Swertiamarin Attenuates Experimental Rat Hepatic Fibrosis by Suppressing Angiotensin II–Angiotensin Type 1 Receptor–ERK Signaling

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Running title: Swe attenuates hepatic fibrosis by modulating RAS

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Number of text pages: 28
Number of tables: 1
Number of figures: 6
Number of references: 50
Number of words in Abstract: 239
Number of words Introduction: 465
Number of words in Discussion: 761

List of abbreviations: alpha-smooth muscle actin (α-SMA), angiotensin II (Ang II), angiotensin type 1 receptor (AT1R), carbon tetrachloride (CCl4), 4',6-diamidino-2-phenylindole (DAPI), dimethylnitrosamine (DMN), Dulbecco's Modified Eagle's medium (DMEM), 5-ethynyl-2'-deoxyuridine (EdU), extracellular matrix (ECM), hepatic stellate cells (HSCs), fetal bovine serum (FBS), phosphate buffered saline (PBS), radioimmunoassay (RIA), renin-angiotensin system (RAS), Traditional Chinese Medicine (TCM), Swertiamarin (Swe)
JPET #234179

Recommended section: Gastrointestinal, Hepatic, Pulmonary, and Renal
Abstract

The rennin–angiotensin system (RAS) is crucial in hepatic fibrosis development, and therapies targeting this system may be a promising treatment for hepatic fibrosis. In this study, we investigated the effects of swertiamarin (Swe), an ethanol extract of Gentiana manshurica Kitag, on hepatic fibrosis and its underlying mechanisms through regulating RAS. Primary rat hepatic stellate cells (HSCs) were isolated and treated with angiotensin II (Ang II) with or without Swe and losartan. The proliferation and activation of HSCs were measured. Rat hepatic fibrosis was induced by intraperitoneal dimethylnitrosamine (DMN) injection for 4 weeks. Rats were treated with Swe or losartan from the 3rd week until the end of the experiment. Hydroxyproline (Hyp) content in liver tissue was assayed with Jamall's method and liver collagen deposition was visualized using sirius red staining. RAS components were analyzed by western blot, immunofluorescent staining, and real time RT-PCR. The results showed that Swe significantly inhibited Ang II-induced HSCs proliferation and activation. Swe also significantly suppressed DMN-induced alpha-smooth muscle actin production in rat liver and improved liver function. Swe partially inhibited Ang II-induced angiotensin type 1 receptor (AT1R) upregulation and suppressed Ang II-induced ERK and c-jun phosphorylation in HSCs. In the DMN-treated rats, Swe treatment significantly inhibited the plasma Ang II levels. DMN-induced AT1R upregulation and phosphorylation of ERK and c-jun in rat liver were also inhibited by Swe. In conclusion, Swe may attenuate hepatic fibrosis through inhibiting HSC activation by regulating the RAS.

Keywords: angiotensin II, hepatic fibrosis, rennin- angiotensin system,
Introduction

Hepatic fibrosis, a dynamic process caused by many chronic liver diseases, such as hepatitis B virus infection and alcohol abuse, can eventually lead to cirrhosis and even malignant tumors (Beljaars et al., 2002). Clinical and laboratory studies have indicated that fibrosis is a reversible process, whereas the reversion of cirrhosis remains unlike (Friedman and Bansal, 2006; Ismail and Pinzani, 2011; Kim do et al., 2013; Czaja, 2014). Therefore, the development of therapies that could reverse fibrosis would have a substantial clinical impact. Hepatic stellate cell (HSC) is a central mediator of hepatic fibrosis and plays a crucial role in the process of hepatic fibrosis (Hao et al., 2014; Tung et al., 2014). After HSC activation, the extracellular matrix (ECM) production is increased, and the degradation of ECM is decreased, eventually leading to abnormal deposition of ECM and hepatic fibrosis (Moreira, 2007; Das and Vasudevan, 2008). In view of the important role of HSC in the production of ECM, HSC is considered as a target in antifibrotic therapy (Safadi and Friedman, 2002).

The rennin-angiotensin system (RAS) is a master regulator of blood pressure and body fluid homeostasis, as well as plays an important role in tissue remodeling (Ruiz-Ortega et al., 2001b). In recent years, RAS was observed to be involved in the process of hepatic fibrosis. Angiotensin II (Ang II), the main effector hormone of RAS, regulates key steps in the tissue fibrosis process through the angiotensin type 1 receptor (AT1R) (Mezzano et al., 2001). In carbon tetrachloride (CCL4)-induced rat hepatic fibrosis model, the mRNA expression of angiotensinogen increases at the early stage of hepatic fibrosis (Lu et al., 2011). In patients with cirrhosis, systemic Ang II is frequently upregulated (Vilas-Boas et al., 2009). Increased systemic Ang II
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induces contraction and proliferation of human HSCs, exacerbates hepatic fibrosis and promotes inflammation, oxidative stress and vascular damage (Bataller et al., 2000; Bataller et al., 2005). Interestingly, activated HSCs also synthesize Ang II and express components of RAS, including AT1R (Bataller et al., 2003b). Accordingly, inhibiting the generation of Ang II and/or blocking the binding of Ang II to AT1R could markedly attenuate hepatic fibrosis (Jonsson et al., 2001; Ohishi et al., 2001; Paizis et al., 2001). Therefore, therapies targeting RAS may represent a promising treatment for hepatic fibrosis in the setting of chronic liver disease.

In our institute, we have focused on developing effective therapeutic methods for the treatment of hepatic fibrosis for many years. *Gentiana manshurica* Kitag, a herb belonging to the Gentianaceae family, is used in Traditional Chinese Medicine (TCM) to treat many liver diseases. *Gentiana manshurica* Kitag can reverse acute alcohol-induced liver steatosis (Lian et al., 2010) and prevent acetaminophen-induced acute hepatic injury in mice by inhibiting JNK/ERK pathways (Wang et al., 2010a). Swertiamarin (Swe), the ethanol extract of *Gentiana manshurica* Kitag, exerts antioxidant and hepatoprotective effect against D-galactosamine-induced liver damage in rats (Jaishree and Badami, 2010). In our preliminary experiments, Swe also showed a significant effect on the proliferation and activation of HSCs induced by Ang II. Therefore, in the present study, we further investigated the anti-hepatic fibrosis effect of Swe. In view of the importance of RAS in liver fibrogenesis, we determined if the mechanism of Swe action occurred by regulation of RAS.
Materials and Methods

Chemicals and Drugs

Swe was obtained from Shanghai winherb Medical Technology Co., Ltd, China. The purity of Swe was 98%, molecular weight 374.34, and molecular formula C\textsubscript{16}H\textsubscript{22}O\textsubscript{10}. Swe was dissolved in dimethylsulfoxide at a concentration of 20 mM and stored at −20 °C for future use. The EdU cell proliferation kit (Cell-Light \textsuperscript{TM} EdU DNA Cell Proliferation Kit, Cat# 10310) was purchased from Guangzhou RiboBio, China. Ang II was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against AT1 (Santa Cruz, sc-1173), pERK1/2 (Santa Cruz, sc-7383), total ERK2 (Santa Cruz, sc-1467), GAPDH (Santa Cruz, sc-166574), α-SMA(Abcam, ab5649), GAPDH (KangChen, KC-5G4), β-tubulin (Cell Signaling Technology, 2146), c-Jun (Cell Signaling Technology, 9165) and p-c-Jun (Cell Signaling Technology, 9261) were used in this study.

HSC Isolation and Culture

Primary HSCs were isolated from normal rat livers by perfusion with pronase, followed collagenase and nycodenz density-gradient centrifugation (Friedman et al., 1992). Primary HSCs were cultured with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS). Primary HSCs were seeded in 6-, 12- and 96-well plates on the 3rd day. The experiments were performed in accordance with the Helsinki Declaration of 1975.

LDH Assay

HSCs were seeded at a density of 5,000 cells per well in 96-well plates. After growing for 24 h under normal growth condition, the medium was replaced with DMEM
containing different concentrations of swertiamarin. The cells were incubated for another 24 h. The activity of LDH in cell-culture medium (extracellular LDH) or intracellular LDH was determined using a commercial LDH kit according to the manufacturer’s instructions. The amount of LDH released into the extracellular medium was expressed as the percentage of the total intra- and extracellular content.

**Cell Proliferation Analysis**

To assess cell proliferation, HSCs were plated on 96-well plates. Upon reaching 60% confluence, cells were treated with 0.1 μM Ang II, different concentrations of Swe, or Losartan, respectively, for 24 h. The cells were incubated under standard conditions in complete media. Cell proliferation was detected using the incorporation of 5-ethynyl-2′-deoxyuridine (EdU) from the EdU Cell Proliferation Assay Kit. Briefly, the cells were incubated with 50 μM EdU for 3 h before fixation, permeabilization and EdU staining, which were performed according to the manufacturer’s protocol. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) at a concentration of 1 μg/mL for 10 min. EdU positive and negative cells were determined by fluorescent imaging. Images were taken using Cellomics ArrayScan VTI HCS Reader and analyzed using Cellomics Cell Health Profiling BioApplication Software.

**Immunofluorescence**

Cells were plated in 96-well plates. After growing for 24 h under normal growth conditions, the medium was replaced with DMEM containing Ang II (0.1 μM) (Osterreicher et al., 2009) and different concentrations of Swe. The cells were incubated for another 24 h. Cells were then washed twice with cold phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 minutes.
Immunofluorescence was performed to detect the F-actin and α-SMA, as described previously (Sohail et al., 2009). Images were taken using Cellomics ArrayScan VTI HCS Reader and analyzed using Cellomics Cell Health Profiling BioApplication Software.

**Animals**

Male wistar rats (6–8 weeks old) were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, and maintained in a room under temperature control at 23 ± 2 °C and a 12-h light-dark lighting cycle. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai University of Traditional Chinese Medicine, China. All animals received humane care during the study with unlimited access to chow and water. The rats were randomly divided into five groups: Control (n=15), DMN (n=15), Swe low dose (Swe 15 mg/kg) (n =15), Swe high dose (Swe 20 mg/kg) (n =15) and Losartan Treatment (L) (n=15). To induce liver fibrosis, 10 μg/kg DMN was given by intraperitoneal injection three consecutive days per week over a period of 4 weeks referred to Ala-kokko (Ala-Kokko et al., 1987) with minor modification (Su et al.; Pines et al., 1997). From the third week of DMN injection, rats in the Swe 15 mg/kg, Swe 20 mg/kg and Losartan groups were treated with Swe at a dose of 15 mg/kg·d, 20 mg/kg·d, and Losartan at a dose of 10 mg/kg·d, respectively. Rats in control and DMN groups were treated with equal amount of vehicle. After 2 weeks of treatment, rats were anaesthetized with 2% pentobarbital sodium and the blood samples were obtained from the vena cava inferior. A portion of each liver was fixed in 10% phosphate-buffered formalin for histological studies after paraffin embedding. The remainder was snap-frozen in liquid nitrogen and stored at -80 °C for western blot.
Hematoxylin-eosin and Sirius red staining

The left lateral lobe of the liver was sliced, and the tissue slices were fixed in 10% buffered-neutral formalin for 24 h. The fixed liver tissue slices were embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. Sections (5 μm) were subjected to hematoxylin-eosin (HE) and Sirius red staining as described previously (Wang et al., 2010b). An arbitrary scope was given to each microscopic field viewed at a magnification of 200×.

Hydroxyproline (Hyp) content Measurement

Hepatic Hyp content was measured with HCl hydrolysis according to Jamall’s methods. Briefly, 100 mg of all liver samples were homogenized and hydrolyzed in 6 M HCl at 110 °C for 18 h. Hydrolysates were filtrated with 3 mm filter paper and dried at 40 °C. The samples were then incubated with Ehrlich’s solution (25% (w/v) p-dimethylaminobenzaldehyde and 27.3% (v/v) perchloric acid in isopropanol) at 50 °C for 90 min and measured at A558 nM. All results were normalized by total protein concentration and calculated using a standard curve.

Serum Biochemical Measurements

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb) and total bilirubin (TBil) levels were measured according to the manual instruction (NanJing JianCheng Bioengineering Institute, China).

Measurement of Plasma Ang II Levels

Blood was collected from the inferior vena cava into a chilled glass tube containing protease inhibitors and Enalapril to prevent ex vivo conversion of Ang I to Ang II. Samples were eluted from the column with 90% methanol, and then dried and
reconstituted for RIA. RIA for Ang II was performed using $^{125}$I-angiotensin II (Perkin-Elmer, Foster City, CA) and rabbit anti-Ang II antibody (Phoenix Pharmaceuticals, Inc., Belmont, CA) with cross-reactivity of <2% for Ang II precursors and its degradation products. After incubation for 2 days at 4 °C, bound and free Ang II was separated using dextran-coated charcoal. The supernatant was measured with a gamma-counter (ICN, Costa Mesa, CA). The ratio B/Bo was corrected for non-specific binding, expressed as a percentage of maximal binding, and read against a standard curve (log–logit transformation).

**Western Blot**

Liver tissue of rats or HSCs were lysed using RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl pH7.4, 1 mM EDTA, 1 mM PMSF, 1× Roche complete mini protease inhibitor cocktail, Roche PhosSTOP phosphatase inhibitor cocktail). The supernatants were collected after centrifugation at 10,000 ×g at 4 °C for 15 min. The protein concentration was determined using a BCA protein assay kit (Thermo Scientific). Equal amounts of protein were separated by 10% SDS gel electrophoresis (SDS-PAGE) under denaturing and non-reducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked with odyssey blocking buffer (LI-COR Bioscience) at room temperature for 1 h and then incubated with primary antibody at 4 °C overnight. After washing in PBS-Tween (PBST), the blots were incubated with fluorescence-coupled secondary antibody. The signals were visualized using the Odyssey Imaging System (LI-COR Bioscience).

**Real-time RT-PCR**

Total RNA was isolated using the Trizol Reagent (Invitrogen, Shanghai, China), according to the manufacturers’ protocol. The RNA concentration was determined.
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using a NanoDrop Spectrophotometer. cDNA was generated using 1 μg of total RNA in a final reaction volume of 20 μl by the first-strand cDNA synthesis kit (TOYOBO, Osaka, Japan), according to the manufacturer's protocol. Quantitative Real-time PCR was performed with an ABI ViiA7 Real-time PCR system. Primers, along with their sequences, are listed in Table 1. PCR mixtures contained 2 μl of cDNA, 10 μl of SYBR® Premix 2× and 0.25 μM of forward and reverse primers, for a total volume of 20 μl. Reactions were started with a polymerase activation step at 94 °C for 10 min; followed by 40 cycles of 94 °C for 10 s, 60 °C for 20 s and 72 °C for 25 s. Fluorescent data were acquired after each cycle. The absence of non-specific products was verified after each run by melting curve analysis. The relative gene quantities were calculated by the $2^{-\Delta\Delta CT}$ method in comparison with the expression levels of GAPDH.

Statistical Analysis

Data are expressed as means ± standard deviation (S.D). Data were analyzed using one-way analysis of variance, as well as the least significant difference test, and $P <0.05$ was considered statistically significant.
Results

Swe Inhibited HSCs Proliferation

Primary HSCs were incubated with 5 to 100 μM of for 24 h and then assayed using LDH method (Fig 1A). The results showed that 15 μM Swe had no toxic effect on HSCs. HSCs proliferation was determined by the EdU cell proliferation assay (Pipparelli et al.). After 24 h incubation, Ang II significantly promoted the proliferation of HSCs (by 60%) when compared with control (Figs 1B and 1C). To determine the effect of Swe on HSCs proliferation, cells were co-treated with Ang II and Swe (2.4 μM, 6 μM or 15 μM). Swe significantly suppressed Ang II-induced HSCs proliferation at 6 and 15 μM. However, 2.4 μM of Swe showed little effect on the HSCs proliferation. Losartan also significantly suppressed the proliferation of HSCs (Figs 1B and 1C).

Swe Inhibited the Activation of HSCs

To evaluate the effect of Swe on Ang II-induced HSCs activation, we examined the expression of α-SMA. As shown in Figs 2A - 2D, immunofluorescence staining and western blot showed that α-SMA expression was significantly enhanced by Ang II and was reversed by losartan and Swe treatments, in a concentration-dependent manner (Figs 2A-D). Real time RT-PCR also showed that Ang II-induced upregulation of α-SMA mRNA was significantly suppressed by 6 and 15 μM of Swe and 1 μM of losartan (Fig 2E). The actin cytoskeleton plays an important role in defining cell shape and morphology. In this study, we also examined F-actin reorganization during the conversion from quiescent HSCs to myofibroblasts after Ang II administration. The results revealed that Ang II induced significant F-actin
reorganization (Figs 2F and 2G), forming abundant long stress fibers in HSCs.

Different concentrations of Swe blocked these processes.

**Swe Regulated RAS-related Molecules**

To explore the mechanisms of the inhibition of Ang II-induced HSCs activation by Swe, we tested the expression of RAS-related molecules by western blot and real time RT-PCR. Ang II treatment significantly upregulated the expression of AT1R (Figs 3A and 3B). Losartan and Swe treatment partly reversed Ang II-induced AT1R upregulation. Real time RT-PCR showed that Ang II treatment significantly increased TGF-β1 mRNA level (Fig 3C). Both Losartan and Swe suppressed the upregulation of TGF-β1 mRNA induced by Ang II (Fig 3C).

The activation of ERK and AP1 is associated with cell growth. Therefore, we next examined the effect of Swe on the phosphorylation of ERK and AP1. Ang II treatment induced a rapid phosphorylation of ERK1/2 (Figs 3D and 3E). Treatment of Ang II for 3 h also induced phosphorylation of c-jun, a subunit of AP1. Swe significantly suppressed Ang II-induced phosphorylation of ERK1/2 and c-jun, in a dose-dependent manner (Figs 3D, E). Furthermore, the nuclear translocation of c-jun was also inhibited by Swe (Fig 3F).

**Swe Ameliorated Liver Fibrosis Induced by DMN in Rats**

The activation of HSCs plays a vital role in the progress of hepatic fibrosis, and the above results suggested that Swe might be an effective agent in the treatment of hepatic fibrosis. Therefore, we further investigated the effect of Swe on DMN-induced rat hepatic fibrosis. The excessive and disorganized deposition of collagens is a major pathogenic feature of fibrotic diseases. As shown in Figs 4A and 4B, DMN treatment induced a significant increase of collagen expression, as
determined by Sirius red staining. Treatment with Swe for 2 weeks resulted in an alleviation of collagen deposition (Figs 4A and 4B). Losartan also attenuated DMN-induced collagen overexpression in rat livers. Western blot showed that α-SMA expression in DMN-treated rats was significantly increased and was then inhibited by Swe and Losartan (Figs 4C and 4D). In DMN-treated rats, the hydroxyproline (Hyp) content was also significantly elevated compared with that of the control (Fig 4E). Treatment with Swe or Losartan improved the Hyp content significantly (Fig 4E).

**Swe Improved DMN-induced Rat Hepatic Injury**

DMN significantly decreased the body weight and liver/body ratio of rats compared with the control (Figs 5A and 5B). The rat body weight and liver/body ratio in the Swe and losartan groups were significantly increased compared with those in the DMN group (Figs 5A and 5B). As shown by HE staining, the liver showed normal cellular architecture in the control group (Fig 5C). However, DMN induced severe histopathological changes in the rat liver, such as centrilobular hepatic necrosis and infiltrating lymphocytes. Treatment with Swe and losartan partly prevented the histopathological changes associated with DMN-induced hepatotoxicity (Fig 5C). DMN treatment also significantly decreased the albumin (Alb) level and increased the alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBiL) levels in rat serum (Figs 5D–5G). Swe and losartan treatment significantly improved DMN-induced liver function injury.

**Swe Regulated the RAS Signaling Molecules in vivo**

To further confirm whether Swe could also regulate RAS in vivo, we determined the levels of RAS-associated molecules in rats. In the DMN-induced rat hepatic fibrosis
model, the expression of AT1R was significantly upregulated (Figs 6A and 6B).

Losartan, a blocker of AT1R, significantly decreased that of AT1R compared with the DMN group. Swe treatment also suppressed DMN-induced abnormal expression of AT1R. Phosphorylated ERK and c-jun were also significantly elevated in DMN alone treated rats (Figs 6A, 6C and 6D). Co-treatment with Swe or Losartan inhibited DMN-induced ERK and c-jun phosphorylation. In the DMN group, the Ang II level in rat serum was significantly increased compared with control group (Fig 6E). In the Swe high dose and low dose groups, Ang II levels were significantly downregulated compared with the DMN group (Fig 6E). However, the Ang II level of the Losartan group was higher than that of the DMN group (Fig 6E).
Discussion

Hepatic fibrosis, a reversible woundhealing response, is characterized by abnormal ECM deposition. It is a multifunctional process that involves HSC, cytokines, chemokines and various growth factors and results from a disruption of homeostatic mechanisms that maintain the liver ecosystem (Friedman, 2008; Malhi and Gores, 2008). HSCs, called lipid storage cells, lipocytes or Ito’s cells, are perisinusoidal cells that normally reside in the space of Disse and contain numerous retinoid and lipid droplets (Geerts, 2001; Iredale, 2007).

In response to injury, quiescent HSCs, involved in the depletion of vitamin A storage and lowering of retinol chains expression, acquire contractility and activate into collagen type I, α-SMA myofibroblasts. HSC is the main cell type responsible for excessive deposition of connective tissue components in response to liver injury. The activation of HSC is the central event in the development of hepatic fibrosis. Therefore, many effective therapeutic approaches focus on regulating HSC activation and proliferation.

In the process of hepatic fibrosis, the components of RAS have attracted increasing attention (Zhang et al., 2013). RAS accelerates inflammation, tissue repair, and fibrogenesis through Ang II and its receptor AT1R (Kisseleva and Brenner, 2011). Prolonged administration of Ang II directly activates HSCs and causes hepatic fibrosis in rodents (Bataller et al., 2005; Zhang et al., 2014), while AT1R knockout may protect mice from hepatic fibrosis (Kanno et al., 2003; Yang et al., 2005). This makes RAS an attractive target for antifibrotic therapy. In our study, we showed that Swe treatment inhibited Ang II-induced HSC proliferation and activation. Furthermore, Swe treatment suppressed Ang II-induced TGF-β1 mRNA
Thus, the in vitro results indicate that Swe may be an effective agent for treating hepatic fibrosis. Therefore, we studied the effects of Swe on hepatic fibrosis in vivo. In DMN-induced rat hepatic fibrosis model, Swe significantly inhibited the expression of α-SMA and collagen, and improved the rat liver function, exhibiting a good therapeutic effect. These data suggest that Swe is a good candidate for the treatment of hepatic fibrosis.

The profibrotic activity of Ang II is mainly mediated by AT1R (Wang et al., 2005; Yang et al., 2005; Li et al., 2007). Consistent with previous studies (Lu et al., 2011; Dasuri et al., 2013; Shahid et al., 2013), we also demonstrated that the AT1R level was upregulated in Ang II-treated HSCs and the livers of DMN-treated rats (Li et al., 2012). In the present study, Swe inhibited Ang II- and DMN-induced AT1R upregulation in HSCs and rat liver, respectively. In addition, Swe downregulated plasma Ang II levels in DMN-treated rats. These results indicate that the downregulation of Ang II–AT1R signaling is involved in the antihepatic fibrosis effect of Swe.

The Ang II intracellular signaling pathway also involves the transcription factor nuclear factor-kappaB, AP-1, and mitogen-activated protein kinase (Ruiz-Ortega et al., 2001a; Bataller et al., 2003a). Ang II treatment induces a rapid phosphorylation of ERK1/2 in cultured HSCs and rat livers (Bataller et al., 2003a; Li et al., 2007). In addition, Ang II substantially increases the activity of AP-1 and expression of AP-1 target genes via ERK1/2 pathway, such as procollagen α1 and TGF-β1 (Vogt, 2001; Li et al., 2007). However, Ang 1–7 inhibits Ang II-induced ERK1/2 phosphorylation in many cell types, such as human endothelial cells, vascular smooth muscle cells, and rat aortic cells (Zhu et al., 2002; Su et al., 2006). Our study also
showed that Ang II stimulation produced a fast phosphorylation of ERK1/2 and c-jun. Swe treatment significantly inhibited Ang II-induced and DMN-induced ERK and c-jun phosphorylation in HSCs and rat livers, respectively. These results suggest that Swe may exert antihepatic fibrosis effects by suppressing Ang II–AT1R signaling and consequently suppressing ERK and c-jun phosphorylation.

Although we showed that Swe ameliorated DMN-induced hepatic fibrosis and inhibited the Ang II and AT1R in rats, the direct correlation between the antihepatic fibrosis effects of Swe and the RAS remains unclear. In addition to the RAS, many signaling pathways are involved in hepatic fibrosis induced by DMN or other chemicals; nevertheless, our Ang II-induced hepatic fibrosis model appears to be the most useful for verifying the direct action of Swe on the RAS.

In conclusion, our study demonstrated that Swe is an effective agent to treat experimental hepatic fibrosis. Swe inhibited Ang II-induced HSCs activation and proliferation. Swe also attenuated DMN-induced rat hepatic fibrosis and improved liver functions. The mechanisms involved in Swe’s action include downregulating Ang II and AT1R expression, and repressing ERK and c-jun activation. Our results indicate that Swe is a potentially effective agent for treating hepatic fibrosis through the regulation of RAS.
Acknowledgments

The authors declare no conflicts of interest.
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Authorship Contributions

Participated in research design: Tao and Liu

Conducted experiments: Li and Wang

Performed data analysis: Li

Wrote or contributed to the writing of the manuscript: Li, Tao, and Liu
References


Jaishree V, Badami S (2010) Antioxidant and hepatoprotective effect of swertiamarin from
Enicostemma axillare against D-galactosamine induced acute liver damage in rats. *J Ethnopharmacol* **130**: 103-106.


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Footnotes:

This work was supported by grants from the National Natural Science Foundation of China [Nos. 81173405, 81473404 and 81270053]; the National Science and Technology Major Project [2014ZX10005001]; and the International S&T Cooperation Program of China [2014DFA31440].

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Figure legends

Figure 1. Swe inhibited the proliferation of HSCs

(A) The amount of LDH released into the extracellular medium was expressed as the percentage of the total intra- and extracellular content compared with the control group. HSCs were treated with Angiotensin II (Ang II, 0.1 μM) with or without Swe (2.4, 6, 15 μM) and losartan (1 μM) for 24 h. The proliferation of HSCs was determined using a commercial EdU cell proliferation kit. (B) The typical images of EdU staining (× 200, Red: DAPI; Blue: EdU). (C) Quantitative analysis of the fluorescence intensity of EdU staining. Each bar represents the means ± S.D. (n=6). #p<0.05, ##p<0.01, compared with the control group; **p<0.01, compared with the Ang II group.

Figure 2. Swe inhibited the activation of HSCs

HSCs were treated with Ang II (0.1 μM) with or without Swe (2.4, 6, 15 μM) and losartan (1 μM) for 24 h. The expression of α-SMA was determined by (A) immunofluorescence staining, (B) western blot and (E) real time RT-PCR. (F) The expression of F-actin was examined by immunofluorescence staining. [C, D and G] Quantitative data of α-SMA and F-actin expression, respectively. Each bar represents the means ± S.D. (n=3). ##p<0.01, compared with the control group; *p<0.05, **p<0.01, compared with the Ang II group.

Figure 3. Effect of Swe on RAS-related molecules

HSCs were treated with Ang II (0.1 μM) with or without Swe (2.4, 6, 15 μM) and losartan (1 μM) for 24 h. (A) Effect of Swe on the expression of AT1R as determined by western blot. (B) Quantitative data of AT1R expression. (C) Effect of Swe on the mRNA levels of TGFβ-1. (D) Effect of Swe on the phosphorylation of ERK and
c-jun. (E) Quantitative data of ERK and c-jun phosphorylation. (F) Effect of Swe on the nuclear translocation of c-jun. Each bar represents the means ± S.D. (n=3). # 
P<0.01, compared with the control group; * P<0.05, ** P<0.01, compared with the Ang II group.

**Figure 4. Effect of Swe on rat hepatic fibrosis**

(A) Sirius red staining of collagen deposition in the rat liver (original magnification × 200). (B) Quantitative data of collagen deposition in the rat liver. (C) Western blot analysis of α-SMA expression in the rat liver. (D) Relative expression of α-SMA normalized to GAPDH. (E) The hydroxyproline content in the rats liver. Each bar represents the means ± S.D. # 
P<0.01, compared with the control group; * P<0.05, ** P<0.01, compared with the DMN group.

**Figure 5. Effect of Swe on body weight and liver function**

(A) Body weight; (B) Liver/body weight ratio; (C) HE staining of the rat liver (original magnification × 200); (D–G). Plasma albumin (Alb), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBiL) levels, respectively. Each bar represents the means ± S.D. (n=15). # 
P<0.01, compared with the control group; ** P<0.01, compared with the DMN group.

**Figure 6. Effect of Swe on RAS-related molecules in vivo**

(A). The AT1R, pERK1/2, ERK1, p-c-jun and c-jun expression in rat livers; (B, D). Relative levels of AT1R, pERK and ERK1 normalized to GAPDH, respectively; (C). Relative levels of p-c-jun normalized to c-jun; (E) Plasma Ang II levels determined by radioimmunoassay. Each bar represents the means ± S.D. # 
P<0.01, compared with the control group; ** P<0.01, compared with the DMN group.
Table 1. The primers used in this study for real time PCR

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Figure 4
Figure 6