Original Paper

Antioxidant and Hepatoprotective Effect of Swertiamarin on Carbon Tetrachloride-Induced Hepatotoxicity via the Nrf2/HO-1 Pathway

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Key Words

Swertiamarin • Carbon tetrachloride • Nrf2 • Oxidative stress • Inflammation • Efflux transporters

Abstract

Background/Aims: Swertiamarin (STM), the main bioactive component in *Swertia mussotii* Franch (Gentianaceae), has been shown to exert hepatoprotective effects on experimental liver injury. However, the effects and exact mechanisms of STM on carbon tetrachloride (CCl₄) causing hepatotoxicity are still unknown. This study investigated the potential protective effects and mechanisms of STM on CCl₄-induced liver injury in rats. **Methods:** Adult male Sprague-Dawley (SD) rats were exposed to CCl₄ with or without STM co-administration for consecutive eight weeks. **Results:** STM significantly ameliorated CCl₄-induced increase in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels and histopathological changes in the liver. Hepatic oxidative stress was repressed by STM, as evidenced by the decrease in malondialdehyde (MDA), with concomitant increase in antioxidant activity (e.g. superoxide dismutase (SOD); glutathione peroxidase (GPx)), glutathione (GSH) level. STM also obviously attenuated inflammatory response in CCl₄-lesioned livers as evidenced by the decrease in inflammatory cytokines/chemokines (e.g. inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β)). Additionally, STM significantly induced the expression of CYPs, efflux transporters and PDZK1 as compared with the CCl₄ group. Moreover, co-administration of STM with CCl₄ remarkably up-regulated the expression of Nrf2, HO-1 and NQO1 compared with the CCl₄ group. **Conclusions:** The present study demonstrates that STM exerts a protective effect against CCl₄-induced liver injury and inflammation with its antioxidant effects and induction of hepatic detoxification enzymes and efflux transporters expression, at least in part, via the Nrf2/HO-1 pathway in rats.

© 2017 The Author(s)
Published by S. Karger AG, Basel**Introduction**

Swertiamarin (STM) is the main bioactive component in *Swertia mussotii* Franch (Gentianaceae), a commonly used Tibetan medicine, exerting broad pharmacological

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activities, especially hepatoprotective effect [1-3]. It has been previously reported to attenuate experimental hepatic fibrosis by suppressing the rennin-angiotensin system [4]. However, the effects and mechanisms of STM on carbon tetrachloride (CCl₄)-induced liver injury remain unclear.

Carbon tetrachloride (CCl₄) is a well-known hepatotoxin that is widely used to induce experimental liver fibrosis and cirrhosis in rodents [5]. CCl₄-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl₄ by cytochrome P450 (CYP) to the trichloromethyl radicals ($\bullet\text{CCl}_3$ and/or $\text{CCl}_3\text{OO}\bullet$), which lead to membrane lipid peroxidation and finally to cell necrosis [6, 7]. The second phase of CCl₄-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of proinflammatory factors [8]. Thus, CCl₄ at a high dosage can cause liver injury and fibrosis through cellular necrosis, oxidative stress and inflammation.

It is well known that cell damage induced by reactive oxygen species (ROS) is an important mechanism of liver injury. Naturally, cells are induced to develop some adaptive responses by intrinsic and extrinsic oxidants and electrophiles to counteract the environmental stresses [9]. The redox sensitive transcription factor, nuclear E2-related factor 2 (Nrf2) is an essential transcription factor that regulates an array of detoxifying and antioxidant defense genes expression through the antioxidant response element (ARE) in the liver [10]. Nrf2 controls the basal and induced expression of hundreds of ARE-dependent genes to regulate the physiological and pathophysiological outcomes of oxidant exposure. Many researchers have demonstrated that Nrf2 prevented the liver from kinds of hepatotoxins and Nrf2 signaling pathway represented an important target in protecting the liver against the damage induced by ethanol [11-13].

In this study, we mainly focused on the protective effects of STM against CCl₄-induced liver injury. Furthermore, we measured the expression of Nrf2 and some detoxifying enzymes (HO-1, NQO1) to elucidate the antioxidant pathway of STM.

Materials and Methods

Reagents and antibodies

Swertiamarin (purity>98%, dissolved in 1% Tween-20 saline) was provided by Xi'an Jiatian Biotechnology Co., Ltd (Xi'an, China). Carbon tetrachloride was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Anti-CYP2E1 antibody, anti-CYP3A antibody, anti-Bsep antibody, anti-Mrp2 antibody, anti-Mrp3 antibody, anti-Mrp4 antibody, anti-PDZK1 antibody, anti-Nrf2 antibody, anti-NQO1 antibody and anti-HO-1 antibody were purchased from Santa Cruz Biotechnology Inc (California, USA). Anti-GAPDH antibody was purchased from Beijing Biosynthesis Biotechnology, Ltd (Beijing, China). Anti-Histone antibody was purchased from Santa Cruz Biotechnology Inc (California, USA). BCA assay kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Animals and experimental protocol

This study was carried out in strict accordance with the guideline of the Council on Animal Care of Academia Sinica. The protocol was approved by the Ethical Committee on Animal Experimentation of Puai Hospital, Huazhong University of Science and Technology, China. Adult male SD rats weighing 250-280 g were obtained from the Center of Experimental Animal of Hubei Province (Wuhan, China). All animals were kept under the same laboratory conditions of temperature (25 ± 2°C) and lighting (12:12 h light: dark cycle), and were given free access to standard laboratory chow and tap water. All rats were allowed to acclimatize for one week before experiment. The animals were randomly assigned to five experimental groups (n=12), namely:

- i. Control group, rats were given 1% Tween-20 saline by gavage once per day for consecutive eight weeks with co-administration of vehicle (peanut oil, solvent of Carbon tetrachloride, 0.3 mL/100g, s. c. twice a week).
- ii. STM 200mg/kg group, rats were given STM dissolved in 1% Tween-20 saline (200 mg/kg B.W.) by gavage once per day for consecutive eight weeks with co-administration of vehicle (peanut oil, solvent of

Carbon tetrachloride, 0.3 mL/100g, s. c. twice a week).

iii. CCl₄ group, rats were given 1% Tween-20 saline by gavage once per day for consecutive eight weeks with co-administration of 40% CCl₄ mixed peanut oil solution (0.3 mL/100g, s. c. twice a week).

iv. CCl₄+STM 100mg/kg group, rats were treated with STM dissolved in 1% Tween-20 saline (100 mg/kg B.W.) by gavage once per day for consecutive eight weeks with co-administration of 40% CCl₄ mixed peanut oil solution (0.3 mL/100g, s. c. twice a week).

v. CCl₄+STM 200mg/kg group, rats were treated with STM dissolved in 1% Tween-20 saline (200 mg/kg B.W.) by gavage once per day for consecutive eight weeks with co-administration of 40% CCl₄ mixed peanut oil solution (0.3 mL/100g, s. c. twice a week).

Rats were fasted for 12 h after the last dose of agents before they were anaesthetized with pentobarbital sodium (30 mg/kg B.W., intraperitoneally). The blood samples were collected from abdominal aorta for the assays of serum enzymes. One piece of the liver was immediately fixed in 10% formaldehyde for histopathology assay and immunostaining. The rest was frozen with liquid nitrogen, and stored at -80°C until use.

Histological evaluations

Liver tissues were fixed with 10% formalin for 24 h, dehydrated, and embedded in paraffin. The paraffin-embedded samples were sectioned at 5 µm thickness and processed for hematoxylin-eosin (H&E) and Masson's trichrome staining.

Assessment of hepatotoxicity

The blood samples were centrifuged at 4°C for 15 minutes to separate serum. Liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were estimated in serum by using commercial test kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions.

Liver oxidative damage assay

Malondialdehyde (MDA), a degrading product of lipid peroxidation, was determined according to the thiobarbituric acid method using a MDA test kit (Jiancheng Institute of Biotechnology, Nanjing, China). Liver glutathione (GSH), a non-enzymatic antioxidant of hepatic antioxidant defense system, was assayed using commercial test kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions.

Liver inflammation assessment

The level of myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and nitric oxide (NO) were determined by using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China). The level of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were determined by using ELISA kits (Neobioscience, Beijing, China) according to the manufacturer's instructions.

Hepatic antioxidative defense potentials

Hepatic antioxidative defense enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) were assayed using commercial test kits (Nanjing Jiancheng Bioengineering Institute, China).

Western blotting

The protein levels of detoxification enzymes, efflux transporters and Nrf2 were determined in the liver by western blotting analysis. The whole liver lysate was prepared to evaluate the expression level of CYP2E1, CYP3A, PDZK1, HO-1 and NQO1. The membrane protein fractions of liver tissue were prepared to investigate the levels of Bsep, Mrp2, Mrp3 and Mrp4. The nuclear extracts of liver tissue were prepared to evaluate the expression level of Nrf2 in nucleus. The protein concentration was determined using the bicinchoninic acid (BCA) assay. Equal amounts of protein were separated with 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After electrophoresis, the gels were transferred onto polyvinylidene difluoride membranes, which were blocked with Tris-buffered saline containing 5% nonfat milk at 4°C. Then, the membranes were incubated overnight at 4°C in solution containing 0.1% Tween 20,

5% nonfat milk and the following primary antibodies: CYP2E1 (1:500); CYP3A (1:500); PDZK1 (1:500); HO-1 (1:500); NQO1 (1:500); Bsep (1:500); Mrp2 (1:500); Mrp3 (1:500); Mrp4 (1:500); Nrf2 (1:500); GAPDH (1:20000); Histon H3 (1:1000). Immunoreactive bands were quantified using the Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD). GAPDH or Histone H3 was used as an internal index.

Statistical Analysis

Data were expressed as mean $\pm$ S.D. The significant differences between groups were assessed with SPSS version 13.0. The differences between group means were calculated by one-way ANOVA with LSD post hoc analysis. Difference was considered statistically significant when $p < 0.05$, and extremely significant when $p < 0.01$.

Results

Effect of STM on histopathology of liver

The hepatic samples of the control and STM 200 groups presented a normal cellular architecture with hepatocytes in ordered arrangement. However, liver tissues from rats treated with CCl_4 showed extensive histopathological changes, characterized by hepatic lobule impairment, severe hepatocytes degeneration/necrosis, fatty changes, inflammatory cell infiltration and congestion. The histopathological lesions in the CCl_4 + STM groups were improved notably compared with those in the CCl_4 group. This indicated that STM attenuated the intensity of CCl_4 -induced liver damage (Fig. 1A).

To confirm hepatic fibrosis histologically, a Masson's trichrome staining was performed (Fig. 1B). The staining revealed that CCl_4 increased hepatic fibrosis when compared with the control group. And hepatic fibrosis was attenuated by STM administration at 100 and 200mg/kg.

Effect of STM on liver injury markers

To evaluate the effects of STM on liver injury induced by CCl_4 in rats, the serum levels of ALT, AST and ALP were measured. As shown in Figure 2A-C, the levels of ALT, AST and ALP were significantly ($p < 0.01$) increased in the CCl_4 -treated group. After the administration of STM, there were a significant reduction in ALT, AST and ALP ($p < 0.05$ or $p < 0.01$) compared

Fig. 1. Histological images of liver samples. Control and STM 200mg/kg: normal liver histology without fibroplasia and inflammatory cell infiltration; CCl_4 : The normal structure of hepatic lobules was damaged with many inflammatory cells infiltration; hepatocytes degeneration/necrosis, fatty changes, infiltration of lymphocytes and severe collagen fibers presented with pseudo-lobe were observed; CCl_4 +STM 100mg/kg: comparatively low degree of fibrotic changes with STM treatment (100 mg/kg B.W.); CCl_4 +STM 200mg/kg: relieved liver histopathology with STM treatment (200 mg/kg B.W.). (A. H-E; B. Masson, original magnification $\times 200$).

Fig. 3. Effects of STM on CCl₄-induced hepatic oxidative stress. A. Level of MDA. B. Level of GSH. C. Level of SOD. D. Level of CAT. E. Level of GPx. F. Level of GST. Data are represented as means ± S.D. for 7-12 animals per group. * *p* < 0.05 versus control, ** *p* < 0.01 versus control; # *p* < 0.05 versus CCl₄, ## *p* < 0.01 versus CCl₄ by one-way ANOVA and LSD post hoc test.

with the CCl₄ group. Liver injury seemed to attenuate more frequently in rats treated with higher concentrations of STM than that with a lower one.

Fig. 4. Effect of STM on inflammatory response in liver A. Level of MPO. B. Level of iNOS. C. Level of NO. D. Level of IL-1 β . E. Level of IL-6. F. Level of TNF- α . Data are represented as means $\pm$ S.D. for 7-12 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

Effect of STM on oxidative stress in liver

MDA is the marker of lipid peroxidation and end product of oxidative decomposition of polyunsaturated fatty acid. The effects on the levels of MDA in the liver of different groups are shown in Figure 3A. CCl₄ significantly ($p < 0.01$) increased the hepatic MDA levels in the CCl₄ group. The STM treatment at higher as well as lower doses significantly lowered the MDA levels in the liver tissue in comparison with the CCl₄ group.

CCl₄ treatment significantly decreased the levels of GSH and SOD in the liver tissue as compared to the control group ($p < 0.01$ and $p < 0.05$). STM treatment significantly ($p < 0.05$) increased the GSH levels at 200 mg/kg dose in comparison with the CCl₄ group. The SOD levels were also improved significantly ($p < 0.05$) by STM treatment at 200 mg/kg in comparison with the CCl₄ group (Fig. 3B, C).

CCl₄ treatment significantly ($p < 0.01$) decreased the activities of CAT, GPx and GST in the liver tissue as compared to the control group. STM treatment markedly restored the activities of CAT, GPx at 100 as well as 200 mg/kg dose in comparison with the CCl₄ group ($p < 0.05$ or $p < 0.01$). And STM significantly ($p < 0.05$) increased the activity of GST at 200 mg/kg in comparison with the CCl₄ group (Fig. 3D, E, F).

Effect of STM on inflammatory response in liver

The results showed that MPO activity in the CCl₄-treated group, an index of neutrophil infiltration, was increased significantly compared with that of the control group (Fig. 4A). Following STM (100 and 200mg/kg) treatment, MPO activity was decreased remarkably as compared to the CCl₄ group ($p < 0.01$). The activity of iNOS was increased in the CCl₄ group

Fig. 5. Effect of STM on detoxification enzymes expression in liver. A. CYP2E1 in CCl₄-treated rats with different doses of STM for eight weeks. B. CYP3A in CCl₄-treated rats with different doses of STM for eight weeks. Data are represented as means $\pm$ S.D. for 3-4 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

Fig. 6. Effect of STM on efflux transporters expression in liver. A. Level of Bsep. B. Level of Mrp2. C. Level of Mrp3. D. Level of Mrp4. E. Level of PDZK1. Data are represented as means $\pm$ S.D. for 3-4 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

obviously compared to the control group ($p < 0.01$) (Fig. 4B). In the treatment group with STM and CCl₄, the activity of iNOS was decreased markedly compared to the CCl₄ group ($p < 0.01$). In addition, CCl₄ also elevated the hepatic NO level notably ($p < 0.01$) compared with that of the control group (Fig. 4C). The hepatic NO level was significantly decreased following treatment with STM compared to the CCl₄ group ($p < 0.01$).

The content of pro-inflammatory cytokines, IL-1 β , IL-6 and TNF- α in the liver displays the degree of inflammatory response in liver. The IL-1 β , IL-6 and TNF- α content in liver tissue in STM-treated groups was significantly lower than that in the CCl₄ group ($p < 0.01$), indicating the alleviation of liver inflammation with STM treatment (Fig. 4D-F).

Effect of STM on detoxification enzymes expression in liver

Western blot analysis was performed to examine the effect of STM on CYP2E1 and CYP3A protein expression. Compared to the control rats, rats receiving CCl₄ alone showed a remarkable decrease in CYP2E1 protein level ($p < 0.05$). In rats co-treated with 200 mg/kg STM, an obvious higher level of CYP2E1 ($p < 0.05$) was found as compared with the rats intoxicated with CCl₄ alone. The restoration of CYP2E1 protein content by STM treatment implied that STM could have a hepatoprotective effect, which led to rapid recovery from CCl₄-induced liver injury. Western blotting results also demonstrated that the protein level of CYP3A was significantly reduced by CCl₄ intoxication ($p < 0.05$). STM (200 mg/kg) treatment elevated the protein level of CYP3A notably ($p < 0.05$) compared with the CCl₄ only group (Fig. 5).

Fig. 7. Effects of STM on CCl₄-induced hepatic Nrf2/HO-1 pathway. A. Relative density analysis of the Nrf2 protein bands in nucleus. B. Relative density analysis of HO-1. C. Relative density analysis of NQO1. GAPDH and Histon H3 were probed as the internal control in relative density analysis. Data are represented as means $\pm$ S.D. for 3-4 animals per group. * $p < 0.05$ versus control, ** $p < 0.01$ versus control; # $p < 0.05$ versus CCl₄, ## $p < 0.01$ versus CCl₄ by one-way ANOVA and LSD post hoc test.

Effect of STM on efflux transporters expression in liver

CCl₄ intoxication reduced the expression of canalicular Bsep and Mrp2 significantly compared with the control group ($p < 0.05$). The protein levels of Bsep and Mrp2 at the canalicular membrane of hepatocytes were markedly increased in CCl₄+STM 200mg/kg group compared with the CCl₄ group ($p < 0.01$). CCl₄ significantly reduced the expression of basolateral Mrp3 and Mrp4 ($p < 0.05$ and $p < 0.01$). The protein levels of Mrp3 and Mrp4 were notably elevated in CCl₄+STM groups compared with the CCl₄ group. On the other hand, CCl₄ repressed the protein expression of PDZK1 markedly ($p < 0.01$) compared with the control group. And the protein expression of PDZK1 was remarkably up-regulated in CCl₄+STM groups compared with the CCl₄ group (Fig. 6).

Effect of STM on Nrf2/HO-1 pathway in liver

The accumulated evidence showed that Nrf2 is a basic leucine zipper redox-sensitive transcriptional factor that plays a center role in ARE-mediated induction of phase II detoxifying and antioxidant enzymes. To further investigate the molecular mechanism of oxidative stress in rat liver, we measured the expression levels of Nrf2, heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase-1 (NQO1). Nrf2 expression levels in nuclear fractions were significantly decreased in CCl₄-treated rats as compared with the controls ($p < 0.01$). Co-treatment with STM (100 and 200mg/kg) strongly increased the translocation of Nrf2 from the cytosol to the nuclear fraction compared with the CCl₄ group. The levels of HO-1 and NQO1 were decreased in rat treated with CCl₄ alone as compared with controls ($p < 0.01$ and $p < 0.05$). STM treatment obviously increased the expression levels of HO-1 and NQO1 in rat liver as compared with the CCl₄ group (Fig. 7).

Discussion

The main finding of this study was that STM, an iridoid compound from *Swertia mussotii* Franch (Gentianaceae), was instrumental in attenuating liver injury in rats induced by a chronic CCl₄ exposure. In the present study, CCl₄ treatment resulted in a significant elevation in serum marker enzymes, ALT, AST and ALP, indicating the liver damage. Besides, severe liver lesion was also induced by CCl₄. All of the above-mentioned pathological changes were significantly abrogated with STM treatment, suggesting that STM was able to attenuate CCl₄-induced liver injury in rats.

CCl₄-induced hepatotoxicity was a common animal model for investigating the potential hepatoprotective activity [14]. Lipid peroxidation is one of the principal causes of CCl₄-induced liver injury and is mediated by the free-radical derivatives of CCl₄. Lipid peroxidation

can be monitored by measuring the content of liver MDA. Hepatic antioxidant defense system disruption is characterized by increased lipoperoxides (LPO), MDA and/or altered enzymatic and non-enzymatic antioxidants including SOD, CAT, GPx, GST and GSH. The superoxide radicals are converted to hydrogen peroxide (H_2O_2) and O_2 by the action of SOD, which is further detoxified to water by CAT or GPx. GST plays a physiological role in initiating the detoxification of alkylation of lipid, protein, and nucleic acid, as well as providing cellular resistance to lipid peroxidation by elevating the conjugation of toxic electrophiles with GSH. Thus, the activities of these enzymes have been used to assess oxidative stress in cells [15-17]. And the excessive reactive oxygen species (ROS) induced by CCl_4 would break the balance between ROS production and antioxidant defense system. In the present study, CCl_4 notably decreased the hepatic SOD, CAT, GPx and GST activities, as well as GSH content. Interestingly, STM markedly restored the activities of those antioxidant enzymes and the content of GSH in the liver of CCl_4 -treated rats. It suggested that STM could attenuate oxidative stress at least partly by renewing the activities of antioxidant enzymes and increasing the content of GSH in liver.

A series factors are believed to be involved in the pathogenesis of the liver injury, including oxidative stress, inflammation and immune reactions. Excessive ROS induced by CCl_4 can induce the nuclear factor kappa B (NF- κ B) activation and its subsequent nuclear translocation, which are responsible for modulation of liver injury by regulating inflammatory cytokine production, such as TNF- α , and the induction of inflammation associated enzymes, including iNOS [18, 19]. CCl_4 exposure generates excessive levels of nitric oxide (NO) through activation of iNOS, thus contributing to damage in hepatic tissue [20]. Hepatic injury in rats treated with CCl_4 also developed with an increase in the activity of MPO, an index of hepatic neutrophil infiltration [21]. Our results revealed that STM significantly inhibited MPO and iNOS activity consistent with decreased NO level. In addition, proinflammatory cytokines including IL-1 β , and IL-6 and TNF- α have been the focus of investigations of inflammatory organ injury because the uncontrolled and prolonged action of these proteins is potentially harmful [22]. Interestingly, STM significantly reduced inflammatory cytokine levels (IL-1 β , IL-6 and TNF- α), thus alleviated inflammatory response in the liver. We preliminarily speculated that STM-mediated hepatoprotection might also benefit from its anti-inflammatory action.

It has been reported that STM treatment could induce detoxification enzymes and efflux transporters in rats [23]. A significant induction of the expression of the basolateral efflux transporters Mrp3 and Mrp4 and canalicular transporter Mdr1 was observed in rats treated with STM compared with the controls. And the expression of detoxification enzymes Cyp3a, Ugt2b, Sult2a1 and Gsta1 in rats treated with STM was also significantly higher than that in the controls. The induced detoxification enzymes thus increase the water solubility of hydrophobic bile acids through hydroxylation (Cyp3a), glucuronidation (Ugt2b), and sulphation (Sult2a1) [24-26]. Generally, CCl_4 is metabolized to highly reactive trichloromethyl free radicals mainly by CYP2E1 [27]. On the other hand, the trichloromethyl radicals generated may bind either at the heme group of CYP or at the active site of the enzyme near the heme group, leading to the inactivation of CYP [28]. Total CYP and CYP isoenzyme concentrations were found to be decreased in rats with severe cirrhosis [29]. In acute liver injury induced by CCl_4 , there were drastic decreases in the activities of the main liver P450 isoenzymes such as CYP1A2, CYP2C6, CYP2E1 and CYP3A2, as well as their protein expressions due to oxidative stress [30]. And chronic CCl_4 -induced liver injury was also characterized by dysregulation of numerous genes involved in metabolism [31]. In this study, considerable decreases in hepatic CYP3A and CYP2E1 protein level were found in rats treated with CCl_4 alone. In contrast, in rats treated with STM, CYP3A and CYP2E1 protein content was less suppressed following CCl_4 administration. Our data suggested that STM might restore the hepatic CYP3A and CYP2E1 expression and lead to a rapid recovery from CCl_4 -induced liver injury. Moreover, bile salt export pump (Bsep) and multidrug resistance-associated proteins (Mrps) are members of the ATP-binding cassette family of membrane transporters mainly locating in the canalicular membrane of hepatocytes. Bsep mediates the

concentrative transport of monovalent bile salts into the canaliculus [32]. Mrps transport relatively hydrophilic compounds and drugs [33]. Among them, Mrp2 mainly expresses in hepatocytes acting as a host defense mechanism preventing the influx of harmful substrates from the apical side and facilitating its efflux out of cells. It has been demonstrated that the up-regulation of Bsep and Mrp2 contributed to the reduced hepatic bile acids and the protective effects of alisol B 23-acetate against CCl₄-induced hepatotoxicity in mice [34]. The two basolateral transporters Mrp3 and Mrp4 are bile acids efflux transporters that eliminate conjugated bile acids from hepatocytes and readily influenced by liver toxicants also. In CCl₄-lesioned rodent model, Mrp3 and Mrp4 were differentially regulated according to animal species and/or experimental methods [35, 36]. Besides, PDZK1 is a PSD-95/discs large/ZO-1 (PDZ)-based adaptor protein that regulates several membrane-transporting proteins in epithelia [37]. It has been demonstrated that PDZK1 could regulate the expression and function of Mrp2 and Mrp4 [38, 39]. Recently, the expression of PDZK1 was found to be significantly down-regulated during cytokine-induced inflammation [40, 41]. In this study, CCl₄ also repressed the protein expression of PDZK1, which was abolished by STM treatment, suggesting a novel regulatory mechanism of hepatic transporters during liver injury. Our results implied that STM could stimulate Bsep and Mrps expression to excrete toxic substances and reduce the hepatotoxicity, highlighting the protective role for STM in liver damage.

The role of the Nrf2/ARE signaling pathway in liver pathophysiology and the potential application of Nrf2 as a therapeutic target to treat viral hepatitis, alcoholic and nonalcoholic liver disease and fibrosis are well documented [42]. Several natural antioxidants exert hepatoprotective effects not only by scavenging the free radical, but also by augmenting the expression of cytoprotective and/or antioxidant genes via Nrf2 signaling pathway [43]. Normally, Nrf2 is tethered in the cytoplasm by the Kelch-like ECH-associated protein 1 (Keap1) for subsequent proteasomal degradation. Upon stimulation by inducers, Nrf2 dissociates from Keap1 and translocates into the nucleus where it binds to the ARE of target genes coding for a number of antioxidant enzymes and phase II detoxifying enzymes. This will lead to activation of Phase II detoxifying enzymes such as glutathione-S-transferase (GST), quinone oxidoreductase 1 (NQO1), hemeoxygenase-1 (HO-1) that efficiently protect mammalian cells from various toxicants [44, 45]. In our study, Nrf2 expression was notably increased by STM treatment compared with the CCl₄ group, suggesting the activation of Nrf2 defense pathway. And STM treatment obviously enhanced expression of the signaling components, such as HO-1 and NQO1. HO-1 is a rate-limiting enzyme in the catabolism of heme belonging to a family of cytoprotective and detoxification genes that possess AREs in their regulatory regions. Excessive oxidative stress has been suggested as a reason for the upregulation of HO-1, as this enzyme is known to be readily inducible upon such stressors [46]. HO-1 plays a key role in maintaining antioxidant homeostasis and limits hepatotoxicity during chronic liver diseases [47-49]. In our study, the expression of HO-1 protein was significantly increased after CCl₄ treatment. And STM treatment further augmented HO-1 protein expression following CCl₄ treatment, which suggests that a strong inductive response of HO-1 by STM is to protect hepatocytes from CCl₄-induced oxidative cellular injuries. NQO1 is a phase II detoxifying enzyme which can be used for catalyzing the two-electron reduction and detoxification of quinones and other redoxcycling endogenous and exogenous chemicals [50]. In our study, STM treatment also reversed the expression of NQO1 down-regulated by CCl₄. These results indicated that the protective effects of STM might link with the activation of the Nrf2 pathway and up-regulation of HO-1 and NQO1.

In conclusion, STM effectively ameliorates the CCl₄-induced liver injury and inflammation in rats through alleviation of oxidative stress and induction of hepatic detoxification enzymes and efflux transporters expression, at least in part, via activation of the Nrf2/HO-1 pathway. And there are different signaling pathways, such as the ERK and PI3K/Akt pathways, involved in the beneficial effect of anti-fibrotic natural agent [51]. Further studies on the possible effect of STM on any other cell signaling pathways involved in the pathogenesis of CCl₄-induced hepatotoxicity are still ongoing.

Abbreviations

ALT (Alanine aminotransferase); AST (Aspartate aminotransferase); ALP (Alkaline phosphatase); CCl_4 (Carbon tetrachloride); SOD (Superoxide dismutase); GPx (Glutathione peroxidase); CAT (Catalase); GST (Glutathione S-transferase); GSH (Reduced glutathione); LPO (Lipoperoxides); MDA (Malondialdehyde); ROS (Reactive oxygen species); MPO (myeloperoxidase); iNOS (inducible nitric oxide synthase); NO (nitric oxide); IL-1 β (interleukin-1 β); IL-6 (interleukin-6); TNF- α (tumor necrosis factor alpha); NF- κ B (nuclear factor kappa B); HO-1 (Hemeoxygenase-1); NQO1 (NAD(P)H: quinone oxidoreductase-1); Nrf2 (Nuclear factor E2-related factor 2); Keap1 (Kelch-like ECH-associated protein-1); ARE (Antioxidant response elements); STM (swertiamarin); H&E (Hematoxylin and eosin); CYP (Cytochrome P450); Bsep (bile salt export pump); Mrps (multidrug resistance-associated proteins).

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Disclosure Statement

None of the authors reports a duality of interest.

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