Acta Pharmacologica Sinica (2018) 39: 1473–1482

www.nature.com/aps

Article

Proteomics analysis reveals a potential new target protein for the lipid-lowering effect of Berberine8998

Cheng-yin YU[1, 2, #, *], Gang-yi LIU[3, #], Xiao-hui LIU[4], Yu-zhou GUI[1], Hai-ming LIU[3], Hong-chao ZHENG[3], Darek C GORECKI[2], Asmita V PATEL[2], Chen YU[3], Yi-ping WANG[1]

[1]*State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China;* [2]*School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth PO1 2DT, UK;* [3]*Xuhui Central Hospital, Shanghai Clinical Center, Chinese Academy of Sciences, Shanghai 200031, China;* [4]*Department of Proteomics Research, College of Life Sciences and Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China*

Abstract

Berberine8998 is a newly synthesized berberine derivative with better lipid-lowering activity and improved absorption. The objective of this study was to investigate the effects of berberine8998 on serum cholesterol and lipid levels *in vivo* and to examine the mechanisms involved. Hamsters on high-fat diet (HFD) were administered berberine or berberine8998 (50 $mg \cdot kg^{-1} \cdot d^{-1}$, ig) for 3 weeks. Berberine8998 administration significantly lowered the total cholesterol, triglycerides and LDL-C levels in HFD hamsters. Bioinformatics revealed that berberine and berberine8998 shared similar metabolic pathways and fatty acid metabolism was the predominant pathway. Western blot validation results showed that peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) and long-chain fatty acid—CoA ligase 1 (ACSL1), two proteins involved in fatty acid metabolism, were expressed differently in the berberine8998 group than in the untreated group and the berberine treatment group. Biochemistry results showed that berberine8998 significantly lowered the non-esterified fatty acid (NEFA) levels, which may lead to a reduction in TG levels in the berberine8998 treatment group and the differences observed in proteomics analyses. Pharmacokinetic analysis conducted in rats. After administration of berberine or berberine8998 (50 mg/kg, ig), berberine8998 exhibited a remarkably improved absorption with increasing bioavailability by 6.7 times compared with berberine. These findings suggest that berberine8998 lowers cholesterol and lipid levels via different mechanisms than berberine, and its improved absorption makes it a promising therapeutic candidate for the treatment of hypercholesterolemia and obesity.

Keywords: berberine; berberine8998; proteomics; lipid-lowering; cholesterol; triglycerides; LDL-C; fatty acid metabolism; hypercholesterolemia; obesity

Acta Pharmacologica Sinica (2018) 39: 1473–1482; doi: 10.1038/aps.2017.200; published online 12 Apr 2018

Introduction

Berberine is an isoquinoline alkaloid (Figure 1A) and the main active constituent of *Coptis chinensis* Franch[1]. Berberine-containing herbs are used in traditional Chinese medicine to treat various conditions and diseases, including diarrhoea, cancer, depression, hypertension, hypercholesterolemia, and diabetes mellitus[2]. Berberine also exhibits significant lipid-lowering activity. Treatment of hypercholesterolemic patients with orally administered berberine reduced serum levels of cholesterol and low-density lipoprotein (LDL) by 29% and 25%, respectively. Berberine treatment reduced serum total cholesterol (TC) by 40% and LDL by 42% in hyperlipidemic hamsters[3].

However, the effect of berberine on triglycerides (TGs) and its associated mechanisms are not fully understood, but serum TG concentrations and risk for coronary heart disease are issues of great interest as well[4]. Fatty acids (FAs) are important metabolic substrates for energy production. Excess FAs and unesterified cholesterol are stored in lipid droplets within triacylglycerol (TG)[5].

High serum LDL is a major risk factor for atherosclerosis and coronary heart disease[6, 7], and is the main target of lipid-lowering drugs[8]. Berberine increases the abundance of LDL receptor (LDLR)[3] in the liver via transcript stabilization[9]. Upregulation of LDLR expression on the hepatocyte surface by genetic or pharmacological means has been shown to increase hepatic clearance and reduce serum levels of LDL

[#] These authors contributed equally to this work.
[*] To whom correspondence should be addressed.
E-mail chengyin.yu@port.ac.uk (Cheng-yin YU)
Received 2017-07-13 Accepted 2017-11-08

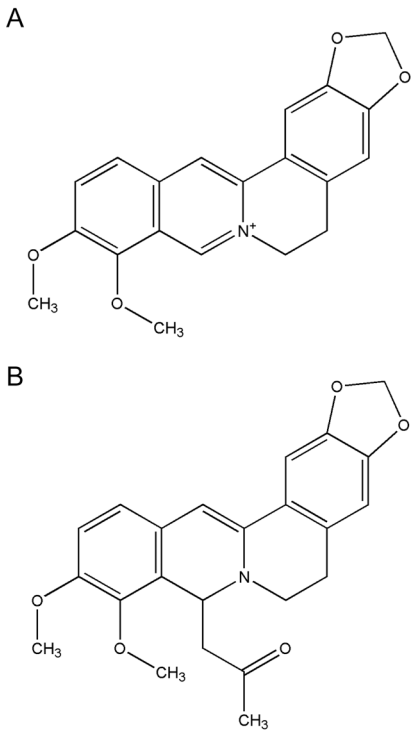

Figure 1. Chemical structures of (A) berberine and (B) berberine8998.

cholesterol[10-12]. LDLR activity is therefore considered a key factor in determining LDL cholesterol concentrations in the systemic circulation.

Berberine and its derivatives reduce lipid levels via multiple mechanisms. Berberine reduces serum LDL-c via the induction of LDLR expression in the liver. Signaling pathway studies show that berberine activates AMPK, ERK and other pathways. Berberine also upregulates liver X receptor α (LXRα) and peroxisome proliferator-activated receptor α/δ (PPARα/δ) expression and downregulated PPARγ expression in the liver[13].

The isobaric Tags for Relative and Absolute Quantitation (iTRAQ) combined with 2D LC-MS/MS is one of the most powerful methodologies in quantitative proteomics, which has been used and reviewed in a variety of reports[14, 15]. Proteomics has a great potential in mechanism identification. Moreover, proteomics analysis permits effective expression measurements in large sample sets. Therefore, we used proteomics to analyze the mechanisms of the lipid-lowering effect of berberine.

One obstacle for berberine use in the treatment of hypercholesterolemia is its low bioavailability. An oral dose of berberine is typically 1.0 g/day (10 tablets/day), which limits its clinical application[16]. Therefore, identification of berberine derivatives with improved bioavailability will facilitate therapeutic applications.

In this study, a newly synthesized berberine derivative, berberine8998 (Figure 1B) was characterized. *In vivo* studies showed that berberine8998 significantly lowered the TC, LDL and TG levels in the hamster model, while berberine only lowered TC and TG levels. In-depth iTRAQ proteomics revealed that peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) and long-chain-fatty acid—CoA ligase 1 (ACSL1), proteins involved in fatty acid metabolism, were expressed differently in the berberine8998 group compared to the control and the berberine treatment groups. Mechanistic studies showed that berberine8998 significantly lowered non-esterified fatty acid (NEFA) levels, which may lead to a reduction in TG levels in berberine8998 treatment groups. Pharmacokinetic analyses showed that berberine8998 had remarkably improved absorption (by 7.74 times) compared to berberine. These findings suggest that berberine8998 lowered cholesterol and lipid levels via different mechanisms compared to berberine, and it is a promising therapeutic candidate for the treatment of hypercholesterolemia and obesity.

Materials and methods

Chemicals and reagents

Berberine (purity >98%) was purchased from Northeast Pharm (China). Berberine8998 (purity >97%) was synthesized by You-hong HU (Shanghai Institute of Materia Medica, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone Laboratories (Logan, UT, USA). Fetal bovine serum (FBS) was obtained from ThermoFisher Scientific (Carlsbad, CA, USA). Provastatin was purchased from Sigma-Aldrich (St Louis, MO, USA).

Animals and procedures

Six-week-old hamsters were obtained from the Animal Center of Shanghai Institute of Materia Medica. Hamsters were randomly divided into four groups: normal diet, model, berberine, and berberine8998 groups. Berberine and berberine8998 were dissolved in CMC-Na prior to administration. Hamsters on the normal diet received carboxymethylcellulose sodium (CMC-Na) as the negative control group (n=3). Hamsters on a high-fat diet (HFD) (0.15% cholesterol and 18% fat) were administered CMC-Na, berberine or berberine8998 (50 mg/kg, n=12) daily intragastrically for 3 weeks. Body weight was monitored.

All hamsters were fasted overnight (12 h) at 7, 14 and 21 days post-dosing, and blood samples were drawn from the retro-orbital plexus into tubes containing 3.8% sodium citrate (w/v) as an anti-coagulant. Blood samples (0.3–0.5 mL) were collected weekly after a 12 h fast and centrifuged for 15 min at 3000 ×g to obtain serum. Serum lipid profiles, including TC, LDL-c and TG levels, were measured using an automatic analyzer (Hitachi, Tokyo, Japan).

Animal experiments were performed according to the National Research Council's Guidelines, and the Institutional Ethical Committee of Shanghai Institute of Materia Medica approved all experimental protocols and procedures.

Protein extraction and iTRAQ reagent labeling

To determine the protein expression differences between the berberine and berberine8998 treatment groups, livers of hamsters from the different treatment groups were collected, snap-frozen in liquid nitrogen, and stored at -80 °C. The samples

were dissolved in the lysis buffer composed of 7 mol/L urea, 2 mol/L thiourea, 65 mmol/L dithiothreitol, and 0.1 mmol/L phenylmethylsulfonyl fluoride at 4 °C, sonicated three times at 70 W for 5 s at 10 s intervals, and homogenized three times using a whirlpool mixer at 10 min intervals. Samples were centrifuged at 20 000×*g* for 30 min at 4 °C, and the protein concentration in the supernatant was determined using a Bradford assay. BSA was used to generate the calibration curve.

Trypsin digestion and iTRAQ labeling were performed. Liver proteins (150 μg) from each hamster were reduced, alkylated and digested overnight at 37 °C with trypsin (MS grade; Promega, Fitchburg, WI, USA). Samples were labeled with iTRAQ reagent (Applied Biosystems) as follows: berberine, iTRAQ reagent 113/114; berberine8998, reagent 115/116; and control, reagent 117/118. Two sets of six isobaric tags were used for the 12 digested protein samples.

Two-dimensional LC-MS/MS analysis based on Triple TOF 5600

Mixed peptides were fractionated using strong cation exchange chromatography on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) and a polysulfoethyl column (Nest Group, Southborough, MA, USA) with the following dimensions: 2.1 mm×100 mm, 5 μm, and 300 Å. Mixed peptides were desalted using a Sep-Pak Cartridge (Waters, Milford, MA, USA), diluted with loading buffer (10 mmol/L KH_2PO_4 in 5% acetonitrile [ACN], pH 2.8), and loaded onto the column. Buffer A was identical in composition to the loading buffer. Buffer B was the same as Buffer A except that it contained 350 mmol/L KCl. Peptide separation was performed using a linear binary gradient of 0% to 50% Buffer B in Buffer A at a flow rate of 200 μL/min for 1 h. Absorbances at 214 and 280 nm were monitored. Thirty strong cation exchange fractions were collected along the gradient, dried, dissolved in Buffer C (5% ACN and 0.1% formic acid [FA]), and analyzed on a Triple TOF 5600 mass spectrometer (Applied Biosystems). Peptides were separated on a Zorbax 300SB-C18 reverse-phase column (Agilent Technologies, Santa Clara, CA, USA) with the following dimensions: 0.1 mm×15 mm, 5 μm, and 300 Å with a gradient of 5% to 35% Buffer D (95% ACN and 0.1% FA) in Buffer C at a flow rate of 0.2 μL/min for 65 min. Survey scans were acquired from *m/z* 400 to 1800, with ≤10 precursors selected for MS/MS from *m/z* 100–2000 using a dynamic exclusion of 30 S for selected ions. iTRAQ-labeled peptides were fragmented under collision-induced dissociation conditions to yield reporter ions at 113.1, 114.1, 115.1, 116.1, 117.1, and 118.1. The ratios of their peak areas reflected the relative abundance of the peptides and proteins in the samples. Larger sequence information-rich fragment ions were also produced under the same MS/MS conditions and provided the identity of the protein from which the peptide originated.

Bioinformatics analysis

Protein Pilot v4.5 (Applied Biosystems) was used to identify and quantify iTRAQ-labeled peptides. This software was also used to determine the minimum number of identified peptides via removal of redundant hits. MS/MS data were searched using UniProt. The rat database was used because the hamster database is not available. The precursor and iTRAQ fragment tolerance values were set to 100 ppm and 0.6 Da. The following parameters were used for data analysis: sample type = iTRAQ (peptide-labeled); Cys alkylation = methyl methanethiosulfonate; digestion = trypsin; instrument = time-of-flight 5600 ESI; species = RAT; ID focus = biological modifications; and database = Swissprot Rat (35 672 entries).

A decoy database search strategy was used to estimate an FDR <1% for peptide and protein identification and minimize the occurrence of false-positive results. At least one peptide with a confidence interval of 95% was included. Data were considered reliable at $P<0.05$ and error factor <2. The P value from Student's t test was used to evaluate the significance of the change in protein expression level. Fold-change ratios <0.7 (downregulated) or >1.3 (upregulated) were selected as cutoff values. Gene Ontology (GO) analysis (http://www.geneontology.org/) was used to verify the function of proteins that were up- or down-regulated by berberine or berberine8998 treatment. A search of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html) identified the signaling pathways of the proteins. MATLAB (MathWorks, Natick, MA, USA) was used for mathematical modeling and analysis of KEGG pathways.

Western blotting of the candidate proteins

Liver tissue lysates (20 μg) were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (0.22 μm) using a semi-dry transfer system (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% BSA in buffer composed of 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween 20 at room temperature for 2 h. Membranes were incubated with mouse monoclonal antibodies against LDLR (1:2000) and ACOX1 (1:200) (both from Abcam, Cambridge, UK) at 4 °C overnight. Membranes were washed and incubated with anti-mouse IgG (1:2000; Bio-Rad) at room temperature for 2 h. Protein bands were detected using a Clarity Enhanced Chemiluminescence Western Blot Substrate kit (Bio-Rad). Densitometry was performed using Quantity One software (Bio-Rad). The ratio of gray values of target proteins represented the relative expression levels for each group. Each experiment was repeated three times.

Measurement of NEFA levels

Serum samples from 60 hamsters were divided into berberine-, berberine8998-treated and untreated (control) groups (n=20 each). NEFA levels were determined using an assay that measures the conversion of fatty acids to acyl-CoA in the presence of acyl-CoA synthetase. Acyl-CoA is oxidized following the addition of acyl-CoA oxidase, which generates hydrogen peroxide and causes oxidative condensation of 3-methyl-*N*-ethyl-*N*-(β-hydroxyethyl)-aniline with 4-aminoantipyrine in the presence of a peroxidase to yield a product with an absorbance at 550 nm on an Automatic Biochemical Analyzer. The NEFA-HA Test Kit (Wako, Japan) was used for this measurement.

Measurement of DiI-LDL uptake by HepG2 cell LDLRs

HepG2 cells were maintained in Dulbecco's modified Eagles' medium (DMEM) (HyClone) containing 10% (*v*/*v*) fetal bovine serum (FBS). Cells were incubated under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37 °C at a density of 10^6 cells per mL. HepG2 cells were incubated with DiI-LDL (Biotium, Hayward, CA, USA) and the following test compounds: pravastatin (positive control, 5 μmol/L) (Sinopharm, Shanghai, China), berberine (40 μmol/L), or berberine8998 (20 or 40 μmol/L). DiI-LDL uptake was determined as follows. The culture medium was removed, and plates were washed three times with 0.4% BSA in phosphate-buffered saline. Isopropanol (0.5 mL/well) was applied to extract DiI-LDL from the cells. A 200-μL volume of supernatant was transferred to a black fluorescent screen to determine DiI-LDL absorption at excitation/emission wavelengths of 520/570 nm. Total DiI-LDL uptake was determined at 37 °C using a SpectraMax M2e fluorescence detector (Molecular Devices, Silicon Valley, CA, USA). The hepatic hydrogen peroxide level was measured using a hydrogen peroxide testing kit (Beyotime Biotech, Haimen, China). The hepatic lipoperoxide level was tested using a malondialdehyde (MDA) testing kit (Nanjing Jiancheng Biotech, Nanjing, China).

Pharmacokinetic study of berberine8998

Male Sprague-Dawley (SD) rats (clean class, 180–200 g, *n*=10) were provided by the Animal Center of Shanghai Institute of Materia Medica and randomly divided into two groups. After twelve hours of food deprivation, berberine and berberine8998 (50 mg/kg, free base) were administered to the respective groups intragastrically (ig). The washout interval between the two treatment periods was 7 d after the final blood collection. Rats received the other compound during the second treatment period. The sample collection protocol was identical in the two treatment periods. Blood samples (0.5 mL) were collected pre-dose and at 0.5, 1, 2, 3, 5, 6, 7, 8, 12, 24, and 48 h after dosing in each treatment period. The collected blood samples were centrifuged at 3000×*g* for 15 min at 4 °C within 30 min of collection. Serum samples were fractionated by chromatography on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) using a reverse-phase column (CAPCELL PAK C18). Chromatographic data were processed using Phoenix® WinNonlin® 6.3 to calculate the pharmacokinetic parameters.

Similar procedures were used in Syrian hamsters. Syrian hamsters (clean class, 90–120 g, *n*=6) were provided by the Animal Center of Shanghai Institute of Materia Medica and randomly divided into two groups. Hamsters were food deprived for 12 h, and berberine or berberine8998 (50 mg/kg, free base) were administered intragastrically (ig). Blood samples (0.5 mL) were collected pre-dose and at 0.5, 1.5, 3, 6, 8 h after dosing in each treatment period.

Results

Berberine8998 ameliorates high-fat diet-induced hypercholesterolemia in hamsters

We initially evaluated the effects of berberine8998 on lipid levels *in vivo*. Six-week-old male hamsters on a high-fat diet received 50 mg·kg^{-1}·d^{-1} berberine, 50 mg·kg^{-1}·d^{-1} berberine8998 or CMC-Na for 3 weeks. Hamsters on chow diet were administered CMC-Na as the normal diet control group. Serum lipid levels, including total cholesterol, low-density lipoprotein cholesterol, triglyceride and high-density lipoprotein cholesterol, were analyzed weekly.

Hypercholesterolemia developed slowly in the normal diet group (Figure 2A–2C), and serum lipid levels in the high-fat diet (HFD) group increased significantly (Figure 2A–2C). Notably, berberine8998 treatment significantly reduced total cholesterol (Figure 2A) and LDL-c (Figure 2B) levels at week 2 and week 3 compared with the HFD group (P<0.05). In contrast, berberine did not alter the LDL-c levels after three weeks of treatment (Figure 2B). Mean LDL-c levels at week 2 and week 3 were 10.30±3.63 and 13.15±7.34 nmol/L, respectively, for the HFD group; and 6.00±1.85 and 8.55±2.59 nmol/L, respectively, for the berberine8998 group (Figure 2B).

Given that triglyceride (TG) is an important component of lipids, the serum TG levels in hamsters were evaluated. The results showed that berberine8998 treatment ameliorated the HFD-induced elevated TG serum levels reducing it by 64% (Figure 2C). Triglyceride levels were significantly attenuated by week 3 in the berberine (P<0.05) and berberine8998 groups (P<0.01) (Figure 2C). Body weight was not significantly different between the treatment, control and normal diet groups, as shown in Figure 2D.

GO analysis indicates similar cellular protein locations in the berberine and berberine8998 treatment groups

Hamster liver samples from the berberine, berberine8998 and high-fat diet model groups were analyzed using iTRAQ shotgun proteomics. High-abundance proteins were depleted prior to analysis. A total of 2049 and 2020 proteins were identified by the first and second iTRAQ experiments, respectively, with a false discovery rate lower than 1%. Combined results of the two replicates identified 2444 proteins, of which 2073 proteins were quantified.

To elucidate the location and function of these proteins, gene ontology analysis and KEGG analysis were conducted. GO analysis revealed that proteins were located in similar cellular components in the high-fat diet group, berberine treatment group and berberine8998 group. The top five enriched cellular components were cell part, organelle part, organelle, macromolecular complex and membrane-enclosed lumen. The top five molecular functions included binding, catalytic activity, structural molecule activity, transporter activity and enzyme regulator activity (Figure 3A).

KEGG analysis indicates similar metabolic pathways affected in berberine and berberine8998 treatment groups

A total of 442 differentially expressed proteins were screened in both runs in the berberine8998 group, and a total of 268 differentially expressed proteins were screened in both runs in the berberine group. To filter the non-significant changes in liver protein expression levels between sample groups,

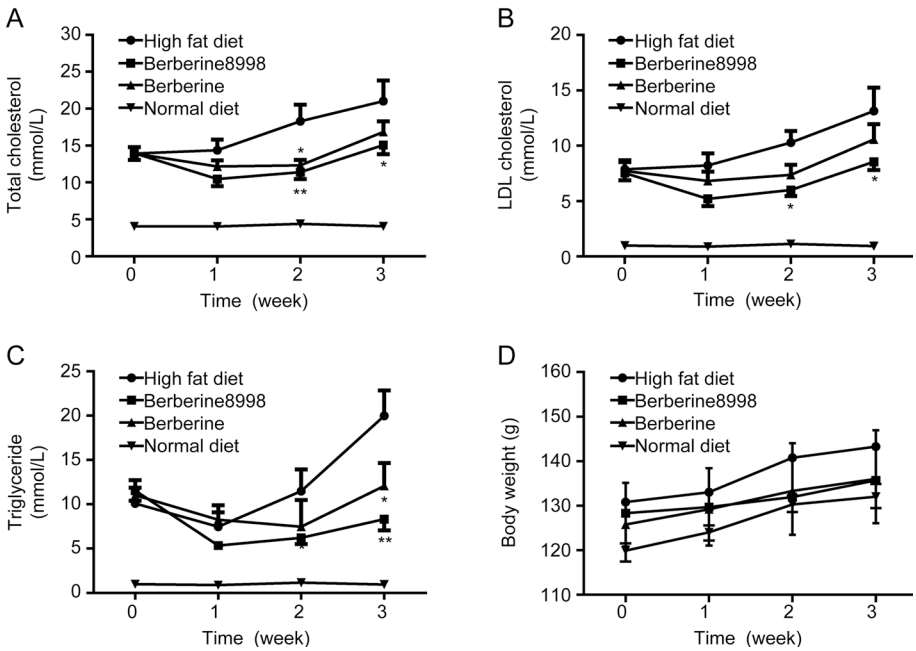

Figure 2. Analysis of (A) LDL and serum (B) TC and (C) TG levels in hamsters treated with berberine or berberine8998 or left untreated over 3 weeks (*P<0.05, **P<0.01 compared with high-fat diet group).

fold-changes in median ratios <0.7 (downregulated) or >1.3 (upregulated) were selected as cutoff values, and ratios were assessed using Student's t test. The median values of the ratios between the two groups were also calculated. A total of 48 proteins exhibited significant changes in the berberine8998 group; 16 proteins were upregulated, and 32 proteins were downregulated. A total of 53 proteins exhibited significant changes in the berberine group; 23 proteins were upregulated, and 30 proteins were downregulated. The top ten most significant changes in protein expression after berberine8998 and berberine treatment are summarized in Table 1 and Table 2.

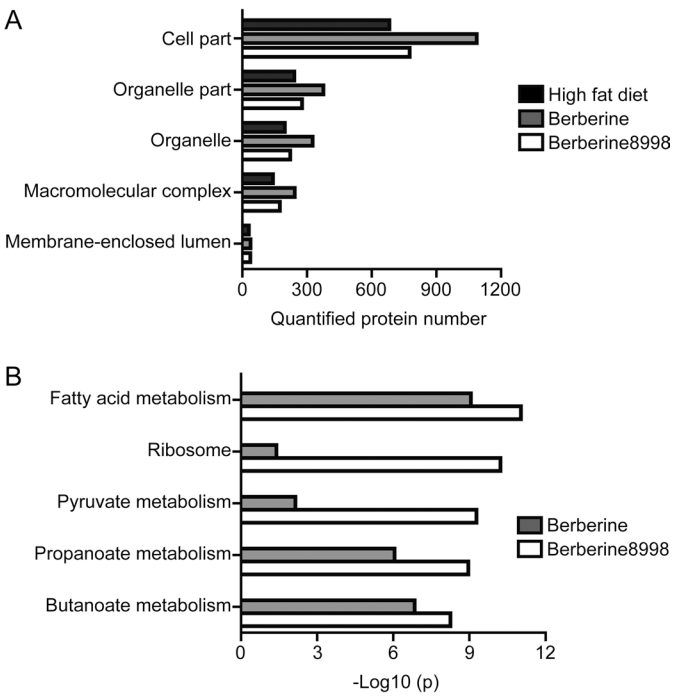

Figure 3. (A) GO analysis of cellular components. (B) Biological processes associated with differentially expressed proteins in BBR (berberine-treated) and BBR-8998 (berberine8998-treated) hamsters.

KEGG metabolic pathways were analyzed to determine the major pathways involved in berberine and berberine8998 treatment and statistical analysis determined the pathways represented in the berberine and berberine8998 treatment groups. As shown in Figure 3B, the major KEGG pathways were fatty acid metabolism, ribosome, pyruvate metabolism, propanoate metabolism and butanoate metabolism. The most significant metabolic pathway was fatty acid metabolism. These results suggest that berberine8998 and berberine lowered serum lipid levels via similar metabolic pathways.

Fatty acid metabolism is the major pathway affected by berberine8998

Although the major metabolic pathways were similar in berberine and berberine 8998 treatment groups, the differences in the magnitude of the target proteins were examined. Bioinformatics analysis revealed that fatty acid metabolism was the most significant pathway involved in the lipid-lowering effects of these agents. Therefore, two of the differentially expressed proteins, ACOX1 and ACSL1 were selected for validation by Western blotting. ACOX1 and ACSL1 expression in the liver was downregulated in the berberine and berberine8998 treatment groups compared to the control group (Figure 4A). The protein expression of ACOX1 and ACSL1 was significantly decreased by berberine8998 treatment by approximately 2- to 3-fold while the berberine treatment decreased ACOX1 and

Table 1. List of differentially expressed proteins after berberine8998 treatment in hamsters. The proteins were identified by TOF-AB 5600 (↑, up-regulated; ↓, down-regulated).

Protein name	Gene name	*P*-value	Fold of change	Protein function
Peroxisomal carnitine O-octanoyl transferase	Crot	1.57×10^{-5}	0.69↓	Beta-oxidation of fatty acids. The highest activity concerns the C6 to C10 chain length substrate.
Alpha-N-acetyl galactosaminidase	Naga	3.58×10^{-5}	1.71↑	Removes terminal alpha-N-acetylgalactosamine residues from glycolipids and glycopeptides.
Heterogeneous nuclear ribonucleoprotein K	Hnrnpk	4.13×10^{-5}	0.67↓	One of the major pre-mRNA-binding proteins.
Peroxisomal acylcoenzyme A oxidase 1	Acox1	1.24×10^{-3}	0.52↓	Catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs.
Acetyl-CoA acetyltransferase	Acta2	1.28×10^{-3}	1.55↑	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.
Apolipoprotein B-100	Apob	1.45×10^{-3}	0.75↓	Apolipoprotein B is a major protein constituent of chylomicrons (apo B-48), LDL (apo B-100) and VLDL (apo B-100).
1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-1	Plcd1	1.58×10^{-3}	1.37↑	The production of the second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) is mediated by activated phosphatidylinositol-specific phospholipase C enzymes.
Guanine nucleotide binding protein subunit beta-2-like 1	Gnb2l1	2.16×10^{-3}	0.69↓	Involved in the recruitment, assembly and/or regulation of a variety of signaling molecules.
Uricase	Uox	2.34×10^{-3}	0.54↓	Catalyzes the oxidation of uric acid to 5-hydroxyisourate, which is further processed to form (S)-allantoin.
40S ribosomal protein S15	Rps15	2.50×10^{-3}	0.52↓	Liver regeneration. Ribosomal small subunit assembly.
Long-chain-fatty-acid—CoA ligase 1	ACSL1	2.10×10^{-3}	0.50↓	Activation of long-chain fatty acids for both synthesis of cellular lipids, and degradation via beta-oxidation. Preferentially uses oleate, arachidonate, eicosapentaenoate and docosahexaenoate as substrates.

ACSL1 expression by approximately 1- to 2-fold. Therefore, the magnitude of ACOX1 and ACSL1 protein expression varied between the berberine and berberine8998 treatment groups.

ACOX1 is involved in the β-oxidation of fatty acid metabolism. Serum non-esterified fatty acid levels were analyzed because of the reduced TG levels *in vivo* (Figure 2C). Serum NEFA levels exhibited similar changes (4.07 *vs* 4.93 mmol/L; $P<0.05$; Figure 4B) compared to the high-fat diet group. These results suggest that berberine8998 reversed the HFD-induced increase in serum TG and NEFA levels.

To further elucidate the mechanisms associated with decreased serum LDL levels by berberine8998, a Dil-LDL uptake experiment was performed in HepG2 cells. Berberine8998 (40 μmol/L) dose-dependently stimulated LDL uptake in HepG2 cells compared with berberine (2.26 *vs* 1.22, $P<0.01$) (Figure 4C). The LDLR mediates LDL uptake in HepG2 cells. Therefore, the LDLR expression was evaluated in HepG2 cells by Western blotting. As shown in Figure 4D, LDLR was upregulated dose-dependently by treatment with both berberine and berberine8998, and berberine8998 induced a greater effect. These results further confirmed that berberine8998 promoted the uptake of LDL via increasing the expression of LDLR.

To elucidate the mechanisms involved in the effect of ACOX1 on fatty acid metabolism, hepatic malondialdehyde (MDA) and peroxide levels were analyzed. Additionally, mitochondrial fatty acid oxidation was analyzed via PGC-1α and UCP-1 expression. The results showed that hepatic malondialdehyde (MDA) and peroxide levels were inhibited, though not significantly. RNA levels of PGC-1 α and UCP-1 expression increased following treatment (Figure 4E–4G).

Berberine8998 shows significantly improved bioavailability

The pharmacokinetics of berberine8998 and berberine were further evaluated in SD rats. The mean serum concentration *vs* time profile is presented in Figure 5. The principal pharmacokinetic parameters of berberine and berberine8998 are summarized in Table 3. The maximum concentrations of berberine and berberine8998 in the serum (C_{max}) were 55.11 ng/mL and 230.3 ng/mL, while the half-lives of berberine and berberine8998 in serum ($t_{1/2}$) were 29.18 h and 10.97 h, respectively. The areas under the curve ($AUC_{0\text{-}48}$) were 312.4 ng/mL·h and 2418 ng/mL·h for berberine and berberine8998, respectively. These results suggest that the bioavailability of berberine improved significantly by 6.7 times in SD rats as a result of the structural modification to that of berberine8998.

Similar results were also observed in hamsters (Figure 5B and Table 4), with the bioavailability enhance by 3.6 times in hamsters.

Discussion

This research simultaneously compared the proteome profiles

Table 2. List of differentially expressed proteins after berberine treatment in hamsters. The proteins were identified by TOF-AB 5600 (↑, up-regulated; ↓, down-regulated).

Protein name	Gene name	*P*-value	Fold of change	Protein function
Cytosol aminopeptidase	Lap3	1.79×10^{-4}	1.41 ↑	Catalyzes the removal of unsubstituted N-terminal amino acids from various peptides.
Ras-related protein Rab-14	Rab14	4.09×10^{-4}	1.62 ↑	Involved in membrane trafficking between the Golgi complex and endosomes.
Signal recognition particle	Srp54	6.17×10^{-5}	0.63 ↓	Binds to the signal sequence of presecretory protein when they emerge from the ribosomes and transfers them to TRAM (translocating chain-associating membrane protein).
Guanine nucleotide-binding protein subunit beta-2-like 1	Gnb2l1	1.17×10^{-3}	0.63 ↓	Involved in the recruitment, assembly and/or regulation of a variety of signaling molecules.
Acetyl-CoA acetyltransferase	Acta2	1.43×10^{-3}	2.25 ↑	2acetyl-CoA = CoA + acetoacetyl-CoA.
Alpha-actinin-4	Actn4	1.78×10^{-3}	0.65 ↓	F-actin cross-linking protein which is thought to anchor actin to a variety of intracellular structures.
Misshapen-like kinase 1	Mink1	2.23×10^{-5}	0.68 ↓	Serine/threonine kinase which acts as a negative regulator of Ras-related Rap2-mediated signal transduction to control neuronal structure and AMPA receptor trafficking.
Carbonyl reductase [NADPH] 1	Cbr1	2.34×10^{-3}	1.35 ↑	Catalyzes the reduction of a wide variety of carbonyl compounds includingquinones, prostaglandins, menadione, plus various xenobiotics.
Ras-related protein Rab-2A	Rab2a	2.40×10^{-3}	1.33 ↑	Required for protein transport from the endoplasmic reticulum to the Golgi complex.
Short-chain specific acyl-CoA dehydrogenase	Acads	2.54×10^{-3}	0.65 ↓	Introduces a double bond at position 2 in saturated acyl-CoAs of short chain length.
Long-chain-fatty-acid—CoA ligase 1	ACSL1	1.30×10^{-3}	0.52 ↓	Activation of long-chain fatty acids for both synthesis of cellular lipids, and degradation via beta-oxidation. Preferentially uses oleate, arachidonate, eicosapentaenoate and docosahexaenoate as substrates.
Peroxisomal acyl-coenzyme A oxidase 1	ACOX1	5.49×10^{-3}	0.64 ↓	Catalyzes the desaturation of acyl-CoAs to 2-*trans*-enoyl-CoAs. Isoform 1 shows highest activity against medium-chain fatty acyl-CoAs and activity decreases with increasing chain length. Isoform 2 is active against a much broader range of substrates and shows activity towards very long-chain acyl-CoAs. Isoform 1 shows optimum activity with a chain length of 10 carbons while isoform 2 exhibits optimum activity with 14 carbons.

of high-fat hamster liver tissues samples with or without berberine8998 treatment. This study was the first time that the iTRAQ method was coupled with 2D LC-MS/MS (TripleTOF 5600) to investigate the mechanisms of action of berberine and berberine8998. Two differentially expressed proteins (ACOX1 and ACSL1) were identified by this proteomics approach and selectively validated further using Western blotting and biochemical analyses. The differences in protein expression may be the result of the improvement in the bioavailability of berberine8998.

The effects of berberine8998 on serum lipids were determined for the first time and compared with those of berberine. The results in Figure 2 illustrate a significant reduction in LDL, total TC and TG levels following berberine8998 treatment. Berberine reduced TC and TG but with reduced potency. To elucidate the possible mechanisms involved in this improvement, the function, interplay and changes in the abundance of proteins in response to internal and external cues were investigated[15]. The iTRAQ method combined with two-dimensional LC–MS/MS was used to analyze protein expression in the liver tissues of mice maintained on a high-fat diet treated with either berberine or berberine8998.

Previous studies investigated the proteomic effects of berberine on multi-target antimicrobial[13] and breast cancer cells[17]. The proteomic profiles of berberine8998 treatment on hamsters have not been investigated previously. This technique is one of the most powerful methodologies in quantitative proteomics[18]. The iTRAQ method identified a total of 48 proteins as significantly changed in the berberine8998 group: 16 proteins were upregulated, and 32 proteins were downregulated. While a total of 53 proteins were significantly changed in the berberine group: 23 proteins were upregulated, and 30 proteins were downregulated.

GO analysis showed that the differentially expressed proteins in the treatment and control groups had similar cellular locations. KEGG pathway enrichment suggested that fatty acid metabolism was specifically activated by the treatment. Hepatic ACOX1 expression was significantly lower in the

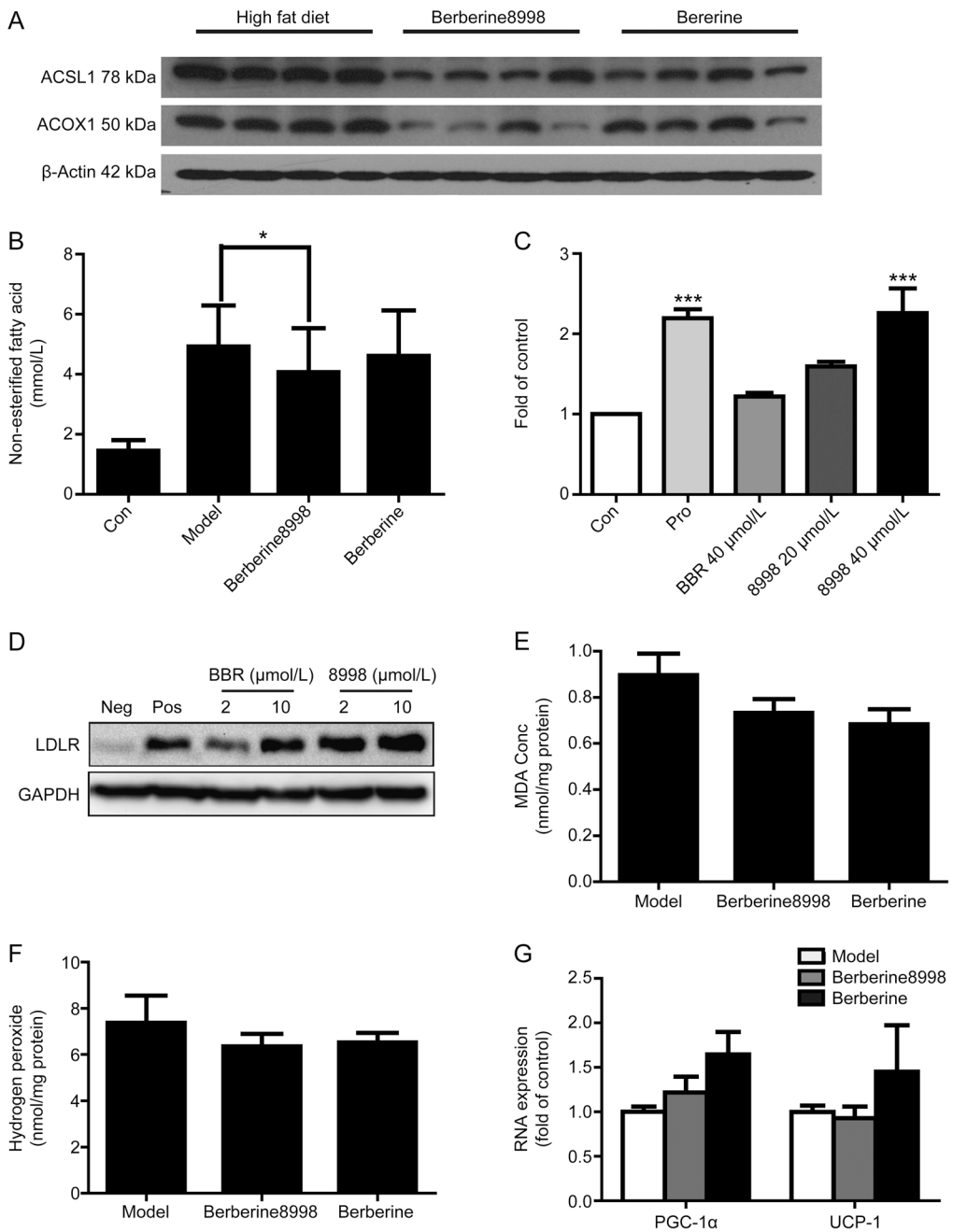

Figure 4. (A) Western blot analysis of ACOX1 expression in hamsters treated with BBR (berberine) or berberine8998 (BBR-8998) *vs* untreated controls fed a high-fat diet. All results are from four independent experiments. (B) Serum levels of NEFA. Hamsters were maintained on a normal or high-fat diet. In high-fat diet hamsters, one group received no treatment, and the other two groups were treated with either berberine or berberine8998 (50 mg/kg each). (C) Average uptake amount of Dil-LDL in HepG2 cells incubated with test compounds. Pro, pravastatin; BBR, berberine; 8898, berberine8998. (D) Western blot analysis of LDLR expression level. Neg, blank; POS, pravastatin 5 μmol/L; BBR, berberine 2 μmol/L and 10 mol/L; 8998, berberine8998 2 μmol/L and 10 μmol/L. (E) MDA levels. (F) Peroxide levels (G) Hepatic RNA expression of PGC-1α and UCP-1. All results are from three independent experiments.

berberine8998 group. Western blotting results confirmed that berberine8998 altered the levels of ACOX1 and other related proteins. These results suggest that berberine8998 reduces serum NEFA levels via modulation of ACOX1 expression. iTRAQ proteomics revealed that the effect of berberine8998 on TG was mediated by NEFA and hepatic ACOX1 expression. NEFA was downregulated by berberine8998. This ACOX1-regulated mechanism suppressed TG and NEFA levels *in vivo*, which is a newly defined lipid-lowering mechanism of berberine8998. Further mechanistic studies revealed that berberine8998 enhanced hepatic LDL uptake by increasing LDLR expression. Consistent with the lipid lowering effect,

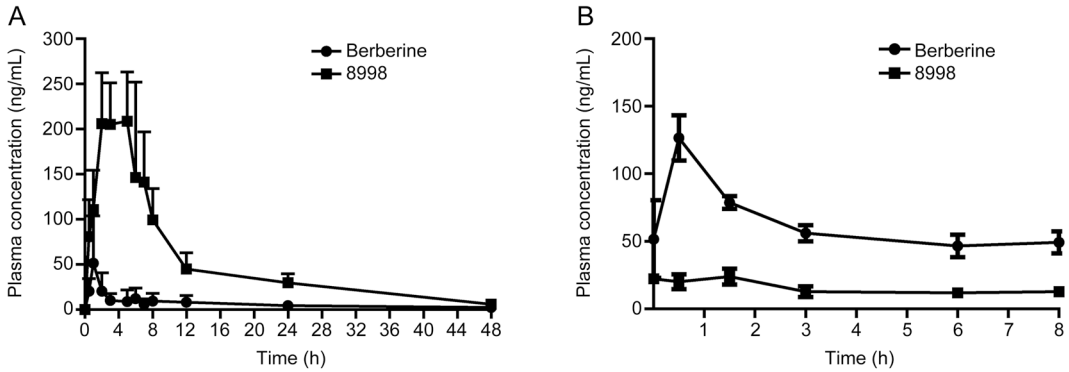

Figure 5. The mean concentration–time profile following a single oral administration of 70 mg/kg berberine and berberine8998 to SD rats.

Table 3. Pharmacokinetic parameters of berberine and berberine8998 in SD rats.

Drug	Dose (mg)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{inf} (h*ng/mL)
Berberine	70	1.5±1.0	55.1±49.3	312.4±165.2	397.6±170.4
Berberine8998	70	3.0±1.7	230.3±70.2	2418.0±644.3	2519.0±653.5

Table 4. Pharmacokinetic parameters of berberine and berberine8998 in hamsters.

Drug	Dose (mg)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{inf} (h*ng/mL)
Berberine	50	0.5±0.3	42.3±6.6	125.6±48.1	119.7±118.5
Berberine8998	50	0.6±0.2	126.9±47.2	457.2±132.9	601.7±625.4

berberine8998 exhibited increased bioavailability in SD rats compared with berberine, and the mechanisms involved require further investigation.

Reductions in total cholesterol and LDL were observed in the berberine and berberine8998 groups. The mechanisms of cholesterol lowering by berberine8998 were similar to those of berberine. Increased uptake of LDL in HepG2 cells and the upregulation of LDLR expression were observed in both treatment groups. Consistent with these findings, we found that berberine and, to a greater extent, berberine8998 stimulated the uptake of LDL in HepG2 cells, which is similar to the cholesterol-lowering agent pravastatin. The intensity (a.u.) of Dil-LDL in HepG2 cells was measured to compare the lipid uptake amounts of a positive control group (Pravastatin) to the berberine and berberine8998 groups. Western blot analyses showed that the upregulation of LDLR expression was greater in cells treated with berberine8998 than in those treated with berberine, which indicates that berberine8998 has superior lipid-lowering effects to the parent molecule *in vitro*. As shown in Figure 4E–4G, hepatic malondialdehyde (MDA) and peroxide levels were inhibited, though this difference did not reach statistical significance. The RNA levels of PGC-1α and UCP-1 expression were increased by treatment (Figure 4E–4G). ACOX1 inhibition was reported to improve hepatic lipid and reactive oxygen species metabolism[13], which was similarly observed in the present study. Therefore, inhibition of ACOX1 appears involved in the lipid-lowering effects of berberine8998.

The berberine derivatization into berberine8998 greatly improved drug absorption. This high availability of berberine8998 at least partially contributed to its lipid-lowering efficacy. The mechanisms of the absorption-improving of berberine8998 will be examined in future studies.

In conclusion, berberine8998 significantly lowered the LDL and total cholesterol levels via upregulation of hepatic LDLR expression. The reduction in TG levels in berberine8998-treated hamsters was associated with lower NEFA levels. iTRAQ proteomic studies revealed that ACOX1 was significantly inhibited in the berberine8998 treatment group. The effect of berberine8998 on TG was mediated by NEFA and hepatic ACOX1 expression. This ACOX1-regulated mechanism suppressed the TG and NEFA levels *in vivo*, which is a newly defined mechanism of the lipid-lowering effect of berberine8998. The improved bioavailability of berberine8998 is of significant importance and will be examined in future studies.

Abbreviations

LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; LDLR, LDL receptor; Dil-LDL, 1,1′-dioctadecyl-

3,3,3′,3′-tetramethylindocarbocyanine iodide labeled LDL; ACOX1, peroxisomal acyl-coenzyme A oxidase 1; NEFA, non-esterified fatty acids; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SD, Sprague-Dawley.

Acknowledgements

This work was supported by a grant from Xuhui Central Hospital, Shanghai Clinical Center and the Chinese Academy of Sciences (Shanghai, China) through support for Science and Technology Commission of Shanghai Municipality by the Foundation under grant 10431903400. This work was also supported by a grant from the Shanghai Municipal Commission of Health and Family Planning by the Foundation under grant ZK2012A39.

Author contribution

Cheng-yin YU, Chen YU, Darek C GORECKI, Asmita V PATEL and Yi-ping WANG designed the experiments; Cheng-yin YU, Gang-yi LIU, Xiao-hui LIU and Yu-zhou GUI performed the experiments; Cheng-yin YU, Hai-ming LIU, Darek C GORECKI, Asmita V PATEL and Hong-chao ZHENG analyzed the experimental results; Cheng-yin YU, Darek C GORECKI and Asmita V PATEL wrote the manuscript.

Reference

1 Yin J, Zhang H, Ye J. Traditional chinese medicine in treatment of metabolic syndrome. Endocr Metab Immune Disord Drug Targets 2008; 8: 99–111.
2 Pirillo A, Catapano AL. Berberine, a plant alkaloid with lipid- and glucose-lowering properties: From *in vitro* evidence to clinical studies. Atherosclerosis 2015; 243: 449–61.
3 Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, *et al.* Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. Nat Med 2004; 10: 1344–51.
4 Cheng Z, Chen AF, Wu F, Sheng L, Zhang HK, Gu M, *et al.* 8,8-Dimethyldihydroberberine with improved bioavailability and oral efficacy on obese and diabetic mouse models. Bioorg Med Chem 2010; 18: 5915–24.
5 Li YH, Li Y, Yang P, Kong WJ, You XF, Ren G, *et al.* Design, synthesis, and cholesterol-lowering efficacy for prodrugs of berberrubine. Bioorg Med Chem 2010; 18: 6422–8.
6 Canto JG, Iskandrian AE. Major risk factors for cardiovascular disease: debunking the "only 50%" myth. JAMA 2003; 290: 947–9.
7 Rader DJ, Daugherty A. Translating molecular discoveries into new therapies for atherosclerosis. Nature 2008; 451: 904–13.
8 Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011; 473: 317–25.
9 Li H, Chen W, Zhou Y, Abidi P, Sharpe O, Robinson WH, *et al.* Identification of mRNA binding proteins that regulate the stability of LDL receptor mRNA through AU-rich elements. J Lipid Res 2009; 50: 820–31.
10 Defesche JC. Low-density lipoprotein receptor--its structure, function, and mutations. Semin Vasc Med 2004; 4: 5–11.
11 Fass D, Blacklow S, Kim PS, Berger JM. Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. Nature 1997; 388: 691–3.
12 Jeon H, Blacklow SC. Structure and physiologic function of the low-density lipoprotein receptor. Annu Rev Biochem 2005; 74: 535–62.
13 Zeng J, Deng S, Wang Y, Li P, Tang L, Pang Y. Specific inhibition of Acyl-CoA Oxidase-1 by an acetylenic acid improves hepatic lipid and reactive oxygen species (ROS) metabolism in rats fed a high fat diet. J Biol Chem 2017; 292: 3800–9.
14 Kristensen LP, Larsen MR, Mickley H, Saaby L, Diederichsen AC, Lambrechtsen J, *et al.* Plasma proteome profiling of atherosclerotic disease manifestations reveals elevated levels of the cytoskeletal protein vinculin. J Proteomics 2014; 101: 141–53.
15 Ray S, Reddy PJ, Jain R, Gollapalli K, Moiyadi A, Srivastava S. Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. Proteomics 2011; 11: 2139–61.
16 Zhang Y, Li X, Zou D, Liu W, Yang J, Zhu N, *et al.* Treatment of type 2 diabetes and dyslipidemia with the natural plant alkaloid berberine. J Clin Endocrinol Metab 2008; 93: 2559–65.
17 Chou HC, Lu YC, Cheng CS, Chen YW, Lyu PC, Lin CW, *et al.* Proteomic and redox-proteomic analysis of berberine-induced cytotoxicity in breast cancer cells. J Proteomics 2012; 75: 3158–76.
18 Hou Q, Tan HT, Lim KH, Lim TK, Khoo A, Tan IB, *et al.* Identification and functional validation of caldesmon as a potential gastric cancer metastasis-associated protein. J Proteome Res 2013; 12: 980–90.