

# Euphornin L promotes lipid clearance by dual regulation of LDLR and PCSK9

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**Abstract.** Our previous study identified euphornin L as an active lipid-lowering compound in high-fat diet-fed Golden Syrian hamsters. The aim of the present study was to investigate the mechanisms underlying the lipid-lowering effects of euphornin L. Euphornin L in HepG2 cells was assessed via DiI-LDL uptake assays and found to increase LDL-uptake and LDLR protein levels. RNA interference assays demonstrated that its LDL-uptake effects were LDLR-dependent. Dual luciferase reporter and mRNA stability assays revealed that euphornin L had little effect on *LDLR* mRNA transcription but lengthened the half-life of *LDLR* mRNA by activating ERK protein in cells. Euphornin L decreased the secretion of PCSK9 protein and alleviated PCSK9-mediated LDLR protein degradation. *In vivo* experiments in hamsters, which were treated with euphornin L (30 mg/kg/day) for 3 weeks, confirmed these findings. LDLR protein levels in liver tissue were upregulated, while PCSK9 protein levels in serum were downregulated. Altogether, the present study demonstrated that euphornin L increased LDLR protein levels by dual regulation of *LDLR* mRNA and PCSK9 protein, and represented an active compound for lipid-lowering drug development.

## Introduction

Low density lipoprotein cholesterol (LDL-C) has been considered a key predictor for cardiovascular disease, which is at present the leading cause of mortality and morbidity worldwide (1-3). Circulating LDL is mainly cleared by the LDL receptor (LDLR) on the cell membrane of hepatocytes. The mature LDLR is a transmembrane glycoprotein of 839 amino acids, with five major structural domains: the ligand-binding domain (LBD); an EGF precursor homology domain, which includes the EGF-A domain, the EGF-B domain, a 6-bladed  $\beta$ -propeller and the EGF-C domain; an O-linked sugar domain; a transmembrane domain; and a cytoplasmic tail (4,5). The LDL particles are recognized and bound by the LBD of LDLR and then internalized by endocytosis. At the low pH found in endosomes, the LDLR/LDL complex dissociates and LDLR returns to the cell surface, while the released LDL proceeds to lysosomes and is degraded (6-8). Therefore, elevation of LDLR protein has been the main strategy to reduce LDL-C (9).

The expression of LDLR protein is tightly regulated at the transcriptional, post-transcriptional and post-translational levels. In hepatocytes, the transcription of *LDLR* is negatively regulated by cellular cholesterol through the sterol responsive element 1 (SRE1) motif located in the promoter region of the *LDLR* gene (10). When the cellular cholesterol level is low, SRE1 binding proteins, which are located in the endoplasmic reticulum (ER) membrane, are activated by two-step proteolytic cleavage and released into the nucleus to bind with the SRE1 motif, resulting in the activation of *LDLR* transcription (11). However, the newly synthesized *LDLR* mRNA is short-lived because of the three adenosine and uridine-rich elements, AU-rich elements (AREs) in the 3' untranslated region (UTR) of *LDLR* mRNA (12,13). The half-life of *LDLR* mRNA is ~30 min (12). The activation of ERK protein has been found to be involved in *LDLR* mRNA stabilization at the post-transcriptional level (4,14-16).

Proprotein convertase subtilisin/kexin type 9 (PCSK9) protein, a secretory protein mainly expressed and secreted by the liver tissues, functions at the post-translational level by promoting the degradation of LDLR protein (17-19). PCSK9 is a single peptide and includes a prodomain, a catalytic domain

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*Abbreviations:* LDL, Low density lipoprotein; LDL-C, Low density lipoprotein-cholesterol; LDLR, LDL receptor; SRE1, sterol responsive element 1; ARE, adenosine and uridine-rich element; PCSK9, Proprotein convertase subtilisin/kexin type 9

*Key words:* euphornin L, lipid-lowering activity, low density lipoprotein receptor, proprotein convertase subtilisin/kexin type 9, extracellular signal-regulated kinase protein, secretion

and a C-terminal domain (CTD) (20). Following autocleavage between Gln-152 and Ser-153, the prodomain remains noncovalently attached to the catalytic domain (5). PCSK9 serves as a natural eliminator of LDLR by binding to the EGF-A extracellular domain of LDLR protein, triggering its degradation in lysosomes and thus increasing the level of circulating LDL-C (21). The first humanized PCSK9 antibodies, alirocumab and evolocumab, were approved by the US FDA in 2015 and introduced a significant reduction in cardiovascular death (22,23). However, the daunting cost and the troublesome subcutaneous administration are evident drawbacks for the two antibodies. Therefore, small, orally available compounds that act as PCSK9 regulators and have excellent efficacy and low cost are desired.

Our previous study identified euphornin L, a jatrophone macrocyclic diterpenoid extracted from *Euphorbia helioscopia*, as an active compound for promoting LDL uptake and improving LDLR protein levels in HepG2 cells. An *in vivo* study showed that treatment with euphornin L in high-fat diet-fed Golden Syrian hamsters significantly reduced serum LDL-C by 38.9% (24). The current study investigated the mechanisms underlying the lipid-lowering effects of euphornin L, which is designated C21 in this article, in HepG2 cells.

## Materials and methods

**Materials and reagents.** Dulbecco's modified Eagle's medium (DMEM; cat. no. SH30243.01) was purchased from HyClone (Cytiva). Fetal bovine serum (FBS; cat. no. 10091-148) and Lipofectamine<sup>®</sup> 3000 (cat. no. L3000-015) were obtained from Thermo Fisher Scientific, Inc. Atorvastatin calcium salt trihydrate (Atorvastatin; cat. no. PZ0001; ≥98%-HPLC), Phorbol 12-myristate 13-acetate (PMA; cat. no. P1585; ≥99%-HPLC) and DMSO (cat. no. D2650; ≥99.7%-HPLC) were purchased from Sigma-Aldrich; Merck KGaA.

Rabbit anti-human LDLR monoclonal antibody (cat. no.; ab52818; 1:1,000) and rabbit anti-human PCSK9 monoclonal antibody (cat. no.; ab181142; 1:2,000) were purchased from Abcam. Rabbit anti-human/hamster p-ERK polyclonal antibody (cat. no. 9101; 1:2,000), rabbit anti-human/hamster ERK monoclonal antibody (cat. no. 4695; 1:2,000) and mouse anti-human/hamster β-actin monoclonal antibody (cat. no. 4967; 1:3,000) were purchased from Cell Signaling Technology, Inc. Rabbit anti-hamster LDLR polyclonal antibody (cat. no. 3839; 1:2,000) was purchased from BioVision, Inc. (cat. no. 3839; 1:2,000). Rabbit anti-hamster PCSK9 polyclonal antibody was a kind gift from laboratory of Professor Jingwen Liu (Department of Veterans' Affairs, Palo Alto Health Care System).

Hamster serum triglyceride (cat. no. CH0101151; Maccura Biotechnology Co., Ltd.), total cholesterol (cat. no. L873; Shino-Test Corporation), high density lipoprotein cholesterol (cat. no. AQ986; FujiFilm Wako Pure Chemical Corporation), LDL-C (cat. no. AG251; FujiFilm Wako Pure Chemical Corporation) and the activities of aspartate aminotransferase (AST; cat. no. A871; Shino-Test Corporation) and alanine aminotransferase (ALT; cat. no. B871; Shino-Test Corporation) were measured with commercial kits and the Hitachi 7020 autoanalyzer (Hitachi, Ltd.).

**Compound extraction and isolation.** *Euphorbia helioscopia* was harvested in March 2015 from Hebei (China) and identified by Professor Heming Yang (Shanghai Institute of Materia Medica, China). A voucher specimen (no. SIMM 238) has been deposited in the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

The air-dried, powdered *E. helioscopia* (10 kg) was extracted three times with 95% aqueous ethanol (EtOAc) at room temperature. A crude extract (934 g) was obtained after concentration *in vacuo*, which was dissolved in water (2l) and then extracted with EtOAc to get the EtOAc-soluble fraction (377 g). The latter was subjected to a silica gel column and eluted with petroleum ether-acetone (100:0-0:100) to obtain eight fractions A-H. Fractions D1-D9 were yield from fraction D after eluting with petroleum ether (PE)/EtOAc (20:1 to 1:1). Sephadex LH-20 gel column was then used to get two fractions (D9A-D9B) from fraction D9. Then fractions D9B1-D9B3 were acquired by eluting with PE/EtOAc (8:1-3:1) from fraction D9B. Fraction D9B2 was then put into a RP-C18 column methanol (MeOH)/H<sub>2</sub>O, 75:25 to 95:5) to obtain fractions D9B2A-D9B2D, among which C21 (812.5 mg) was harvested from fraction D9B2C by preparative HPLC (MeOH/H<sub>2</sub>O, 85:15). C21 was identified as Euphornin L by comparison of the <sup>13</sup>C NMR spectra with the data reported previously (25).

Euphornin L (C21): <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ: 15.8, 17.1, 20.3, 20.6, 21.2, 21.3, 21.4, 22.6, 22.8, 32.4, 36.6, 39.7, 39.8, 41.9, 43.6, 73.2, 74.0, 74.0, 83.0, 92.2, 121.3, 128.4 (x2), 129.4, 129.4 (x2), 130.5, 132.7, 133.0, 136.0, 166.2, 169.4, 169.6, 170.0, 170.5. HR-ESI-MS: *m/z* 565.2770 (M + Na)<sup>+</sup> (calculated for C<sub>31</sub>H<sub>42</sub>O<sub>8</sub>Na, 565.2772). HPLC analysis: MeOH-H<sub>2</sub>O (0.1% formic acid; 90:10), eluted at 3.52 min, 97.77% purity.

**Cell culture.** The liver cancer cell line HepG2 was obtained from the American Type Culture Collection (cat. no. HB-8065) and maintained in DMEM with 10% FBS (v/v) and incubated under a humidified atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. Cells were sub-cultured once every two days. Cells within 4 to 11 passages were used for experiments.

**Cell viability analysis.** HepG2 cells were seeded in 96-well plates (1.0x10<sup>4</sup> cells/well) for 24 h prior to treatment with indicated concentrations of C21. After another 24 h, cell viability was detected with Enhanced Cell Counting Kit-8 (cat. no. C0043; Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

**Lipoprotein isolation and DiI-LDL preparation.** Human plasma (200 ml) was obtained from Shanghai Xuhui Central Hospital, China (healthy donors, n=4, male, 20-40 years old), after informed consent and approval by the Ethics Committee of Shanghai Xuhui Central Hospital (approval no. 2018-038). The procedures conformed to the principles outlined in the Declaration of Helsinki (26). LDL and lipoprotein-deficient serum (LPDS) were separated from the pooled plasma by ultracentrifugation. Briefly, density of the pooled plasma was adjusted to 1.019 g/ml with NaBr before ultracentrifugation at 4°C, 250,000 x g for 4 h. After removing the first layer of chylomicron and VLDL, the density of the remaining part was adjusted to 1.063 g/ml with NaBr before ultracentrifugation at 4°C, 250,000 x g for 24 h. The resulting first layer was

LDL. After removing LDL, density of the remaining part was adjusted to 1.21 g/ml with NaBr before ultracentrifugation at 4°C, 250,000 x g for 48 h; the bottom layer was LPDS. LDL and LPDS were dialyzed in dialysis buffer and PBS for 24 h and 48 h, respectively. LDL was then labeled with the fluorescent probe 1, 1'-dioctadecyl-3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI; Biotium) as previously described, with some modifications (27). Briefly, DiI was dissolved in DMSO (15 mg/ml) and added into the LDL/LPDS mixture (1:2 v/v) to a final concentration of 300 mg DiI/mg LDL protein and incubated for 18 h at 37°C. Density of the mixture was adjusted to 1.063 g/ml with NaBr before ultracentrifugation at 10°C, 250,000 x g for 24 h to obtain the first layer of DiI-labeled LDL (DiI-LDL), followed by dialyzing in dialysis buffer and PBS for 24 and 48 h, respectively. The obtained DiI-LDL was sterilized using 0.45 µm filters (EMD Millipore).

**DiI-LDL uptake assay.** DiI-LDL uptake assays were conducted as described previously (27), with minor modifications. Briefly, HepG2 cells were seeded in 24-well plates. At 12 h later, the culture medium was changed to 2% LPDS DMEM (v/v). After incubation for 24 h, the drugs were added into the medium and incubated with cells for another 24 h. Then the culture medium was changed to DiI-LDL DMEM (20 µg/ml) and incubated for 3 h at 37°C in the dark. Subsequently, the cells were rinsed twice with ice-cold PBS buffer containing 0.4% albumin (Sigma-Aldrich; Merck KGaA) and washed twice with ice-cold PBS buffer. Then, 500 µl of isopropanol was added into each well followed by a 20-min incubation in the dark at room temperature with constant shaking. Aliquots (200 µl) were used for fluorescence detection with SpectraMax M2e Microplate Reader at 520-578 nm (Molecular Devices, LLC).

**Western blot analysis of proteins in hamster liver tissues and in HepG2 cells.** Western blotting with HepG2 cell lysates was described previously (24). HepG2 cells were cultured in 6-well plates. After 12 h, the culture medium was changed to 2% LPDS DMEM (v/v). Following incubation for 24 h, the drugs were added into the medium and incubated with cells for another 24 h. The cells were washed three times with ice-cold PBS buffer and total cellular proteins were extracted using 100 µl/well lysis buffer containing protease and phosphatase inhibitor cocktail (cat. no. 539134; Calbiochem; Merck KGaA) and centrifuged at 12,000 x g, 4°C for 10 min.

Proteins in liver tissues were extracted using 200 µl lysis buffer containing protease and phosphatase inhibitor cocktail from 20-40 mg frozen liver tissues.

Protein concentrations were determined with BCA Protein Assay kit (cat. no. P0010; Beyotime Institute of Biotechnology). A total of 30 µg protein was loaded per lane in 8% SDS-PAGE and then transferred onto PVDF membranes (cat. no. 162-0177; Bio-Rad Laboratories, Inc.), blocked with 5% skimmed milk (cat. no. 232100; Becton, Dickinson and Company) for 2 h at room temperature and incubated with primary antibodies overnight at 4°C. Membranes were then washed three times in TBST solution followed by incubation with secondary antibodies (cat. no. 1706515 or 1706516; Bio-Rad Laboratories, Inc.) for 2 h and visualized using Clarity Western ECL Blotting Substrates (cat. no. 170-5061; Bio-Rad Laboratories,

Table I. Real-time QPCR primers.

Gene name	Accession no.	Primer (5'-3')
Human		
<i>LDLR</i>	NM 000527.4	ctgaaatgccgtgttactg gccaatcccttgcacatct
<i>PCSK9</i>	NM 174936.3	ccaagcctcttcttctcacc gcatcgcttgcacatcact
<i>GAPDH</i>	NM 002046.3	aagaaggtggtgaagcagg aggtggaggagtggtgtcgc
Hamster		
<i>LDLR</i>	NM 010700	ttgggttgattccaaactcc gattggcactgaaaatggct
<i>PCSK9</i>	NM 153565	tgctccagaggtcatcacag gtccactctgtgacatgaag
<i>GAPDH</i>	DQ 403055	aactttggcattgtggaagg ggatgcaggatgatgttct

LDLR, low-density lipoprotein receptor; PCSK9: proprotein convertase subtilisin/kexin type 9; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Inc.). Values were normalized to the housekeeping protein β-actin. Quantity One (v4.6.2, Bio-Rad Laboratories, Inc.) was used for densitometry.

**RNA interference.** Small interfering RNA (siRNA; 1,500 ng/well) was transfected into HepG2 cells in 12-well plates using Lipofectamine 3000 reagent according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h post transfection, cell culture medium was discarded and replaced with fresh DMEM with 10% FBS (v/v). At 24 h later, cells were used for subsequent experiments. siRNAs against human LDLR (forward: 5'-CGGCUUAAGAACAACAACAdTdT-3', reverse: 5'-UGUUGAUGUUCUUAAGCCGdTdT-3') and scrambled siRNA were synthesized by Shanghai GenePharma Co., Ltd.

**RNA isolation and reverse transcription-quantitative (RT-q) PCR.** Total RNA was extracted from HepG2 cells after treatment with drugs for 6 h, or from hamster liver tissues, using TRIzol® Reagent (cat. no. 15596-018; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA (cat. no. RR036A; Takara Biotechnology Co., Ltd.). RT-qPCR was performed using SYBR Green PCR Supermix (cat. no. 172-5125; Bio-Rad Laboratories, Inc.) with specific primers as listed in Table I. The relative signal intensity was measured by CFX Real-time PCR Detection System (Bio-Rad Laboratories, Inc.). The following thermocycling conditions were used: Initial denaturation at 95°C for 2 min; followed by 40 cycles of 95°C for 15 sec, 58°C for 25 sec and 72°C for 25 sec. Values were normalized to the housekeeping gene GAPDH and then analyzed in Bio-Rad CFX manager (v3.0) using the mode for normalized expression ( $2^{-\Delta\Delta Cq}$ ) (28). Results were presented as relative fold changes to the control group.



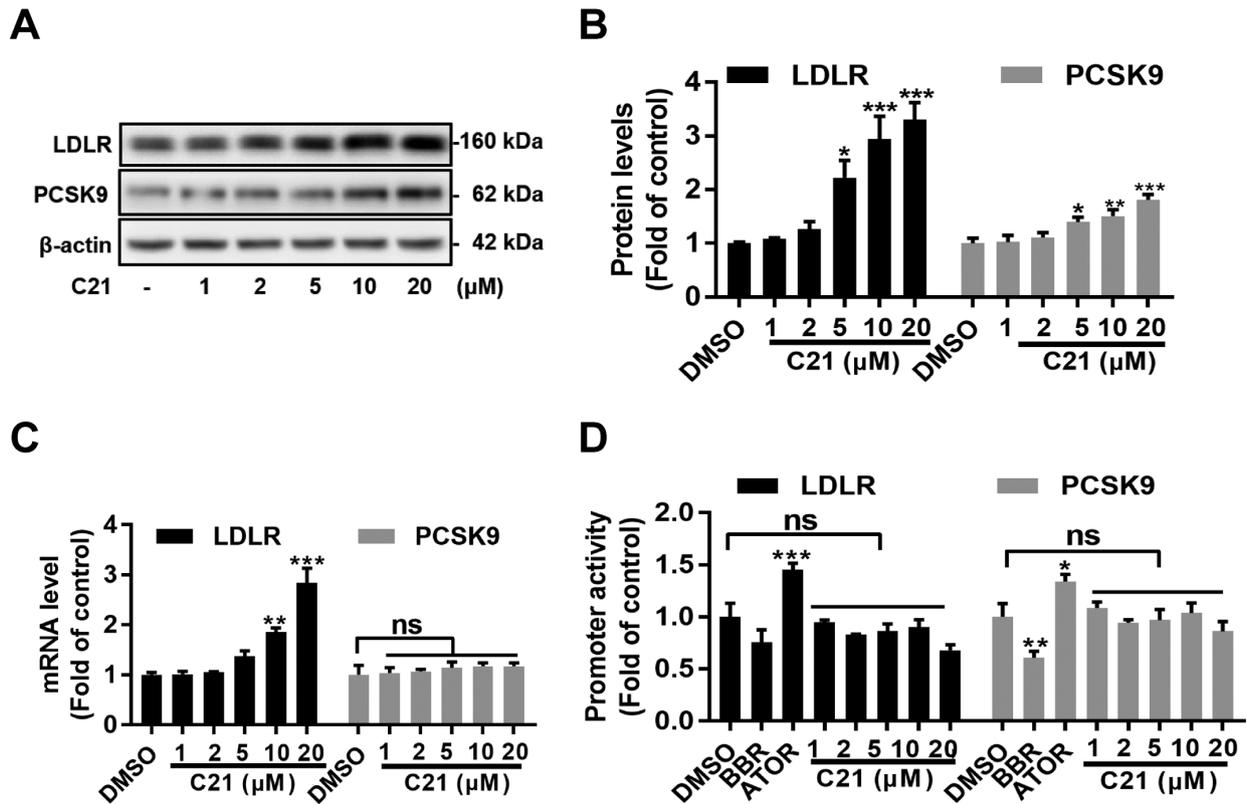


Figure 2. C21 has little effect on the promoter activities of *LDLR* and *PCSK9*. (A and B) C21 elevated LDLR and PCSK9 protein levels in HepG2 cells after treatment for 24 h. (C) C21 boosted *LDLR* mRNA levels dose-dependently after treatment for 6 h but had little effect on *PCSK9* mRNA levels. (D) C21 induced no significant effects on *LDLR* and *PCSK9* promoter activities. Results are presented as the means  $\pm$  standard error of the mean,  $n \geq 5$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. vehicle control by one-way ANOVA (with Dunnett's multiple comparisons test). C21, euphornin L; LDL, Low density lipoprotein; LDLR, LDL receptor; PCSK9, Proprotein convertase subtilisin/kexin type 9; BBR, berberine; ATOR, atorvastatin; ns, no significance.

**Statistical analysis.** For each experiment, the values were expressed as the mean  $\pm$  standard error of the mean. All data were obtained from at least three independent experiments. Statistical analysis was performed using GraphPad Prism (v7.0, GraphPad Software Inc.). Student's *t*-test was used to determine if two sets of data were significantly different from each other. One-way ANOVA was used to compare the statistical differences between at least three groups of data and Dunnett's multiple comparison test was used to compare all groups with the control group.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**C21 improves LDL uptake by increasing LDLR protein levels in HepG2 cells.** Since C21 (Fig. 1A) had little effect on the viability of HepG2 cells at experimental concentrations in this study (1-50  $\mu$ M; Fig. 1B), DiI-LDL uptake assays and western blot assays were conducted to verify the effect of C21 on LDL uptake and LDLR protein. In line with our previous study (24), C21 dose-dependently promoted DiI-LDL uptake and LDLR protein abundance in HepG2 cells (Fig. 1C-E). In addition, knocking down LDLR with a specific siRNA abolished the C21-induced increase in DiI-LDL uptake (Fig. 1D-F). These results suggested that the promotion of DiI-LDL uptake by C21 was LDLR-dependent and that C21 improved LDL uptake by increasing LDLR protein levels in HepG2 cells.

**C21 has little effect on the promoter activities of *LDLR* and *PCSK9* genes.** As mentioned above, PCSK9 serves an important role in the post-translational regulation of LDLR protein. The present study further assessed whether PCSK9 was involved in C21-induced LDLR elevation in HepG2 cells using western blot assay. As expected, LDLR protein levels increased dose-dependently. Notably, PCSK9 protein levels were also dose-dependently increased in HepG2 cells after treatment with C21 (Fig. 2A and B). To further investigate the effects of C21 on mRNA levels and promoter activities of both *LDLR* and *PCSK9*, RT-qPCR assays and dual luciferase reporter assays were conducted. It was found that C21 dose-dependently increased *LDLR* mRNA levels but had little effect on *PCSK9* mRNA levels (Fig. 2C). Notably, C21 induced no significant difference in the promoter activities of both *LDLR* and *PCSK9* genes (Fig. 2D). These results indicated that post-transcriptional regulation of LDLR and PCSK9 might be induced by C21 to elevate LDLR and PCSK9 protein levels in HepG2 cells.

**C21 increases the half-life of *LDLR* mRNA and reduces the *PCSK9* protein abundance in cell culture medium.** Given the instability of *LDLR* mRNA, the half-life of *LDLR* mRNA in HepG2 cells was evaluated after treatment with C21. C21 (20  $\mu$ M) increased the half-life of *LDLR* mRNA by almost 3-fold (Fig. 3A, DMSO group,  $T_{1/2} = 31.49$  min; C21-20  $\mu$ M group,  $T_{1/2} = 92.45$  min).

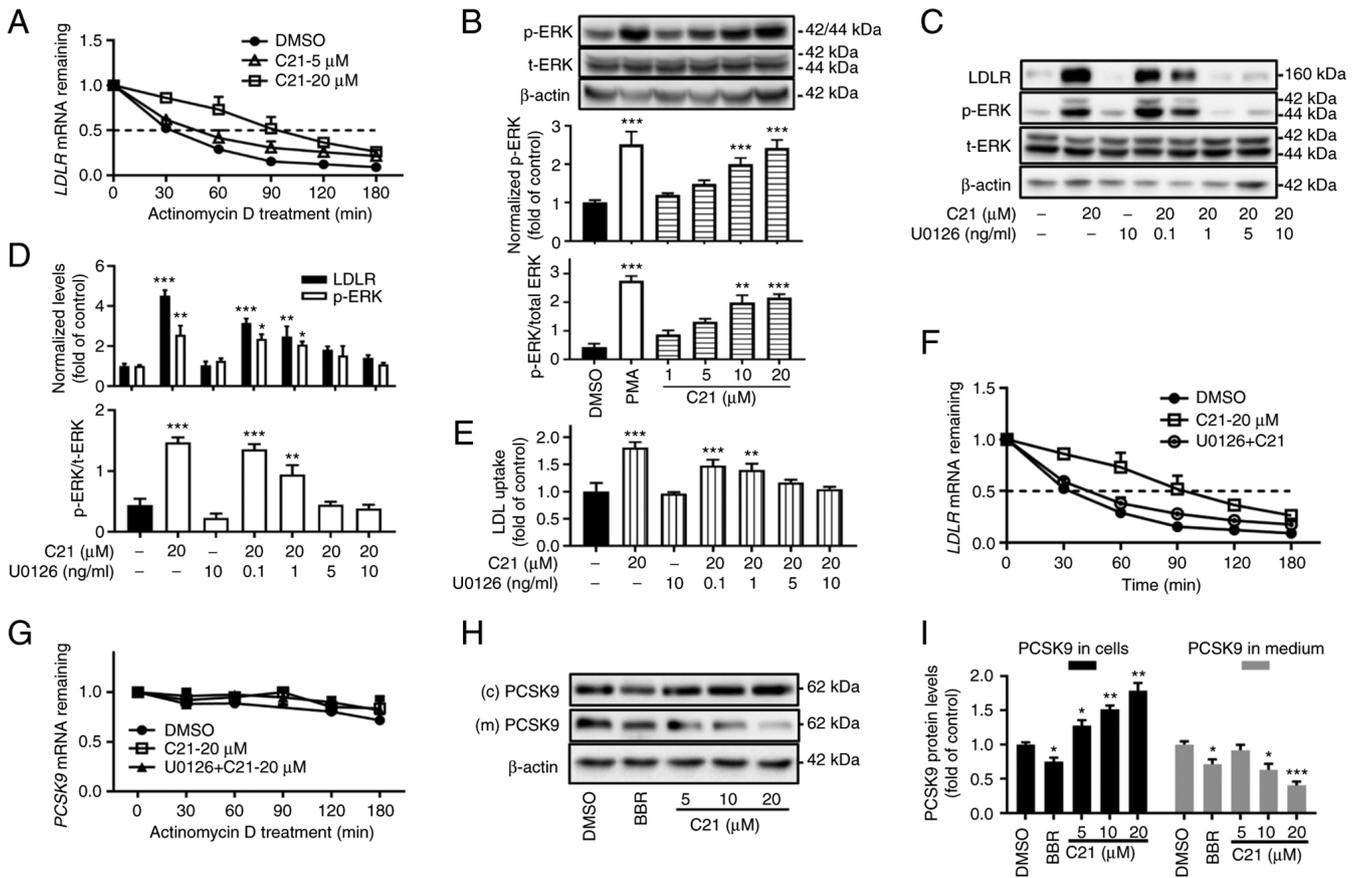


Figure 3. C21 increases the half-life of *LDLR* mRNA and reduced the secretion of PCSK9 protein. (A) C21 increased the half-life of *LDLR* mRNA. (B) After treatment for 2 h, C21 dose-dependently promoted the phosphorylation of ERK protein. PMA (5 ng/ml) was used as a positive control. (C-F) The ERK activation inhibitor U0126 counteracted the effects of C21 on *LDLR* protein, LDL uptake and half-life of *LDLR* mRNA in HepG2 cells. (G) C21 had little effect on the half-life of *PCSK9* mRNA. (H and I) C21 decreased the secretion of PCSK9 protein into cell culture medium. BBR (40 μM) was used as a positive control. Results are presented as the means ± standard error of the mean, n≥5. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. vehicle control by one-way ANOVA (Dunnett's multiple comparisons test). C21, euphornin L; LDL, Low density lipoprotein; LDLR, LDL receptor; PMA, phorbol 12-myristate 13-acetate; p-, phosphorylated; t-, total; PCSK9, Proprotein convertase subtilisin/kexin type 9; (c) PCSK9, PCSK9 protein in whole cell lysates; (m) PCSK9, PCSK9 protein in cell culture medium; BBR, berberine.

Since the activation of ERK protein has been found to be involved in *LDLR* mRNA stabilization (4,14-16), the effect of C21 on ERK was assessed. The results showed that treatment with C21 for 2 h dose-dependently elevated the phosphorylation of ERK (Fig. 3B). Furthermore, when cells were pretreated with U0126, a specific inhibitor of mitogen-activated protein kinase (MAPKK or MEK), the effects of C21 on *LDLR* protein (Fig. 3C and D), LDL uptake (Fig. 3E) and *LDLR* mRNA half-life (Fig. 3F) were abolished, which demonstrated that C21 boosted *LDLR* expression by stabilizing *LDLR* mRNA and the activation of ERK was essential in this post-transcriptional regulation.

C21 had little effect on the half-life of *PCSK9* mRNA (Fig. 3G). However, unexpectedly, following treatment with C21 (5-20 μM), PCSK9 protein levels in cell culture medium were decreased even though PCSK9 protein levels in whole cell lysates were increased. The plant-derived compound berberine, which has been reported to transcriptionally suppress PCSK9 expression in HepG2 cells (4), was used as a positive control. As C21 evoked little effect on the promoter activity and mRNA level of *PCSK9* in HepG2 cells, it was hypothesized that C21 might inhibit the secretion of PCSK9 protein.

*C21 upregulates LDLR and PCSK9 protein levels in hamster liver tissues and downregulates PCSK9 protein levels in hamster serum.* Consistent with the results obtained from HepG2 cells, *LDLR*, PCSK9 and p-ERK protein levels as well as *LDLR* mRNA levels in hamster liver tissues were significantly elevated (P<0.05 or P<0.001, Fig. 4A-C, E and F). In addition, compared with the HF group, the C21 group showed a significant reduction in PCSK9 protein abundance in serum (P<0.05, Fig. 4D) but little change in *PCSK9* mRNA levels in liver tissues (Fig. 4C).

In addition, treatment with C21 caused no obvious damage to hamster liver tissues since no significant differences in serum ALT and AST activities, nor in the food intake of hamsters, were observed between the two groups (Fig. 4G-I).

**Discussion**

The current study explored the mechanisms underlying the lipid-lowering effect of C21 in HepG2 cells. It demonstrated that C21 increased *LDLR* protein levels by stabilizing *LDLR* mRNA and inhibiting the secretion of PCSK9 protein.

Following treatment with C21 for 6 h, the *LDLR* mRNA in HepG2 cells was dose-dependently increased (Fig. 2C).

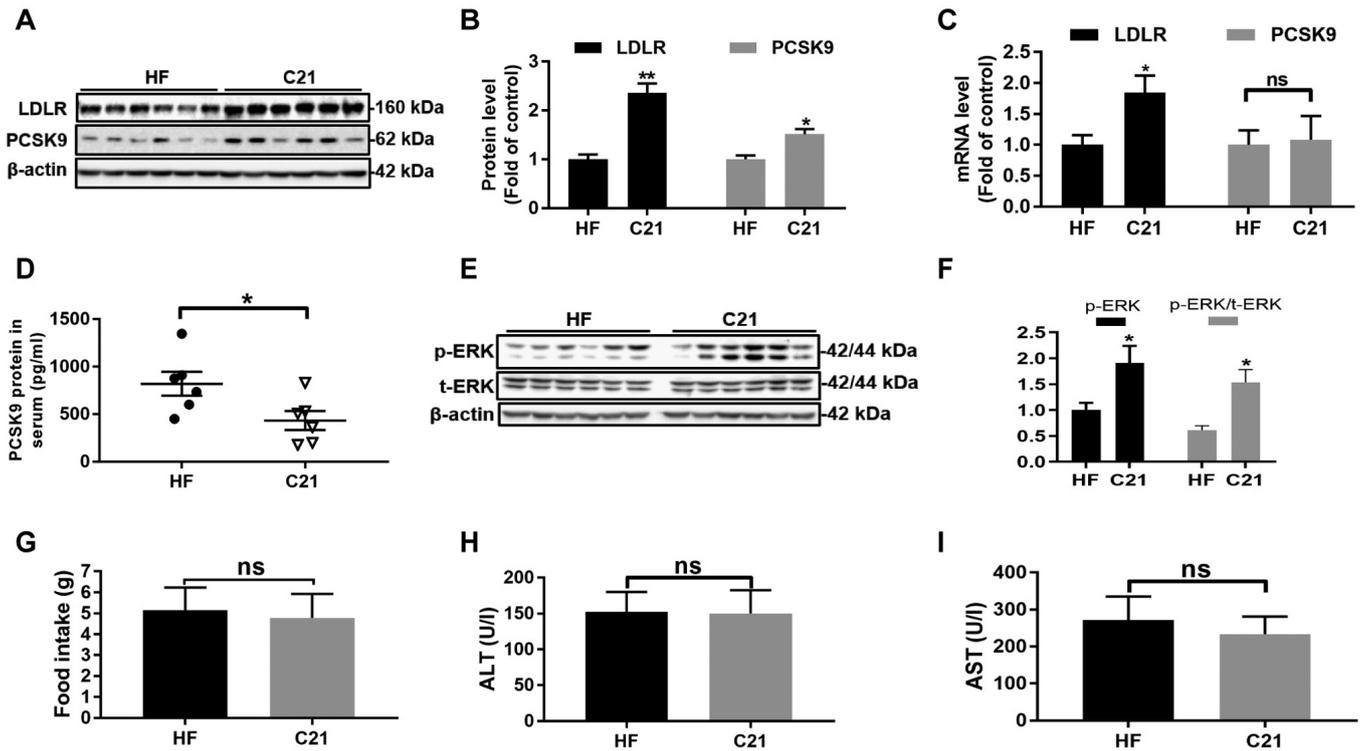


Figure 4. C21 upregulated LDLR and PCSK9 protein levels in hamster liver tissues and downregulated PCSK9 protein levels in hamster serum. (A-C) mRNA and protein levels of LDLR and PCSK9 in hamster liver tissues (n=6/group). (D) PCSK9 protein levels in hamster serum were significantly reduced after 3 weeks of treatment with C21 (30 mg/kg/d). (E and F) C21 promoted the phosphorylation of ERK protein in hamster liver tissues. (G) The food intake of hamsters in the two groups was not significantly different. (H and I) The activities of ALT and AST in hamster serum were not significantly different between the two groups. Results are presented as the means  $\pm$  standard error of the mean, n=6. \*P<0.05, \*\*P<0.01 vs. vehicle control by Student's t-test. C21, euphornin L; LDL, Low density lipoprotein; LDLR, LDL receptor; PCSK9, Proprotein convertase subtilisin/kexin type 9; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HF, high-fat diet; p-, phosphorylated; t-, total; ns, no significance.

However, in our previous study, HepG2 cells were treated for 24 h with C21 (1-10  $\mu$ M) and the *LDLR* mRNA levels were not significantly changed (24). It is hypothesized that 24 h may not be an appropriate time to assess the effects of C21 on *LDLR* mRNA since the *LDLR* mRNA half-life was 92.45 min following treatment with 20  $\mu$ M C21.

The half-life of ARE-containing mRNAs is mainly regulated by ARE-BPs (ARE binding proteins) (34). Some ARE-BPs, such as TTP, ZFP36, AUF1/hnRNP D and KSRP proteins, have been shown to promote the degradation of mRNA by binding with AREs (35,36) and other ARE-BPs, such as ELAVL1/HuR protein, prolong the half-life of mRNA (34,37). The activation of ERK protein has been found to be involved in *LDLR* mRNA stabilization (4,14-16). The mechanism remains to be elucidated, but the phosphorylation of ERK may influence the binding of specific ARE-BPs, such as ZFP36L1 and ZFP36L2 (36) as well as HuR protein (37), to the SRE1 motif in the *LDLR* promoter. The present study found that the activation of ERK protein was essential in the stabilization of *LDLR* mRNA induced by C21. It is planned to determine whether and which ARE-BPs are involved downstream of ERK activation in future studies.

Following treatment with C21, PCSK9 protein in HepG2 cells increased while PCSK9 protein levels in cell culture medium decreased without a significant influence on the promoter activity and mRNA level of *PCSK9*. Therefore, it was hypothesized that the secretion of PCSK9 might be inhibited by C21. The inhibition was confirmed when the serum PCSK9 level was decreased

while the hepatocellular PCSK9 protein level was increased in hamsters from the C21 group. During the cellular transportation of secretory proteins, coat protein complex II, consisting of SAR1 gene homolog B (SAR1B) GTPase, SEC23/SEC24 (heterodimers) and SEC13/SEC31 (heterotetramers), is responsible in the early secretion phase from the ER to the Golgi bodies (38). It has been reported that genetic deficiency of SEC24A in mice results in decreased secretion of PCSK9 protein (39). However, SEC24A cannot interact directly with PCSK9 since the former is located outside of the ER membrane while the latter is limited to the luminal side (40). A study published in 2018 proposed that SURF4 protein, an ER cargo receptor, might serve as a primary mediator of PCSK9 in the ER, as knocking down SURF4 results in an accumulation of PCSK9 in the ER (40). Sortilin 1 (SORT1), a trans-membrane trafficking receptor localized to the trans-Golgi network and early endosomes (41), has been demonstrated to be significantly associated with coronary artery disease, LDL-C levels and abdominal aortic aneurysm (41-43). In 2014, Gustafsen *et al* (44) reported that SORT1 promotes the secretion of PCSK9. Furthermore, a SORT1 inhibitor, AF38469, decreases plasma cholesterol levels in Western diet-fed mice, suggesting that SORT1 may serve as a potential target for hyperlipidemia treatment (45). The current study found that PCSK9 protein levels in cell culture medium or hamster serum decreased after treatment with C21, without any significant changes in *PCSK9* promoter activity or *PCSK9* mRNA levels. Thus, the secretion process of PCSK9 might be inhibited by C21. However, how C21

is involved in this process remains to be elucidated, which will be attempted in future studies.

In conclusion, the present study demonstrated that euphornin L increased LDLR protein levels in HepG2 cells by dual regulation of *LDLR* mRNA and PCSK9 and represented an active compound for lipid-lowering drug development.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

LX and HL designed the study. HL, JuL and XZ performed the experiments and drafted the manuscript. JiL, CX, WW and YL were involved in data analysis and interpretation. All authors confirm the authenticity of all data. All authors have read and approved the final manuscript.

### Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shanghai Institute of Materia Medica (approval no. 2018-08-WYP-30) and the Ethics Committee of Shanghai Xuhui Central Hospital (approval no. 2018-038). Informed consents were signed by the healthy volunteers.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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