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

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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;
ABSTRACT

Aims
The objective of this study was to investigate the effect of a berberine derivative, berberine8998, on serum cholesterol and lipid levels in vivo and to explore the mechanisms involved.

Methods
Lipid lowering effect of berberine and berberine8998 was determined by high fat diet model on hamsters. Total cholesterol, low-density lipoprotein and triglycerides were analyzed. The potential target proteins on hamster liver were screened by iTRAQ labeled proteomics. The effect of berberine and berberine8998 on ACOX1 and ACSL1 was determined by western blot. Non-esterified fatty acids were analyzed to demonstrate its effect on triglyceride and fatty acid. Pharmacokinetics of berberine 8998 was conducted on SD rats.

Results
Berberine8998 significant lowered the total cholesterol, triglycerides (TG) and LDL-c levels in hamsters. Bioinformatics revealed that berberine and berberine8998 shared similar metabolic pathways where fatty acid metabolism was the most dominant one. Western blot validation results showed that peroxisomal acyl-coenzyme A oxidase 1 (ACOX1), Long-chain-fatty-acid—CoA ligase 1 (ACSL1) a protein involved in fatty acid metabolism, expressed differently in berberine8998 group comparing with both model group and berberine treatment group. Further biochemistry results showed that berberine8998 significantly lowered the non-esterified fatty acid (NEFA), which may
lead to the reduction of TG levels in berberine8998 treatment groups and the
difference in proteomics. Pharmacokinetic study showed that berberine8998, could
remarkably improve absorption by 6.7 times, comparing with berberine.

**Conclusion**

These findings suggest that berberine8998 lowers both cholesterol and lipid with
different mechanisms from berberine and is a promising therapeutic candidate for
treating hypercholesterolemia and obesity.

**Keywords:** Berberine8998, Proteomics, Lipid-lowering, Fatty acids.
**Introduction**

Berberine is an isoquinoline alkaloid (Fig. 1A) and the main active constituent of Coptis deltoids\textsuperscript{[1]}. In traditional Chinese medicine, berberine-containing herbs are used to treat various conditions and diseases, including diarrhea, cancer, depression, hypertension, hypercholesterolemia, and diabetes mellitus\textsuperscript{[2]}. It has also been shown to have 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; in hyperlipidemic hamsters, berberine treatment reduced serum total cholesterol (TC) by 40% and LDL by 42%\textsuperscript{[3]}.

However, the effect of berberine on triglycerides (TG) and its associated mechanisms were not fully understood. Serum TG concentrations and risk for coronary heart disease has been an issue of great interest \textsuperscript{[4]}. Fatty acids (FAs) are important metabolic substrates for energy production. Excess FAs and unesterified cholesterol are stored in lipid droplets within triacylglycerol (TG)\textsuperscript{[5]}.

LDL is a major risk factor for atherosclerosis and coronary heart disease\textsuperscript{[6-7]} and is the main target of lipid-lowering drugs\textsuperscript{[8]}. Berberine increases the abundance of LDL receptor (LDLR) \textsuperscript{[3]} in the liver by stabilizing its transcript\textsuperscript{[9]}. Upregulating LDLR expression on the hepatocyte surface by genetic or pharmacologic means has been shown to increase hepatic clearance and reduce serum levels of LDL-cholesterol\textsuperscript{[10-12]}. LDLR activity is therefore considered as a key factor determining LDL-cholesterol concentration in the systemic circulation.
Berberine and its derivatives have multiple mechanisms involved in its lipid lowering effect. Berberine reduced serum LDL-c by inducing LDLR expression in liver. Signaling pathway studies showed that berberine activated AMPK, ERK and other pathways. Berberine also up-regulated liver X receptor a (LXRα), peroxisome proliferator-activated receptor α/δ (PPARα/δ) expression and down-regulated PPARγ expression in the liver.\textsuperscript{[13]}

The isobaric tags for relative and absolute quantitation (iTRAQ) method combined with 2D LC-MS/MS is one of the most powerful methodologies in quantitative proteomics which has been applied and reviewed in a variety of reports\textsuperscript{[14, 15]}. Proteomics has been showing a great potential in mechanism research. The advent of proteomics analysis has permitted effective expression measurement of large set of samples. Therefore, proteomics may applied to analyze the mechanisms of lipid lowering effect of berberine.

Another obstacle for application of berberine in the treatment of hypercholesterolemia was its low bioavailability. The oral dose of berberine is typically 1.0 g/day (10 pills/day) which limited its clinical application\textsuperscript{[16]}. Therefore, screening of berberine derivatives will facilitate the improvement of its bioavailability.

In this study, a newly synthesized berberine derivative, berberine8998 (Fig. 1B) was well characterized. In-vivo study showed that berberine8998 significant lowered the total cholesterol, low-density-lipoprotein and triglycerides levels in hamsters while berberine only lowered total cholesterol and triglycerides. In-depth iTRAQ proteomics unveiled that peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) and
long-chain-fatty-acid—CoA ligase 1 (ACSL1), a protein involved in fatty acid metabolism, expressed differently in berberine treatment group comparing with both model group and berberine treatment group. Mechanistic study showed that berberine significantly lowered the non-esterified fatty acid (NEFA), which may lead to the reduction of TG levels in berberine treatment groups. Further pharmacokinetic study showed that berberine, could remarkably improve absorption by 7.74 times, comparing with berberine. These findings suggest that berberine lowers both cholesterol and lipid with different mechanisms from berberine and is a promising therapeutic candidate for treating hypercholesterolemia and obesity.

**Materials and methods**

**Chemical and reagents**

Berberine (purity >98%) was purchased from Northeast Pharm (China).

Berberine8998 (purity >97%) was synthesized by Youhong Hu (Shanghai Institute of Materia Medica, Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from HyClone Laboratories (Logan, Utah, USA). Fetal bovine serum (FBS) was obtained from ThermoFisher Scientific (Carlsbad, California, USA). Provastatin was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Non-esterified fatty acids

**Animals and procedures**

Hamsters, clean class at 6 weeks of age, were obtained from the Animal Center of Shanghai Institute of Materia Medica. Hamsters were randomly divided into four
groups (normal diet, model, berberine, berberine8998 groups). Berberine and berberine8998 were dissolved in CMC-Na before administered. 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, as berberine and berberine8998 respectively, n=12) daily in an intragastric manner for 3 weeks. Body weight was monitored.

At the every week in the treatment period (7, 14 and 21 days post-dose), all hamsters were fasted overnight (12 h) and blood samples, anti-coagulated with 3.8% sodium citrate (w/v), were drawn from the retro-orbital plexus. Blood samples (0.3–0.5 mL) were collected weekly from the orbits after a 16 h fasting, and centrifuged for 15 min at 3000 × g to obtain serum. Serum lipid profiles, including total cholesterol (TC), LDL cholesterol (LDL-C) and triglyceride (TG) levels, were measured using an automatic analyzer (Hitachi, Tokyo, Japan).

The animal experiments were conducted according to the National Research Council’s Guidelines. All experimental protocols and procedures were approved by the Institutional Ethical Committee of Shanghai Institute of Materia Medica.

**Protein extraction and iTRAQ reagent labeling**

To determine the protein expression differences in berberine and berberine8998 treatment groups, livers of hamsters from different treatment groups were collected, snap-frozen in liquid nitrogen, and stored at -80°C. The samples were dissolved in lysis buffer composed of 7 M urea, 2 M thiourea, 65 mM dithiothreitol, and 0.1 mM
phenylmethylsulfonyl fluoride at 4°C, and sonicated three times at 70 W for 5 s at 10 s intervals, followed by three rounds of homogenization using a whirlpool mixer at 10 min intervals. Samples were centrifuged at 20000g for 30 min at 4°C and the protein concentration in the supernatant was determined by the Bradford assay, with BSA used to generate the calibration curve.

Trypsin digestion and iTRAQ labeling were carried out. Liver proteins (150 µg) from each hamster were reduced and alkylated and then 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 applied to the 12 digested protein samples.

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

Mixed peptides were fractionated by strong cation exchange chromatography on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) using an polysulfoethyl column (Nest Group, Southborough, MA, USA) with the following dimensions: 2.1 × 100 mm, 5 µm, and 300 Å. Mixed peptides were desalted with a Sep-Pak Cartridge (Waters, Milford, MA, USA), diluted with loading buffer (10 mM KH₂PO₄ 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 mM KCl. Peptide separation was carried out using a linear binary gradient of 0%–50% Buffer B in Buffer A at a flow rate of 200 µL/min for 1 h. Absorbance at 214 and 280 nm was monitored, and 30
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 reversed-phase column (Agilent Technologies, Santa Clara, CA, USA) with the following dimensions: 0.1 × 15 mm, 5 µm, and 300 Å with a gradient of 5%–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. The 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 therefore of the 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.

**Bioinformatic 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 by removing redundant hits. MS/MS data were searched with UniProt. The rat database was used since the hamster database was not available. The precursor and iTRAQ fragment tolerance values were set to 100 ppm and 0.6 Da. Parameters for data analysis were: sample type = iTRAQ (peptide-labeled); Cys alkylation = methyl methanethiosulfonate; digestion = trypsin;
instrument = time-of-flight 5600 ESI; species = RAT; ID focus = biological modifications; database = Swissprot Rat (35672 entries).

To minimize the occurrence of false positive results, a decoy database search strategy was adopted to estimate the FDR < 1% for peptide and protein identification. At least one peptide with a confidence interval of 95% was included. Data were considered reliable for P < 0.05 and error factor < 2. The P value from the 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 downregulated by berberine or berberine8998 treatment. The signaling pathways of proteins were identified by searching the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html). MATLAB (MathWorks, Natick, MA, USA) was used for mathematical modeling and for analyzing KEGG pathways.

**Western blotting of the candidate protein**

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 mM Tris-HCl, 150 mM 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. After
washing, membranes were incubated with anti-mouse IgG (1:2000; Bio-Rad) at room
temperature for 2 h, followed by detection with a Clarity Enhanced
Chemiluminescence Western Blot Substrate kit (Bio-Rad). Densitometry was carried
out using Quantity One software (Bio-Rad). The ratio of gray values of target proteins
represented the relative expression levels for each group. The experiment was
repeated three times.

**Measurement of NEFA levels**

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

**Measurement of Dil-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% O2 and 5% CO2 at 37 ºC at a density of 10^6 cells
per mL. HepG2 cells were incubated with Dil-LDL (Biotium, Hayward, CA, USA)
and the following test compounds: pravastatin (positive control, 5 μM) (Sinopharm,
Shanghai, China), berberine (40 µM), or berberine8998 (20 or 40 µM). 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 then 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). Hepatic hydrogen peroxide level was measured by a hydrogen peroxide testing kit (Beyotime Biotech., Haimen, China). 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, 180-200g, n=10, provided by the Animal Center of Shanghai Institute of Materia Medica) were randomly divided into two groups. After twelve hours of food deprivation, rats were administered berberine and berberine8998 (50 mg/kg, free base) intragastrically (i.g). The washout interval between two treatment periods was 7 days after the final blood collection. After the washout period, rats were given the alternating compound in the second period. Sample collection protocol was identical in two treatment periods. Blood samples (0.5 mL) were collected pre-dose and at 0.5 h, 1 h, 2 h, 3 h, 5 h, 6 h, 7 h, 8 h, 12 h, 24 h, 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 the
collection time. Serum samples were fractionated by chromatography on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) using an reverse phase column (CAPCELL PAK C18, 2.0perfor. 5hromatogralical data was processed by Phoenix® WinNonlin® 6.3 to calculate pharmacokinetics parameters.

Similar procedures were adopted in Syrian hamsters. Syrian hamsters (clean class, 90~120g, n=6, provided by the Animal Center of Shanghai Institute of Materia Medica) were randomly divided into two groups. After twelve hours of food deprivation, hamsters were administered berberine and berberine8998 (50 mg/kg, free base) intragastrically (i.g). Blood samples (0.5 mL) were collected pre-dose and at 0.5 h, 1.5 h, 3 h, 6 h, 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 were randomized to receive berberine (50mg/kg/d), berberine8998 (50mg/kg/d) 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) was analyzed every week.

Hypercholesterolemia was developed slowly in the normal diet group (Figure 2A-2C) while serum lipid levels in the high fat diet (HFD) model group were significantly
increased (Figure 2A-2C). Notably, berberine treatment (50 mg/kg/d) significantly reduced total cholesterol (Figure 2A) and LDL-c (Figure 2B) on week 2 and week 3, compared with the HFD group ($P< 0.05$). In particular, berberine did not alter the LDL-c levels in three weeks treatment (Figure 2B) while berberine attenuated LDL-c levels on week 2 and week 3 ($P<0.05$). Mean levels of week 2 and week 3 were 10.30±3.63 and 13.15±7.34 (nM, Mean± SD) for HFD and 6.00±1.85, 8.55±2.59 (nM, Mean± SD) for berberine (50mg/kg) groups, respectively (Figure 2B).

Given that triglyceride (TG) is an important component of lipid, the serum TG levels in hamsters was evaluated with bio-chemistry analyzer. TG levels had similar changes to TC and LDL-c. The results showed that berberine ameliorated the elevated serum TG levels induced by high fat diet. TG was decreased by 64% after using berberine (Fig. 2C). Triglyceride levels were significantly attenuated on week 3 in both berberine ($P< 0.05$) and berberine groups ($P< 0.01$) (Figure 2C). Body weight was not significantly different among treatment groups, model and normal diet groups as shown in Figure 2D.

**GO analysis indicates similar cellular locations in berberine and berberine treatment group**

Hamster liver samples from the berbreine, berberine and high fat diet model groups were analyzed by iTRAQ shot-gun proteomics. High-abundant proteins were depleted before the 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%. Upon combing results of the two replicates, a total of 2444 proteins were
identified and 2073 proteins were quantified.

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

**KEGG analysis indicates similar metabolic pathways in berberine and
berberine8998 treatment group**

A total of 442 differentially expressed proteins were screened in both runs in
berberine8998 group and a total of 268 differentially expressed proteins were
screened in both runs in berberine group. In order to filter the non-significant changes
in liver protein expression level between sample groups, fold change in median
ratios < 0.7 (down-regulated) or > 1.3 (upregulated) were selected as cutoff values
and the ratio was assessed with the Student’s t test and the median value of the ratio
between two groups was also calculated. In berberine8998 group, a total of 48
proteins were determined as significant changes, were 16 upregulated, and 32 were
downregulated. In berberine group, a total of 53 proteins were determined as
significant changes, 23 were upregulated (Table 2), and 30 were downregulated. The
top ten of the most significant changes in protein expression after berbrine8998 and berberine treatment were summarized in Table 1 and Table 2.

KEGG metabolic pathways were analyzed to determine the major pathways involved in berberine and berberine8998 treatment. P values were determined for pathways represented in the treatment groups (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 significantly metabolic pathway was fatty acid metabolism. These results suggested that berberine8998 and berberine lowered the serum lipids through 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. According to bioinformatics analysis, fatty acid metabolism was the most significant pathway involved in the lipid lowering effects. Therefore, we selected two of the differentially expressed proteins, ACOX1 and ACSL1, for validation by western blotting. ACOX1 and ACSL1 expression in the liver was down regulated in berberine and berberine8998 treatment groups as compared to the control group (Fig. 4A). The protein expression of ACOX1 and ACSL1 significantly decreased by berberine 8998 treatment by approximately 2~3 fold. The protein expression of ACOX1 and ACSL1 significantly decreased by berberine treatment by approximately
1~2 fold. Therefore, the magnitude of ACOX1 and ACSL1 protein expression varied in berberine and berberine8998 treatment groups.

ACOX1 involves in the β-oxidation of fatty acid metabolism. Given to the reduction of TG levels \textit{in vivo} (Figure 2C), serum non-esterified fatty acid levels were analyzed. Serum NEFA levels exhibited similar changes (4.07 vs. 4.93 mmol/L; P < 0.05; Fig. 4B) when compared with high fat diet group. These results suggest that berberine8998 reverses the increase in serum TG and NEFA levels induced by a high-fat diet.

To further unveil the associated mechanisms of lowering serum LDL levels with berberine8998, a Dil-LDL uptake experiment was conducted in HepG2 cells. Berberine8998 (40 µM, 2.26) dose-dependently stimulated in LDL uptake in HepG2 cells comparing with berberine (2.26 vs 1.22 P<0.01) (Fig. 4C). Since the LDL-uptake is mediated by LDL receptor in HepG2 cell, we evaluated LDLR expression in HepG2 cells by western blotting. As shown in Fig 4D, LDLR was upregulated dose-dependently by treatment with berberine and berberine8998, with the latter inducing a greater effect. These results further confirm that berberine8998 promotes the uptake of LDL by increasing the expression of LDLR.

To unveil the mechanisms involved in the effect of ACOX1 on fatty acid metabolism. Hepatic malondialdehyde (MDA) and peroxide levels were analyzed. Besides, mitochondrial fatty acid oxidation was analyzed by PGC-1α and UCP-1 expression.

The results showed that hepatic malondialdehyde (MDA) and peroxide levels was inhibited though not significant. The RNA levels of PGC-1α and UCP-1 expression were increased through treatment (Figure 4E, 4F and 4G).
Berberine8998 significantly improved bioavailability in SD rats and hamsters

The pharmacokinetics of berberine8998 and berberine was further evaluated in SD rat. The mean serum concentration versus time profile was presented in Fig. 5. The principal pharmacokinetic parameters of berberine and berberine8998 are summarized in Table 3. The maximum concentration of berberine and berberine8998 in serum ($C_{\text{max}}$) was 55.11 ng/mL and 230.3 ng/mL. The half-life of berberine and berberine8998 in serum ($t_{1/2}$) was 29.18 h and 10.97 h. The area under curve ($\text{AUC}_{0-48}$) was 312.4 ng/mL·h and 2418 ng/mL·h for berberine and berberine8998, respectively. These results suggested that the bioavailability of berberine was significantly improved by 6.7 times in SD rats.

Similar results were also observed in hamsters. As shown in Figure 5B and Table 4. The bioavailability of berberine was significantly improved by 3.6 times in hamsters.

Discussion

In this research, proteomes profiles of high fat hamster liver tissues samples with or without berberine8998 were simultaneously compared. It was the first time that iTRAQ method was coupled with 2D LC-MS/MS (TripleTOF 5600) to investigate the mechanisms of how berberine and berberine8998 produce their action. Two differentially expressed proteins (ACOX1 and ACSL1) identified by the proteomics approach were further selectively validated using western blotting and biochemical
analysis. The protein expression differences may be result from the improvement of bioavailability of berberine8998.

The results of berberine8998 on serum lipids were determined for the first time and was compared with berberine. The results in Figure 2 illustrated a significant reduction in LDL, total TC and TG levels while berberine took effect on TC and TG, with a weaker extent. In order to elucidate the possible mechanisms involved in this improvement, the function, the interplay and the changes in abundance of proteins are in response to internal and external cues[15]. The iTRAQ method combined with two-dimensional LC–MS/MS was used to analyze protein expression in the liver tissue of mice maintained on a high-fat diet treated with 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 were remained unknown. This is among the most powerful methodologies in quantitative proteomics[18]. Using the iTRAQ method, in berberine8998 group, a total of 48 proteins were determined as significant changes, were 16 upregulated, and 32 were downregulated. In berberine group, a total of 53 proteins were determined as significant changes, 23 were upregulated, and 30 were downregulated.

The GO analysis showed that proteins that were differentially expressed in the treatment relative to the control group had similar cellular locations; the KEGG pathway enrichment suggested that fatty acid metabolism were specifically activated by the treatment. The hepatic ACOX1 expression was significantly lower in
berberine8998 group. Western blotting assay results confirmed berberine8998-induced change in levels of ACOX1 and other related proteins. These results suggested that berberine8998 could reduce serum NEFA levels by modulating ACOX1 expression. With iTRAQ proteomics, the effect of berberine8998 on TG was mediated by NEFA and hepatic ACOX1 expression. NEFA was down-regulated by berberine8998. This ACOX1-regulated mechanism suppresses the in-vivo TG and NEFA levels, which is a newly defined mechanism of berberine8998 in its lipid lowering effect. Further mechanism studies revealed that berberine8998 enhanced hepatic LDL uptake by increasing LDLR expression. Consistent with the lipid lowering effect, berberine8998 up-regulated bio-availability in SD rats comparing with berberine, and the mechanisms involved may require further investigations. The reduction of total cholesterol and LDL were observed in both berberine and berberine8998. The mechanisms of cholesterol lowering by berberine8998 were similar with that of berberine. The uptake of LDL in HepG2 cells and the upregulation of LDLR expression were observed in both berberine 8998 and berberine 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 screened to compare the lipid uptake amount of positive control group (Pravastatin), berberine group and berberine8998 group. The western blot analysis showed that the upregulation of LDLR expression was greater in cells treated with berberine8998 as compared to berberine, indicating 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 was inhibited though not significant. The RNA levels of PGC-1 α and UCP-1 expression were increased through treatment (Figure 4E, 4F and 4G). The inhibition ACOX1 was reported to improve hepatic lipid and reactive oxygen species metabolism[13], which was similar in this study. Therefore, inhibition of ACOX1 involved in the lipid lowering effects of berberine8998.

Berberine derivative berberine8998 could greatly improve the absorption of berberine by 7.74 times (AUC$_{0-48}$ of berberine8998 / AUC$_{0-48}$ of berberine). High availability of berberine8998 contributed at least in part of its lipid lowering efficacy. The mechanisms of absorption improving effect of berberine8998 will be explored in future.

In conclusion, berberine8998 significantly lowered LDL, total cholesterol levels in hamsters via upregulation of hepatic LDLR expression. The reduction of TG levels in berberine 8998 treated hamsters was associated with lower NEFA levels. The iTRAQ proteomic studies revealed that ACOX1 was significantly inhibited in berberine8998 treatment group. The effect of berberine8998 on TG was mediated by NEFA and hepatic ACOX1 expression. This ACOX1-regulated mechanism suppresses the in-vivo TG and NEFA levels, which is a newly defined mechanism of berberine8998 in its lipid lowering effect. The improvement of bioavailability in berberine8998 will be explored in the future.
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Author contribution

Chengyin Yu, Chen Yu, Darek C Gorecki, Asmita V. Patel and Yiping Wang designed experiments; Chengyin Yu, Gangyi Liu, Xiaohui Liu and Yuzhou Gui carried out experiments; Chengyin Yu, Haiming Liu, Darek C Gorecki, Asmita V. Patel and Hongchao Zheng analyzed experimental results. Chengyin Yu, Darek C Gorecki and Asmita V. Patel wrote the manuscript.
Reference:


10 Defesche JC. Low-density lipoprotein receptor--its structure, function, and mutations. Semin Vasc Med 2004; 4: 5-11.


Tables

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

Table 2. List of differentially expressed proteins after berberine8998 treatment in hamsters. The proteins were identified by TOF-AB 5600. (↑, up-regulated; ↓, down-regulated).

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

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

Figure legends

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

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).

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.

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 NEFAs. Hamsters were maintained on a normal or high-fat diet. In high fat diet hamsters, one group received no treatment, while the other two groups were treated with berberine
or berberine8998 (50 mg/kg each). (C) Average uptake amount of Dil-LDL in HepG2 cell incubated with test compounds. Pro, pravastatin; Berberine; Berberine8998. (D) Western blot analysis of LDLR expression level. Neg (blank); POS, (pravastatin 5uM); BBR (Berberine 2uM and 10uM); 8998 (Berberine8998 2uM and 10uM). (E) MDA levels. (F) Peroxide levels (G) Hepatic RNA expression of PGC-1α and UCP-1. All results are from three independent experiments.

Figure 5. The mean concentration–time profile following a single oral administration of 70 mg/kg berberine and berberine8998 to SD rats
Figure 1

A

B

106x176mm (300 x 300 DPI)
Figure 2

A. Total Cholesterol (nmol/L)

B. LDL Cholesterol (nmol/L)

C. Triglyceride (nmol/L)

D. Body Weight (g)

Legend:
- High Fat Diet
- Berberine 9998
- BBR
- Normal Diet

Different letters indicate significant differences among groups.

146x119mm (300 x 300 DPI)
Figure 4

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>High fat diet</th>
<th>Berberine8998</th>
<th>Berberine</th>
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<tbody>
<tr>
<td>ACS1 78kD</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ACOX1 50kD</td>
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<tr>
<td>β-actin 42kD</td>
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</table>

B

Non-esterified fatty acid (mM)

C

Fold of Control

D

<table>
<thead>
<tr>
<th>BBR (μM)</th>
<th>8998 (μM)</th>
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</thead>
<tbody>
<tr>
<td>Neg</td>
<td>2</td>
</tr>
<tr>
<td>Pos</td>
<td>2</td>
</tr>
</tbody>
</table>

LDLR

GAPDH

E

MDA Conc.

F

Hydrogen Peroxide

G

RNA expression

229x331mm (300 x 300 DPI)
Figure 5

A

B

82x37mm (300 x 300 DPI)
Appendix I

A

B

C

242x390mm (300 x 300 DPI)