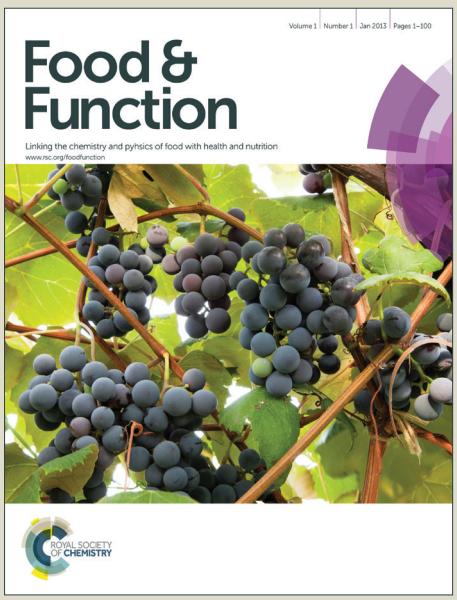
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Protective effects of alisol B 23-acetate from an edible botanical *rhizoma alismatis* against carbon tetrachloride-induced hepatotoxicity in mice

Qiang Meng, Xinli Chen, Changyuan Wang, Qi Liu, Huijun Sun, Pengyuan Sun, Xiaokui Huo, Zhihao Liu and Kexin Liu*

Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, China

* Corresponding author at: Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, 9 West Section, Lvshun South Road, Dalian 116044, China. Fax: + 86 411 8611 0407.

E-mail address: kexinliu@dlmedu.edu.cn (K.X. Liu)

ABSTRACT

Carbon tetrachloride (CCl_4)-induced hepatotoxicity is a common syndrome with simultaneous severe hepatocyte deaths and acute cholestasis. The purpose of the present study is to investigate the hepatoprotective effect of alisol B 23-acetate (AB23A), a natural triterpenoid from an edible botanical *rhizoma alismatis*, on acute hepatotoxicity induced by CCl_4 in mice, and further to elucidate the involvement of farnesoid X receptor (FXR) and signal transducers and activators of transcription 3 (STAT3) in the hepatoprotective effect. H&E staining, BrdU immunohistochemistry and TUNEL assay were used to identify the amelioration of histopathological changes, hepatocyte proliferation and apoptosis. Real-time PCR and Western blot assay were used to elucidate the mechanisms underlying AB23A hepatoprotection. The results indicated that AB23A treatment dose-dependently protected against hepatotoxicity induced by CCl_4 via FXR activation. Through FXR activation, AB23A promoted hepatocyte proliferation via an induction in hepatic levels of FoxM1b, Cyclin D1 and Cyclin B1. AB23A also reduced hepatic bile acids through decreasing hepatic uptake transporter Ntcp, bile acid synthetic enzymes Cyp7a1, Cyp8b1, and increasing efflux transporter Bsep, Mrp2 expression. In addition, AB23A induced STAT3 phosphorylation, STAT3 target genes Bcl-xl and SOCS3, resulting in decreased hepatocyte apoptosis. In conclusion, AB23A produces protective effect against CCl_4-induced hepatotoxity, due to FXR and STAT3-mediated gene regulation.

Keywords: Alisol B 23-acetate; CCl_4; FXR; STAT3; Transporters; Enzymes

Introduction

The liver represents the primary target for chemical toxic reaction due to its critical role in detoxification of both endogenous and exogenous substances. Therefore liver is more easily impaired than other organs. Toxicants, environmental pollutants, ionizing radiations and drug overdose can damage hepatocytes' membrane and organelles. Improper liver repair will lead to fibrogenesis and cirrhosis, which may eventually result in either liver failure or hepatocellular carcinoma.[1, 2] Carbon tetrachloride (CCl_4) is a hepatotoxicant widely used in rodents to study the mechanisms of toxin-induced liver injury.[3] CCl_4 injection model of hepatotoxicity and repair is closed to the clinical liver injury cases with simultaneous severe hepatocyte deaths and acute cholestasis.

Many genes and signaling pathways have been shown to regulate liver repair. Farnesoid X receptor (FXR) belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors and is highly expressed in liver, intestine, kidney, and adrenal glands.[4, 5] FXR has been shown to play a critical role in liver repair,[6, 7] and many target genes of FXR have been identified to be involved in this process. For example, forkhead box M1b (FoxM1b), a direct FXR target gene, is adequate to accelerate hepatocytes proliferation in liver regeneration and liver repair.[8] In addition, FXR is the primary bile acid receptor that acts as a master regulator of bile acid homeostasis.[9-12] Bile acids are potentially toxic, and substantial increases in hepatic bile acids levels will induce hepatocytes apoptosis and necrosis and promote liver fibrosis and cirrhosis, thereby the levels of bile acids need to be tightly controlled.[13, 14] FXR, through down-regulating bile acid synthetic genes and up-regulating bile acid transport genes expression, is served as the intrinsic sensor of liver to sense and controls bile acid

levels.[15-17]

Signal transducers and activators of transcription 3 (STAT3) is a cytoplasmic signal transcription factor that belongs to the signal transducers and activators of transcription family (STATs).[18] STAT3 has been reported to have positive properties of promoting liver regeneration, modulating apoptosis and lipid homeostasis.[19, 20] In a recent study, bile acid showed a specific effect on STAT3 activation after liver injury. The hepatic excessive bile acid can repress the tyrosine phosphorylation of STAT3 which is the active form of STAT3.[6] Therefore, attenuating bile acid accumulation and activating STAT3 phosphorylation may be beneficial strategies against CCl_4-induced acute hepatotoxicity.

Alisol B 23-acetate (AB23A), is a natural triterpenoid isolated from *rhizoma alismatis* which is an edible and medicinal plant widely used as a traditional Chinese medicine for a long history. Its chemical structure is shown in Fig. 1A. Several pharmacological studies have revealed that AB23A has several pharmacological activities, such as anti-hepatitis virus, anti-proliferative activity of cancer cell lines and antibacterial effects.[21-23] We have recently demonstrated that AB23A has promotive effect on liver regeneration in mice after 70% partial hepatectomy through FXR activation.[24] There is also hepatocyte proliferation in liver repair process after CCl_4-induced hepatotoxicity.[6] Therefore an important and intriguing question arises whether AB23A has promotive effect on hepatocyte proliferation and whether AB23A has hepatoprotective effect on CCl_4-induced hepatotoxicity. Another further question is that whether FXR or STAT3 and their target genes contribute to its hepatoprotection if AB23A possesses protective effect against CCl_4-induced hepatotoxicity.

In the present study, we aimed to investigate the hepatoprotective effects of AB23A

against CCl_4-induced hepatotoxicity in mice, and further to explore whether FXR or STAT3 and their target genes contribute to its hepatoprotection.

Material and methods

Chemicals

AB23A (purity > 98%) was purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Chenodeoxycholic acid (CDCA) was purchased from Sigma-Aldrich (St. Louis, MO). All biochemical indicators kits and other chemicals were commercially available.

Animals and treatments

Male C57BL/6 mice (8-9 weeks) used in this study were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number: SCXK 2008-0002). Mice were housed in laboratory animal facilities under a standard 12-hr light/dark cycle. All animal maintenance and treatment protocols were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China. AB23A (10, 20 or 40 mg/kg) or vehicle (10%HPBCD in 500mM phosphate pH 7.0) alone was treated to mice by oral gavage once daily for 7 days. On the 5th or 6th day, four hours after AB23A or vehicle treatment, mice were injected intraperitoneally with 750 μl/kg of CCl_4 in olive oil. On the 7th day, 4 hours after AB23A or vehicle treatment, mice were sacrificed under anesthesia (pentobarbital sodium, 65 mg/kg, intraperitoneal injection),

and liver were collected (n=10).

Serum biochemical and hepatic bile acids analysis

The plasma was collected from suborbital veins into heparinized tubes and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bile acids were determined. For bile acids measurement in liver, liver tissues samples (50 mg) were mixed in phosphate buffer saline (0.01 mol/L, pH 7.0). After centrifugation, total bile acids in supernatant were analyzed using a commercial kit (Bio-Quant, San Diego) according to the manufacturer's protocol. The result obtained were the mean of ten different animals liver per time point.

Liver histology

Small pieces from different lobes of the livers were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin & eosin (H&E). Morphologic changes were observed and the percentage of hepatocytes undergoing mitosis was calculated. For 5-bromo-2-deoxyuridine (BrdU) staining, mice were injected intraperitoneally with the BrdU solution (50 mg/kg body weight) 2 hours before being sacrificed.[25] Liver sections were prepared and stained using a BrdU staining kit (Roche, Indianapolis, IN). The number of positively stained cells was counted in at least 5 randomly selected fields for each tissue section.

TUNEL assay

Liver tissue samples embedded in paraffin were prepared, and used for TUNEL assay.

Apoptosis detection kit was used according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Fluorescein, Roche, Basle, Sweden). Ten microscopic fields within the view were randomly selected, and the positive cells (green spots) were counted. The extent of damage was evaluated using the average number of positive cells.

Quantitative real-time PCR

Total hepatic RNA from mice liver tissues was extracted using Trizol reagent according to the manufacturer's instruction (Invitrogen, Grand Island, NY). 1 μg RNA was purified and randomly reverse-transcribed to cDNA using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotech, Kyoto, Japan). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix and an ABI prim 7500 Sequence Detection System (Applied Biosystems, USA). The primers for genes are shown in Table 1. The quantity of mRNA was normalized with an internal standard mouse β-actin.

Protein isolation and Western blot

Liver tissues were homogenized in protein lysis buffer containing 1 mM PMSF. After the determination of protein concentration by BCA method (Thermo Scientific, Rochford, IL), 50 μg of total protein were resolved by 10% SDS-PAGE, transferred to PVDF membrane. After blocking with 5% nonfat dry milk in Tris-buffered saline, membranes were incubated overnight with primary antibodies, including Cyclin D1 (ab7958, 1/1000, Abcam, Cambridge, MA), Cyclin B1 (H-433, 1/1000), FoxM1b (sc-376621, 1/1000) (Santa Cruz Biotechnology, Santa Cruz, CA) and STAT3, p-STAT3 (Bioworld Technology, USA). Specific bands were detected by an enhanced chemiluminescence

(ECL) method using Bio-Spectrum Gel Imaging System (UVP, USA).

Statistical analysis

Data are expressed as means ± S.D. Statistical analysis between two groups were performed by a Student's t test and multiple comparisons were performed by a one-way ANOVA. $P< 0.05$ was considered statistically significant.

Results

Effects of AB23A on serum liver functional enzymes and total bile acids

To test whether AB23A has any effect on hepatotoxicity induced by CCl_4, male C57BL/6 mice were orally administered AB23A (10, 20 and 40 mg/kg), CDCA (40 mg/kg), a known FXR agonist used as a positive drug, or vehicle for 7 days until sacrifice. On the 5th or 6th day at 4 hours after AB23A, CDCA or vehicle treatment, mice were injected intraperitoneally CCl_4, and were sacrificed on the 7th day. The serum activities of ALT and AST, the diagnostic markers for the presence of hepatotoxicity induced by CCl_4, were substantially increased at the time points of 24 and 48 h after CCl_4 administration. In contrast, AB23A treatment dose-dependently reversed the CCl_4-induced increases in ALT and AST. 40 mg/kg of CDCA also reduced serum activities of ALT and AST, which was similar to the effect of AB23A 20 mg/kg group (Fig. 1B-C). The maximum increase in these biochemical indicators was observed at 48 h after CCl_4 administration, suggesting that liver injury was most serious at 48 h. Therefore, the time point of 48 h was chosen for the following study. In addition, we determined the changes in serum total bile acids. Fig. 1D illustrated that serum total bile acids were increased by CCl_4 to 136 μmol/L,

which was 3.4 fold higher than that of vehicle group, whereas AB23A and CDCA reduced CCl_4-induced increase in serum total bile acids. Taken together, these data clearly demonstrated that AB23A can protect against CCl_4–induced hepatotoxicity.

Effects of AB23A on liver histopathological changes and hepatocyte apoptosis

To further evaluate the liver histopathological changes, the liver sections were stained by H&E. Severe congestion in the portal area, sinusoids, numerous vacuoles and necrosis were observed in the livers from vehicle-treated mice with CCl_4 administration. These liver histopathological changes were ameliorated by AB23A and CDCA treatment (Fig. 2A). Then, we further determined apoptotic hepatocytes stained by TUNEL. The number of apoptotic cells in vehicle-treated mice after CCl_4 administration was significantly increased, indicating an increase in apoptotic degeneration of hepatocytes as a result from CCl_4 intoxication. AB23A and CDCA treatment decreased the TUNEL-positive cells (Fig. 2B-C). However, we didn't observe any difference between the vehicle only group and the AB23A only group, indicating that AB23A may not impair the liver. Therefore, these results suggested that AB23A can provide remarkable protection against CCl_4-induced hepatotoxicity.

AB23A promotes hepatocyte proliferation in mice after CCl_4 administration

The survived hepatocytes proliferation has been demonstrated to be critical for liver repair in response to CCl_4–induced hepatotoxicity and FXR plays a key role in the process of liver repair after toxicant injury.[6, 26] To clarify the mechanism that AB23A attenuated hepatotoxicity induced by CCl_4, we stained liver sections for BrdU and

determined the percentage of positively stained hepatic cells. A significant dose-dependent increase in the number of BrdU-positive hepatocytes was observed in mice treated with AB23A (Fig. 3A-B), suggesting that AB23A increased hepatocyte DNA synthesis in mice. In addition, the mitotic index in AB23A-treated mice was significantly higher than that in vehicle-treated mice (Fig. 3C). Taken together, these results suggested that AB23A can attenuate hepatotoxicity induced by CCl_4 via enhancing hepatocyte proliferation in mice.

AB23A up-regulates FoxM1b and cell cycle genes and protein expression in mice

To investigate the mechanism underlying increased cell proliferation in AB23A-treated mice, we determined the expression levels of the transcription factor FoxM1b which is a FXR direct target gene and is essential for regulating hepatocyte DNA replication and mitosis at first. A dose-dependent increase in FoxM1b expression was observed in AB23A-treated mice (Fig. 4A). We further determined the expression of FoxM1b downstream target genes Cyclin D1 that is essential for G1/S phase transition, and Cyclin B1 which is critical for G2/M phase transition. Dose-dependent increases in Cyclin D1 and Cyclin B1 gene and protein were observed in AB23A-treated mice with a maximal increase at the dose of 40 mg/kg (Fig. 4B-D). These results showed that AB23A through activating FXR up-regulated FoxM1b, Cyclin D1 and Cyclin B1 expression, resulting in accelerated hepatocyte proliferation after CCl_4 administration.

AB23A reduces hepatic bile acid levels through regulating hepatic transporters involved in bile acid transport and bile acid synthetic enzymes

Bile acids are synthesized specifically from cholesterol in the liver and subsequently excreted into bile. A significant increase in serum bile acid levels after CCl_4 administration was observed in Fig. 1D, suggesting that hepatotoxicity induced by CCl_4 may impair enterohepatic circulation and bile acid homeostasis. Therefore, we further determined hepatic bile acid levels. Hepatic bile acid levels were increased in mice after CCl_4 administration and were significantly reduced by AB23A treatment in a dose-dependent manner (Fig. 5A). To understand the mechanism underlying reduced hepatic bile acids in mice treated with AB23A, we first examined the expression of hepatic bile acids transporters, including Na+/taurocholate cotransporting polypeptide (Ntcp) which is responsible for taking up bile acids from portal blood into hepatocyte, bile salt export pump (Bsep) and multidrug resistance-related protein 2 (Mrp2) both of which are located on canalicular membrane and are responsible for excreting bile acids into bile. Bsep is a direct downstream target gene of FXR. The transcription of Ntcp and Mrp2 are also regulated by FXR. As shown in Fig. 5B, CCl_4 administration increased Bsep, Mrp2 and decreased Ntcp expression. This may be an adaptive response to CCl_4–induced hepatotoxicity. AB23A treatment further increased Bsep, Mrp2 and decreased Ntcp expression in a dose-dependent manner. Besides the above transporters, bile acid synthetic enzymes are also involved in bile acid homeostasis. We determined the gene expression of cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting enzyme in bile acid synthesis. Cyp7a1 was decreased by 69% in mice after CCl_4 administration. CCl_4 also decreased another bile acid synthetic enzyme sterol-12α-hydroxylase (Cyp8b1) expression. AB23A treatment further reduced Cyp7a1 and Cyp8b1 gene expression. In addition, a dose-dependent increase in gene expression of small heterodimer partner (Shp)

was observed in mice treated with AB23A (Fig. 5C). It has been demonstrated that Shp induction can suppress Cyp7a1 and Cyp8b1 expression and FXR directly regulates Shp. The above results suggested that AB23A through activating FXR, induced Shp expression resulting in decreased Cyp7a1 and Cyp8b1 mRNA. Taken together, these data indicated that AB23A reduced hepatic bile acid levels via an induction of Bsep, Mrp2 and an inhibition of Ntcp, Cyp7a1 and Cyp8b1 expression.

Effect of AB23A on STAT3 phosphorylation after CCl_4 administration

Hepatocytes normally exist in a highly differentiated quiescent G_0 phase. Upon injury, the remaining hepatocytes are primed by acute phase response factor STAT3. Bile acids are known to directly modulate phosphorylation of STAT3 after liver injury. The overload of hepatic bile acids can repress the tyrosine phosphorylation of STAT3 which is the active form of STAT3. To evaluate the effects of AB23A on the activation of STAT3, the expression of STAT3 tyrosine phosphorylation was assessed by Western blotting. Results indicated a higher level of STAT3 tyrosine phosphorylation after AB23A treatment with a constant total STAT3 protein expression (Fig. 6A). To further confirm the positive role that AB23A plays in STAT3 activation, we determined the expression of the two main STAT3 target genes, Bcl-xl and SOCS3. As expected, the expression of Bcl-xl and SOCS3 in mice was significantly up-regulated by AB23A (Fig. 6B-C). The higher expression of the antiapoptotic gene Bcl-xl, may contribute to the decreased cell deaths in mice treated with AB23A.

Discussion

Liver is known as the main organ in the metabolism of toxic chemicals and drugs.[27] It is also the most vulnerable organ to most toxic chemicals, usually resulting in its damage. Therefore it poses a major health problem to people's well being. Previous studies have demonstrated that various chemical substances such as CCl_4, acrolein and 2,4,6-trinitrotoluene can cause severe liver injury.[28-30] CCl_4-induced hepatotoxicity is a widely used animal experimental model to investigate the mechanism underlying liver injury or screen hepatoprotective agents.[31, 32] In the present study, CCl_4 administration caused acute liver damage in mice, which was evidenced by the elevated levels of serum ALT, AST, TUNEL-positive cells and classic histopathological changes. We demonstrated that AB23A had at least two roles in protection against CCl_4-induced hepatotoxicity. One role is to promote hepatocyte proliferation through an induction in hepatic levels of various genes which are essential for regulating hepatocyte DNA replication and mitosis in a dose-dependent manner. The other role is to reduce bile acids in liver through decreasing hepatic uptake, synthesis and increasing efflux of bile acid. We also clarified the involvement of FXR signaling pathway in these functions of AB23A.

After the acute liver injury, the necrotic or apoptotic hepatocytes were replaced by regenerated hepatocytic parenchymal cells.[33] Normal hepatocytes proliferation plays a key role in liver repair. FoxM1b which belongs to an extensive family forkhead box transcription factors, is essential for the regulation of cellular proliferation.[8] FoxM1b stimulates hepatocyte DNA replication and mitosis by increasing the expression of numerous cell cycle regulatory genes such as the S-phase promoting Cyclin D1, Cyclin A2, and M-phase promoting Cyclin B1, Cyclin F.[34, 35] Cyclin D1 facilitates hepatocytes entry and progression through S phase while Cyclin B1 mediates cell cycle progression

from the G_2 phase to mitosis. It has been demonstrated that FoxM1b is a FXR direct downstream target gene by binding to IR-0 of FXR response element (FXRE) which serves as an enhancer of intron 3 of FoxM1b gene.[7, 8] We have shown that AB23A is an exogenous activator of FXR in the previous studies.[24] The present study demonstrated that through activating FXR, AB23A up-regulated the expression of FoxM1b, Cyclin D1 and Cyclin B1, therefore leading to an increase in hepatocyte proliferation.

CCl_4-induced hepatotoxicity disrupts bile acid enterohepatic circulation and causes the accumulation of bile acids in the liver.[6] These negative effects result in hepatocellular apoptosis, necrosis and consequently aggravating liver fibrosis and cirrhosis. A variety of transporters and enzymes have been demonstrated to play crucial roles in hepatic bile acids homeostasis.[36-38] Hepatic uptake of bile acids takes place at the basolateral membrane of hepatocytes and is mainly mediated through Ntcp in mice.[39] Canalicular excretion of bile acids constitutes the rate-limiting step in hepatic bile acids excretion and Bsep, a member of the canalicular ATP-binding cassette (ABC) transporter superfamily, is the major canalicular bile acids transporter. Mrp2 is responsible for transporting bile acids or bilirubin conjugated with glucuronide or sulfate into bile to protect against hepatotoxicity in mice. This study demonstrated that CCl_4-induced hepatotoxicity resulted in the adaptive changes in hepatic gene expression, which was in line with previous study.[6] Through activating FXR, AB23A increased expression of bile acid export transporter Bsep which is a FXR direct downstream gene and Mrp2, another FXR downstream gene, and decreased hepatic uptake transporters Ntcp expression. Besides transporters, bile acid synthetic enzymes including Cyp7a1 and Cyp8b1 also play important roles in bile acids homeostasis. AB23A treatment reduced FXR downstream

target genes Cyp7a1 and Cyp8b1 expression via FXR-Shp axis, leading to suppressing bile acids synthesis. In fact, we observed a transient increase of FXR mRNA levels shortly after CCl_4 administration (data not shown). This may be due to the fact that FXR activation by increased bile acids in liver up-regulates the transcription of FXR.

In this study, we also observed that AB23A promoted STAT3 phosphorylation and activated the expression of Bcl-xl and SOCS3, the two main target genes of STAT3. STAT3 signaling pathway, usually activated in the acute phase of liver injury, is an important pathway for survival and repair of liver cells.[40] STAT3 is also involved in mitigating exaggerated inflammatory reaction after hepatocyte necrosis. Bcl-xl inhibits hepatic caspase-3-like activity, therefore reduces hepatocyte apoptosis and liver fibrotic responses.[41] SCOS3 can protect liver by preventing liver apoptosis and hemorrhagic necrosis.[42] These effects contribute to AB23A activity against hepatocyte apoptosis. However, the evidence of a direct effect of FXR on STAT3 phosphorylation had never been observed. The overload of bile acids in liver has been reported to lead to reduced STAT3 phosphorylation to hepatotoxicity. Therefore, we suggested that AB23A activated STAT3 phosphorylation by relieving the stress of bile acids overload in the liver, as evidenced in Fig. 4. The effects of AB23A on activation of STAT3 phosphorylation, Bcl-xl and SCOS3 resulted in decreased hepatocyte apoptosis.

The toxic effects of CCl_4 strongly depend on its reductive metabolism to free radicals and the consequent oxidative stress, which are responsible for the damage in different cell components and functions. Maybe AB23A has other pharmacological roles in hepatoprotection such as the effect of antioxidative stress via interfering with the metabolism of CCl_4 in the liver. However, this hypothesis is

not unlikely given that AB23A was administered for several days prior to intoxication and appears to be a lipophilic substance.

To preferably observe the hepatoprotective effect of AB23A, we selected three doses (10, 20 and 40 mg/kg) by repeated administration for 7 consecutive days until mice sacrifice. The dose of 10 mg/kg is the minimal dose of AB23A that can play roles in liver protection in mice following CCl_4 administration. We observed that the hepatoprotection of AB23A were in a dose-dependent manner. This result may support the potential therapeutic use of AB23A.

In conclusion, this study demonstrated the hepatoprotective effect of AB23A against CCl_4–induced hepatotoxicity. The mechanism of action is promotion of hepatocyte proliferation and reduction of hepatic bile acids. These effects are in association with FXR activation and STAT3 phosphorylation. The protective and healing properties of AB23A need further investigation. It will be meaningful to develop AB23A as a new natural medicine for treatment of acute hepatotoxicity.

Disclosure of potential conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This present study was financially supported by a grant from the National Natural Science Foundation of China (Nos. 81302826, 81273580).

References

1 B. C. Fuchs, Y. Hoshida, T. Fujii, L. Wei, S. Yamada, G. Y. Lauwers, C. M. McGinn, D. K. DePeralta, X. Chen, T. Kuroda, M. Lanuti, A. D. Schmitt, S. Gupta, A. Crenshaw, R. Onofrio, B. Taylor, W. Winckler, N. Bardeesy, P. Caravan, T. R. Golub and K. K. Tanabe, *Hepatology*, 2014, **59**, 1577-1590.
2 K. Yamasaki, M. Tateyama, S. Abiru, A. Komori, S. Nagaoka, A. Saeki, S. Hashimoto, R. Sasaki, S. Bekki, Y. Kugiyama, Y. Miyazoe, A. Kuno, M. Korenaga, A. Togayachi, M. Ocho, M. Mizokami, H. Narimatsu and H. Yatsuhashi, *Hepatology*, 2014, **60**, 1563-1570.
3 C. Yu, F. Wang, C. Jin, X. Wu, W. K. Chan and W. L. McKeehan, *Am. J. Pathol.*, 2002, **161**, 2003-2010.
4 B. M. Forman, E. Goode, J. Chen, A. E. Oro, D. J. Bradley, T. Perlmann, D. J. Noonan, L. T. Burka, T. McMorris, W. W. Lamph, R. M. Evans and C. Weinberger, *Cell*, 1995, **81**, 687-693.
5 Y. D. Wang, W. D. Chen, D. D. Moore and W. Huang, *Cell Res.*, 2008, **18**, 1087-1095.
6 Z. Meng, Y. Wang, L. Wang, W. Jin, N. Liu, H. Pan, L. Liu, L. Wagman, B. M. Forman and W. Huang, *Mol. Endocrinol.*, 2010, **24**, 886-897.
7 L. Zhang, Y. D. Wang, W. D. Chen, X. Wang, G. Lou, N. Liu, M. Lin, B. M. Forman and W. Huang, *Hepatology*, 2012, **56**, 2336-2343.\
8 W. D. Chen, Y. D. Wang, L. Zhang, S. Shiah, M. Wang, F. Yang, D. Yu, B. M. Forman and W. Huang, *Hepatology*, 2010, **51**, 953-962.
9 M. Makishima, A. Y. Okamoto, J. J. Repa, H. Tu, R. M. Learned, A. Luk, M. V. Hull, K. D. Lustig, D. J. Mangelsdorf and B. Shan, *Science*, 1999, **284**, 1362-1365.
10 D. J. Parks, S. G. Blanchard, R. K. Bledsoe, G. Chandra, T. G. Consler, S. A. Kliewer, J. B. Stimmel, T. M. Willson, A. M. Zavacki, D. D. Moore and J. M. Lehmann, *Science*, 1999, **284**, 1365-1368.
11 J. K. Kemper, *Biochim. Biophys. Acta*, 2011, **8**, 842-850.
12 L. Adorini, M. Pruzanski and D. Shapiro, *Drug Discov Today*, 2012, **17**, 988-997.
13 S. Fiorucci, E. Antonelli, G. Rizzo, B. Renga, A. Mencarelli, L. Riccardi, S. Orlandi, R. Pellicciari and A. Morelli, *Gastroenterology*, 2004, **127**, 1497-1512.
14 Y. Lu, Z. Zhang, X. Xiong, X. Wang, J. Li, G. Shi, J. Yang, X. Zhang, H. Zhang, J. Hong, X. Xia, G. Ning and X. Li, *Gastroenterology*, 2012, **143**, 1630-1640.
15 Y. Liu, J. Binz, M. J. Numerick, S. Dennis, G. Luo, B. Desai, K. I. MacKenzie, T. A. Mansfield, S. A. Kliewer, B. Goodwin and S. A. Jones, *J. Clin. Invest.*, 2003, **112**, 1678-1687.
16 B. Kong, L. Wang, J. Y. Chiang, Y. Zhang, C. D. Klaassen and G. L. Guo, *Hepatology*, 2012, **56**, 1034-1043.
17 Y. M. Vanwijngaerden, J. Wauters, L. Langouche, S. Vander Perre, C. Liddle, S. Coulter, S. Vanderborght, T. Roskams, A. Wilmer, G. Van den Berghe and D. Mesotten, *Hepatology*, 2011, **54**, 1741-1752.
18 B. Gao, *Cell Mol Immunol*, 2005, **2**, 92-100.
19 F. Bohm, U. A. Kohler, T. Speicher and S. Werner, *EMBO Mol Med*, 2010, **2**, 294-305.
20 F. Hong, S. Radaeva, H. N. Pan, Z. Tian, R. Veech and B. Gao, *Hepatology*, 2004, **40**, 933-941.
21 Z. Y. Jiang, X. M. Zhang, F. X. Zhang, N. Liu, F. Zhao, J. Zhou and J. J. Chen, *Planta Med.*, 2006, **72**, 951-954.
22 H. G. Jin, Q. Jin, A. Ryun Kim, H. Choi, J. H. Lee, Y. S. Kim, D. G. Lee and E. R. Woo, *Arch. Pharm. Res.*, 2012, **35**, 1919-1926.
23 Y. H. Xu, L. J. Zhao and Y. Li, *World J Gastroenterol*, 2009, **15**, 2870-2877.
24 Q. Meng, X. Chen, C. Wang, Q. Liu, H. Sun, P. Sun, J. Peng and K. Liu, *Biochem. Pharmacol.*, 2014, **30**, 009.
25 N. Meng, X. Liu, X. Fu, Y. D. Wang, W. D. Wang, L. Chen, B. M. Zhang, Forman and W. Huang, *J. Hepatol.*, 2011, **55**, 885-895.
26 P. Borude, G. Edwards, C. Walesky, F. Li, X. Ma, B. Kong, G. L. Guo and U. Apte, *Hepatology*, 2012, **56**, 2344-2352.
27 Y. Yue, S. Wu, H. Zhang, X. Zhang, Y. Niu, X. Cao, F. Huang and H. Ding, *Food Chem. Toxicol.*, 2014, **26**, 76-84.
28 J. Q. Ma, J. Ding, L. Zhang and C. M. Liu, *Food Chem. Toxicol.*, 2014, **64**, 41-48.
29 M. K. Mohammad, D. Avila, J. Zhang, S. Barve, G. Arteel, C. McClain and S. Joshi-Barve, *Toxicol. Appl. Pharmacol.*, 2012, **265**, 73-82.
30 P. Leffler, E. Brannas, D. Ragnvaldsson, H. Wingfors and R. Berglind, *J Toxicol Environ Health A*, 2014, **77**, 1183-1191.
31 A. T. Williams and R. F. Burk, *Semin. Liver Dis.*, 1990, **10**, 279-284.
32 M. Y. Wang, G. Anderson, D. Nowicki and J. Jensen, *Plant Foods Hum. Nutr.*, 2008, **63**, 141-145.

33 R. Bataller and D. A. Brenner, *J. Clin. Invest.*, 2005, **115**, 209-218.
34 X. Wang, N. J. Hung and R. H. Costa, *Hepatology*, 2001, **33**, 1404-1414.
35 X. Wang, E. Quail, N. J. Hung, Y. Tan, H. Ye and R. H. Costa, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 11468-11473.
36 W. A. Alrefai and R. K. Gill, *Pharm. Res.*, 2007, **24**, 1803-1823.
37 W. Huang, K. Ma, J. Zhang, M. Qatanani, J. Cuvillier, J. Liu, B. Dong, X. Huang and D. D. Moore, *Science*, 2006, **312**, 233-236.
38 H. Yang, K. Ramani, M. Xia, K. S. Ko, T. W. Li, P. Oh, J. Li and S. C. Lu, *Hepatology*, 2009, **49**, 1982-1991.
39 S. Gundala, L. D. Wells, M. T. Milliano, V. Talkad, B. A. Luxon and B. A. Neuschwander-Tetri, *Arch. Toxicol.*, 2004, **78**, 68-73.
40 A. Moh, Y. Iwamoto, G. X. Chai, S. S. Zhang, A. Kano, D. D. Yang, W. Zhang, J. Wang, J. J. Jacoby, B. Gao, R. A. Flavell and X. Y. Fu, *Lab. Invest.*, 2007, **87**, 1018-1028.
41 T. Takehara, T. Tatsumi, T. Suzuki, E. B. Rucker, 3rd, L. Hennighausen, M. Jinushi, T. Miyagi, Y. Kanazawa and N. Hayashi, *Gastroenterology*, 2004, **127**, 1189-1197.
42 D. Jo, D. Liu, S. Yao, R. D. Collins and J. Hawiger, *Nat. Med.*, 2005, **11**, 892-898.

Legend to figures

Fig. 1 Hepatoprotection of AB23A against CCl_4-induced hepatotoxicity. Biochemical indicators in mice orally administered vehicle, 10, 20 or 40 mg/kg of AB23A, 40 mg/kg of CDCA respectively, were determined at the time points of 24 and 48 hours after CCl_4 administration. (A) The chemical structure of AB23A. Serum ALT (B) and AST (C) activities elevated by CCl_4 at 24 and 48 h, were significantly reduced by treatment with different doses of AB23A. Serum total bile acids (D) increased in mice after CCl_4 administration, were dose-dependently reduced in AB23A-treated mice. Data are the mean ± S.D. (n=10). *$p<0.05$ versus Vehicle only; #$p<0.05$ versus Vehicle + CCl_4.

Fig. 2 Effects of AB23A on liver histopathological changes and hepatocyte apoptosis. (A) The images of representative H&E stained liver sections (100 ×magnification) at 48 h after CCl_4 administration were shown. Areas of severe liver necrosis were marked by arrows. (n=5) (B) The protective effect of AB23A against CCl_4–induced hepatocyte apoptosis through TUNEL method (100 ×magnification). Green fluorescence indicates apoptotic cell. (C) Statistical analysis of TUNEL fluorescence images in mice (n=10). Data are presented as the mean ± S.D. *$p<0.05$ versus Vehicle only; #$p<0.05$ versus Vehicle + CCl_4.

Fig. 3 AB23A treatment (10, 20, 40 mg/kg, p.o.) promotes hepatocyte proliferation after CCl_4–induced hepatotoxicity. (A) The images of immunohistochemical detection for BrdU at 48 h after CCl_4 administration in mice administered vehicle or AB23A; original magnification, ×400. BrdU-positive hepatocytes were marked by arrows. (B)

Quantification of BrdU-positive hepatocytes after CCl_4 administration in vehicle- or AB23A-treated mice. The value of mice with vehicle at 48 h after CCl_4 administration is normalized to 100%. AB23A increased the number of BrdU-positive hepatocytes in mice. (C) The number of mitotic liver cells at 48 h after CCl_4 administration in mice. AB23A increased the number of mitotic liver cells. Data are presented as the mean ± S.D. *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl_4 (n=5).

Fig. 4 AB23A increases the hepatic expression of FoxM1b, Cyclin D1 and Cyclin B1 in mice. Quantitative real-time PCR analysis was performed to measure hepatic expression of proliferation-related genes FoxM1b (A), Cyclin D1 (B) and Cyclin B1 (C) in mice. AB23A treatment significantly induced gene expression of FoxM1b, Cyclin D1 and Cyclin B1. Data are the mean ± S.D. (n=8). * p<0.05 versus Vehicle only; # p<0.05 versus Vehicle + CCl_4. (D) The expression of FoxM1b, Cyclin D1 and Cyclin B1 protein in liver tissue from mice were determined by Western blotting analysis. Increases in expression of FoxM1b, Cyclin D1 and Cyclin B1 protein were observed in AB23A-treated mice after CCl_4 administration in a dose-dependent manner.

Fig. 5 AB23A reduces hepatic bile acid levels through regulating hepatic transporters involved in bile acid transport and bile acid synthetic enzymes. (A) Hepatic bile acids were increased in mice after CCl_4 administration and were reduced by AB23A treatment in a dose-dependent manner. Quantitative real-time PCR analysis was performed to measure the gene expression of (B) Ntcp, Bsep and Mrp2. AB23A treatment decreased Ntcp and increases Bsep, Mrp2 expression. In addition, (C) AB23A reduced bile acid

synthetic enzymes Cyp7a1, Cyp8b1 and increased Shp expression. Data are presented as the mean ± S.D. *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl_4 (n=8).

Fig. 6 AB23A treatment (10, 20, 40 mg/kg, p.o.) activates STAT3 phosphorylation. (A) Effect of AB23A on expression of p-STAT3 in mice. (B) Effect of AB23A on expression of Bcl-xl in mice. (C) Effect of AB23A on expression of SOCS3 in mice. Data are presented as the mean ± S.D. *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl_4 (n=8).

Table 1 The primer sequences used for real-time PCR assay in mice

Gene	GenBank accession	Forward primer (5'-3')	Reverse primer (5'-3')
FoxM1b	NM_008021.4	ACCTGGAGCAGAATCGGGTTA	CAGTGCTGTTGATGGCAAACTGTA
Cyclin D1	NM_007631.2	TACCGCACAACGCACTTTC	AAGGGCTTCAATCTGTTCCTG
Cyclin B1	NM_172301.3	CACCCCTGGVATCTTCTCCTT	AGCGTCTTCAGAGACAGCCAG
Ntcp	U95132.1	GCATGATGCCACTCCTCTTATAC	TACATAGTGTGGCCTTTTGGACT
Bsep	NM_021022.3	AGCAGGCTCAGCTGCATGAC	AATGGCCCGAGCAATAGCAA
Mrp2	NM_013806.2	AACTGCCTCTTCAGAATCTTA	GCCAGCCACGGAACCAGCTGCT
CYP7a1	NM_007824.2	CAAGAACCTGTACATGAGGGAC	CACTTCTTCAGAGGCTGCTTTC
Cyp8b1	NM_010012.3	CCCCTATCTCTCAGTACACATGG	GACCATAAGGAGGACAAAGGTCT
Shp	NM_011850.2	GTCTTTCTGGAGCCTTGAGCTG	GTAGAGGCCATGAGGAGGATTC
Bcl-xl	NM_009743.4	GAGAGGCAGGCGATGAGTTT	CGATGCGACCCCAGTTTACT
SOCS3	NM_007707.3	GCGGGCACCTTTCTTATCC	GGAACTGGCTGCGTGCTT
β-actin	NM_007393.3	TATTGGCAACGAGCGGTTC	ATGCCACAGGATTCCATACCC

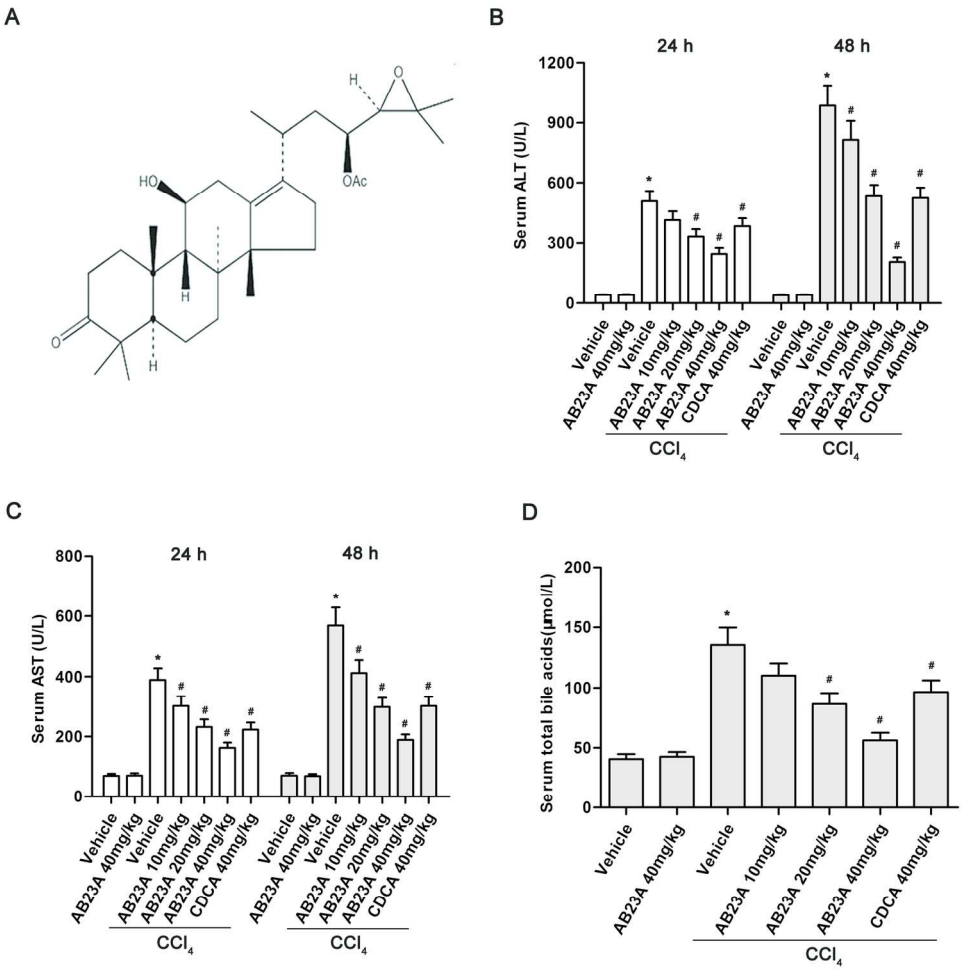


Fig. 1 Hepatoprotection of AB23A against CCl4-induced hepatotoxicity. Biochemical indicators in mice orally administered vehicle, 10, 20 or 40 mg/kg of AB23A, 40 mg/kg of CDCA respectively, were determined at the time points of 24 and 48 hours after CCl4 administration. (A) The chemical structure of AB23A. Serum ALT (B) and AST (C) activities elevated by CCl4 at 24 and 48 h, were significantly reduced by treatment with different doses of AB23A. Serum total bile acids (D) increased in mice after CCl4 administration, were dose-dependently reduced in AB23A-treated mice. Data are the mean ± S.D. (n=10). *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl4.
160x160mm (300 x 300 DPI)

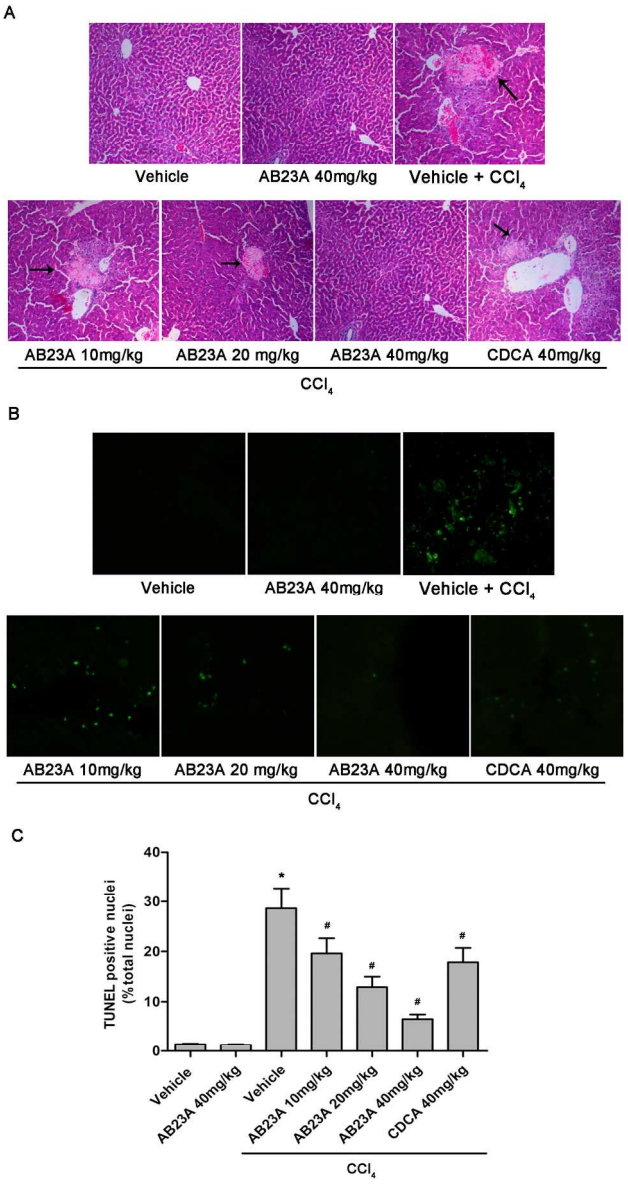


Fig. 2 Effects of AB23A on liver histopathological changes and hepatocyte apoptosis. (A) The images of representative H&E stained liver sections (100 ×magnification) at 48 h after CCl4 administration were shown. Areas of severe liver necrosis were marked by arrows. (n=5) (B) The protective effect of AB23A against CCl4–induced hepatocyte apoptosis through TUNEL method (100 ×magnification). Green fluorescence indicates apoptotic cell. (C) Statistical analysis of TUNEL fluorescence images in mice (n=10). Data are presented as the mean ± S.D. *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl4. 245x434mm (300 x 300 DPI)

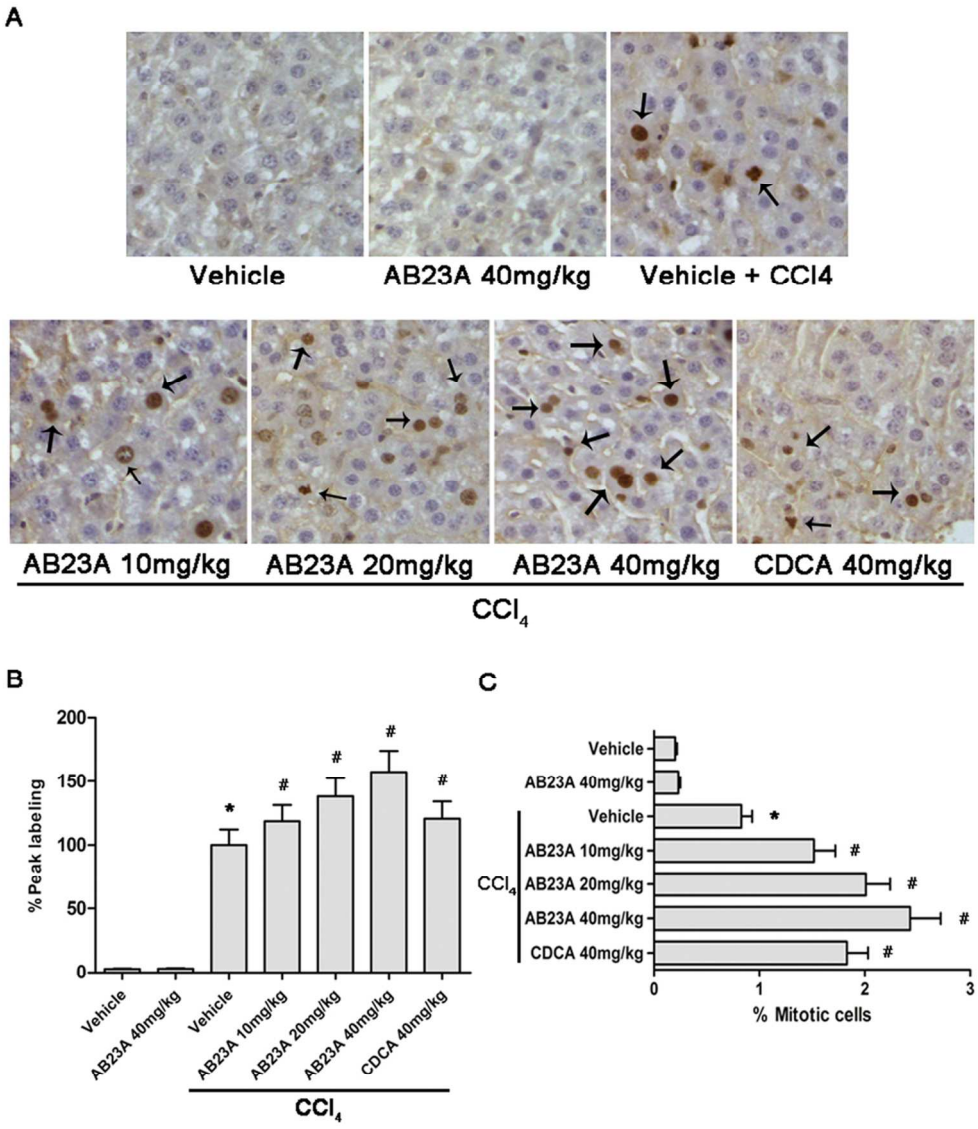


Fig. 3 AB23A treatment (10, 20, 40 mg/kg, p.o.) promotes hepatocyte proliferation after CCl4–induced hepatotoxicity. (A) The images of immunohistochemical detection for BrdU at 48 h after CCl4 administration in mice administered vehicle or AB23A; original magnification, ×400. BrdU-positive hepatocytes were marked by arrows. (B) Quantification of BrdU-positive hepatocytes after CCl4 administration in vehicle- or AB23A-treated mice. The value of mice with vehicle at 48 h after CCl4 administration is normalized to 100%. AB23A increased the number of BrdU-positive hepatocytes in mice. (C) The number of mitotic liver cells at 48 h after CCl4 administration in mice. AB23A increased the number of mitotic liver cells. Data are presented as the mean ± S.D. *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl4 (n=5).
94x112mm (300 x 300 DPI)

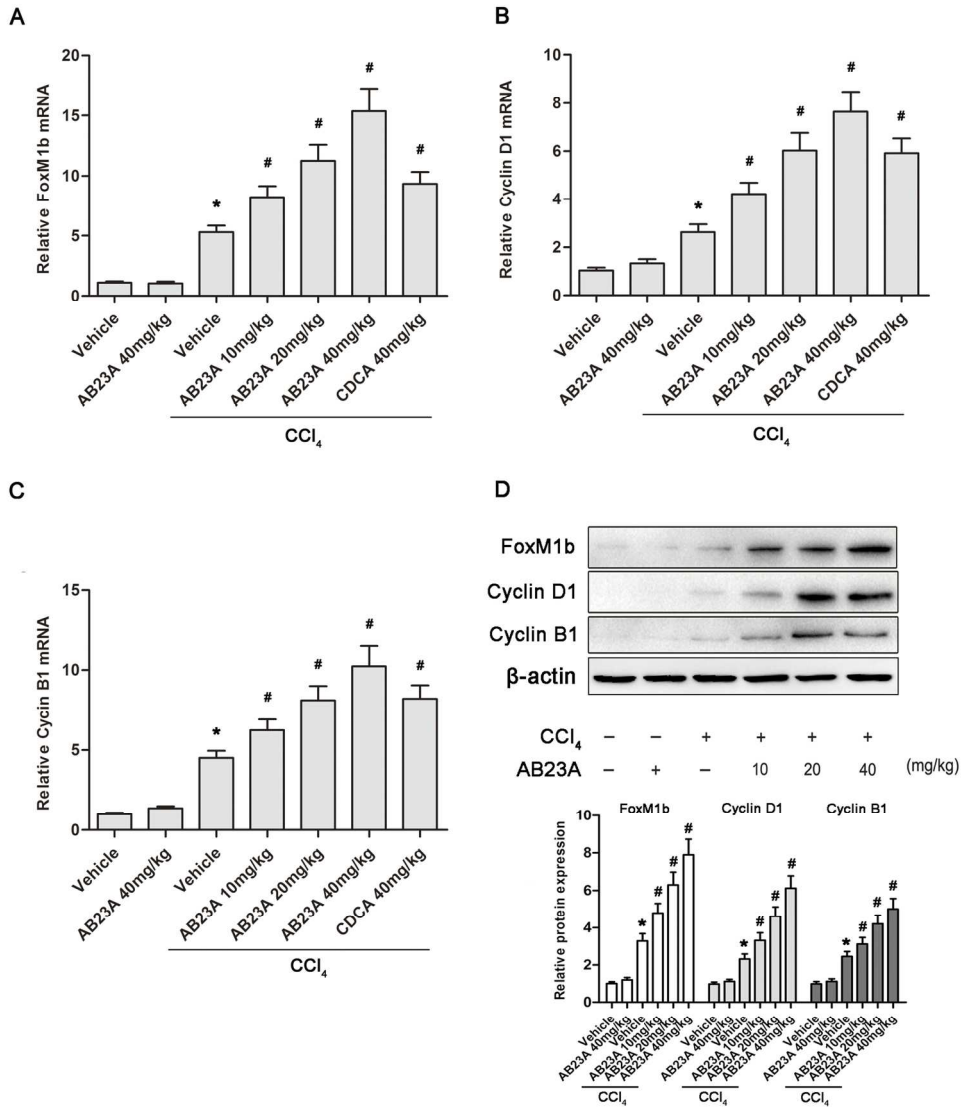


Fig. 4 AB23A increases the hepatic expression of FoxM1b, Cyclin D1 and Cyclin B1 in mice. Quantitative real-time PCR analysis was performed to measure hepatic expression of proliferation-related genes FoxM1b (A), Cyclin D1 (B) and Cyclin B1 (C) in mice. AB23A treatment significantly induced gene expression of FoxM1b, Cyclin D1 and Cyclin B1. Data are the mean ± S.D. (n=8). * $p<0.05$ versus Vehicle only; # $p<0.05$ versus Vehicle + CCl4. (D) The expression of FoxM1b, Cyclin D1 and Cyclin B1 protein in liver tissue from mice were determined by Western blotting analysis. Increases in expression of FoxM1b, Cyclin D1 and Cyclin B1 protein were observed in AB23A-treated mice after CCl4 administration in a dose-dependent manner.
180x202mm (300 x 300 DPI)

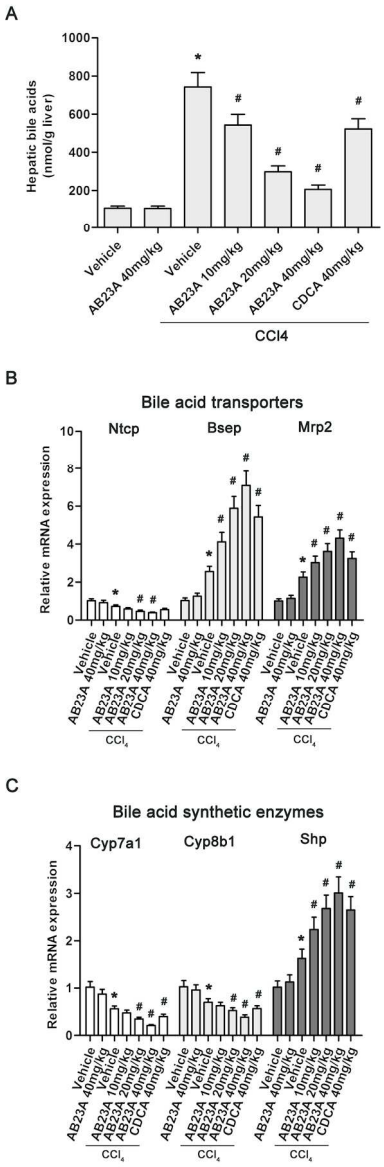


Fig. 5 AB23A reduces hepatic bile acid levels through regulating hepatic transporters involved in bile acid transport and bile acid synthetic enzymes. (A) Hepatic bile acids were increased in mice after CCl4 administration and were reduced by AB23A treatment in a dose-dependent manner. Quantitative real-time PCR analysis was performed to measure the gene expression of (B) Ntcp, Bsep and Mrp2. AB23A treatment decreased Ntcp and increases Bsep, Mrp2 expression. In addition, (C) AB23A reduced bile acid synthetic enzymes Cyp7a1, Cyp8b1 and increased Shp expression. Data are presented as the mean ± S.D. *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl4 (n=8).
234x688mm (300 x 300 DPI)

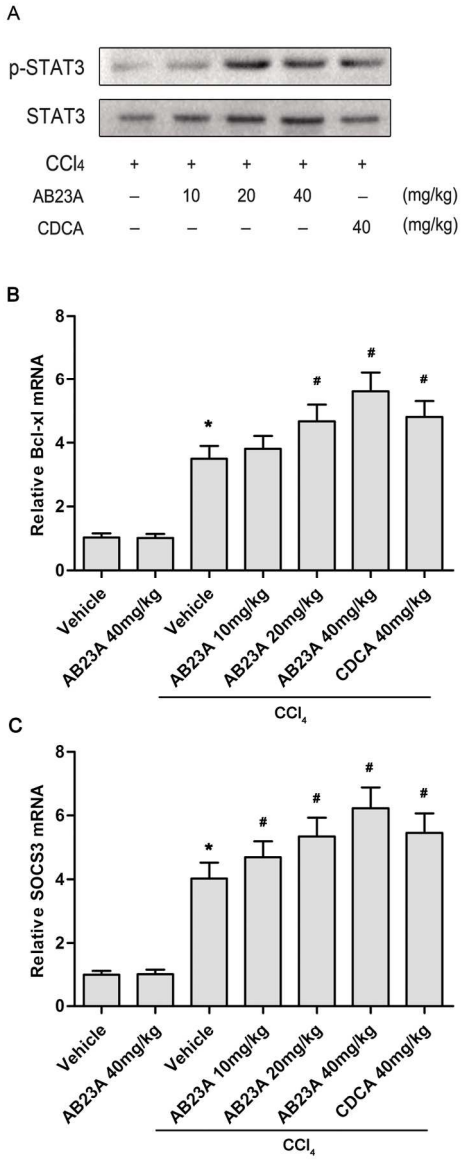
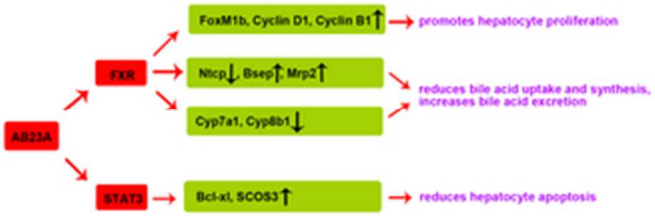


Fig. 6 AB23A treatment (10, 20, 40 mg/kg, p.o.) activates STAT3 phosphorylation. (A) Effect of AB23A on expression of p-STAT3 in mice. (B) Effect of AB23A on expression of Bcl-xl in mice. (C) Effect of AB23A on expression of SOCS3 in mice. Data are presented as the mean ± S.D. *p<0.05 versus Vehicle only; #p<0.05 versus Vehicle + CCl4 (n=8).
218x545mm (300 x 300 DPI)



Alisol B 23-acetate protects against CCl4-induced hepatotoxicity via FXR and STAT3-mediated gene regulation in mice.
28x10mm (300 x 300 DPI)