23,24-Dihydrocucurbitacin B promotes lipid clearance by dual transcriptional regulation of LDLR and PCSK9

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23,24-Dihydrocucurbitacin B (designated as C95 in this article) is a cucurbitane triterpenoid that has been shown to possess a variety of pharmacological activities, such as anti-inflammatory and anti-HIV-1 activities etc. In this study, we investigated the effects of 23,24-dihydrocucurbitacin B on lipid regulation. We showed that 23,24-dihydrocucurbitacin B (1–5 μM) dose-dependently promoted Dil-LDL uptake in HepG2 cells by upregulating low-density lipoprotein receptor (LDLR) protein. In HepG2 cells, 23,24-dihydrocucurbitacin B (1–10 μM) dose-dependently enhanced LDLR promoter activity by elevating the mature form of SREBP2 (sterol regulatory element binding protein 2) protein levels on one hand, and inhibited PCSK9 (proprotein convertase subtilisin/kexin type 9) promoter activity by attenuating HNF1α (hepatocyte nuclear factor-1α) protein levels in nuclei on the other hand. Consequently, the expression of LDLR protein markedly increased, whereas the PCSK9-mediated LDLR protein degradation decreased. In a high-cholesterol LVG golden Syrian Hamster model, administration of 23,24-dihydrocucurbitacin B (30 mg·kg⁻¹·d⁻¹, intragastric, for 3 weeks) significantly decreased the serum LDL-cholesterol (LDL-C) levels. PCSK9 protein levels in the serum and liver tissues were significantly decreased, whereas LDLR protein levels in liver tissues were significantly increased in the treated animals as compared with the control animals. In conclusion, our study demonstrates for the first time that 23,24-dihydrocucurbitacin B exhibits dual transcriptional regulation of LDLR and PCSK9 in HepG2 cells by increasing SREBP2 protein levels and decreasing HNF1α protein levels in the nuclei. These results propose a new strategy to simultaneously manage LDLR and PCSK9 protein expression and provide a promising lead compound for drug development.

Keywords: 23,24-dihydrocucurbitacin B; lipid-lowering agent; LDLR; PCSK9; HNF1α; SREBP2

INTRODUCTION

LDL-cholesterol (LDL-C) has been considered a key predictor for cardiovascular disease, which is presently the leading cause of mortality and morbidity worldwide [1–3]. At present, statins, which are HMG-CoA reductase (HMGCR) inhibitors, are the mainstays for lipid-lowering treatment. HMGCR is the rate-limiting enzyme in the cholesterol synthesis pathway. Depletion of intracellular cholesterol activates the proteolytic cleavage of sterol regulatory element binding protein 2 (SREBP2) and releases the mature form of SREBP2 into the cell nucleus. By binding with sterol regulatory element 1 (SRE1) in the LDLR promoter region, SREBP2 upregulates LDLR transcription, resulting in LDL-C reductions in the blood [4].

In addition to low-density lipoprotein receptor (LDLR), statins also promote the expression of the proprotein convertase subtilisin/kexin type 9 (PCSK9) protein [5]. PCSK9 acts as a natural eliminator of LDLR by binding to the EGF-A extracellular domain of the LDLR protein, triggering its degradation in lysosomes and thus increasing the levels of circulating LDL-C [6]. Both the LDLR and PCSK9 genes harbor the positive regulatory motif SRE1 in their promoter regions [5, 7, 8]. Statins upregulate the expression of LDLR and PCSK9 through the SREBP2-SRE1 pathway in HepG2 cells [5]. Upregulation of PCSK9 is believed to counteract the effects of statins to further reduce LDL-C levels in patients [5]. Another positive regulator of PCSK9 transcription is the hepatocyte nuclear factor-1α (HNF1α) protein. HNF1α promotes PCSK9 transcription by binding with the HNF1 motif, which is located upstream of SRE1 in the PCSK9 promoter [9]. The plant-derived compound berberine (BBR) has been reported to transcriptionally suppress PCSK9 expression by attenuating nuclear SREBP2 and HNF1α protein abundance [9]. Given the posttranslational regulation of LDLR by PCSK9, PCSK9 elimination and interruption of LDLR-PCSK9 interactions have been demonstrated to be effective strategies to combat dyslipidemia [10]. The first humanized PCSK9 antibodies, alirocumab, and evolocumab, significantly reduce cardiovascular mortality [11, 12]. However, their daunting costs and troublesome subcutaneous administration are evident drawbacks. Therefore, small orally available compounds acting as PCSK9 regulators with excellent efficacy and low cost are desired.

23,24-Dihydrocucurbitacin B, designated as C95 in this article, is a cucurbitane triterpenoid extracted from the roots of Trichosanthes cucumeroides. C95 has been reported to possess multiple pharmacological activities, such as delayed type hypersensitivity-inhibiting [13], antitumor [14–16], anti-inflammatory [17], and...
anti-HIV-1 activities [18]. However, few reports are available about the lipid-regulating effects of C95.

Herein, we evaluated the effects of C95 on LDL uptake in HepG2 cells in vitro, explored the underlying mechanisms, and assessed the lipid-lowering efficacy of C95 in high-fat diet (HFD)-fed hamsters in vivo. We found that C95 dose-dependently promoted Di-LDL uptake through an LDLR-dependent pathway in HepG2 cells. Further investigations revealed that C95 dose-dependently enhanced LDLR protein levels by promoting LDLR mRNA expression and attenuating PCSK9-mediated LDLR protein degradation. Furthermore, C95 potently improved the lipid profile in a high-cholesterol golden Syrian hamster model. Our findings demonstrate, for the first time, that 23,24-dihydrocucurbitacin B exerts dual regulation of LDLR and PCSK9 through a unique mechanism.

**MATERIALS AND METHODS**

**Materials and chemicals**

Dulbecco’s modified Eagle’s medium (DMEM, catalog number: SH30243.01) was purchased from HyClone Laboratories (Logan, UT, USA). Fetal bovine serum (FBS, catalog number: 10091-148) and Lipofectamine 3000 (catalog number: L3000–015) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Atorvastatin (atorvastatin calcium salt trihydrate, catalog number: P20001, ≥98% HPLC) and the β-actin antibody (catalog number: A5441, 1:3000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for human LDLR (catalog number: ab52818, 1:1000) and PCSK9 (catalog number: ab181142, 1:2000) were purchased from Abcam (Cambridge, UK), and the Lamin B1 antibody (catalog number: 66095–1, 1:2000) was purchased from Proteintech (Chicago, IL, USA).

Antibodies for hamster proteins: the LDLR antibody was purchased from BioVision (Mountain View, CA, USA, catalog number: 3839, 1:2000). The PCSK9 antibody was a kindly gift from the laboratory of Prof. Jing-wen Liu (Department of Veterans Affairs, Palo Alto Health Care System, Palo Alto, CA, USA). The HNF1α antibody was obtained from Santa Cruz (catalog number: sc-6547, 1:2000). The SREBP2 antibody was obtained from Abcam (catalog number: ab30682, 1:2000).

Hamster serum triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), LDL-C, and the activities of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured with commercial kits (Shino-Test Co., Ltd., Shanghai, China) and the Hitachi 7020 autoanalyzer (Japan).

**Extraction and isolation**

C95 was extracted and isolated from the roots of *T. cucumeroides*, which were collected from Yunnan (China) and identified by professor He-ming Yang (Shanghai Institute of Materia Medica). The air-dried, powdered roots (10.0 kg) of *T. cucumeroides* were extracted three times with 95% aqueous ethanol at room temperature. The combined extracts were concentrated to yield a crude extract, which was partitioned between EtOAc and H2O. The EtOAc layer was added to a silica gel column and eluted with petroleum ether-acetone (1:0 to 0:1). Nine major fractions (A–I) were subjected to different treatments for the indicated time points. Then, the cells were washed three times with ice-cold PBS buffer, and total cellular protein was extracted using 100 μL/well lysis buffer containing a protease and phosphatase inhibitor cocktail (catalog number: 539134, Calbiochem, Frankfurt, HE, Germany) and centrifuged at 12000 × g at 4 °C for 10 min. Cell nuclear and cytoplasmic proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer’s instructions (catalog number: P0013B, Beyotime Biotechnology).

**High-performance liquid chromatography (HPLC) analysis**

MeOH-H2O (0.1% formic acid) (65:35), eluted at 5.45 min, 95.6% purity. C95 was dissolved in dimethyl sulfoxide and serially diluted to predetermined concentrations.

**Cell culture**

HepG2 cells (catalog number: HB-8065, ATCC, Manassas, VA, USA) were maintained in DMEM with 10% FBS (v/v) and incubated under a humidified atmosphere of 95% O2 and 5% CO2 at 37 °C. The cells were subcultured once every 2 days. Cells within 4–11 passages were used for experiments.

**Cell viability analysis**

HepG2 cells were seeded in 96-well plates (1.0 × 104 cells/well) for 24 h before treatment with the indicated concentrations of C95. After another 24 h, cell viability was detected with the Enhanced Cell Counting Kit-8 (Enhanced CCK-8, catalog number: C0043) according to the manufacturer’s instructions (Beyotime Biotechnology, Shanghai, China). The viability of cells treated with vehicle control dimethyl sulfoxide (DMSO) was expressed as 1.0.

**Lipoprotein isolation and DiI-LDL preparation**

Human plasma was obtained from Shanghai Xuhui Central Hospital, China, after informed consent was obtained and approval was granted by the Ethics Committee. The procedures conformed to the principles outlined in the Declaration of Helsinki [20]. LDL and lipoprotein-deficient serum (LPDS) were separated from the pooled plasma of healthy volunteers by ultracentrifugation and were then dialyzed in dialysis buffer and phosphate-buffered saline (PBS). LDL was then labeled with the fluorescent probe Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, catalog number: 60010, Biotium, CA, USA) as previously described [21], with some modifications. In brief, Dil was dissolved in DMSO (15 mg/mL) and added into the LDL/LPDS mixture (v/v, 1:2) to a final concentration of 300 mg Dil/mg LDL protein, and the mixture was incubated for 18 h at 37 °C. The mixture was subjected to ultracentrifugation to obtain Dil-labeled LDL (DiI-LDL) and then dialyzed in dialysis buffer and PBS. The obtained DiI-LDL was sterilized using 0.45 μm filters (Millipore, MA, USA).

**DiI-LDL uptake assay**

DiI-LDL uptake assays were conducted as described previously [21], with minor modifications. In brief, HepG2 cells were seeded in 24-well plates. After specific treatments, the culture medium was changed to DiI-LDL DMEM (20 μg/mL), and the cells were incubated for 3 h at 37 °C in the dark. Then, the cells were rinsed twice with ice-cold PBS buffer containing 0.4% albumin (Sigma-Aldrich) and washed twice with ice-cold PBS buffer. Then, 500 μL of isopropanol was added into each well, and the cells were incubated for 20 min in the dark at room temperature with constant shaking. Aliquots of 200 μL were used for fluorescence detection with the SpectraMax M2e Microplate Reader (520–578 nm, Molecular Devices, San Jose, CA, USA).

**Western blot analysis of proteins in hamster liver tissues and HepG2 cells**

HepG2 cells were cultured in six-well plates for 12 h. Then, the medium was changed to 2% LPDS for 24 h, after which the cells were subjected to different treatments for the indicated time points. Then, the cells were washed three times with ice-cold PBS buffer, and total cellular protein was extracted using 100 μL/well lysis buffer containing a protease and phosphatase inhibitor cocktail (catalog number: 539134, Calbiochem, Frankfurt, HE, Germany) and centrifuged at 12000 × g at 4 °C for 10 min. Cell nuclear and cytoplasmic proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer’s instructions (catalog number: P0013B, Beyotime Biotechnology).
Proteins in liver tissues were extracted using 200 μL of lysis buffer containing the protease and phosphatase inhibitor cocktail from 20–40 mg of frozen liver tissues.

The protein concentrations were determined with the BCA Protein Assay kit (catalog number: P0010, Beyotime Biotechnology). In total, 30 μg/lane of proteins were subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (catalog number: 162–0177, Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated with primary antibodies overnight at 4 °C. The membranes were then washed three times in TBST solution, incubated with secondary antibodies (catalog number: 1706515 or 1706516, Bio-Rad) for 2 h and visualized using Clarity Western ECL incubated with secondary antibodies (catalog number: 1706515 or 1706516, Bio-Rad) for 2 h and visualized using Clarity Western ECL.

RNA interference
Small interfering RNA (siRNA) was transfected into HepG2 cells at 50%–60% confluence using Lipofectamine 3000 reagent according to the manufacturer’s instructions (Invitrogen, Thermo Fisher Scientific). siRNAs against human LDLR (forward: 5′-CGGCUUAGAACAUCAACaGdTdT-3′, reverse: 5′-UGUUGAUGUUGCUAACGCCgTdT-3′), PCSK9 (forward: 5′-GGUCUGAAGUAAGCAAGGgCdTdT-3′, reverse: 5′-UGACUUUUGCAGCAgGACCdTdT-3′) and a scrambled siRNA were synthesized by GenePharma Inc. (Shanghai, China). After transfection, the cells were cultured in fresh medium for another 24 h before treatment.

RNA isolation and real-time QPCR
Total RNA from HepG2 cells or hamster liver tissues was extracted using TRIzol Reagent (catalog number: 15596–018, Thermo Fisher Scientific) and reverse transcribed into cDNA (Takara, Dalian, China). Real-time QPCR was performed using SYBR Green PCR Supermix (catalog number: 172–5125, Bio-Rad) with the specific primers listed in Table 1. The relative signal intensities were measured with the CFX Real-Time PCR Detection System (Bio-Rad). The values were normalized to those for the housekeeping gene β-actin. After normalizing, the cells were cultured in fresh medium for another 24 h before treatment.

Transient transfections of luciferase reporter constructs
Wild-type or mutant LDLR and PCSK9 promoter reporter constructs were co-transfected with the Renilla luciferase vector, which was constitutively expressed as a transfection control, into HepG2 cells using Lipofectamine 3000 reagent. Luciferase activity was evaluated according to the manufacturer’s instructions (Promega Inc., Madison, WI, USA). The data were normalized to the Renilla luciferase activity.

Animals and drug treatment
Male LGV golden Syrian hamsters (strain code: 501), with body weights of 80–90 g, were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd., China. All hamsters were housed in ventilated cages under a 12 h light–dark cycle with free access to water. The animals were fed a normal diet for the first week to allow them to become acclimated to the environment, and the normal diet was then switched to a HFD containing 0.5% cholesterol and 10% lard oil until the end of the experiment. After feeding of the HFD for another week, blood was collected via the retro-orbital sinus from hamsters and subjected to centrifugation at 4000 × g for 10 min at room temperature to obtain serum. Then, lipid profile analyses, including analyses of TC, HDL-C, LDL-C, and TG, were performed. The hamsters were then grouped into two groups with similar lipid profiles: a HF group (n = 6) and a C55 group (n = 6), which were treated with vehicle and C95 (30 mg·kg⁻¹·d⁻¹), respectively, in an intragastric (i.g.) manner for 21 days (D1–21). The serum lipid profiles and body weights were measured at four time points: the day before the switch to the HFD, one week after the switch to the HFD, on D10 after treatment and on D21 after treatment. Food intake was monitored every day during treatment (D1–21). At the end of the administration period (D21), the hamsters were anaesthetized with inhaled isoflurane (2%) and sacrificed to obtain liver samples and serum. The liver samples were weighed and stored at −80 °C in parts. The serum PCSK9 protein levels and serum ALT and AST activities were assessed.

During the experiments, all procedures were conducted under the guidelines of Directive 2010/63/EU of the European Parliament for the protection of animals used for scientific purposes [22] and were approved by the Institutional Ethics Committee of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Enzyme-linked immunosorbent assay (ELISA)
At the end of the administration period (D21), the hamsters were anaesthetized with inhaled isoflurane (2%). Blood was collected via the retro-orbital sinus from hamsters and subjected to centrifugation at 4000 × g for 10 min at room temperature to obtain serum. The PCSK9 protein levels in serum were measured using the Quantikine ELISA Kit for Mouse Proprotein Convertase 9/PCSK9 according to the manufacturer’s instructions (catalog number: MPC900, R&D Systems Inc., Minneapolis, MN, USA).

Statistical analysis
All statistical analyses were performed following the recommendations for experimental design and analyses in pharmacology [23]. The values are presented as the mean ± S.D. All data were obtained from at least three independent experiments.

Student’s t test was used to determine two sets of data which were significantly different from each other. One-way ANOVA was used to analyze differences among at least three groups of data, and Dunnett’s multiple comparisons test was used to compare all groups with the control group. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).
To our surprise, C95-induced additional increases in LDLR protein culture medium in a dose-dependent manner (Fig. 2a). We then assessed whether PCSK9 was involved in the C95-induced LDLR posttranslational regulation of the LDLR protein. We further decreased PCSK9 protein expression in HepG2 cells. In cells transfected with the SRE-mut construct (Fig. 3e), which indicated that the HNF1 motif was the major functional site through which C95 inhibited the effect of C95 on LDL uptake activity in HepG2 cells. Results are presented as means ± S. D., n ≥ 5. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle control by one-way ANOVA (Dunnett's multiple comparisons test).

**RESULTS**

**C95 elevates DiI-LDL uptake by increasing LDLR protein levels in HepG2 cells**

C95 exhibited little effect on the viability of HepG2 cells within the experimental concentrations (1–20 μM), and the viability of cells in all groups was above 95% (Fig. 1b). DiI-LDL uptake assays were then conducted to evaluate the effects of C95 on LDL uptake in vitro. C95 dose-dependently promoted DiI-LDL uptake in HepG2 cells (Fig. 1c). Accordingly, LDLR protein levels increased dose-dependently (Fig. 1d). As LDL uptake can be mediated in both LDLR-dependent and LDLR-independent ways [24], the involvement of LDLR in C95-induced LDL uptake was evaluated by siRNA-mediated knockdown. Knockdown of LDLR with the specific siRNA abolished the C95-induced elevation of DiI-LDL uptake (Fig. 1e, f).

Therefore, we concluded that C95 regulated DiI-LDL uptake by increasing LDLR protein levels in HepG2 cells.

C95 upregulates LDLR mRNA levels and attenuates PCSK9 mRNA levels in HepG2 cells

As mentioned above, PCSK9 has an important role in the posttranslational regulation of the LDLR protein. We further assessed whether PCSK9 was involved in the C95-induced LDLR elevation in HepG2 cells. To this end, after treatment with C95, whole-cell lysates and cell culture medium were subjected to Western blot assays. As expected, LDLR protein levels increased dose-dependently (Fig. 1d). As LDL uptake can be mediated in both LDLR-dependent and LDLR-independent ways [24], the involvement of LDLR in C95-induced LDL uptake was evaluated by siRNA-mediated knockdown. Knockdown of LDLR with the specific siRNA abolished the C95-induced elevation of DiI-LDL uptake (Fig. 1e, f).

Therefore, we concluded that C95 regulated DiI-LDL uptake by increasing LDLR protein levels in HepG2 cells.

C95 inhibits PCSK9 promoter activity through the HNF1 motif and upregulates LDLR promoter activity through the SRE1 motif

To determine the mechanism by which C95 downregulated PCSK9 mRNA levels and simultaneously upregulated LDLR mRNA levels in HepG2 cells, luciferase reporter assays were carried out to analyze the effects of C95 on PCSK9 and LDLR promoter activities. In line with its effects on protein and mRNA levels, C95 dose-dependently inhibited PCSK9 promoter activity and enhanced LDLR promoter activity (Fig. 3a, b).

To define the specific sites in the PCSK9 and LDLR promoters that were responsible for C95-induced regulation, we transfected HepG2 cells with PCSK9 and LDLR promoter luciferase reporter constructs (Fig. 3c, d) to evaluate promoter activity changes. Consistent with previous research [9], mutations in the HNF1 or SRE1 motif in the PCSK9 promoter induced dramatic reductions in promoter activity. Moreover, C95 induced additional increases in cells transfected with the SRE-mut construct (P < 0.05 vs. vehicle control in the same group) but not in cells transfected with the HNF-mut construct (P > 0.05, Fig. 3e), which indicated that the HNF1 motif was the major functional site through which C95 decreased PCSK9 protein expression in HepG2 cells. In cells transfected with LDLR promoter luciferase reporter constructs, increases in promoter activity induced by atorvastatin or C95 were abolished by mutation of the SRE1 motif in the LDLR promoter.
Fig. 2  C95 upregulates LDLR mRNA levels and attenuates PCSK9 mRNA levels in HepG2 cells. a  C95 downregulated PCSK9 protein levels in both HepG2 cell lysates (C-PCSK9) and culture medium (M-PCSK9). b  The effects of C95 on LDLR protein abundance and LDL uptake activity in HepG2 cells transfected with PCSK9-siRNA. HepG2 whole-cell lysates were subjected to Western blot assays to detect the LDLR and PCSK9 protein levels. *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated and un-transfected cells group, #P < 0.05, ##P < 0.001 vs. untreated cells transfected with PCSK9-siRNA by one-way ANOVA (Dunnett’s multiple comparisons test). e  The effects of C95 on LDLR mRNA (left column) and PCSK9 mRNA (right column) levels in HepG2 cells. Results are presented as means ± S.D., n ≥ 5. ***P < 0.001 vs. vehicle control by one-way ANOVA (Dunnett’s multiple comparisons test).

(Fig. 3f), which proved that C95 upregulated LDLR promoter activity through the SRE1 motif.

These results implied that C95 inhibits PCSK9 promoter activity mainly through the HNF1 motif and upregulates LDLR promoter activity mainly through the SRE1 motif.

C95 influences both HNF1α and SREBP2 protein abundance in HepG2 cell nuclei

Considering that the SRE1 and HNF1 motifs are the functional sites for SREBPs and HNF1α, which are transcription factors, to regulate their target genes, the protein levels of HNF1α, SREBP2, LDLR, and PCSK9 in HepG2 cell lysates were monitored after exposure to C95 at 5 μM for various durations (0–24 h). Intriguingly, we found that the levels of the mature form of the SREBP2 protein were elevated within the first 3 h of treatment and then returned to normal afterwards. However, the HNF1α protein levels declined until the end of treatment (24 h), and at 24 h, the protein level was approximately 0.51 times that at 0 h (P < 0.05). Accordingly, the PCSK9 protein levels decreased until the end of treatment (24 h), at which time the protein abundance was ~0.44 times that at 0 h (P < 0.05). In contrast, the LDLR protein levels continuously increased within this period (Fig. 4a). In addition, LDLR and PCSK9 mRNA levels were quantified in HepG2 cells with the same treatments during the same time periods (1–24 h). Similarly, C95 induced a continuous increase in LDLR mRNA in the first 12 h of treatment, with a slight decrease at 24 h. Interestingly, the PCSK9 mRNA levels slightly but nonsignificantly increased within the first 3 h of treatment and then tended to decrease until the end of treatment (24 h), at which time it was only 0.59 times that at 0 h (P < 0.05) (Fig. 4b). Based on the time-related changes in the HNF1α and SREBP2 proteins during the treatment process, we next detected these two proteins in HepG2 cells after exposure to different concentrations of C95 for 24 h and 3 h, respectively, at which time the two protein levels were changed significantly (Fig. 4c). The results showed that C95 (1–10 μM) dose-dependently decreased HNF1α protein levels in both the cytoplasm and nuclei after treatment for 24 h (Fig. 4c) and dose-dependently (1–10 μM) elevated the levels of the mature form of the SREBP2 protein after treatment for 3 h (Fig. 4d). BBR, which has been demonstrated to decrease HNF1α protein levels after 24 h of treatment in HepG2 cells [9], was used as a positive control (Fig. 4c).

C95 improves lipid profiles in HFD-fed hamster serum

To evaluate the lipid-lowering effects of C95 in vivo, HFD-fed golden Syrian hamsters were treated with C95 (i.g., 30 mg · kg⁻¹ · d⁻¹, n = 6) or vehicle (n = 6) for 21 days (D1–21).

After 10 days of treatment, TC (Fig. 5a), TG (Fig. 5b), and LDL-C (Fig. 5c) levels in the treatment group were significantly decreased compared with those in the HF group (P < 0.01 or P < 0.001). These effects were even more significant at the end of the treatment period (D21, P < 0.001 vs. HF group). No significant differences were induced in serum HDL-C (Fig. 5d). The activities of serum ALT and AST were assessed to evaluate the possible damage to hamster liver tissues. We found that the activities of the two enzymes were similar between the two groups (P > 0.05, Fig. 5e, f), as were the body weights and food intake (P > 0.05, Supplementary Figure S1).

In addition, consistent with the results obtained from HepG2 cells, LDLR mRNA (Fig. 5g), LDLR protein (Fig. 5h), and SREBP2 protein (mature form, Fig. 5k) levels in hamster liver tissues were significantly elevated after treatment. PCSK9 mRNA (Fig. 5h), PCSK9 protein (Fig. 5j), and HNF1α protein (Fig. 5k) levels in
hamster liver tissues significantly declined after administration of C95 at 30 mg·kg\(^{-1}\)·d\(^{-1}\) for 3 weeks, as were PCSK9 protein levels in serum at D21 (Fig. 5i).

Furthermore, in addition to those of LDLR, the mRNA levels of HMGCR and HMGCS1, two other target genes of SREBP2, were detected by real-time QPCR to assess whether the SREBP2 pathway was activated by C95 in hamster liver tissues. Compared with those in the HF group, the mRNA levels of both HMGCR and HMGCS1 in hamster liver tissues were significantly elevated in the C95 treatment group (Fig. 5l), which confirmed that the SREBP2 pathway was indeed activated by C95 in hamster liver tissues. These results proved that C95 improves lipid profiles in HFD-fed hamster serum and validated that C95 is a promising lipid-lowering compound in vivo.

**DISCUSSION**

In this study, for the first time, we evaluated the effects of 23,24-dihydrocucurbitacin B on LDL uptake in vitro and the lipid-lowering effects in vivo and explored the mechanism of action of this compound in lipid regulation. We demonstrated that 23,24-dihydrocucurbitacin B promotes LDLR expression and diminishes PCSK9 expression at the transcriptional level by increasing SREBP2 protein while decreasing HNF1\(\alpha\) protein in HepG2 cell nuclei.

BBR has been reported to dually regulate LDLR and PCSK9 [9]; BBR upregulates LDLR expression by stabilizing LDLR mRNA and reduces the protein levels of both HNF1\(\alpha\) and SREBP2 in the nucleus to suppress transcription of PCSK9. In contrast, 23,24-dihydrocucurbitacin B upregulates LDLR expression by increasing LDLR mRNA levels through promotion of SREBP2 protein levels and downregulates PCSK9 expression by decreasing HNF1\(\alpha\) protein levels in the cell nucleus. We found that the levels of the mature form of the SREBP2 protein were elevated within the first 3h of treatment, whereas HNF1\(\alpha\) protein levels decreased throughout the 24h of treatment (Fig. 4a) in HepG2 cells. The elevations in the levels of the SREBP2 protein (mature form) induced by C95 at 3h were dose-dependent (Fig. 4d), as was the inhibition of HNF1\(\alpha\) protein at 24h (Fig. 4c). Moreover, the C95-mediated upregulation of SREBP2 protein and downregulation of HNF1\(\alpha\) protein were also confirmed in hamster liver tissues (Fig. 5k). However, in the current study, we did not focus on why and how these time-related changes occurred in HepG2 cells or whether the changes in SREBP2 and HNF1\(\alpha\) proteins in hamster liver tissues were time-related. We will try to answer these questions in our future studies.

However, we noticed that the 1.5- to 2.0-fold increases in SREBP2 protein and LDLR promoter activity (Figs. 4a, 3b) were inconsistent with the increase in LDLR mRNA after exposure to 5 \(\mu\)M C95, which reached a level approximately six-fold greater than that in the control group (Fig. 4b). Moreover, the change trends between LDLR mRNA and SREBP2 protein were inconsistent as well. The decline in LDLR mRNA (Fig. 4b) occurred several hours later than the decline in SREBP2 protein (Fig. 4a). Considering that LDLR mRNA is unstable because of the three AU-rich elements (AREs) in its 3\(^{\text{′}}\)-UTR and that the half-life of LDLR mRNA is approximately 30 min [29], we suspected that C95 might induce posttranscriptional regulation of LDLR mRNA, such as
regulation of the stability of LDLR mRNA, so that the half-life of LDLR mRNA was lengthened. To comprehensively evaluate the effects of C95 on LDLR expression, more experiments are warranted and should be conducted in future studies. Consistent with the decline in HNF1α protein, PCSK9 protein levels decreased during the treatment (Figs. 4a, c, 5j, k). This decline in PCSK9 protein may have contributed to an elevation in LDLR protein such that LDLR protein levels increased continuously during the 24 h of treatment (Fig. 4a), even though the LDLR mRNA levels were decreased at 24 h (Fig. 4b).

The mRNA levels of HMGCR and HMGCS1, the target genes of the SREBP2 protein, were also promoted by C95 in hamster liver tissues, which indicated that the SREBP2 pathway was activated by C95. Given that LDLR and PCSK9 share the common SRE1 motif in their promoter regions, the activation of the SREBP2 pathway might also have promoted the transcription of PCSK9 mRNA. However, the slight increase in PCSK9 mRNA within the first 3 h of treatment did not produce a statistically significant difference in the treatment group compared with the control group (Fig. 4b). We speculated that the possible increase in PCSK9 mRNA caused by SREBP2 activation may have been counteracted by the attenuation of HNF1α protein levels. It has been reported that the upregulation of PCSK9 promoter activity mediated by the SREBP2 protein relies at least partially on the HNF1α protein, since
SREBP2 induces no significant increases in PCSK9 promoter activity if the HNF1 motif is mutated [9].

In conclusion, our study evaluated the lipid regulation efficacy of 23,24-dihydrocucurbitacin B both in vitro and in vivo and demonstrated that 23,24-dihydrocucurbitacin B transcriptionally regulates both LDLR and PCSK9 expression. Our study also revealed that 23,24-dihydrocucurbitacin B exhibits a remarkable lipid-lowering effect in vivo. This study expands understanding of the bioactivities of 23,24-dihydrocucurbitacin B, proposes another strategy to regulate LDLR and PCSK9 protein expression and provides a promising lead compound for drug development.

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AUTHOR CONTRIBUTIONS
LJX designed this research and revised the manuscript; HHL performed the research and drafted the article; JL and XJZ extracted and isolated the compound used in this article; JML and CX contributed analytical tools and data analysis; WQW analyzed the structure and purity of the compound used in this article; and YLL contributed critical reagents.
ADDITIONAL INFORMATION
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