifuged for 20 min at 10 000 x g. The pellets were re-suspended using the nuclear extraction buffer, and centrifuged for 5 min at 20 000 x g to collect the supernatant as nuclear proteins. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels (6% for Bsep protein separation), and then transferred onto PVDF membranes (Merck Millipore, Darmstadt, Germany). Membranes were incubated with antibodies against β-Actin (1:1000) (Santa Cruz, California), Cyp7a1 (1:1000) (ab65596, Abcam, Cambridge, Massachusetts), Fxr (1:200) (sc-13063, Santa Cruz, California), Shp (1:1000) (sc-30169, Santa Cruz, California), p-AMPK (1:1000) (25355, Cell signaling, Danvers, Massachusetts), AMPK (1:1000) (25325, Cell Signaling Technology, Danvers, Massachusetts), Bsep (sc-74500, Santa Cruz, California), Fgf15 (sc-27177, Santa Cruz, California), histone H3 (1:1000) (no. 9715, Cell Signaling Technology, Danvers, Massachusetts), and NF-κB p-65 (1:1000) (no. 4764, Cell Signaling Technology, Danvers, Massachusetts). We validated the antibodies for p-AMPK, Fxr, Shp, and Cyp7a1 using either positive control-treated or knockdown cells (Supplementary Figure 2). The other antibodies were confirmed by the technical support from the corresponding suppliers. The protein bands were visualized with the Amersham Imager 600 after reacting with ECL Western blotting detection reagents (Merck Millipore, Darmstadt, Germany). Fluorescence immunostaining was performed according to our previous method (Lu et al., 2018).

Statistical analysis. Data are expressed as means ± SEM. Statistical analysis was performed using GraphPad Prism software, version 7.0. The differences between groups were analyzed using a 1-way analysis of variance (ANOVA) followed by 2-by-2 comparisons using Tukey’s test. Differences were considered significant at p < 0.05.

RESULTS

Emodin Protected Against ANIT-Induced Cholestatic Liver Injury in Mice

A single dose of 50 mg/kg ANIT was selected to induce cholestasis in this study (Figure 1A). ANIT caused a decrease in body and liver weights (Figure 1B). Its hepatotoxicity was evidenced by a marked elevation in serum levels of ALT, ALP, and total bilirubin, which were markedly decreased by emodin (Figure 1C). GSH conjugation is the first step for ANIT to produce liver injury (Dietrich et al., 2001). However, no alterations in hepatic GSH were observed in ANIT-treated mice (Figure 1D). Pathological analysis exhibited multifocal necrosis, neutrophil infiltration adjacent to necrotic area, biliary hyperplasia, and distorted nuclei near bile ducts in livers of ANIT-treated mice (Figure 1E).

Emodin markedly attenuated neutrophil infiltration as well as necrotic and degenerative changes in livers of ANIT-treated mice, suggesting a protective effect of emodin on ANIT toxicity.

Emodin Demonstrated Anti-inflammatory and Anti-oxidative Activities in ANIT-Treated Mice

Inflammation and oxidative stress have been suggested to be associated with the pathogenesis of ANIT-induced cholestasis (Ohta et al., 1999). In the present study, ANIT increased mRNAs of 5 inflammatory factors, such as interleukin-1β (IL-1β), IL-6, IL-10, tumor necrosis factor alpha (TNFα), as well as two inflammation-related genes, namely inducible nitric oxide synthase (iNOS) and α-smooth muscle actin (αSMA) (Figure 2A).
Emodin markedly decreased IL-1β, IL-6, IL-10, and α-SMA in livers of ANIT-treated mice, which was associated with inhibition of NF-κB p65 protein in nuclear extracts (Figure 2B). ANIT increased mRNAs of several anti-oxidative genes, such as quinone dehydrogenase 1 (Nqo1), heme oxygenase-1 (HO-1), glutathione peroxidase 2 (Gpx2), glutathione S-transferases (Gstα1/2 and Gstm3), and growth arrest and DNA-damage-inducible protein (Gadd45α) (Figure 2C). Emodin decreased mRNAs of HO-1, Gpx2,
Gst\(\alpha1/2\), and Gadd45\(\alpha\) in livers of ANIT-treated mice. Therefore, emodin alleviates ANIT toxicity in mice through its anti-inflammatory and anti-oxidative activities.

**Emodin Had Little Effect on Hepatic Accumulation of BAs in ANIT-Treated Mice**

Cholestatic liver injury is usually attributed to the accumulation of BAs in the liver (Zhang et al., 2012). In the present study, ANIT markedly increased BAs in both serum and livers, whereas it significantly decreased BAs in ilea (Figure 3A). Emodin decreased BAs in serum of ANIT-treated mice, whereas it had little effect on BAs in livers and ilea. Additionally, it should be noted that emodin alone showed a tendency to increase BAs in both serum and livers, but decrease BAs in ilea.

ANIT-treated mice underwent some adaptive responses to hepatic BA retention, including a decrease in BA synthesis...
(Cyp7a1, 8b1, 27a1, and 7b1) and BA uptake (Ntcp and Oatp1b2) (Figs. 3B and 3C). In contrast, ANIT showed mixed effects on efflux transporters, including an increase in Mrp4 and Ostb, as well as a decrease in Bsep, Mrp2, Mrp3, and Ostx. Emodin increased mRNAs of Ntcp, Oatp1b2, and Bsep, whereas it decreased mRNA of Mrp4 in livers of ANIT-treated mice. Notably, emodin alone significantly decreased Cyp27a1, Cyp7b1, Mrp2, Mrp3, and Bsep in livers of mice (Figs. 3B and 3C). Bsep is the rate-limiting step that mediates the efflux of BAs from liver to bile. Western blot was performed to further confirm that emodin suppressed Bsep under the normal condition, but induced Bsep under the cholestatic condition (Supplementary Figure 3B).

Emodin Inhibited Bsep in Both Mouse Livers and Hepatocytes

The effect of emodin on Bsep was further investigated in livers of mice treated with emodin at 3 doses (30, 90, and 300 mg/kg) for either 4 or 14 days. Both mRNA and protein of Bsep were decreased by emodin at the high dose (Figure 4A, and Supplementary Figure 3C). Four-day treatment of emodin showed little effect on other BA transporters, whereas 14-day treatment of emodin at the high doses decreased Mrp2 and Ostx. Despite of its inhibitory effect on mRNAs of Bsep and Mrp2, emodin showed little effect on bile flow or biliary excretion of BAs in rats (Figure 4B).

Bsep is a known direct target gene of Fxr and is induced by Fxr activation. Compared with the ANIT group, the mRNA and protein expressions of Fxr and its target gene Shp were not altered in the ANIT + Emodin group, suggesting that Fxr was not involved in emodin-mediated induction of Bsep under the cholestatic condition (Figure 4C). It should be noted that emodin alone decreased both mRNA and protein of Fxr (Figure 4C). Emodin alone increased Shp mRNA, but had little effect on Shp.

**Figure 3.** Emodin had little effect on hepatic accumulation of BAs in ANIT-treated mice. (A) Total BA concentrations in serum, livers, and ilea in mice treated with emodin and ANIT. (B) The mRNA expression of BA synthetic enzymes in livers. (C) The mRNA expression of transporters in livers. Relative mRNA levels were calculated as fold change normalized to controls. The data are expressed as mean ± SE, n = 6-8. *p < .05 versus Control group. #p < .05 versus ANIT group.

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Figure 4. Emodin inhibited Bsep in both mouse livers and hepatocytes. (A) Mice (n = 6–8/group) were divided to one control group and three emodin groups gavaged with emodin at 30 (Emodin-L), 90 (Emodin-M), and 300 mg/kg (Emodin-H) for either 4 days or 14 days. The mRNA expression of major transporters in livers of mice after 4-day and 14-day treatments of emodin. (B) Rats were treated with emodin (150 mg/kg, p.o.) for 7 days, and the effect of emodin on bile flow and biliary excretion of BAs was investigated. (C) The mRNA expression of Fxr and Shp in livers, as well as Western blot analysis of Fxr, Shp, p-AMPK, and AMPK in livers of mice treated with Emodin, ANIT, and ANIT + Emodin. (D) Primary mouse hepatocytes were pretreated with 5 μM of compound C for 30 min before starting emodin treatment (10 μM) for 6 h. The expression of Bsep was quantified. Relative mRNA levels were calculated as fold change normalized to controls. The data are expressed as mean ± SE, n = 3. *p < .05 versus Control group.
protein. Several studies report that emodin is an activator of AMPK, which is a suppressor of Fxr transcriptional activity (Chen et al., 2012; Song et al., 2013; Tzeng et al., 2012). The current data showed that emodin activated AMPK in livers of mice (Figure 4C). Further study in primary mouse hepatocytes showed that emodin-mediated inhibition of Bsep could be reversed by co-treatment of compound C, a potent inhibitor of AMPK (Figure 4D and Supplementary Figure 3D). Collectively, the AMPK-Fxr crosstalk appears to contribute to the inhibitory effect of emodin on Bsep under the normal condition.

**Emodin-Containing Herbs Aggravated ANIT-Induced Cholestatic Liver Injury in Mice**

Emodin is a major anthraquinone in PM and SC, which have been widely consumed as either herbal medicine or tea (Ahn et al., 2016; Dong et al., 2017). Our preliminary data showed that PM at a dose equivalent to 9 times the human consumption significantly increased serum levels of ALT and total bilirubin in mice given 75 mg/kg ANIT (Figure 5A). Because deaths were observed in mice cotreated with SC and 75 mg/kg ANIT (data not shown), we chose 50 mg/kg ANIT to induce cholestasis in mice to investigate the aggravating mechanisms of SC. As shown in Figure 5B, SC markedly increased serum ALT and total bilirubin of ANIT-treated mice in a dose-dependent manner. Consistently, the two ANIT + SC groups showed more severe morphological changes than the corresponding ANIT groups (Figure 5C). In contrast, SC had little effect on GSH concentration (Figure 5D), anti-oxidative genes (Ngo-1, Gstz1/2, Gstm3, and Gpx2) (Figure 5E), and inflammatory genes (IL-6, IL-10, TNFα, and scl-SMA) (Figure 5F) in livers of ANIT-treated mice. Therefore, the aggravating effect of SC on ANIT toxicity is not due to increased oxidative stress or inflammation.

**SC Increased BA Accumulation in ANIT-Treated Mice in a Dose-Dependent Manner**

As shown in Figure 6A, SC alone had little effect on serum or liver BAs. In contrast, SC increased BAs in both serum and livers of ANIT-treated mice in a dose-dependent manner. Further analysis revealed that the BAs accumulated in the liver were majorly the primary conjugated BAs. SC alone tended to increase Cyp7a1 mRNA (Figure 6B). In contrast, SC at the high dose significantly decreased Cyp7a1 mRNA in livers of ANIT-treated mice (Figure 6B). Western blot analysis confirmed the mixed effects of SC on Cyp7a1 under normal and cholestastic conditions (Figure 6C). Compared with the ANIT group, the ANIT + SC-H group showed lower expressions of 2 BA uptake transporters (Ntcp and Oatp1b2) and 2 canalicular BA efflux transporters (Bsep and Mrp2), but higher expressions of two transporters (Ntcp and Oatp1b2) and 2 canalicular BA efflux transporters (Bsep and Mrp2). Among these transporters, only Bsep was decreased by SC among these transporters, only Bsep was decreased by SC. SC alone tended to increase Cyp7a1 mRNA in livers of ANIT-treated mice (Figure 6B). In contrast, SC at the high dose significantly decreased Cyp7a1 mRNA in livers of ANIT-treated mice (Figure 6B). Western blot analysis confirmed the mixed effects of SC on Cyp7a1 under normal and cholestastic conditions (Figure 6C). Compared with the ANIT group, the ANIT + SC-H group showed lower expressions of 2 BA uptake transporters (Ntcp and Oatp1b2) and 2 canalicular BA efflux transporters (Bsep and Mrp2), but higher expressions of two transporters (Ntcp and Oatp1b2) and 2 canalicular BA efflux transporters (Bsep and Mrp2). Among these transporters, only Bsep was decreased by SC alone. Western blot confirmed the inhibitory effect of SC on Bsep protein (Supplementary Figure 3E). Unlike in livers, both ANIT and ANIT + SC had little effect on major BA transporters (Asbt, Ostx, and Ostβ) in ilea, except that they decreased lbabp mRNA (Figure 6E). Therefore, the aggravating effect of SC on ANIT toxicity appears to be due to Bsep inhibition and thus BA accumulation in the liver.

**SC Interfered With the AMPK-FXR Crosstalk in Livers of Mice**

Activation of AMPK has been shown to inhibit Fxr expression, contributing to the pathogenesis of cholestasis induced by ANIT and estrogen (Li et al., 2016a, 2017b; Lien et al., 2014). In the present study, SC had little effect on mRNAs of Fxr and Shp in livers of ANIT-treated mice, except that Shp mRNA was increased in the ANIT + SC-H group (Figure 7A). Nonetheless, Western blot analysis revealed that SC alone increased AMPK phosphorylation and decreased Fxr protein in livers of mice (Figure 7B). Indeed, compared with the control group, AMPK phosphorylation was increased in all other treatment groups. Given the fact that SC is a commonly consumed tea, mice were treated with SC at two doses for 14 days to evaluate its long-term effect. Both the low and high dose of SC decreased Bsep mRNA and protein, whereas they had little effect on the other BA transporters in livers of mice (Figure 7C and Supplementary Figure 3F). It should be noted that long-term treatment of SC had little effect on Fxr and Shp mRNAs, but significantly decreased G6P, a target gene of AMPK in livers of mice (Figure 7D). Additionally, long-term SC treatment showed little effect on Fxr protein, but increased AMPK phosphorylation in livers of mice (Figure 7E). Further analysis revealed that long-term treatment of SC markedly altered the intestinal Fxr-Fgf15 signaling (Supplementary Figure 7). This suggests a time-dependent effect of SC on Fxr signaling in mice. Taken together, the aggravating effect of SC on ANIT toxicity appears to be due to its interference with the AMPK-Fxr crosstalk.

**ANIT Markedly Increased Hepatic Retention of Emodin in SC-Treated Mice**

Our previous study identified the major SC constituents in the blood of rodents after SC treatment, including 2 naphthopyrones (cassiaside and nor-rubrofuscinarin) and 5 anthraquinones (emodin, aurantoio-obtusin, phycion, obtusifolin, and obtusin) (Figure 8A) (Wang et al., 2018). In the present study, only 3 compounds (emodin, aurantoio-obtusin, and obtusin) were detected with significant amounts in livers of mice after SC treatments (Supplementary Table 2). Compared with the SC-H group, the relative concentration of emodin was significantly increased about 140% in livers of the ANIT + SC-H group (Figure 8B). Additionally, aurantoio-obtusin and obtusin also tended to increase (50%–90%) in livers of the ANIT + SC-H group. Next, HepG2 cells were either treated with 5 μM compounds for 30 min, or with 1 mM metformin for 2 h as a positive control. Five compounds (emodin, obtusin, phycion, nor-rubrofuscinarin, and obtusifolin) increased AMPK phosphorylation with emodin showing the strongest activity (Figure 8C). In contrast, cassiaside and aurantoio-obtusin exhibited a suppressive effect on AMPK phosphorylation. Interestingly, none of these compounds was able to activate Fxr in HepG2 cells as shown by the Fxr reporter assay (Figure 8D). Due to the low expression of BSEP in HepG2 cells, primary mouse hepatocytes were used to evaluate the effect of these compounds on Bsep. Four compounds (emodin, obtusin, phycion, and cassiaside) were shown to decrease Bsep mRNA (Figure 8E). Collectively, SC-mediated interference with the AMPK-Fxr crosstalk in livers of ANIT-treated mice appears to be due to the increased hepatic accumulation of emodin and other anthraquinones.

**DISCUSSION**

Exposure of rodents to ANIT is an established intrahepatic cholestatic model for the identification of possible cholestasis therapies. This study confirmed the hepatoprotective activity of emodin in the ANIT-induced cholestatic mouse model. Unexpectedly, 2 emodin-containing herbs (PM and SC) were shown to markedly aggravate the cholestatic liver injury in ANIT-treated mice.
Figure 5. Emodin-containing herbs aggravated ANIT-induced cholestatic liver injury in mice. (A) Serum ALT and total bilirubin in mice treated with Polygonum multiflorum (PM) and ANIT. Mice were randomly divided into 4 groups (Control, PM, ANIT, and ANIT + PM). PM was orally given at a dose (20 g/kg) equivalent to 9 times of human consumption (6–12 g/day). ANIT was given at 75 mg/kg (p.o.). (B) Serum ALT and total bilirubin in mice treated with SC and ANIT. Mice were randomly assigned into 6 groups (Control, SC-L, SC-H, ANIT, ANIT + SC-L, and ANIT + SC-H). SC-L and SC-H were given SC at 10 g/kg and 30 g/kg, respectively. A single oral dose of 50 mg/kg ANIT was used to induce cholestasis in mice. Blood and tissues were collected 48 h after ANIT treatment. (C) Histological analysis of liver sections of mice treated with SC and ANIT. (D) Concentration of GSH in livers of mice. GSH was quantified using HPLC-UV. (E) The mRNA expression of genes involved in anti-oxidative stress in livers of mice. (F) The mRNA expression of genes involved in inflammation in livers of mice. Relative mRNA levels were calculated as fold change normalized to controls. The data are expressed as mean ± SE, n = 6-8. *p < .05 versus Control group. #p < .05 versus ANIT group.
Figure 6. SC increased BA accumulation in ANIT-treated mice in a dose-dependent manner. Mice were randomly assigned into 6 groups (Control, SC-L, SC-H, ANIT, ANIT + SC-L, and ANIT + SC-H). SC-L and SC-H were given SC at 10 g/kg and 30 g/kg, respectively. (A) The concentrations of total BAs (T-BAs), total conjugated BAs (C-BAs), total unconjugated BAs (U-BAs), total primary BAs (1^oBAs), and total secondary BAs (2^oBAs) in serum and livers, respectively. BAs were quantified by HPLC-MS/MS. (B) The mRNA expression of BA synthetic enzymes in livers of mice. (C) Western blot analysis of Cyp7a1 in livers of mice. (D) The mRNA expression of transporters in livers of mice. (E) The mRNA expression of BA transporters in ilea of mice. Relative mRNA levels were calculated as fold change normalized to controls. The data are expressed as mean ± SE, n = 6–8. *p < .05 versus Control group. #p < .05 versus ANIT group.
Figure 7. SC interfered with AMPK-Fxr in livers of mice. (A) The mRNA expression of Fxr, Shp, and Fgfr4 in livers of mice treated with SC and ANIT. Mice were randomly assigned into 6 groups (Control, SC-L, SC-H, ANIT, ANIT + SC-L, and ANIT + SC-H). SC-L and SC-H were given SC at 10 g/kg and 30 g/kg, respectively. (B) Western blot analysis of Fxr, Shp, p-AMPK, and AMPK in livers of mice treated with SC and ANIT. (C) The effect of 14-day treatment of SC on the mRNA expression of BA transporters in livers of mice. Mice ($n = 6–8$) were orally administered with SC at 10 and 30 g/kg (SC-L and SC-H), corresponding to 3 and 9 times the clinical dose in humans, respectively. (D) The mRNA expression of Fxr, Shp, and G6P in livers of mice after 14-day treatment of SC. (E) Western blot analysis of Fxr, Shp, p-AMPK and AMPK in livers of mice after 14-day treatment of SC. Relative mRNA levels were calculated as fold change normalized to controls. The data are expressed as mean ± SE, $n = 6–8$. * $p < .05$ versus control group. # $p < .05$ versus ANIT group.
Many mechanisms have been shown to be involved in the pathogenesis of ANIT-induced cholestasis. Consistent with previous study in rats, emodin in this study also inhibited inflammation in livers of ANIT-treated mice by suppressing the NF-κB pathway. Previous studies showed contradictory effects of ANIT on GSH concentration in the liver. For instance, 60 mg/kg of ANIT was shown to increase GSH about 70% in livers of rats after 48-h treatment (Ding et al., 2012), whereas 75 mg/kg ANIT
was able to decrease GSH about 40% in livers of mice after 48-h treatment (Ding et al., 2012; Zhao et al., 2017). In the present study, 50 mg/kg of ANIT had little effect on hepatic GSH concentration in the liver. Nonetheless, ANIT caused an increase in oxidative stress, which was suppressed by emodin. Although inhibition of mitochondrial fatty acid β-oxidation (β-FAO) has been suggested to be a major reason for ANIT-induced cholestasis, the current data suggests that emodin has little effect on β-FAO (Supplementary Figure 4). Therefore, emodin exerts its beneficial effect on ANIT-induced cholestasis through its antioxidative and anti-inflammatory activities.

Cholestasis is characterized by BA accumulation in the liver. In this study, ANIT caused a decrease in both BA synthesis (Cyp7a1, Cyp8b1, Cyp27a1, and Cyp7b1) and BA uptake (Ntcp and Oatp1b2), as well as an increase in basolateral BA efflux (Mrp4 and Ostj), which are consistent with previous observations (Gartung et al., 1996; Wagner et al., 2003; Zhang et al., 2012). Emodin decreased BAs in serum of ANIT-treated mice, which could be due to increased BA uptake transporters (Ntcp and Oatp1b2). In contrast, emodin had little effect on hepatic BAs, which might be due to decreased BA efflux transporter (Mrp4). Although emodin alone showed little effect on bile flow in rats, it tended to increase BAs in serum and livers of mice. Additionally, long-term treatment of emodin at high dose showed a suppressive effect on mRNAs of both Mrp2 and Bsep, both of which play important roles in maintaining the bile flow. Previous study showed that emodin could increase serum ALT, AST, and total BAs in rats treated with lipopolysaccharide (LPS), suggesting the potential hepatotoxicity of emodin (Tu et al., 2015). A 2-year National Toxicology Program (NTP) study also showed the hepatotoxic potential of emodin in normal rats and mice, characterized with increased serum levels of ALT and total bilirubin, as well as relative liver-to-body weight ratios (National Toxicology Program, 2001). Taken together, despite of the protective effect of emodin on ANIT toxicity, the current data suggest a procholestatic effect of emodin in normal mice.

The present study suggests that emodin has a suppressive effect on Bsep under the normal condition, but an inductive effect on Bsep under the cholestatic condition. Bsep has been shown to be suppressed by inflammatory cytokines in cholestasis (Hartmann et al., 2002; Kosters and Karpen, 2010). Therefore, emodin-mediated induction of Bsep in ANIT-treated mice could be attributed to its anti-inflammatory activity. Bsep is a direct target gene of Fxr, and could be induced by Fxr activation. Emodin is a known activator of AMPK, which is a negative regulator of Fxr transcriptional activities. In this study, emodin was shown to interfere with the AMPK-Fxr crosstalk in livers of mice. Emodin showed no direct effect on Fxr activity, as revealed by the luciferase assay in HepG2 cells. Additionally, emodin-mediated Bsep induction in primary mouse hepatocytes could be reversed by the AMPK inhibitor. Therefore, emodin-mediated suppression of Bsep under the normal condition appears to be due to its interference with the AMPK-Fxr crosstalk.

The hepatotoxicity of emodin-containing herbs, such as PM and Rheum palmatum L., has been reported in previous studies (Wang et al., 2011; Zhang et al., 2018). However, to the best of our knowledge, the present study demonstrates for the first time that 2 emodin-containing herbs, in particular SC, aggravate the cholestasis and liver injury induced by ANIT in mice. A single dose of 75 mg/kg ANIT is frequently selected to induce cholestasis in mice. However, the liver injury caused by 75 mg/kg ANIT might be too severe and mask the aggravating effect of SC (Supplementary Figure 5). This might explain why there are few studies about the aggravating effect of herbs on ANIT toxicity. Notably, liver injuries induced by these herbs frequently demonstrate some features of idiosyncrasy. For instance, no hepatotoxicity was observed in rats either after a 60-day treatment of PM at a dosage about 200 times that of the clinical dose, or after a 26-week treatment of SC at a dose equivalent to 10 times human consumption (Li et al., 2017a; Pei et al., 2017). LPS-treated mice have been shown to be a model to study the idiosyncrasy-like liver injuries of some drugs (Roth et al., 2003). Our study suggests that ANIT-treated mice might be also a useful model for drug- or herb-induced idiosyncrasy.

Several studies have demonstrated that the AMPK-Fxr crosstalk contributes to the pathogenesis of cholestasis induced by ANIT and estrogen (Li et al., 2016a, 2017b; Lien et al., 2014). Metformin aggravates ANIT-induced cholestasis through its activation of AMPK and thus Fxr inhibition, whereas UDCA alleviates estrogen-induced cholestasis through AMPK inhibition (Li et al., 2016b; Lien et al., 2014). In this study, SC did not exacerbate the effect of ANIT on oxidative stress, inflammation, or β-FAO (Supplementary Figure 6A), whereas it caused a dose-dependent increase in BAs in both serum and livers of ANIT-treated mice. The increased BAs were mostly conjugated primary BAs, especially taurocholic acid (TCA), taurolithocholic acid (TCA-L), and β-muricholic acid (β-MA) (Supplementary Figure 6B), which have been shown to induce pro-inflammatory mediators in hepatocytes (Zhang et al., 2012). SC also increased cytotoxic secondary BAs, such as taurodeoxycholic acid (TDCA), tauromuricholic acid (TMDCA), taurohyodeoxycholic acid (THDCA), and deoxycholic acid (DCA). In addition, SC suppressed tetra-OH BAs in livers, suggesting a strong inhibitory effect of SC on BA hydroxylation, the predominant BA detoxification pathway in rodents (Supplementary Figure 6C). SC-mediated increase of BA accumulation in livers of ANIT-treated mice was associated with an activation of AMPK-Fxr crosstalk and a suppression of Bsep, suggesting that AMPK-Fxr crosstalk was involved in SC-mediated aggravation of ANIT toxicity. Interestingly, SC showed a time-dependent effect on Fxr. Short-term treatment of SC suppressed Fxr protein and induced Cyp7a1 protein in the liver (Figure 7). Long-term treatment of SC did not alter liver Fxr-Shp signaling, but activated intestinal Fxr-Fgf15 signaling (Supplementary Figure 7A), which was accompanied with a decrease in Cyp7a1 mRNA in the liver and alterations in the intestinal microbial community composition (Supplementary Figure 7B). Nonetheless, long-term treatment of SC also increased AMPK phosphorylation and suppressed Bsep in livers of mice.

Herb-induced hepatotoxicity accounts for more than 20% of liver injury cases, but the component responsible for the toxicity is usually unknown or can only be suspected (Navarro et al., 2017). Identification of the responsible constituents and toxicity mechanisms is critical for regulatory oversight of these nonprescription products to guarantee their safety. In this study, emodin auranito-obtusin and obtusin were increased in livers of the ANIT + SC groups. Both emodin and obtusin showed a strong activity to increase AMPK phosphorylation and decrease Bsep in hepatocytes. Although the other ingredients and mechanisms could not be excluded, the current data suggests that emodin and obtusin are potential risk ingredients for SC-induced aggravation on intrahepatic cholestasis. Future studies should be conducted to evaluate the effect of obtusin and other anthraquinones on the AMPK-Fxr crosstalk and ANIT toxicity.

To conclude, the current data highlight a double-edged role of emodin in ANIT-induced cholestasis. Emodin exerts its beneficial effect on ANIT-induced cholestasis through its
anti-oxidative and anti-inflammatory activities. However, emodin interferes with the AMPK-Fxr crosstalk, which appears to cause the aggravating effect of SC on intrahepatic cholestasis. It should be noted that the current study is limited by not considering other minor SC constituents and other possible mechanisms. For example, a recent study suggests a potential role of metabolizing enzymes in the hepatotoxicity of emodin (Wu et al., 2018). Nevertheless, the present study represents our first step to elucidate the responsible constituents and mechanisms for the hepatotoxicity of emodin-containing herbs.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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ETHICAL STANDARDS

The manuscript does not contain clinical studies or participant data.

REFERENCES


