Silibinin A decreases statin-induced PCSK9 expression in human hepatoblastoma HepG2 cells

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Abstract. Hypercholesterolemia is one of the major risk factors for the occurrence and development of atherosclerosis. The most common drugs used to treat hypercholesterolemia are 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, known as statins. Statins induce a beneficial increase in the levels of the low density lipoprotein receptor (LDLR) and additionally upregulate proprotein convertase subtilisin/kexin type 9 (PCSK9), which leads to LDLR degradation. This process causes a negative feedback response that attenuates the lipid lowering effects of statins. Therefore, the development of PCSK9 inhibitors may increase the lipid-lowering functions of statins. In the present study, a drug-screening assay was developed using the human *PCSK9* promoter, based on data from a dual-luciferase reporter assay, and the efficacies of various compounds from Traditional Chinese Medicine were examined. Among the compounds examined, SIL was demonstrated to function by targeting PCSK9. It was identified that SIL treatment decreased the expression levels of PCSK9 in HepG2 cells by decreasing the activity of the *PCSK9* promoter in a dose-and time-dependent manner. Notably, SIL antagonized the statin-induced phosphorylation of the p38 MAPK signaling pathway. The present study suggested that SIL may be developed as a novel PCSK9 inhibitor that may increase the efficiency of statin treatment.

Introduction

Hypercholesterolemia is one of the major risk factors responsible for the occurrence and development of atherosclerosis. This condition has become the primary therapeutic focus of atherosclerosis treatment (1). The most common cholesterol-lowering drugs are statins. These compounds decrease intracellular cholesterol levels by selectively inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (2). This in turn inhibits cholesterol biosynthesis and decreases hepatic cholesterol concentration (2). Statins increase the expression levels of the low density lipoprotein receptor (LDLR) in liver cell membranes by the sterol regulatory element binding protein (SREBP) pathway, leading to an increase in the clearance of LDL particles from the blood circulation (2). However, the use of statins causes several side effects. High doses of statins may increase the incidence and severity of multiple adverse events, including hepatotoxicity and myopathy, which are accompanied by muscle cramps, stiffness and weakness (3). In addition, statins increase the levels of the proprotein convertase subtilisin/kexin type 9 (PCSK9), which leads to LDLR degradation, thereby causing a negative feedback response that attenuates their lipid lowering effect (4). Therefore, the development of PCSK9 inhibitors may, in theory, enhance the lipid-lowering functions of statins.

PCSK9 is a newly identified serine protease that has emerged as a critical regulator in the pathogenesis of hypercholesterolemia and atherosclerosis (5). PCSK9 consists of 692 amino acids and is synthesized in the cytoplasm of hepatocytes. This protein enters the endoplasmic reticulum and the signal peptide of PCSK9 is cleaved. Pro-PCSK9 undergoes autocatalytic cleavage at residue Gln152 and binds to the catalytic domain of the protein to form a mature protein by the secretory pathway (6,7). The mature PCSK9 protein binds directly to the epidermal growth factor repeat A of the LDLR, and the PCSK9: LDLR complex is transferred to the lysosomes for degradation. Therefore, LDLR is no longer recycled back to the cell membrane surface, which leads to elevated LDL levels in the plasma resulting from decreasing binding of the LDL to its receptor (8-10). Previous studies have indicated that functional mutations of *PCSK9* are associated with human hypercholesterolemia (gain-of-function mutation) or hypocholesterolemia (loss-of-function mutation), which are associated with increased and decreased cardiovascular risk, respectively (11-14). In human studies, PCSK9 has been demonstrated to be an important target for the decrease of plasma cholesterol concentration and the decrease in cardiovascular risk (5).

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The United States of America Food and Drug Administration (FDA) has approved the marketing of 2 monoclonal antibodies against PCSK9, namely alirocumab and evolocumab, which are effective in decreasing levels of atherogenic lipoproteins and are well tolerated (15,16). However, these 2 inhibitors are expensive; their estimated cost ranges from $12,000-$15,000 for each patient per year (17). Therefore, the development of low-cost PCSK9 inhibitors will have significant commercial interest. In contrast to these observations, traditional Chinese medicine (TCM) has been used in clinical practice for >2,000 years in China and has exhibited marked beneficial effects on human health (18). Recently, several studies demonstrated a favorable effect of TCM for the treatment of dyslipidemia by the regulation of PCSK9, for example; berberine is a compound isolated from a Chinese herb that has exhibited cholesterol-lowering activity. Berberine inhibits *PCSK9* transcription by downregulating hepatic hepatocyte nuclear factor 1 (HNF1) protein expression via the ubiquitin-proteasome degradation pathway (19,20). Therefore, TCM may serve as a promising candidate to screen functional PCSK9 inhibitors.

In the present study, a novel drug-screening assay was developed based on the human *PCSK9* promoter. The assay used data from a dual-luciferase reporter assay and the compound silibinin A (SIL), a TCM compound, was identified to repress *PCSK9* promoter activity. SIL has been demonstrated to have a broad range of pharmacological activities, including anti-inflammatory, anti-oxidant, anti-cancer, neuroprotective and cardioprotective effects (21-26). Therefore, SIL has been used as a popular dietary supplement due to its optimal tolerability and low toxicity, and its diverse biological functions. The present study suggested that SIL may be developed as a novel PCSK9 inhibitor used in combination with statins, in order to retain their lipid lowering activity.

Materials and methods

Cell culture. The 293 and human hepatoblastoma HepG2 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution. All cells were incubated in a cell culture chamber at 37˚C under a humidified atmosphere with 5% CO_2.

Promoter construction. The promoter of human *PCSK9* (-1,833- +100 bp) was retrieved from the National Centre for Biotechnology Information. As demonstrated previously, PCSK9 transcription is controlled through *cis* regulatory elements located in the proximal promoter region of the PCSK9 gene where the transcription factor Sp1, HNF1 and sterol regulatory element-binding protein 1 (SRE-1) sites are located (-430- -345) (27). Notably, the SRE-1 motif is responsible for the statins-induced PCSK9 transcription (28). Therefore, the region containing all these functional sites was selected for the drug screening protocol. Genomic DNA was extracted from human liver HL7702 cells using a Wizard® Genomic DNA Purification kit (Promega Corporation). The primers were designed using Primer Premier 5 software (Premier Biosoft International), and *Mlu*I and *Xho*I restriction sites were added upstream and downstream of the promoter sequence. The specific sequences were as follows: Forward (F), 5'-TGCAAAAAAGAGTATGCCCGT-3'; reverse (R), 5'-TCC CCAAACAGCGTCAGATTA-3'. The *PCSK9* promoter fragment was amplified by polymerase chain reaction (PCR). PCR was conducted by activating the DNA polymerase at 94˚C for 5 min, followed by 30 cycles of three-step PCR (94˚C for 30 sec, 66.8˚C for 20 sec and 72˚C for 2 min), a final extension at 72˚C for 10 min and holding at 4˚C (PrimeSTAR® HS DNA Polymerase; Takara Bio, Inc.), and the band size was detected by 1% agarose gel electrophoresis. The recovered product and the pGL3-basic vector (MiaoLingPlasmid) were double-digested with *Mlu*I (Takara Bio, Inc.) and *Xho*I (Takara Bio, Inc.) at 37˚C for 4 h, and annealed together using T4 DNA ligase (Takara Bio, Inc.) at 16˚C overnight. The purified product was ligated (the size of the *PCSK9-luc* promoter plasmid was 6,751 bp), and finally the sequence of the pGL3-basic *PCSK9-luc* promoter plasmid was verified by GenScript Biotech Corp.

Drug screening and dual-luciferase reporter assay. TCM compounds (detailed drug information were presented in Table I) were used to construct a library in Jiangsu Key Laboratory for Molecular Medical Biotechnology. These compounds were purchased from Nanjing Cebai Biotechnology Co., Ltd. and Shanghai Aladdin Biochemical Technology Co., Ltd. The treatment doses for these compounds were selected based on previous studies (Table I) (29-66), which confirmed their functional effects for the treatment of various diseases. To analyze the activity of the *PCSK9* promoter, pGL3-*PCSK9-luc* (60 ng) and internal control plasmid pRL-TK (10 ng; MiaoLingPlasmid) was co-transfected into cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following 36 h of culture, 293 or HepG2 cells were incubated with serum-free DMEM containing 1% dimethyl sulfoxide (DMSO) as a negative control, or with the indicated drugs (Table I) for 12 h at 37˚C. The cell lysates were extracted and luciferase signal intensities (ratios of firefly luciferase signal normalized to *Renilla* luciferase) were measured using a Microplate Luminometer (Promega Corporation).

Cytotoxicity assay. Cell viability was examined by the Cell Counting Kit-8 assay (CCK-8; Nanjing Jiancheng Bioengineering Institute Co., Ltd.). To determine the non-toxic concentration for cells, SIL (1, 5, 10, 25, 50, 100 and 200 μM) was added to each well ($1x10^4$ cells/well). The plates were subsequently incubated for 12 h. CCK-8 solution (10 μl/well) was added to each well and the cells were cultured for an additional 2 h. Finally, a microplate reader was used to measure the absorbance at 450 nm.

In situ terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. TUNEL assays were performed to detect DNA strand breaks using a commercial kit provided by the Vazyme. In brief, HepG2 cells were treated as aforementioned and fixed with 4% paraformaldehyde for 15 min at room temperature. Following washing, the cells were permeabilized with 0.1% Triton X-100, and finally incubated

Table I. Potential drugs that decrease proprotein convertase subtilisin/kexin type 9 expression.

No.	Drugs	Concentration	(Refs.)
1	Isoorientin	30 μM	(29)
2	Dihydrokaempferol	690 μM	(30)
3	Hyperin	100 μM	(31)
4	Glycitein	100 μM	(32)
5	Genistein	60 μM	(33)
6	Calycosin	100 μM	(34)
7	Formononetin	200 μM	(35)
8	Irisflorentin	40 μM	(36)
9	Dichotomitin	3 μM	(37)
10	Iristectorigenin A	20 μM	(38)
11	6"-O-xylosyl-glycitein	350 μM	(32)
12	Corylin	30 μM	(39)
13	Kaempferide	50 μM	(40)
14	Isovitexin	20 μM	(41)
15	Naringenin	150 μM	(42)
16	Isofraxidin	70 μM	(43)
17	Oleanolic acid	80 μM	(44)
18	Berberine	50 μM	(45)
19	Resveratrol	20 μM	(46)
20	Rosiglitazone	300 μM	(47)
21	Oridonin	160 μM	(48)
22	Lithium chloride	100 mM	(49)
23	Chlorogenic acid	450 μM	(50)
24	Baicalin	220 μM	(51)
25	Nicotinic acid	10 mM	(52)
26	Cycloastragenol	20 μM	(53)
27	Prim-O-glucosylcimifugin	210 μM	(54)
28	Epimedin C	500 μM	(55)
29	Linarin	10 μM	(56)
30	Vitexin	20 μM	(57)
31	Myricetin	75 μM	(58)
32	Herbacetin	100 μM	(59)
33	Typhaneoside	10 μM	(60)
34	Afzelin	460 μM	(61)
35	Taxifolin	50 μM	(62)
36	Silibinin A	100 μM	(63)
37	Daidzein	10 μM	(64)
38	6"-O-Xylosyltectoridin	100 μM	(65)
39	Schaftoside	2 μM	(66)

with TUNEL labeling reagent for 60 min at 37˚C. The cells were double-stained with 10 μg/ml DAPI for 10 min at 37˚C (Sigma-Aldrich; Merck KGaA). The sections were captured in three different fields per sample with a DP70 digital camera connected to an ECLIPSE Ts2R-FL microscope (Nikon Corporation). Representative images from at least three separate experiments were presented.

Reverse transcription quantitative PCR (RT-qPCR) analysis. Total RNA was isolated using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed with the PrimeScript™ RT reagent kit (Takara Bio, Inc.). The resulting cDNA was amplified by qPCR using SYBR Green (Takara Bio, Inc.) and the LightCycler® 480 System (Roche Diagnostics) under the following thermocycling conditions: Denaturation at 95˚C for 5 min, followed by 95˚C for 10 sec and 60˚C for 30 sec for 40 cycles. The relative expression of each targeted gene was determined using the $2^{-\Delta\Delta Cq}$ comparative method (67). The primers for human *β-actin* were included for normalization. The primer sequences were as follows: *PCSK9* F, 5'-AGGGGAGGACATCATTGGTG-3'; *PCSK9* R, 5'-CAG GTTGGGGGTCAGTACC-3'; *β-actin* F, 5'-CACCCACAC TGTGCCCATCTACGA-3'; *β-actin* R, 5'-CAGCGGAAC CGCTCATTGCCAATGG-3'.

Western blot analysis. Total cellular proteins were isolated from HepG2 cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was determined using the BCA protein assay reagent (Beyotime Institute of Biotechnology). Equal amounts of protein (20 μg/lane) were loaded and separated via 10% SDS-PAGE, and then transferred onto PVDF membranes (EMD Millipore). Subsequently, the membranes were blocked with 5% fat-free dry milk at room temperature for 1 h. Membranes were incubated with rabbit anti-PCSK9 (1:1,000; cat. no. BS71876; Bioworld Technology, Inc.), mouse anti-β-actin (1:1,000; cat. no. AP0060; Bioworld Technology, Inc.), rabbit anti-p38 MAPK (1:1,000; cat. no. 9212S; Cell Signaling Technology, Inc.) or rabbit anti-phosphorylated (p)-p38 MAPK (p-Tyr182; (1:1,000; cat. no. 11253; Signalway Antibody LLC) antibodies in 5% fat-free dry milk overnight on a shaker at 4˚C. Membranes were subsequently washed three times in PBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibodies (1:2,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) or goat anti-rabbit IgG secondary antibodies (1:2,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The immunoreactive bands were visualized using electrochemiluminescence (Tanon-5200 Multi; Tanon Science and Technology Co., Ltd.) and quantified with the AlphaEase FC version 3.1.2 software (Alpha Innotech; Cell Biosciences).

Statistical analysis. Groups of data were presented as mean ± standard deviation. The data were analyzed using one-way analysis of variance followed by Fisher's Least Significant Difference post hoc test. The calculations were performed using Origin 8 software (v8.6, OriginLab Corporation). $P<0.05$ was considered to indicate a statistically significant difference.

Results

SIL decreases the activity of PCSK9-luc in 293 cells. The 293 cells transfected with *PCSK9-luc* were used to screen the active ingredients present in the TCM compounds (specific compounds were presented in Table I), which have been suggested to inhibit the transcriptional activity of the *PCSK9* promoter. The transfected cells were incubated with various drug concentrations for 12 h. Among all the tested drugs,

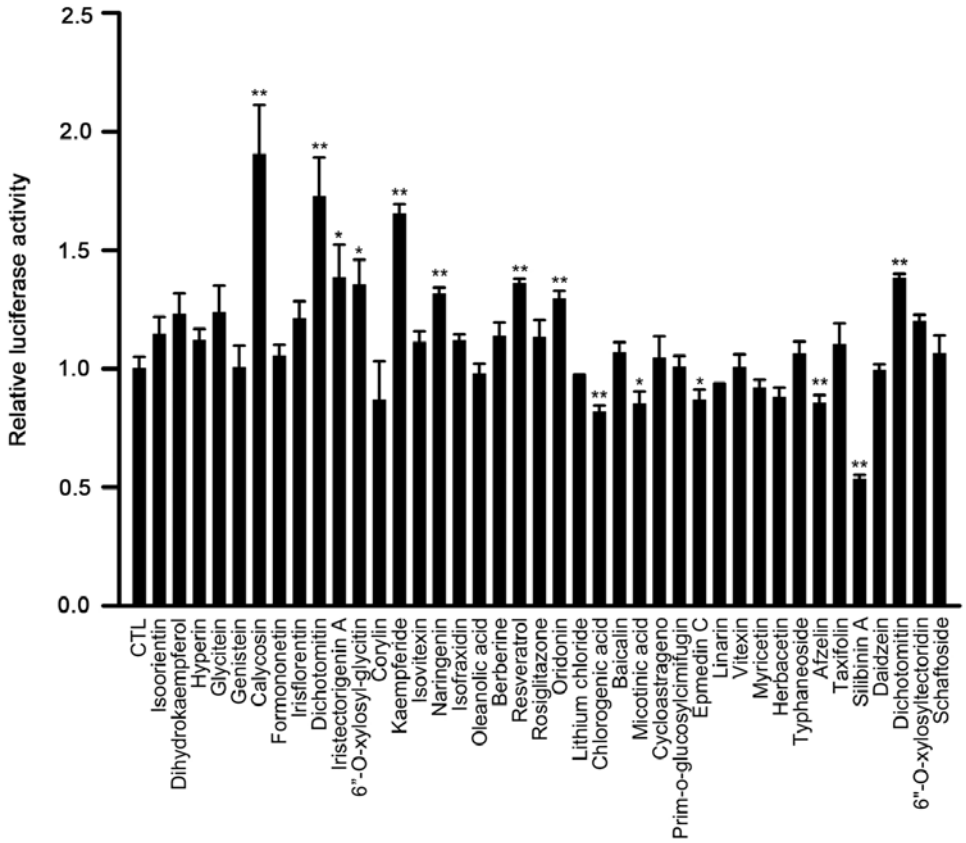

Figure 1. Effect of small molecular weight compounds on the activity of the *PCSK9* promoter. The 293 cells were transfected with *PCSK9-luc* in order to assess the targeting of small molecular weight compounds to *PCSK9*. The cells were treated with vehicle or small molecular weight compounds. The results are presented as mean ± standard deviation of 3 independent experiments. Significant differences were calculated by comparing each treatment of the cells to the cells with vehicle. *P<0.05 and **P<0.01 vs. CTL group. CTL, control; PCSK9, proprotein convertase subtilisin/kexin type 9.

chlorogenic acid, nicotinic acid, epmedin C, linarin, herbacetin, afzelin and SIL decreased the activity of *PCSK9-luc* (Fig. 1). In addition, SIL was the most effective drug, which decreased *PCSK9* promoter activity by 46.6% compared with that measured in the control samples.

Detection of HepG2 cell cytotoxicity and HepG2 apoptosis induction by SIL. The CCK-8 assay was used to assess the potential cytotoxicity of SIL on human HepG2 cells. The data indicated that treatment of SIL at the concentrations of 1, 5, 10 and 25 μM was not cytotoxic to HepG2 cells. SIL at the doses of 50 and 100 μM caused a slight decrease in cell viability, although the differences were not significant (Fig. 2B). To exclude the possibility that the SIL-induced decrease in PCSK9 would induce apoptosis in HepG2 cells, a TUNEL assay was performed and it was identified that 100 μM of SIL did not induce DNA fragmentation in these cells (Fig. 2C). This result indicated that the decrease of PCSK9 expression is not relevant to the apoptosis or cell growth. Therefore, 100 μM SIL was selected as the maximum safe dose for subsequent experiments.

SIL decreases PCSK9 promoter activity and the expression of PCSK9 in a dose-dependent manner. To confirm the results of the drug screening experiments, HepG2 cells were transfected with a firefly luciferase vector containing *PCSK9-luc* and subsequently treated with SIL for 12 h. When compared with the cells treated with DMSO alone, SIL decreases the activity of luciferase in a dose-dependent manner (Fig. 3A). The maximum decrease in the *PCSK9* promoter activity was estimated to be 45.6% for the cells treated with 100 μM SIL.

To examine whether SIL affected the expression levels of *PCSK9* mRNA, HepG2 cells were incubated with increasing concentrations of SIL, and the mRNA levels of *PCSK9* were detected by RT-qPCR. The results indicated that SIL decreased *PCSK9* mRNA levels in a dose-dependent manner (Fig. 3B). At the concentration of 100 μM, SIL inhibited *PCSK9* mRNA levels by 65.8% (P<0.01). The suppressive effects of SIL on the PCSK9 protein expression levels were verified by western blot analysis (Fig. 3C).

SIL decreases PCSK9 mRNA and protein expression levels in a time-dependent manner. Subsequently, the effects of 100 μM SIL on PCSK9 expression in HepG2 cells at different time periods were assessed. PCSK9 mRNA and protein expression levels were significantly decreased following treatment of HepG2 cells with SIL in a time-dependent manner (Fig. 4). Notably, the maximum level of inhibition (62.3% decrease compared with control; P<0.05) was observed at 12 h. A similar trend was observed with regard to the expression of the PCSK9 protein.

SIL antagonizes atorvastatin (ATV)-induced upregulation of PCSK9 expression. It was previously reported that ATV may increase PCSK9 expression in HepG2 cells (68). In the

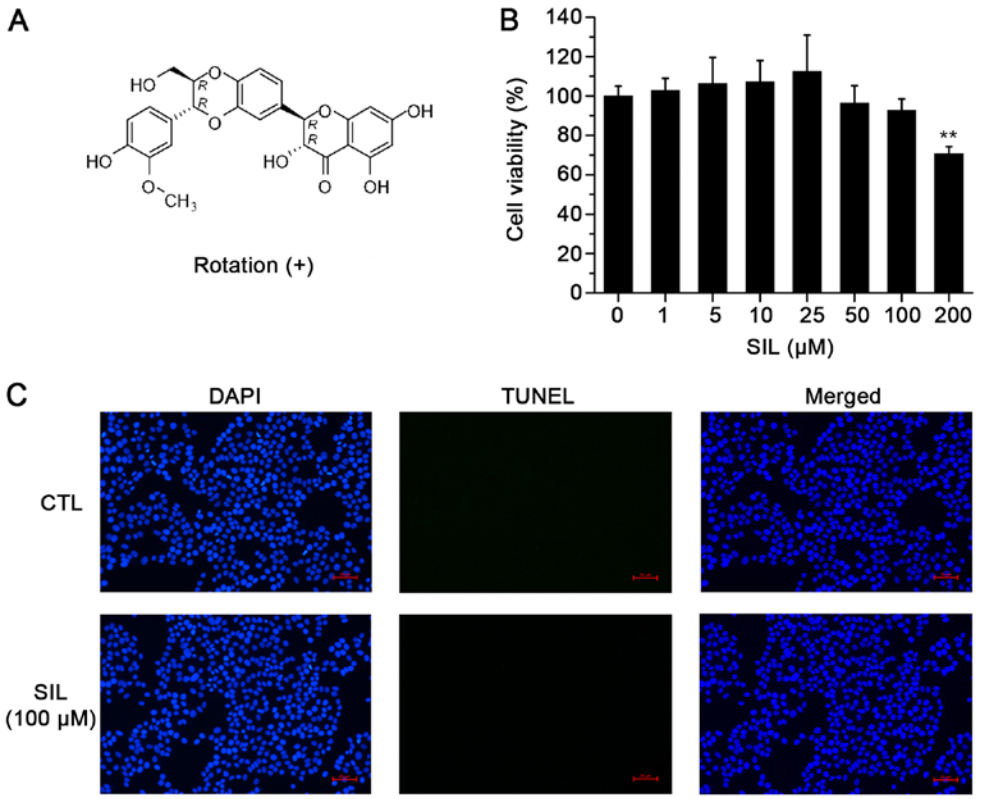

Figure 2. Cytotoxicity and the induction of apoptosis noted in HepG2 cells by SIL treatment. (A) The absolute configuration of the chemical structure of SIL. (B) HepG2 cells were treated with the indicated concentrations of SIL for 12 h. The viability of HepG2 cells was determined by the Cell Counting Kit-8 assay. The results are presented as mean ± standard deviation of 3 independent experiments. **P<0.01 vs. the control group. (C) TUNEL assay. HepG2 cells were treated as indicated in part B. Magnification, x200. Scale bar=25 μm. SIL, silibinin A; CTL, control.

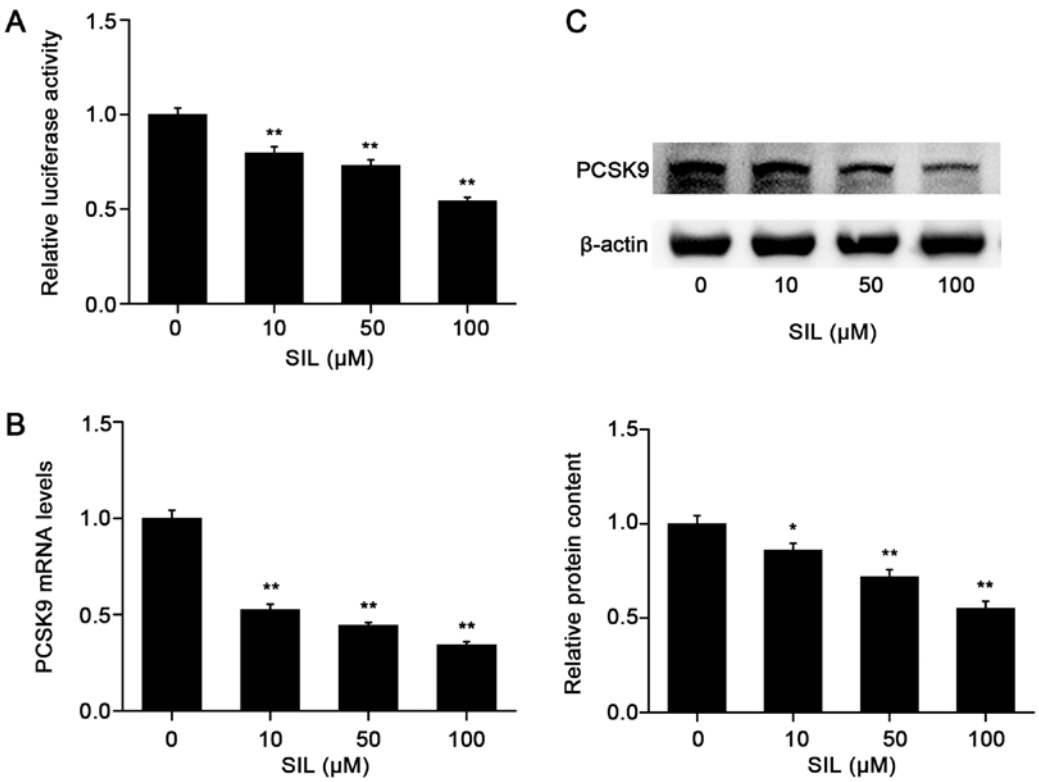

Figure 3. HepG2 cells treated with vehicle or SIL at different concentration intervals for 12 h. (A) HepG2 cells were transfected with *PCSK9-luc* and treated with vehicle or SIL at different concentrations. (B) The effect of increasing concentrations of SIL on the levels of *PCSK9* mRNA. (C) PCSK9 protein expression was detected by western blot analysis. The lower panel represents quantification of the immunoblots by densitometry. The results are presented as mean ± standard deviation of 3 independent experiments. *P<0.05 and **P<0.01 vs. control group. SIL, silibinin A; PCSK9, proprotein convertase subtilisin/kexin type 9.

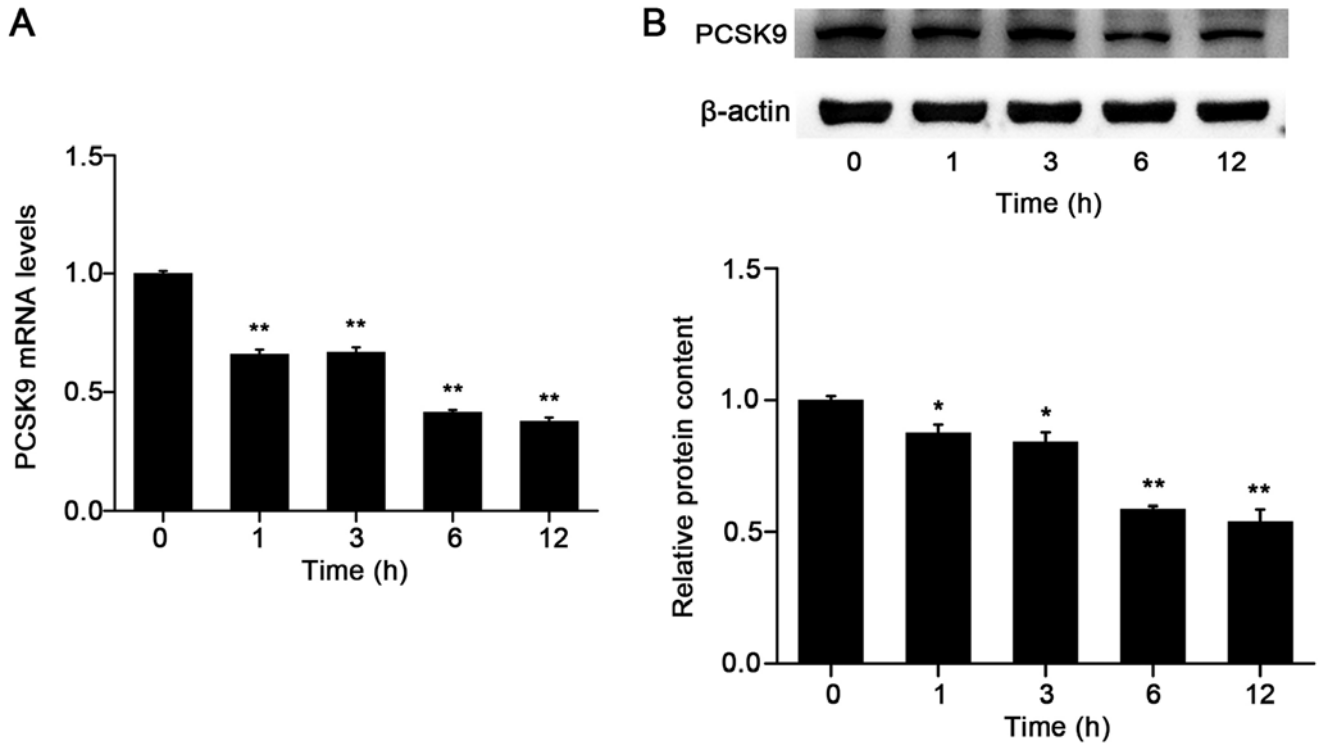

Figure 4. HepG2 cells treated with 100 μM SIL at different time intervals. (A) The effect of 100 μM SIL on the *PCSK9* mRNA levels over time. (B) PCSK9 protein expression was detected by western blot analysis. The lower panel represents quantification of the immunoblots by densitometry. The results are presented as mean ± standard deviation of 3 independent experiments. *P<0.05 and **P<0.01 vs. control group. SIL, silibinin A; PCSK9, proprotein convertase subtilisin/kexin type 9.

present study, *PCSK9* mRNA expression levels were 2.3-fold increased following treatment of HepG2 cells with 10 μM ATV, which was consistent with previous studies (68,69). Notably, co-incubation of HepG2 cells with ATV and SIL for 12 h resulted in an inhibition of the ATV-induced increase of *PCSK9* mRNA levels by 78.1% (Fig. 5A). Similarly, western blot analysis in HepG2 cell lysates revealed that ATV increased PCSK9 protein expression levels by 51.0%, and SIL attenuated this effect by 43.2% (Fig. 5B).

SIL decreases PCSK9 expression by suppressing the p38 mitogen-activated protein kinases (MAPK) pathway. In the liver, the p38 MAPK signaling pathway serves a central role in lipid and cholesterol metabolism, and is induced by statins to cause hepatic oxidative stress and apoptosis (70). To investigate the cellular and molecular mechanism underlying SIL-induced alleviation of ATV-based PCSK9 accumulation, HepG2 cells were treated with ATV (10 μM) and SIL (100 μM) for 0.5 h and 1 h, respectively. As presented in Fig. 6, SIL treatment decreased the phosphorylation of p38 MAPK protein following ATV treatment for 0.5 h by ~34% in HepG2 cells. This effect occurred prior to the induction of p38 phosphorylation triggered by 1 h of ATV treatment. SIL consistently inhibited p38 phosphorylation induced by 1 h of ATV treatment by ~46%. These data indicated that SIL may enhance the lipid-lowering functions of statins.

Discussion

Hypercholesterolemia is a major risk factor for the development of atherosclerosis. Decrease in plasma cholesterol levels is beneficial for the treatment of hypercholesterolemia-associated diseases. In a clinical setting, statins are the most frequently used drugs for lowering blood lipid levels (71). Statins increase LDLR expression by the inhibition of cholesterol synthesis, leading to the clearance of cholesterol in the blood. In addition, they increase *PCSK9* expression at the transcriptional level and cause LDLR degradation within the cell membrane, therefore attenuating their lipid-lowering effects (72). The present study aimed to examine the ability of a novel compound to inhibit PCSK9 expression. Therefore, 39 monomers isolated from TCM studies were screened, and it was identified that SIL inhibited PCSK9 expression in a time- and dose-dependent manner. In addition, SIL inhibited ATV-induced upregulation of PCSK9. These results suggested that SIL may be a promising compound for the attenuation of the negative feedback response of statins with regard to PCSK9 expression.

The monomers isolated from TCM have several advantages, including low cost, low toxicity and optimal isolation procedures. Therefore, the present study focused on the screening of those monomers that targeted PCSK9. Previous studies have indicated that berberine decreases PCSK9 expression and improves the LDL-C uptake from the hepatocytes (19,20,73). However, berberine may also cause detrimental effects, which occur primarily in the digestive system, including nausea, diarrhea, constipation and abdominal pain (74,75). Furthermore, the oral bioavailability of berberine is <1%, due to its poor solubility. In contrast to berberine, the biological half-life time and oral bioavailability levels of SIL are considerably increased, which renders it a good candidate for the

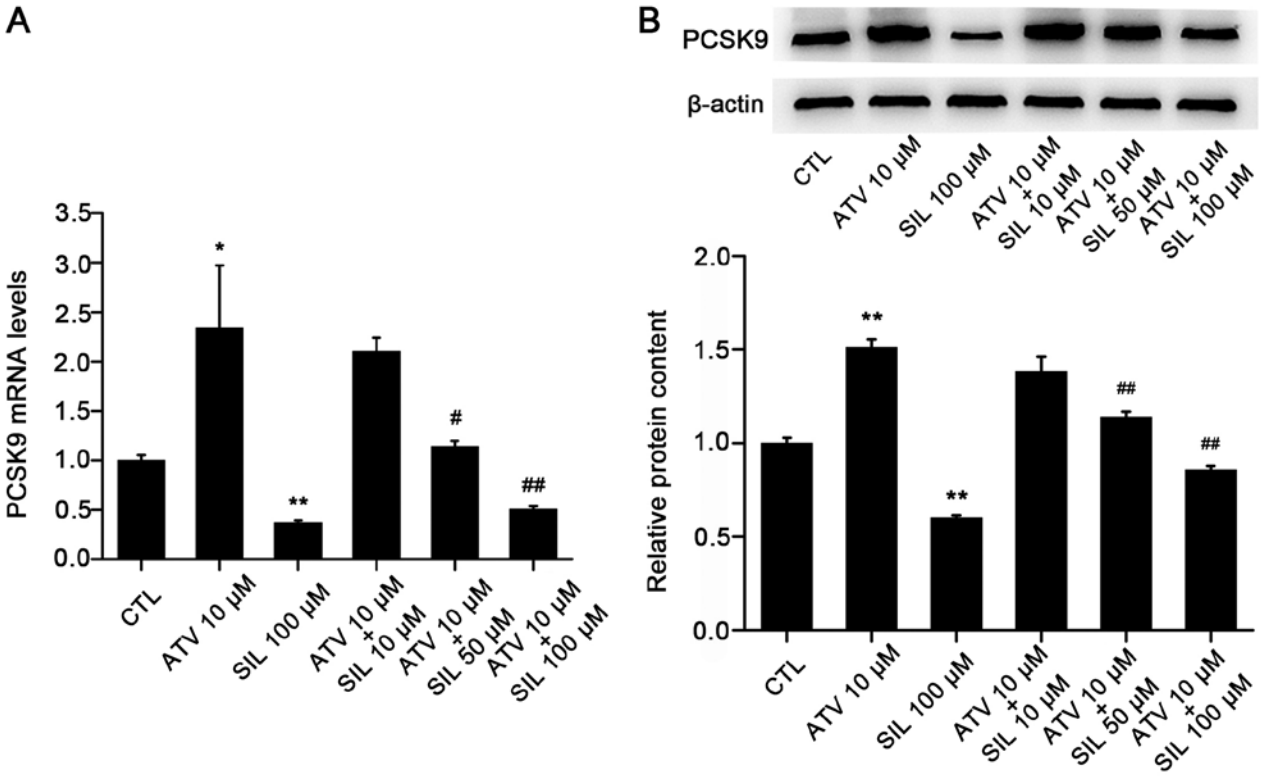

Figure 5. Effects of a combination treatment of 100 μM SIL and 10 μM ATV in HepG2 cells. (A) The effect of SIL and ATV on the levels of *PCSK9* mRNA. (B) The effects of SIL and ATV on PCSK9 protein expression. The lower panel represents quantification of the immunoblots by densitometry. The results are presented as mean ± standard deviation of 3 independent experiments. *P<0.05 and **P<0.01 vs. CTL group. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. ATV 10 group. SIL, silibinin A; ATV, atorvastatin; PCSK9, proprotein convertase subtilisin/kexin type 9; CTL, control.

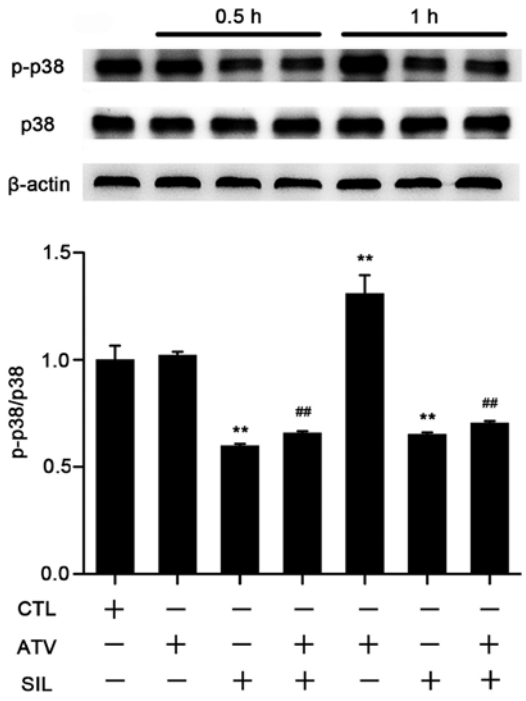

Figure 6. Effects of SIL on p38 MAPK phosphorylation in HepG2 cells. HepG2 cells were co-treated with 10 μM ATV and the indicated concentrations of SIL for 0.5 h and 1 h. p38 MAPK phosphorylation was detected by western blot analysis. The lower panel represents quantification of the immunoblots by densitometry. The results are presented as mean ± standard deviation of 3 independent experiments. **P<0.01 vs. CTL group. $^{\#\#}$P<0.01 vs. ATV group. SIL, silibinin A; p38 MAPK, p38 mitogen-activated protein kinases; ATV, atorvastatin; p-, phosphorylated; CTL, control.

development of novel PSCK9 inhibitors. Indeed, it has been demonstrated that when administrated orally with 120 mg SIL, the peak concentration in the plasma (C_{max}) in patients was 2.7 μM (76). In addition, the recommended oral dosage of SIL in phase I clinical trials (ClinicalTrials.gov Identifier: NCT00487721) for the treatment of prostate cancer is 13 g and C_{max} is 100 μM (77). Taken together, the plasma levels of SIL administrated in the clinical setting were comparable to the concentration used in the present study.

SIL is a polyphenolic that belongs to the flavonoid family of compounds (78). SIL has been used clinically to treat acute and chronic hepatitis, early cirrhosis and poisonous liver injury (22). SIL is a drug with multiple biological targets, including PCSK9, and therefore has potential use in the therapy of lipid metabolism disorders. Notably, the plasma half-life of ATV and SIL is 7 and 6.21 h, respectively. The corresponding time interval to reach C_{max} levels is 1.5 h for ATV, and between 1-2 h for SIL, respectively (79,80). These two important parameters have similar values, suggesting that ATV and SIL may be orally administered simultaneously in order to achieve an improved therapeutic effect of ATV.

The liver serves an important role in the physiological process of lipid synthesis, gluconeogenesis and cholesterol metabolism (81). It has been suggested that ATV induces oxidative stress and apoptotic injury in hepatocytes by increasing the phosphorylation of p38, JNK and ERK MAPK enzymes (70). In HepG2 cells, C-reactive protein increases PCSK9 expression by the activation of the p38 MAPK-hepatocyte nuclear factor 1 homeobox A signaling pathway (82). Similarly, leptin additionally

induced PCSK9 expression by the activation of p38 MAPK (83). Therefore, p38 MAPK is potentially involved in the regulation of PCSK9 expression. In the present study, it was demonstrated that ATV activated p38 MAPK, while SIL suppressed the ATV-induced phosphorylation of p38 MAPK. The activation of the p38 MAPK signaling pathway downregulated peroxisome proliferator-activated receptor α and its transcriptional target genes carnitine palmitoyltransferase 1A and Acyl-coenzyme A oxidase 1, leading to the inhibition of fatty acid β-oxidation (84). Whether other MAPK enzymes are also involved in the inhibition of PCSK9 by SIL remains unknown.

In conclusion, the present study demonstrated that SIL inhibited the ATV-induced PCSK9 upregulation in HepG2 cells, and that it may be used to decrease the adverse effects of statins following simultaneous administration of these 2 drugs to patients with hypercholesterolemia. Future studies are required to confirm the beneficial effects of SIL *in vivo* by the use of hyperlipidemic animal models.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZD designed and performed the study, analyzed the data and wrote the manuscript. WZ and SC performed the experiments and analyzed the data. CL designed the experiments, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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