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Identification of two herbal compounds with potential cholesterol-lowering activity

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ABSTRACT

Low-density lipoprotein receptor (LDLR) plays a pivotal role in the control of plasma LDL-cholesterol level. This occurs predominantly at the transcriptional level through two gene regulation elements, named SRE: sterol-responsive element and SIRE: sterol-independent responsive element. We have developed a high-throughput screening using LDLR promoter activation-based assay to search for cholesterol-lowering compounds from a Chinese herb-based natural compound library. With this approach, we identified two compounds, named Daphnetoxin and Gniditrin, from Chinese herb *Daphne giraldii Nitsche*, which could activate LDLR promoter. Characterization of these compounds showed that they increased the level of LDLR mRNA and consequently up-regulate LDLR expression. The structures of these compounds are different from well-known LDLR promoter activating compounds such as GW707. The results suggested that these herbal compounds could represent good candidates for development of new classes of cholesterol-lowering drugs.

1. Introduction

Increased LDL-cholesterol (LDL-c) is a well-established risk factor for atherosclerosis and the underlying cause of coronary heart diseases and strokes [1]. Low-density lipoprotein receptor (LDLR) is a key regulator of human plasma LDL-c homeostasis [2]. LDLR expressed on the surface of hepatocytes captures LDL-c and internalizes it into the cells, leading to a decrease in circulation LDL-c. Thus the expression level of LDLR directly influences the level of plasma LDL-c [3].

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Abbreviations: LDL, low-density lipoprotein; LDLR, LDL receptor; SRE, sterol-regulatory element; SIRE, sterol-independent regulatory element; HMG-CoA, 3-hydroxy-3-methyglutaryl coenzyme A; Dil-LDL, 3,3′-dioctadecylindocarbocyanine LDL; OM, oncostatin M

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Currently, statins are the best selling cholesterol-lowering drugs [1,4]. They are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis [3]. Statins effectively lower the plasma concentration of LDL-c and reduce mortality and morbidity from coronary artery disease [5,6]. Recent studies showed some additional benefits of statins beyond their cholesterol-lowering effects [7]. However, some patients are unable to tolerate statin treatments due to musculoskeletal symptoms and other side effects [8,9]. In addition, several patients do not achieve their LDL-c lowering goal with statin therapy alone [10]. Consequently, for a more efficient treatment of hypercholesterolemia and to fulfill unmet medical needs, it may be desirable to develop new therapeutic interventions that increase hepatic LDLR expression by mechanisms different from the current statin therapy.

The LDLR expression level is predominantly controlled at transcriptional level by two elements [11]. The first one is called sterol-regulatory element (SRE), which is located in the promoter region upstream of LDLR gene. The SRE controls the expression of LDLR gene at the transcriptional level through a negative feedback mechanism by the intracellular cholesterol pool [12,13]. LDLR transcription can also be regulated through sterol-independent mechanisms (SIRE) which are usually regulated by some growth factors and cytokines [14–18] and the SIRE is located downstream of the SRE [19]. As SRE/SIRE and their regulating protein, SRE binding protein (SREBPs), were identified as key factors to control the expression of LDLR, they have become important indicators in the identification of new classes of cholesterol-lowering compounds and drugs [9].

Natural products always provide significant opportunities for finding novel lead compounds [20,21]. In China, many herbs have been used for treatment in the clinical hyperlipidemia in the practice for many centuries. For example, red yeast rice (*Monascus purpureus*) can lower cholesterol levels through inhibiting HMG-CoA reductase activity [22,23], but the compound responsible for this function has not been identified. Berberine has also been demonstrated to up-regulate cell-surface LDLR level through stabilizing the LDLR mRNA and increasing its half-life *in vivo* [24]. The natural compounds from these herbs could provide a rich source for searching new candidates for cholesterol-lowering drugs. To explore this possibility, we used an in-house made herb-based natural compound library [20] which was screened through a high-throughput SRE/SIRE-transcriptional assay.

In this article, we described in detail our assay development phase, compound screening phase, and the identification of hit compounds, along with some results to prove the biological function of the compounds identified in this process. Our work provides an inside look how to use herb medicine and an alternative way to develop cholesterol-lowering drugs.

2. Materials and methods

2.1. Materials

Cytokine oncostatin M (OM) was purchased from R&D systems (Minneapolis, MN). Dil-LDL was obtained from Biomedical Technologies Inc. (Stoughton, MA). Bright-Glo™ Luciferase Assay solutions were obtained from Promega Corporation (Madison, WI). Lovastatin and 25-hydroxycholesterol were bought from Sigma–Aldrich (St. Louis, MO).

2.2. Plasmid construction

PCR subcloning method was used to construct the human LDLR promoter sequence (270 bp fragment extending from −86 bp through +184 bp of human LDLR promoter, NCBI accession number: L29401) as described previously [25]. Briefly, the primers were 5-GGGGTACCTTGCAGTGAGGTGAAGAC and 5-GACTGCAGGCTTGAGATCTTC. The PCR product was inserted into the pGL3-basic vector, which contains no defined eukaryotic promoter or enhancer sequences (Promega Corp., Madison, WI), with a luciferase reporter gene. The cloned plasmid construct, pLDLR270-luc was verified by restriction mapping and DNA sequencing. p4xSRE-tk-luc plasmid was constructed by inserting a 80 bp-oligo containing four repeats of the SRE fragment into upstream of a minimal thymidine kinase (TK) promoter in a pGL3-basic vector.

2.3. Cell culture, transfection and clone selection

The human embryonic kidney cell line HEK-293 and human hepatocellular liver carcinoma cell line HepG2 were cultured in a humidified 5% CO_2 atmosphere at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin sulfate. Cells were cotransfected with pLDLR270-luc and pcDNA3.1-hygro(+) using lipofectamine2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, Hygromycin was added to the medium (final concentration 200 μg/ml) and the cultures were maintained in Hygromycin-added medium until resistant cell colonies grew up (usually takes 2–3 weeks). Individual clones were picked and analyzed with the stimulation of OM (final concentration 50 ng/ml) followed by a luciferase assay using Bright-Glo™ Luciferase Assay kit (Promega, Madison, WI). The RLU (relative luciferin unit) for each cell clone was measured using Analyst HT microplate reader (Molecular Devices, Sunnyvale, CA). The cell clone (HEK293/pLDLR270-luc) with the best signal to noise ratio was selected and expanded for further assay development and high-throughput compound screening. The HEK293/p4xSRE-tk-luc cell was constructed by the cotransfection of p4xSRE-tk-luc and pcDNA3.1-hygro(+) into HEK293 cells. The Hygromycin selection was done as described above. The cell clones were picked and analyzed by stimulation of U18666A (final concentration 5 μM).

2.4. Plant extraction and natural compound library

One hundred and twenty herbal plants with therapeutic indications in treatment of hyperlipidemia or hypertension based on TCM were collected from Herb Market of Anguo, Northern China, and authenticated in the Department of Systematic Botany, Beijing University of Chinese Medicine. Herbal extraction and HPLC fractionated were performed as described [26]. The fraction samples (16 fractions for each

extraction) were lyophilized and re-dissolved in dimethyl sulfoxide (DMSO) and stored in 96-well sample plates at −80 °C for assays and screening.

2.5. Compound screening

HEK 293 cells transfected with pLDLR270-luc (HEK293/pLDLR270-luc) were seeded in 96-well plates at the density of 2×10^4 cells per well. On day 2, the HPLC fractioned samples were added to the cells at a concentration of approximately 3 μg/ml. After incubation with the samples over night, the expressed luciferase activity was measured in an Analyst HT microplate reader (Molecular Devices, Sunnyvale, CA) using the Bright-Glo™ Luciferase Assay System. The hits that produced more than two-fold induction of luciferase activity were initially identified. Secondary screening with the same assay was subsequently performed to confirm the hits.

2.6. Isolation and identification of hit compounds from herb

The herb from which the hit samples were originated was extracted and HPLC fractionated as described above but with larger scale. The fractions showing activity in primary and secondary screenings were further separated and single compound peaks were collected and subjected to activity assay for stimulation of HEK293/pLDLR270-luc cells. The active fractions with high purity were analyzed by high resolution mass spectrometer (IonSpec4.7 Tesla FTMS-MALDI/DHB) to obtain the molecular formulas and then the structures were analyzed using 2D-NMR (DMX600, Bruck).

2.7. Real-time PCR quantification of relevant RNA

HepG2 cells in 24-well plates were incubated in the presence of vehicle (1% DMSO) or hit compounds over night in DMEM supplemented with 10% fetal bovine serum. Total RNA was extracted and cDNA was produced using TRIzol (Invitrogen, Carlsbad, CA) and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed on ABI-Prism 7300 using SYBR Green I (Applied Biosystem). The primers were AGTTGGCTGCGTTAATGTGAC and TGATGGGTTCATCTGACCAGT for LDLR, GGACCCCTTTGCTTAGATGAAA and CCACCAAGACCTATTGCTCTG for HMG-CoA reductase, ATGGGGAAGGTGAAGGTCG and GGGGTCATTGATGGCAACAATA for GAPDH. Relative abundances of RNA were calculated from the cycle threshold (Ct) using the formula $2^{-\Delta Ct}$ and expressed as arbitrary units.

2.8. LDL-uptake assay

HepG2 cells were pretreated with Daphnetoxin (10 μM) and Gniditrin (1 μM), Lovastatin (1 μM) or 25-hydroxycholesterol (1 μg/ml) over night and then incubated 4 h with 6 μg/ml of fluorescent Dil-LDL. Intracellular fluorescent dye was detected by a fluorescence microscopy using Ix71 Olympus rhodamine filters and photos were taken using a C5060 camera (Olympus, Tokyo, Japan).

3. Results

3.1. Luciferase assays and screening for compounds with induction of LDLR expression

The plasmid pGL3-basic (Promega, Madison, WI) containing an engineered version of firefly luciferase gene but lacking any eukaryotic promoter or enhancer sequences provided the

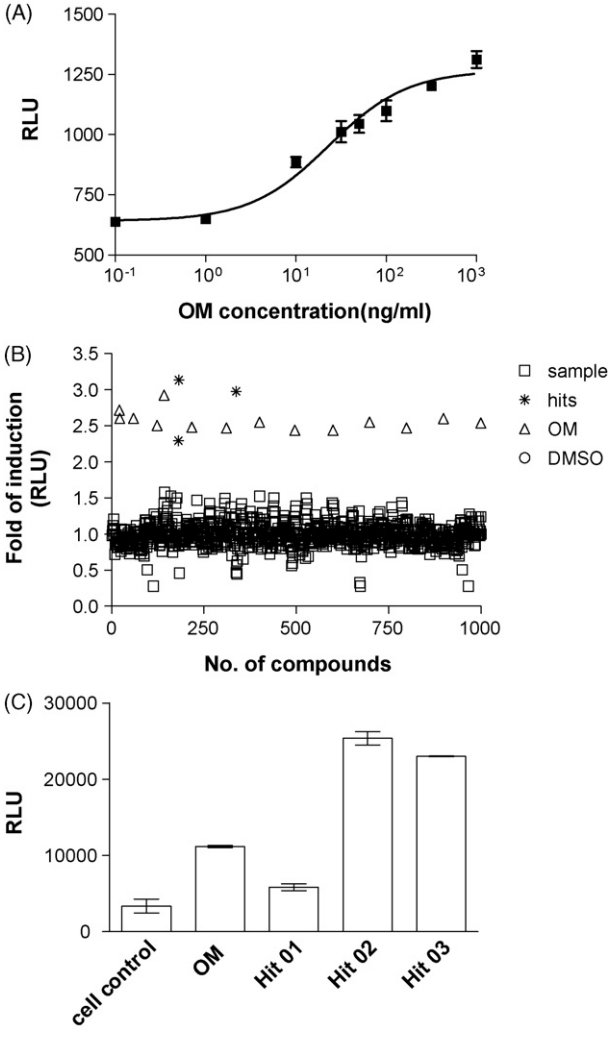

Fig. 1 – Assay development, primary screening and hits confirming. (A) OM dose curve. HEK293/pLDLR270-luc cells were seeded into a 96-well plate at 50,000 cells/well. Different concentrations of OM were added next day. On the day 3 the produced luciferase activity was assayed as described in Section 2. Data shown are means ± S.E.M. of relative luminescence units (RLU) based on duplicate measurements from one of the representative experiments ($n = 3$). (B) A scatter plot of luciferase activity (folds to cell control) from the initial screening of the 1000 fractions. Three hits were identified by these screening. (C) Hits confirmation. The cell culture condition and the treatment were described in the (A). Data shown are means ± S.E.M. of relative luminescence units (RLU) based on duplicate measurements from one of the representative experiments ($n = 3$).

basis construct to build a LDLR-promoter luciferase reporter gene assay. The promoter region of LDLR gene cloned from the human genome contained both SRE and SIRE. With an insertion of a 270 bp LDLR-promoter, the expression of luciferase gene could be regulated by the factors that could affect LDLR gene expression. To identify small molecules that could up-regulate LDLR expression within our herb-based natural compounds, we stably transfected cells using this construct and generated a cell line with a reporter gene, HEK293/p270LDLR-luc. This cell line was positively responsive to the stimulation of OM (cytokine oncostatin M) [27]. Further characterization of this cell line showed that the level of luciferase activity increased in a dose-dependent manner when the cells were treated with OM (Fig. 1A). The EC_{50} value was approximately 23.17 ng/ml, which was consistent with that previously reported observations [27]. OM, a cytokine

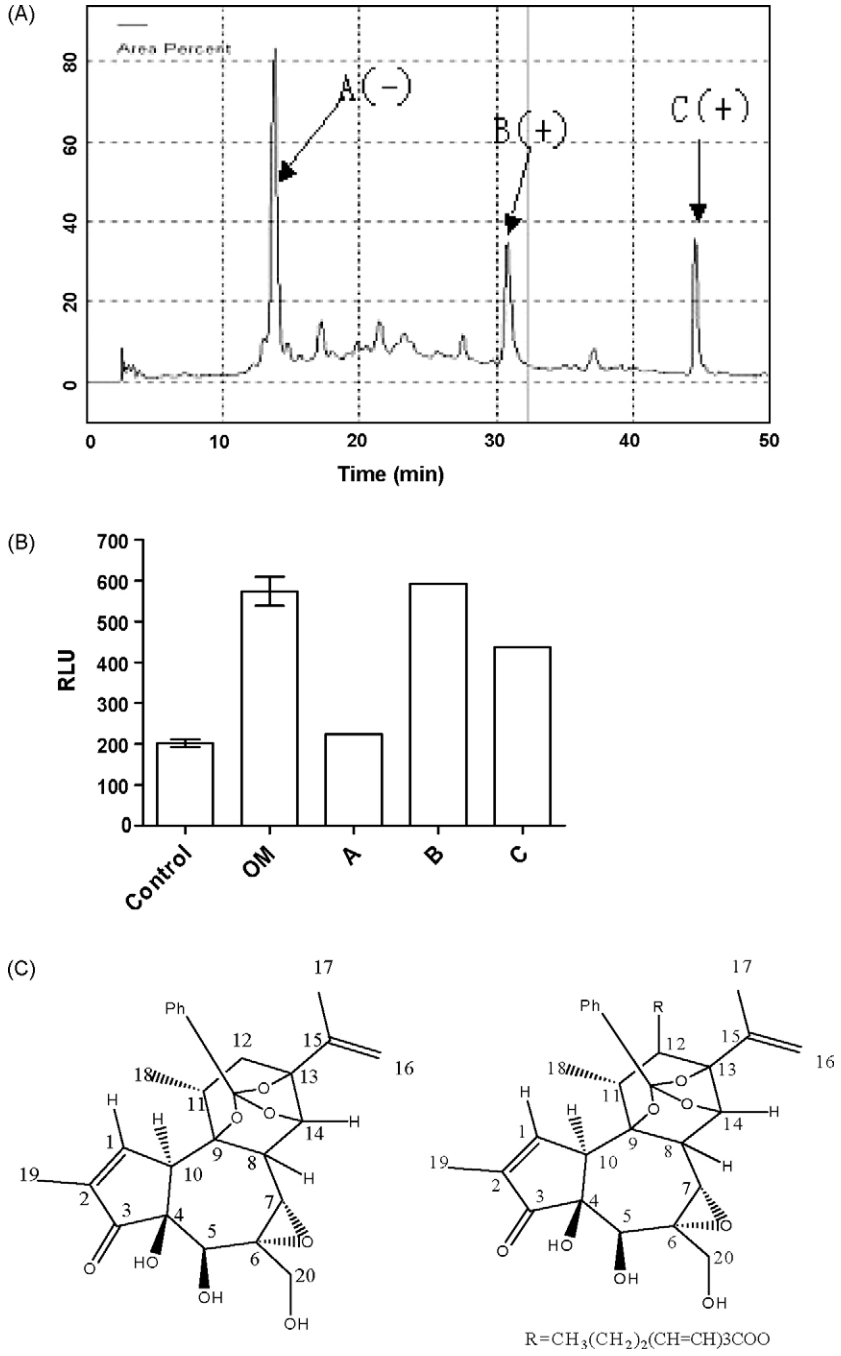

Fig. 2 – Chromatograms activities and chemical structures of Daphnetoxin and Gniditrin. (A) HPLC chromatograms showing the fraction of positive macroporous resin fraction (95:5) (B) HEK293/p270LDLR-luc cells were stimulated with these three HPLC peaks, 24 h after stimulation, the luciferase activity was assayed as described in Section 2. Data shown are means ± S.E.M. of relative luminescence units (RLU) based on duplicate measurements from one of the representative experiments ($n = 3$). (C) Chemical structures of Daphnetoxin and Gniditrin. Both Daphnetoxin and Gniditrin possess the same daphnane-type skeleton. Gniditrin is the archetypal 12-acyloxyderivative of Daphnetoxin.

from the IL-6 subfamily, has been known for its role in up-regulating hepatic LDLR both *in vitro* and *in vivo* [17,28], through the stimulation of SIRE [19,28]. The dose-dependent correlation of our reporter cell line with the treatment of OM indicates that the cloned LDLR-promoter segment controls the expression of luciferase reporter gene in the construction. Furthermore, the luciferase reporter gene expression was indeed regulated through the SRE/SIRE sequences engineered in the structure. With this reporter gene cell line, we searched potential cholesterol-lowering compounds from over 1000 diversified HPLC fractionated samples from our herb extracts using a high-throughput screening approach. As shown in Fig. 1B, out of the 1000 fractionated samples screened, there were 3 hits that induced luciferase activity at a level similar to that by of OM treatment. These hits were selected for further characterization.

3.2. *Isolation of hit compounds that can activate LDLR-promoter*

After our primary screening, hit compounds were selected and a second round of test was performed to confirm their LDLR-promoter activating activities. As shown in Fig. 1C, two of these hit fractions showed LDLR-promoter activation in HEK293/p270LDLR-luc assay system. The activation was dose-dependent (data not shown). We then identified those fractions and found that both fractions were from the herb *Cirald Daphne* Bark. The positive fractions were then further purified by HPLC. As shown in Fig. 2A, three main unique HPLC fractions (A, B, and C) were tested for their activity using HEK293/p270LDLR-luc assay system. As shown in Fig. 2B, fractions B and C were active in the test and were collected for subsequent analysis.

3.3. *Structural identification of isolated active herb compounds*

We performed HR-MS and NMR analysis for structural elucidation of the two isolated compounds. For fraction B: HR-MS: 483.2016 $[M + H]^+$. Formula: $C_{27}H_{30}O_8$ UV: λ_{max} (MeOH): 243 nm. ^{1}H NMR (acetone-d_6, 400 MHz): 7.68 (1H, bs, 1-H), 4.24 (1H, s, 5-H), 3.50 (1H, s, 7-H), 3.11 (1H,d, J = 1.6 Hz, 8-H), 3.96 (1H, bs, 10-H), 2.66 (1H, m, 11-H), 2.41 (1H, m, 12-H), 2.66 (1H, m, 12-H), 4.75 (1H, d, J = 1.6 Hz, 14-H), 4.93 (1H, s, 16-H), 5.14 (1H, s, 16-H), 1.88 (3H, s, 17-H), 1.25 (3H, d, J = 4.8–5.6 Hz, 18-H), 1.76 (3H, bs, 19-H), 3.63 (1H, m, 20-H), 3.96(1H, m, 20-H), 7.43 (3H, m, Ph-H), 7.78 (2H, m, Ph-H).

For fraction C: HR-MS: 669.2690 $[M + Na]^+$. Formula: $C_{37}H_{42}O_{10}$ UV (MeOH): 306 nm. ^{1}H NMR (acetone-d_6, 400 MHz): 7.66 (1H, bs, 1-H), 4.23(1H, bs, 5-H), 3.62(1H, m, 7-H), 3.73(1H, d, J = 1.6 Hz, 8-H), 3.92 (1H, 10-H), 2.68(1H, m, 11-H), 5.18 (1H, m, 12-H), 5.04(1H, m, 14-H), 5.19 (1H, m, 16-H), 5.05 (1H, m, 16-H), 5.90–6.7 (6H, olefinische), 1.89 (3H, s, 17-H), 1.38 (3H, d, J = 2.8 Hz, 18H), 1.76 (3H, s, 19-H), 3.67 (2H, m, 20-H), 0.95 (3H, m, 10-H), 7.44 (3H, m, Ph-H), 7.75 (2H, m, Ph-H).

Based on the above data, we derived their structures (Fig. 2C) and identified the two compounds as Daphnetoxin and Gniditrin, respectively. The two compounds are structurally related. Gniditrin is the archetypal 12-acyloxyderivative of Daphnetoxin.

3.4. *Daphnetoxin and Gniditrin additively activates LDLR-promoter activity with OM*

The next set of experiments was designed to determine which promoter regulatory elements (SRE, or SIRE, or both) were regulated by two hit compounds. When the HEK293/pLDLR270-luc cells were incubated with Daphnetoxin (10 μM), LDLR-promoter activity was increased 8.5-fold comparing with the control. Strikingly, in the presence of OM (50 ng/ml), a 16-fold increase was obtained, although OM by itself only led to a 4.4-fold increase (Fig. 3A), thus reflecting a synergistic effect of both drugs. Gniditrin behaved similarly when it was incubated with OM together (Fig. 3A). A full range of concentration of Daphnetoxon and Gniditrin were tested in the HEK293/p4xSRE-tk-luc cells. Both of them stimulated luciferase expression in a dose-dependent manner (Fig. 3B). The EC_{50} of Daphnetoxin and Gniditrin on HEK293/p4xSRE-tk-luc cells were 4.3 and 0.59 μM, respectively.

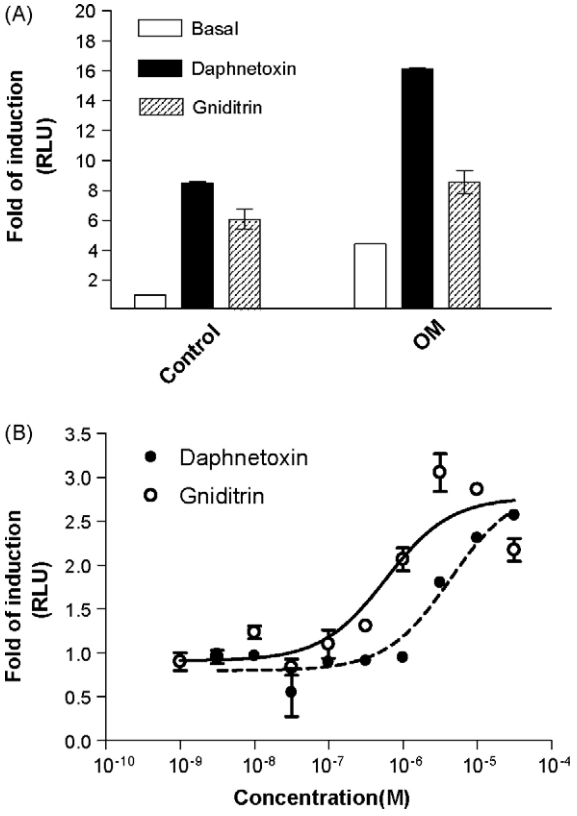

Fig. 3 – SRE is involved in the regulation of LDLR transcription by Daphnetoxin and Gniditrin. (A) HEK293/p270LDLR-luc cells were stimulated with Daphnetoxin (10 μM) or Gniditrin (1 μM) in the absence or presence of OM (50 ng/mL) for 24 h. The normalized luciferase activity is expressed as the fold of luciferase activity in untreated control cells. (B) HEK293/p4xSRE-tk-luc cells were stimulated with Daphnetoxin or Gniditrin, at different concentrations. Twenty-four hours after stimulation, the produced luciferase activity was assayed as described in Section 2. Data shown are means ± S.E.M. of relative luminescence units (RLU) based on duplicate measurements from one of the representative experiments (n = 3).

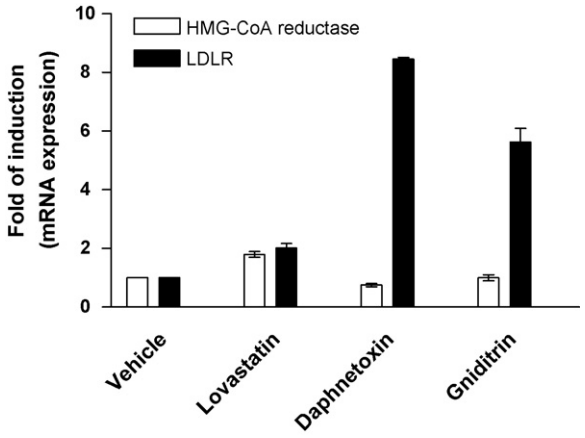

Fig. 4 – Influences of Daphnetoxin and Gniditrin on LDLR and HMG-CoA reductase mRNA expressions. HepG2 cells were treated for 24 h with Lovastatin (1 μM), Daphnetoxin (10 μM) or Gniditrin (1 μM) before total mRNA extraction. Real-time PCR method was used for the quantitation. The relative fold-changes of LDLR and HMG-CoA reductase mRNA after normalization with GAPDH were calculated. The data represent mean ± S.E.M. of four replicates for each treatment.

3.5. *Effects of Daphnetoxin and Gniditrin on LDLR and HMG-CoA reductase mRNA expressions*

Upon incubation of HepG2 cells with Daphnetoxin, Gniditrin or Lovastatin, quantitative real-time PCR showed that Lovastatin induced 2-fold increase for LDLR mRNA expression and 1.8-fold increase for HMG-CoA reductase mRNA expression, whereas Daphnetoxin and Gniditrin induced a 5–8-fold increase for LDLR mRNA expression separately but failed to induce HMG-CoA reductase mRNA expression (Fig. 4). No change was observed for the internal control (GAPDH mRNA) (Fig. 4). The results provide further evidence that the two selected hit compounds specifically up-regulate LDLR expression.

3.6. *Induction of LDL functional receptor by Daphnetoxin and Gniditrin*

To demonstrate that the increase of the LDLR mRNA level correlates with the enhanced activity of the functional LDLR, we developed a LDL-uptake assay on HepG2 cells. When HepG2 cells were treated with different samples, followed by Dil-LDL, the amount of fluorescent dye that accumulates in the lysosomes reflects the cell-surface LDLR activity. From our results, Daphnetoxin or Gniditrin (Fig. 5D and E) led to an

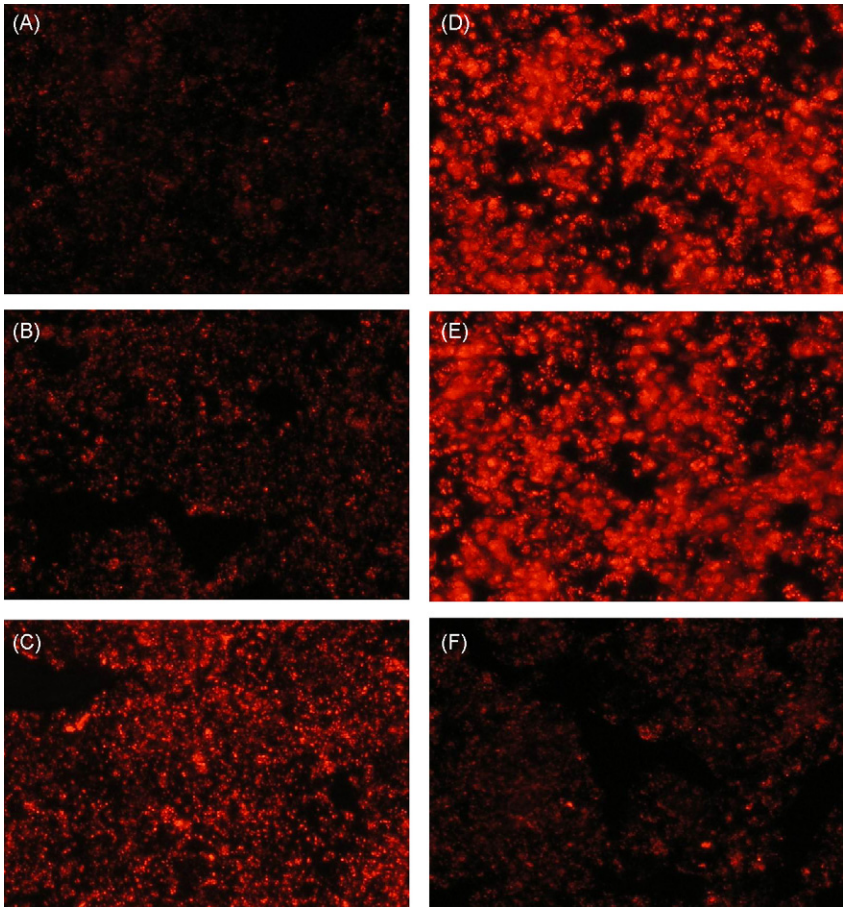

Fig. 5 – Daphnetoxin and Gniditrin increase the uptake of Dil-LDL in HepG2 cells. HepG2 cells were treated with 1 μg/ml 25-hydroxycholesterol (A), 1 μM Lovastatin (B), 50 ng/ml OM (C), 10 μM Daphnetoxin (D), 1 μM Gniditrin (E) or 10 μM vehicle (F), followed by addition of 6 μg/ml Dil-LDL.

increase in the intensity of fluorescence compared to those untreated cells. The increased level was even higher than that treated with the positive controls such as Lovastatin and OM. The fluorescence was reduced when cells were incubated with 25-hydroxycholesterol (Fig. 5A versus Fig. 5F).

4. Discussion

Here, we established a cell-based luciferase assay by measuring the effect on the promoter region of LDLR gene for screening out new compounds, which can increase LDLR transcription, and thus lowering plasma LDL-c. We screened compounds from our anti-hyperlipaemia and anti-hypertension natural herbal compound library. We purified and identified that Daphnetoxin and Gniditrin are major components responsible for increasing of LDLR transcriptional activity. We then confirmed these effects through measuring mRNA and protein expression of the LDLR.

We identified both compounds from the root bark of *Daphne giraldii Nitsche*, which is a traditional Chinese herb, termed as *Cirald Daphne* Bark, and has many pharmacological activities like analgesic, anti-inflammation, inhibiting bacteria, anti-thrombus, anti-tumor and antifertility [29]. Daphnetoxin and Gniditrin are Daphnane-type diterpene orthoesters. They were reported to have anti-leukaemia and piscicidal activities [30,31]. Our research, for the first time reveals that these Daphnane orthoesters can up-regulate expression of LDLR. In addition, Gniditrin has a lower EC_{50} for this LDLR-promoter activation than that of Daphnetoxin suggesting that the acyl moiety at C-12 may act as an activity modulator. Although Daphnetoxin and Gniditrin are reported to have toxicity [30,31], in our study they possess good activities to up-regulate LDLR expression *in vitro*. And it seems that other Daphnane-type diterpene orthoesters may also have the cholesterol-lowering activity from our study, but this hypothesis needs further biomedicine and biochemistry studies.

GW707 and 3-β-[2-(diethylamino)methoxy]androst-5-en-17-one (U18666A) are steroidal LDLR regulators, with mechanisms different from statins. U18666A is known to interfere with intracellular sterol trafficking [32]. GW707 was identified through a screening for compounds regulating LDLR expression through activation of human LDLR-promoter. It was also proposed that it can bind to the SREBP cleavage-activating protein (SCAP) through direct interaction with the sterol-sensing domain of SCAP [25]. Recent studies indicate that ERK signaling cascade is critically involved in the GW707-mediated induction of LDLR expression [33]. Both compounds consist of a planar sterol nucleus with an ether linkage at the C-3 position and an alkyl side chain that contains a tertiary amine. U18666A differs from GW707 in that the sterol side chain is absent from the C-17 position but GW707's mechanism of increasing LDLR mRNA is similar to that of U18666A, which is independent of SCAP function [34].

By using p4xSRE-TK-luc promoter constructs, we demonstrate that the SRE of LDLR-promoter is involved in the activities of Daphnetoxin and Gniditrin. And these compounds show additive activity with OM on pLDLR270-luc promoter constructs. We also investigated the LDLR mRNA and protein expression by real-time PCR and uptake assay. We can see that both compounds can significantly up-regulate the LDLR expression in HepG2 cells whereas fail to increase HMG-CoA reductase mRNA level, suggesting that they might not affect HMG-CoA reductase mRNA expression via SREBP pathway. In addition, our studies indicate that higher levels of LDLR expression and function can be achieved through other compound different from well-known statins, GW707 or U18666A, at least, in cell culture model. The precise mechanisms underlying the up-regulating LDLR expression effects of Daphnetoxin and Gniditrin need to be investigated further. These results hold promise for developing another kind of compound that can treat hypercholesterolemia.

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