Berberine8998, a new derivative of berberine, improves hyperlipidemia through additional mechanisms

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I. Abstract

Berberine (BBR), an herbal originated compound, has lipid-lowering activity. However, its low bioavailability and poor absorption characteristics have limited its clinical application. The aim of this research study was to investigate the lipid lowering effect of a novel berberine derivative, namely berberine8998, and its properties and underlying mechanisms.

For the purpose of this study, the pharmacodynamics of berberine8998 was evaluated on a hamster model of hypercholesterolemia by a high-fat diet. Our results showed that, compared with the other structurally related derivatives of the berberine group, berberine8998 significantly lowered the serum lipid levels in hamsters. Berberine8998 stimulated the uptake of low-density lipoprotein (LDL) in HepG2 cells in a dose-dependent manner. Isobaric tags for the relative and absolute quantitation (iTRAQ) labeling proteome methods was applied, using a TripleTOF 5600 mass spectrometer to compare the pharmacological basis of both berberine and berberine8998. The mechanisms of the actions of these two compounds were investigated by identifying and quantifying the differences in the proteins that were expressed (with respect to the untreated animals) in the liver tissues of hamsters treated with the compounds. The result of proteome study showed that peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) and long-chain fatty acid—CoA ligase 1 (ACSL1), involved in fatty acid metabolism, were regulated by using berberine and berberine8998. Moreover, western blot validation results showed that ACOX1 and ACSL1, which are 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. Furthermore, a pharmacokinetic study was applied to investigate the absorption of two formulations of berberine8998.

These findings suggested that berberine8998 lowered cholesterol and lipid levels via

different mechanisms from berberine, and its improved absorption made it a promising therapeutic candidate for the treatment of hypercholesterolemia and obesity.

II. Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Chengyin Yu

III. Acknowledgement & Recognition

The success and outcome of this project required a lot of guidance and assistance from many people and I am extremely fortunate to have this all along the completion of my project. Whatever I have done is only due to such guidance and assistance and I would not forget to thank them.

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IV. Quotation

Look deep into nature, and then you will understand everything better.

Albert Einstein

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VI. Abbreviations

2D LC-MS/MS, Two-dimensional liquid chromatography-tandem mass spectrometry

ACN, Acetonitrile

ACOX1, Acyl-CoA oxidase 1

ACSL1, Acyl-CoA synthetase long-chain family member 1

Apo, Apolipoprotein

BSA, Bovine serum albumin

CoA, Coenzyme A

DiI, 1,1-Dioctadecyl-3,3,3,3-tetramethylindocarbocyanine iodide

DMEM, Dulbecco's Modified Eagle's Medium

FA, Fatty acid

FBS, Fetal bovine serum

GO, Gene Ontology

HDL, High density lipoprotein

HPLC, High-performance liquid chromatography

iTRAQ, Isobaric tag for relative and absolute quantitation

LDL, Low density lipoprotein

LDLR, Low density lipoprotein receptor

MS, Mass spectrometry

NEFAs, non-esterified fatty acid

RP, Reversed-phase

SCX, Strong cation exchange

1. General Introduction

1.1 Atherosclerosis

At present, the high incidence of atherosclerosis related cardiovascular disease imposes an enormous burden on healthcare systems. Atherosclerosis is a chronic disease of the arterial wall, and is characterized by intimal deposition of lipid in the arteries and its progression into atherosclerotic plaques. The progression of atherosclerosis may cause acute myocardial infarction, stroke and peripheral vascular diseases (Hansson, 2005, Libby et al., 2011). It is the primary cause of coronary artery and cerebrovascular diseases, two of the leading causes of death and morbidity worldwide (Vishram, 2014, Draisci et al., 2001). Lipoproteins that contain apolipoprotein B (apoB) — for example, low-density lipoproteins (LDL) —are the major risk factors for atherosclerosis (Orso and Schmitz, 2017). Experiments and other studies on humans (Hansson, 2005, Libby et al., 2011, Orso and Schmitz, 2017, Vishram, 2014) found that atherosclerosis occurs at sites in the arterial tree where laminar flow is disrupted by atherogenic lipoproteins like LDL. In particular, LDL levels are clearly correlated with the risk of cardiovascular events in humans, as well as individual susceptibility to atherosclerosis and its complications. High plasma LDL levels and modifications, such as oxidation, glycosylation, carbamylation and glycoxidation, have been experimentally shown to be proatherogenic both *in vitro* and *in vivo* (Alique et al., 2015). The arterial wall gradually thickens to form an atherosclerotic plaque, resulting in narrowing of the artery lumen. The process can be summarized into four stages: 1. The initial stage of atherosclerosis includes the entrance of LDLs into tunica intima that is lined by a monolayer of endothelial cells. There they are modified by oxidation or enzymatic activity, and aggregate within the extracellular intimal space, thereby increasing their phagocytosis by macrophages (Fig 1.1a). 2. The migration of vascular smooth muscle cells (SMCs) contribute to the lesion progression by secreting large amounts of extracellular-matrix components like collagen (Fig 1.1b). 3. Plaque macrophages and SMCs can die in the advancing lesions, resulting in release of cellular debris and crystalline cholesterol.

Moreover, SMCs can form a fibrous cap that enables blood coagulation components to come into contact with tissue factors in the plaque's interior (Fig 1.1c). 4. If the plaque does not rupture and the lesion continues to grow, the infiltrating inflammatory cells can interact with the intrinsic arterial cells, promoting lesion formation, etc. The lesion can in turn encroach on the lumen, so that the blood supply to the organ is reduced, resulting in a clinically obstructive disease of (mostly) the heart or brain (Fig 1.1d). Plaques can rupture abruptly, causing blood clots, and often myocardial infarction or stroke (Stary, 2000).

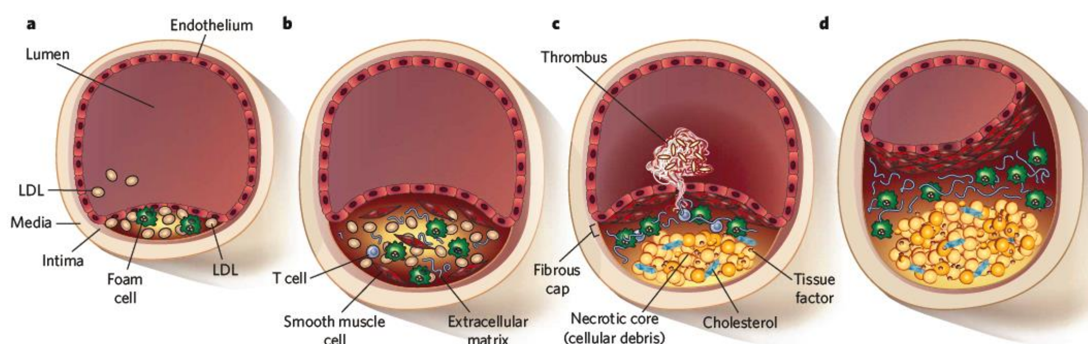

Figure 1.1 Initiation and progression of atherosclerosis. A lesion begins as a fatty streak (a), and develop into an intermediate lesion (b), and then into a lesion that is vulnerable to rupture (c), and finally into an advanced obstructive lesion (d) (Rader and Daugherty, 2008)

However, in the development of atherosclerosis, the pathogenesis of plaques and the progressions of the lesion are highly complex processes, and many aspects of the mechanistic links between lipids and atherogenesis remain unclear. The main approach to threat atherosclerosis is to alleviate hypertension and hyperlipidemia and/or control hemostasis, so that thrombotic complications can be avoided (Weber and Noels, 2011). Observational data and experimental results support the argument that LDL is the major cholesterol-carrying lipoprotein in plasma, and therefore responsible for many forms of coronary heart diseases (Fig 1.2) (Nabel, 2003). After administration of lipid lowering drugs, cardiovascular events as well as progression of atherosclerosis in humans are significantly reduced, regardless of the baseline LDL concentrations (Orso and Schmitz,

2017). Numerous lipid-lowering therapies have established that reducing LDL levels lowers the risk of developing clinical multiple cardiovascular diseases, including atherosclerosis (Weber and Noels, 2011, Nabel, 2003, Lusis, 2000). Accordingly, LDL is the main target in lipid-lowering therapies for cardiovascular diseases and its lowering is the key strategy.

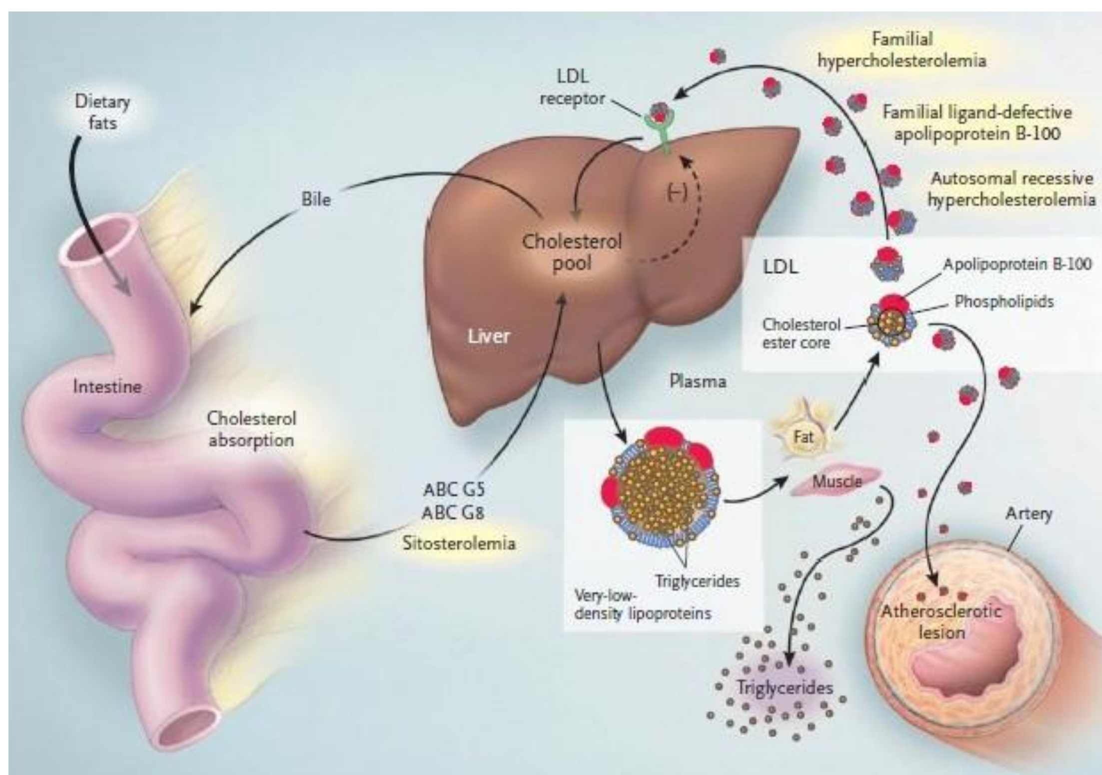

Figure 1.2 Synthesis, metabolism and deposition of cholesterol leading to atherosclerotic lesions by LDL (Nabel, 2003)

1.2 LDLR target lipid lowering drugs (statins)

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, collectively known as statins (Table 1.1), are the most commonly prescribed agents for lipid lowering because of their high efficacy in reducing LDL and excellent tolerability and safety (Simoens and Sinnaeve, 2013). The development of statins has revolutionized the treatment of cardiovascular disease such as atherosclerosis.

Table.1.1 Statins market shares in Belgium (Simoens and Sinnaeve, 2013)

Ranking	Generic Name	Company	Trade Name	Source
1	Atorvastatin	Pfizer & Daiichi Sankyo	Lipitor, Torvast	Synthesis
2	Rosuvastatin	Astra Zeneca & Chiesi	Crestor	Synthesis
3	Simvastatin	Merck & Co EMS	Zocor, Lipex	Natural extraction
4	Pravastatin	Daiichi Sankyo & Nichi-Iko	Pravachol, Selektine, Lipostat	Natural extraction
5	Fluvastatin	Novartis	Lescol, Lescol XL	Synthesis

Studies of familial hypercholesterolemia characterized by very high LDL levels and early coronary heart disease have helped to unravel the pathways regulating plasma cholesterol metabolism. Such understandings are important for the development of cholesterol-lowering drugs. The most convincing evidence for a potential therapeutic target is when a human genetic condition from simple mendelian genetics is associated with an altered risk of the disease. An example is homozygous familial hypercholesterolemia, which is caused by mutations in the gene encoding the low-density lipoprotein receptor (LDLR). The observation that this disease is associated with markedly premature atherosclerosis led to the understanding that increased concentrations of LDL cholesterol in plasma can cause atherosclerosis, and the concept that increasing LDL-receptor expression can reduce LDL concentrations and thus the risk of atherosclerosis.

Statins exert a pleiotropy of anti-inflammatory actions, improve endothelial function and reduce plaque lipids and thrombogenicity, thereby limiting and stabilizing atherosclerotic plaques (Ray and Cannon, 2005). It can inhibit HMG-CoA reductase,

the enzyme that catalyzes the intermediate (mevalonate) which is the rate-limiting step in cholesterol biosynthesis (Endo et al., 1977). Statins also inhibit hepatic synthesis of apolipoprotein B-100 and decrease the synthesis and secretion of triglyceride-rich lipoproteins (Grundy, 1998).

Simultaneously, the primary mechanism for LDL lowering is an accelerated clearance of LDL and LDL precursors from the circulation via LDL receptors (LDLR). The resulting reduction in hepatocyte cholesterol concentration triggers SREBP-2, stimulation of which increases expression of hepatic LDLR (Raal et al., 1997). These lipid lowering mechanisms of statins are illustrated in Fig 1.3.

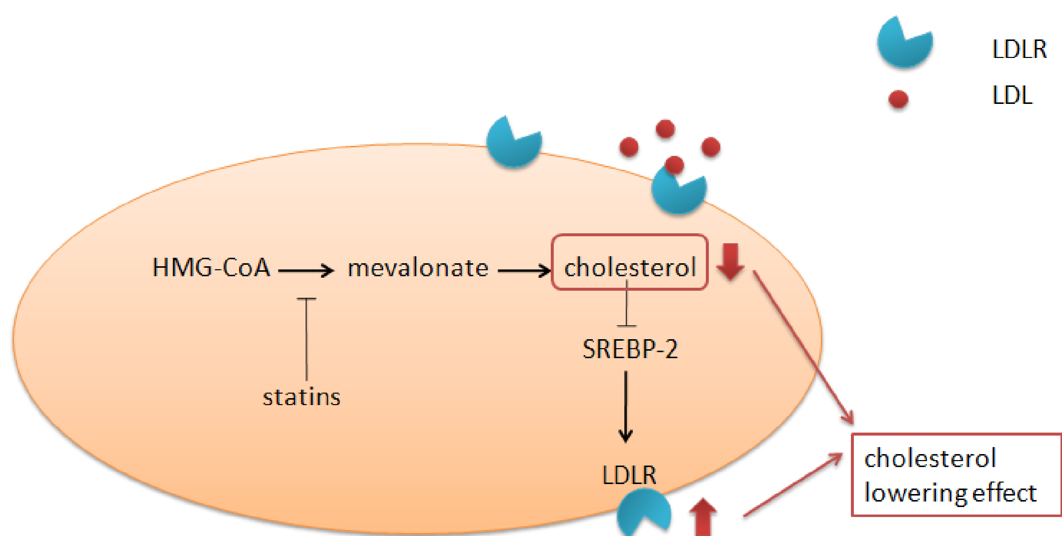

Figure 1.3 The lipid lowering mechanisms of statins

Statins can effectively lower serum LDL levels and modestly raise serum HDL levels. Cholesterol-lowering potency varies between different statins: third-generation statins such as atorvastatin or rosuvastatin are more effective than earlier generation statins, such as the off-patent and less expensive agent simvastatin or pravastatin (Simoens and Sinnaeve, 2013). The comparative efficacy and potency of statins on lipids and lipoproteins in patients without hypertriglyceridemia are shown in table 1.2. In general, LDL is reduced by an additional 7 percent with each doubling of the dose (Maron et al., 2000). Recently there is some concern that statins are not always well tolerated, and

there are known differences in safety. The major adverse effect of statins is myopathy, defined as muscle pain or weakness associated with an elevated serum level of creatine kinase (Sacks et al., 1996). Symptoms may include fever and malaise, and some cases have been associated with elevated serum statin drug levels (Pierce et al., 1990).

In contrast, statins have a number of pleiotrophic beneficial effects leading to reduction in inflammation, slowing the progression of atherosclerosis, and plaque stabilization. At least one-quarter of the high-risk patients who received intensive statin therapy have LDL levels above the guideline mandated goals (Pierce et al., 1990). New biological targets such as Proprotein convertase subtilisin/kexin type 9 (PCSK9) have emerged, and they could lead to incremental lowering of LDL levels to a greater degree than that achieved by high-dose statin therapy.

Table 1.2 Comparative efficacy of six statins on the percentage LDL and HDL changes in patients without hypertriglyceridemia (Maron et al., 2000)

Statin Drug, mg					Change in LDL and HDL	
Atorvastatin	Simvastatin	Lovastatin	Pravastatin	Fluvastatin	LDL	HDL
	10	20	20	40	（+）27%	（+）4-8%
10	20	40	40	80	（+）34%	（+）4-8%
20	40	80			（+）41%	（+）4-8%
40	80				（+）48%	（+）4-8%
80					（+）55%	（+）4-8%

1.3 Development of PCSK9 target lipid lowering drugs

The discovery of Proprotein convertase subtilisin kexin type 9 (PCSK9), initially called NARC1 (Neural Apoptosis Regulated Convertase1), in 2003 and the demonstration of

its unique functional activity to degrade LDL-R has made it a subject of intense research for possible treatment of hypercholesterolemia as an alternative to statin and other drugs (Seidah et al., 2003). Increased level of PCSK9 functional activity leads to an accumulation of cholesterol in the blood - a high risk factor for cardiovascular disease (Basak et al., 2012). Treatment with PCSK9 Inhibitors can reduce cardiovascular inflammation and its consequences (Bonaventura et al., 2017).

Genetic studies have shown that mutations in the gene that encodes PCSK9 augment LDLR levels on cell surfaces, thereby boosting LDL clearance as well as yielding lower serum LDL concentration, and is associated with a form of autosomal dominant hypercholesterolemia (Cohen et al., 2006). Individuals with loss-of-function variants in PCSK9, who are exposed to lower levels of LDL from childhood than those with the common genotype for this enzyme, seem to be protected from atherosclerotic events even when they have other cardiovascular risk factors (Brown and Goldstein, 2006). PCSK9 is thus a highly attractive target for reducing LDL-cholesterol concentrations. Mechanistic studies show that PCSK9 functions by reducing the amount of LDLR at the cell surface of hepatocytes. More specifically, after catalysing its own cleavage, PCSK9 is secreted and binds to the cell-surface LDLR, thereby targeting them for degradation rather than recycling (Horton et al., 2007). A patch of residues in the catalytic domain of PCSK9, that binds to the extracellular domain of the LDLR, is located in the first epidermal growth factor-like repeat homology domain (EGFA) of the LDLR and is closely associated with the molecular interactions within the PCSK9-LDLR complex (Zhang et al., 2007). PCSK9 binds to LDLR by two possible routes, one extracellular, where PCSK9 exits the cell and binds LDLR on the cell surface. The second route may involve an intracellular route where PCSK9 enters the endosome directly from the golgi and binds to LDLR, and then rerouting it to the lysosome, thereby enhancing receptor degradation (Bottomley et al., 2009). Critical observations have provided evidence that increase of PCSK9 disrupts the normal recycling of the LDLR, consequently increasing the serum LDL levels (Lambert et al., 2012). The above

mentioned findings promise PSCK9 to be a potential therapeutic target, and have prompted biopharmaceutical companies to develop interventions for lowering serum LDL levels. (Fig 1.4)

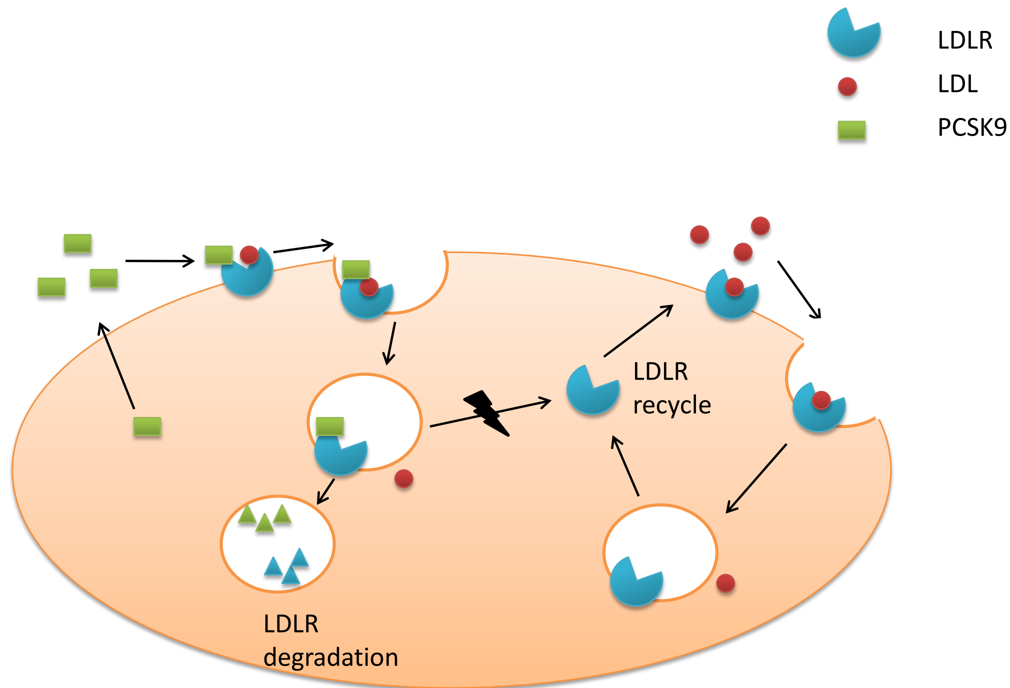

Figure 1.4 Mechanism by which PSCK9 redirects degradation of LDLR. (See text for details)

In recent years, studies have greatly supplemented our understanding of the pathological role of PCSK9 in human biology. Clinical development programs for monoclonal antibodies against PCSK9 have advanced rapidly with completion of comprehensive phase 1 and 2 trials with both REGN727/SAR236553 (REGN727) and AMG 145 (Stein and Swergold, 2013). Completed phase 2 trials in more than 1,500 patients have confirmed the advantage of early lowering of LDL efficacy and demonstrated potential added benefit from reduction of lipid levels.

1.4 Pharmacology of berberine

1.4.1 New perspectives of berberine

Berberine is a quaternary ammonium salt belonging to the protoberberine group of benzylisoquinoline alkaloids (Fig 1.5) and the main active constituent of *Coptis chinensis* French (Yin et al., 2008). As a traditional medicine or dietary supplement in China, some prescriptions contain large concentrations of berberine because in conjunction with its long history of treatment in clinical practice, people have always believed that it has very low toxicity and superior effects for treating many diseases. The berberine-rich herb has been used for the treatment of many chronic and acute diseases including diarrhea, cancer, depression, hypertension, hypercholesterolemia, and diabetes mellitus, as recorded in many ancient Chinese traditional medicinal works (Hashemzaei et al., 2017, Pirillo and Catapano, 2015, Zhang et al., 2008).

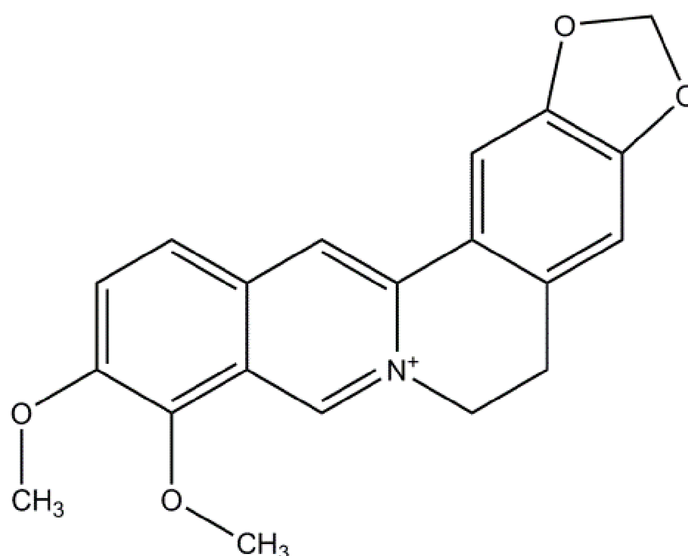

Figure 1.5 Chemical structure of berberine

It has also been shown to have significant lipid-lowering activity. Treatment of hypercholesterolemia patients with orally administered berberine reduced serum levels of cholesterol, triglycerides, and low-density lipoprotein (LDL) by 29%, 35%, and 25%, respectively. For hyperlipidemic hamsters, berberine treatment reduced serum total

cholesterol (TC) by 40% and LDL by 42% (Kong et al., 2004). Berberine has also shown some promise of curing type 2 diabetes and dyslipidemia (Zhang et al., 2008).

1.4.2 Molecular pathways of how berberine regulates LDL

As mentioned, LDL is a major risk factor for atherosclerosis and coronary heart disease, and is the main target of lipid-lowering drugs (Nabel, 2003, Canto and Iskandrian, 2003). Several mechanisms have been proposed for explaining the cholesterol-lowering effects of berberine. Berberine increases the abundance of LDL receptor (LDLR) in the liver by stabilizing its transcription (Kong et al., 2004, Li et al., 2009). LDLR is responsible for the binding and cellular uptake of apolipoprotein (apo)B- and apoE-containing lipoproteins; dysregulation of this process results in hypercholesterolemia. Upregulation of LDLR on the hepatocyte surface by genetic or pharmacological means has been shown to increase hepatic clearance and reduce serum levels of LDL-cholesterol (Defesche, 2004, Fass et al., 1997, Jeon and Blacklow, 2005). The level of LDLR expression is therefore considered as a key factor determining LDL-cholesterol concentration in the circulation.

Berberine also increases the abundance of hepatic LDLRs by reducing the level of PCSK9 which binds the EGFA of the LDLR at the hepatocyte surface, thereby promoting their lysosomal degradation (Cameron et al., 2008, Zhang et al., 2007). Berberine treatment may have also revealed multiple mechanisms by which this compound exerts its cardioprotective effects. Moreover, the advent of proteomics analysis has permitted effective expression measurement of large sets of samples.

1.4.3 Pharmacokinetic studies of berberine8998

There are problems associated with the use of berberine. The hyperlipidemia patients have to take 1 g/ day or above to lower the lipid levels. Patients compliance can play a

role (Zhang et al., 2008). Pharmacokinetic study in animal models has revealed that berberine has a poor bioavailability profile, which means that a relatively high dose is necessary in order for berberine to yield its efficacy *in vivo* (Cheng et al., 2010). To address this problem, the berberine derivatives dihydroberberine (Cheng et al., 2010) and berberrubine (Li et al., 2010) have been synthesized, but their absorption profiles turned out to be limited. Accordingly, there is a need for berberine derivatives having better bioavailability profiles.

1.5 Proteomic research

1.5.1 Protein extraction and sample preparation.

General cell lysis methods for protein extraction are listed in Table 1.3. Lysis buffer contained a chemotropic agent, a reducing agent, a buffer solution and a surface active agent (Ahmed, 2009).

Table 1.3 Methods for performing cell lysis and their impact on the level of breakdown

Lysis intensity	Methods
Low	Osmosis schizolysis, Enzymatic digestion, Eradicator schizolysis
Middle	Homogenate, Freeze-thawing circles, Mechanical crushing
High	Stress break, Bead grinding, Sonication crushing

For archival of formalin-fixed, paraffin-embedded (FFPE) tissues, the protein extraction required tissue boiling in a retrieval solution of Tris-HCl containing 2% SDS (Shi et al., 2006). To extract integral membrane proteins (IMPs), the use of 1-butyl-3-methyl imidazolium tetrafluoroborate (BMIM BF4) cloud significantly improved the large-scale identification of IMPs (Sun et al., 2011).

Systematic detection of low-abundance proteins that may be putative disease

biomarkers is complicated by an extremely wide range of protein abundances. Hence, depletion of major proteins is one potential strategy for enhancing detection sensitivity. Commercialized HPLC columns could be used to remove the high abundant proteins in the blood (Echan et al., 2005). Another method is based on using protein equalizer technology to sharply reduce the concentration of the most abundant components, while simultaneously enhancing the concentration of the most dilute species (Boschetti and Righetti, 2008, Righetti et al., 2006). The Proteo Miner (Bio-Rad) can be applied in the protein equalizer arena as a non-depleting tool for discovering low-abundance species. Digestion and depletion of abundant proteins also can improve proteomic coverage (Fonslow et al., 2013, Fonslow et al., 2014).

Protein digestion is a critical and time consuming step. The current conventional in-solution or in-gel protein digestion has usually been performed using trypsin in a buffered medium overnight, which is considerably slower and limits large scale proteome analysis. New approaches such as microwave assisted technology and pressure cycling technology could accelerate the protein digestion process (Lopez-Ferrer et al., 2008, Lin et al., 2008).

Using multiple proteases for large-scale mass spectrometry-based proteomics on complex samples could improve both protein identification and protein characterization. This method relies on the combined use of different proteases (Table 1.4) to enzymatically digest complex protein mixtures to generate a collection of peptides for mass spectrometric analysis (Swaney et al., 2010). Multiple proteases approach could increase the chances for successful identification of peptides but it is limited to discovery of proteins.

Table 1.4 Summary of proteases cutting sites

Proteases	Proteases cutting site
Trypsin	C terminus of K,R (last amino acid is not P)
Endoproteinase Lys-C	C terminus of K
Endoproteinase Arg-C	C terminus of R

Endoproteinase Asp-N	N terminus of R
Endoproteinase Glu-C	C terminus of E

1.5.2 Column separation strategy for the recovered peptides

For bottom-up proteome analyses of digested peptides obtained from a proteome sample, it is by now generally considered that at least two dimensions of separation (2D LC) are needed to cover a proteome in reasonable depth. The 2D LC separation is usually performed using a SCX column in series with a RP column for reducing the complexity and dynamic range of the samples (Davis et al., 2001). In the course of the analysis, tryptic peptides are eluted stepwise by injecting salt plugs of increasing ionic strength from the SCX column in the first dimension. In the second dimension these peptides are first trapped on a RP enrichment column and finally separated on an analytical RP column by nanoscale liquid chromatography (nanoLC) systems (Yamana et al., 2013). Many different configurations have been developed to optimize RP nanoLC. The most basic design consists simply of the analytical column operating at nanoliter flow rates where a few microliters of the sample are directly loaded onto the column (Masuda et al., 2011, Thakur et al., 2011). By combining two different LC techniques with individual peak capacities between 50 and 100, it should theoretically be possible to achieve total capacities of 2500–10,000 peaks (Nagele et al., 2004).

1.5.3 Conditions for mass detection

In the past three decades, startling new developments in two ionization methods — matrix-assisted laser desorption (MALDI) and electrospray (ESI) — have been introduced by Karas et al. and by Fenn et al., respectively (Karas and Hillenkamp, 1988, Fenn et al., 1989). Their work, which won the Nobel prize in 2002, demonstrated that

these techniques, under appropriate experimental conditions, have high sensitivity and wide mass range, extending to hundreds of thousands of daltons and beyond (Costello, 1997). Thus, these ionization methods can be applied to proteomics studies. MALDI ions source analysis usually requires off-line separation strategy.

Peptide sequencing is the key step in commonly employed approaches within mass spectrometry-based proteomics. The current method of choice for peptide sequencing for MS/MS spectra is collision induced dissociation/collision activated dissociation (CID/CAD). Compared with traditional, ion-trap-based collision-induced dissociation (CID), a more recent method of fragmentation, namely HCD (higher energy collisional dissociation), provides beam type CID tandem MS with detection of fragment ions at high resolution in the orbitrap mass analyzer (Jedrychowski et al., 2011). Development of alternative fragmentation techniques such as electron transfer dissociation (ETD) has extended the possibilities within tandem mass spectrometry (Frese et al., 2011). Moreover, electron-driven dissociation methods, namely by electron capture dissociation (ECD) or electron transfer dissociation (ETD) and their application in phosphor peptide analysis, have also been evaluated (Boersema et al., 2009). These techniques are useful for in large-scale proteomics studies.

The most common fragment spectrum acquisition mode is data dependent acquisition (DDA), which is a serial process of peptide selection and fragmentation. During a DDA experiment, fragmentation spectra are acquired by sequentially isolating and fragmenting peptide ions providing that certain criteria (set *a priori*) related to the precursors have to be satisfied (Shliaha et al., 2013). The fragment ion (MS2) spectra for selected precursor ions detectable in a survey (MS1) scan are generated (Domon and Aebersold, 2006). There is another approach, so called data independent acquisition (DIA) method, which is to fragment a group of precursors covering a mass window of specific size in the cyclic recording throughout the LC time range (Gillet et al., 2012).

1.5.4 The development of labelled peptides proteomics techniques

The small molecule compound exerts its pharmacological effects through binding to proteins directly or affecting its transcription, translation, post-modification indirectly. Therefore, a technique to elucidate these effects may be needed to profile the proteins that involved in its biological effects. Proteomics, which studies proteins on large scale, is a research hotspot in this century (Tubaon et al., 2017). In this type of analysis, MS based isotope labelling proteomic technology is applied for identification and determination of concentration ratios of proteins expressed in animal tissues of different states, especially with respect to effect of a drug against absence of the drug, to understand the function of various proteins.

The first MS-based isotope labeling proteomic technology was ICAT (Gygi et al., 1999). It was quickly commercialized for quantitative proteomics of cells and tissues. The approach of ICAT involves cysteine specific tagging of intact proteins, proteolytic digestion, ICAT peptides labelling, chromatographic separation followed by MS analysis (Yu et al., 2004). However, it has not been widely used because of limitation in determining the cysteine free proteins.

In recent years, the development and commercialization of the alternative chemical labelling method ICPL harnesses NHS ester groups to derivatise primary amino groups in intact proteins. The major advantage of ICPL (in contrast to ICAT) is its reliance on the generally abundant lysine residues in proteins, which results in multiple labelling sites and increasing in MS sensitivity for the labelled peptides (Sarioglu et al., 2006, Wiese et al., 2007).

In summary, the technique involves a reporter group, which is used to identify the amino acids within the digested peptides by means of MS analysis. The iTRAQ reagents attach to stable isotopes, providing derivatives of the digested peptides. The iTRAQ technology also exploits, similar to ICPL, the link to the NHS ester, forming a derivative with the reporter group (Ross et al., 2004). One advantage of having isobaric mass design of the iTRAQ reagents is that differently labelled peptides appear as single peaks

in the MS scans, thus reducing the possibility of peak overlapping (Wiese et al., 2007). There are eight different iTRAQ reagents available, and hence a comparative analysis of a set of two to four samples is feasible within a single MS run. More importantly, the iTRAQ approach is more reliable and effective on cation exchange chromatography separation. The labelled peptides in ICPL, ICAT and iTRAQ techniques all require separation by two-dimensional LC. Adequately labelled peptides digested from each protein are then used to evaluate the results and summarized for analysis of the proteins using statistical methods based on peptide quantization information (Molina et al., 2005).

1.6 Research hypothesis

Berberine8998 (Fig1.6) is a novel berberine derivative. The hypothesis of this study is that berberine 8998 exerts greater LDL-c and TG lowering effect than berberine in vivo. Furthermore, we hypothesize that berberine shares similar mechanisms with berberine in their LDL-c lowering effect. The mechanism of its TG lowering effect will be examined via proteomics.

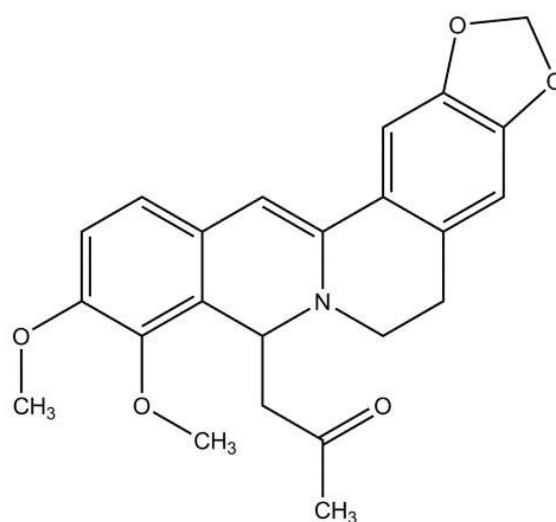

Figure 1.6 Chemical structure of berberine8998

1.7 Research aims and experiment design

1.7.1 Research aims

In the present study, a novel berberine derivative, namely berberine8998, with a view to preparing a compound that will have better pharmacological and bioavailability profile. The aims cover the following:

1. Investigate the acute oral toxicity (Lethal Dosage 50) of berberine8998
2. To evaluate the *in vivo* lipid lowering effects in a hamster model of hypercholesterolemia induced by a high-fat diet.
3. To compare the *in vitro* lipid uptake promoted by berberine and berberine8998 in HepG2 cell line, as determined by the amount of Dil-LDL uptake.
4. Explore the molecular pharmacology mechanisms of berberine and berberine8998 using proteomic methods.
5. Establish a LC/MS/MS method to analyse serum levels of branched amino acids.
6. Measure serum levels of NEFA and validate related expressed proteins.
7. Determine the serum concentration of berberine by LC/MS/MS
8. Compare different formulations of berberine8998.

1.7.2 Experiment design

The main experimental protocol (not including the aims 1, 2, 6 and 8) is shown in Fig 1.7. The pharmacodynamic effects of berberine and berberine8998 were evaluated by measuring the weight, as well as the serum TC, TG, LDL and the non-esterified fatty acids (NEFA) levels in hamsters that were divided into untreated control, berberine and berberine8998 treated groups. Liver tissue samples from hamsters were also collected and the different expression of proteins was analyzed by isobaric tags for the relative and absolute quantitation (iTRAQ) to determine the mechanisms of action of the two

compounds. 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. Western blot analyses were applied to evaluate the LDLR expression.

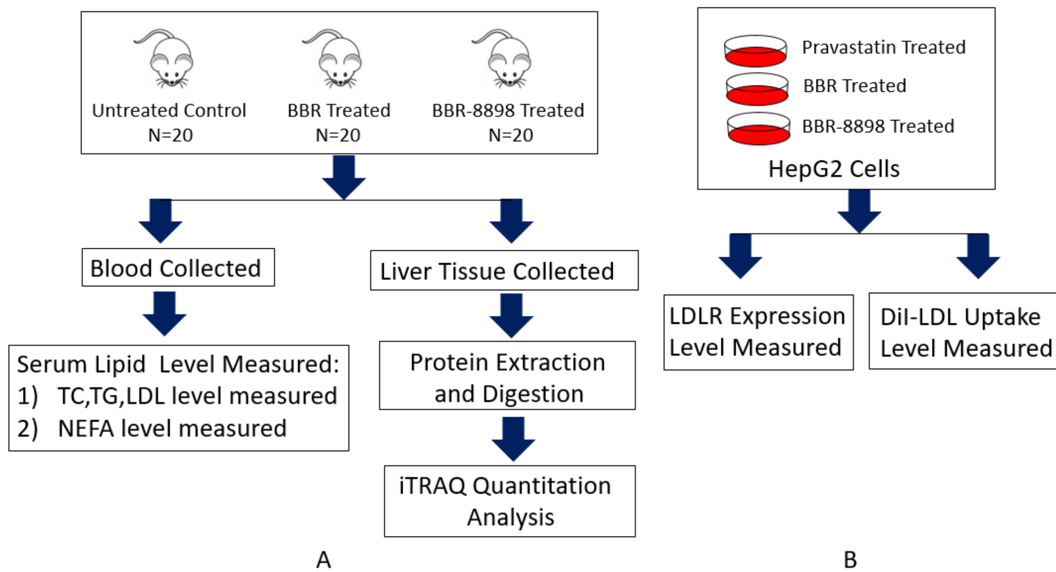

Figure 1.7 Schematic representation of the main experimental protocol. A) Blood and tissue analyses of untreated and BBR- and BBR-B8898-treated hamsters. B) Cell-based analyses of untreated and BBR- and BBR-8898-treated HepG2 cells.

2. Materials and Methods

2.1 Chemicals, regents and instruments

2.1.1 Chemicals and regents

Product name (alphabetical)	Supplier
A	
Amino Acid Analyzer RP C18 Column	AB SCIEX Inc
AA 45/32 amino acid amounts kit	AB SCIEX Inc
Acetonitrile (HPLC grade)	DIKMA Co Ltd
Anti-LDLR	Abcam
Anti-PCSK9	Abcam
Anti-ACOX1	Abcam
Anti- ACSL1	Abcam
B	
BCA assay	Beyotime Ltd
BerberineShifangJubang	Plant Materials Co Ltd.
Berberine8998 (derivative of berberine)	Shanghai Institute of Materia Medica
Protein assay dye reagent concentrate	Bio-Rad
BSA (Bovine Serum Albumin)	Merck Inc
C	
Carvedilol (Internal Standard)	Salutas Pharma GmbH
CAPCELL PAC C18 2.0×100mm，5μm	Thermo Co Ltd
C18 4.0×3.0mm，5μm	Phenomenex Co Ltd
D	
DiI (1,1'-dioctadecy l-3,3,3',3'–tetramethylindocarbocyanine perchlorate)	BiotiumInc
Dialysis bag	Spectrum Co Ltd
Dimethylcarbinol (HPLC grade)	DIKMA Co Ltd
DMEM (Dulbecco's Modified Eagle Medium)	Life (invitrogen) Inc
DMSO (Dimethyl sulfoxide)	AmerscoInc
F	
FBS (Fetal Bovine Serum)	Life (invitrogen) Inc
Fenofibrate	ShangqiuChemry Chemicals Co Ltd
Formic acid (HPLC grade)	CWN Co Ltd

H

High density lipoprotein cholesterol assay kit	Wako Pure Chemical Industries Ltd

I

Isoflurane	Sinopharm Chemical Reagent Co Ltd
Isopropanol	Sinopharm Chemical Reagent Co Ltd
iTRAQ reagent	AB SCIEX Inc

L

Low density lipoprotein cholesterol assay kit	Wako Pure Chemical Industries Co Ltd
NEFA-HA Test kit	Wako Pure Chemical Industries Co Ltd

P

Polysulfoethyl column	Nest Group Inc
PVDF membrane for protein blotting	BioRadInc

S

Sep-Pak Cartridge	Waters Inc
SCX chromatography	The Nest Group Inc
Sodium carboxymethyl cellulose (CMC-Na)	Sinopharm Chemical Reagent Co Ltd

T

Total cholesterol assay kit	Wako Pure Chemical Industries Co Ltd
Triglyceride assay kit	Sichuan Maker Biotechnology Co Ltd
Trypsin	Sigma Aldrich Inc

Z

Zorbax 300SB-C18 reversed-phase column	Agilent Technologies Inc

All the other regents/chemicals were purchased from Sinopharm Chemical Reagent Co Ltd

2.1.2 Instruments

(1) Name: Mass Spectrometer

Model: Applied Biosystems API 3000™ LC/MS/MS System

Manufacturer: Applied Biosystems Co. Ltd.

(2) Name: HPLC system

Model: SIL-HTc Autosampler (X1);

LC-20AD Pump (X2);

DGU-20A3; Auto Degassing apparatus (X1);

CTO-20A column heater (X1)

Manufacturer: Shimadzu Co. Ltd.

(3) Name: Computer

Model: Intel (R) Pentium (R) 4 CPU 3.20/3.19GHz, 1.00GB of RAM

Manufacturer: DELL Co. Ltd

(4) Name: Balance

Model: CP225D

Manufacturer: Sartorius Co. Ltd

(5) Name: Centrifuge

Model: MIKRO 22R (with 1195-L rotor)

Manufacturer: Hettich Co. Ltd

(6) Name: Turbine mixer

Model: Vortex-2

Manufacturer: Scientific Industries Co. Ltd

(7) Name:Pipettor

Model: Eppendorf Research (0.5-10μL，10-100μL，20-200μL，100-1000μL)

Manufacturer: Eppendorf Co.Ltd

(8) Name: Magnetic Stirrers

Model: RCT basic IKAMAG

Manufacturer: IKA

(9) Name: Automatic Biochemical Analyzer

Model: 7020

Manufacturer: Hitachi (China), Ltd.

(10) Name: Filter Unit

Model: Millex-GP, 0.22 μm, polyethersulfone, 33 mm, gamma sterilized

Manufacturer: Millipore Ireland, Ltd.

(11) Name: Ultracentrifuge

Model: CP100MX

Manufacturer: Hitachi (China), Ltd.

(12) Name: Microplate Reader

Model: Spectra Max M2e

Manufacturer: Molecular Devices, Ltd

(12) Name: Electrophoresis tank

Model: Mini-Protean Tetra System

Manufacturer: Biorad, Ltd

(13) Name: Semi-dry transfer unit

Model: Trans-Blot SD Cell

Manufacturer: Biorad Ltd

(14) Name: Electrophoresis Imaging Cabinet

Model: Chemi DOCTM XRS+

Manufacturer: Biorad Ltd

(15) Name: HPLC system (For 2.5.2)

Model: 20AD HPLC system

Manufacturer: Shimadzu Inc

(16) Name: Nano LC system (For 2.5.2)

Model: Ekspert™ nanoLC 415

Manufacture: Eksigent Technologies Inc

(17) Name: Mass Spectrometer (For 2.5.3)

Model: TripleTOF 5600 mass spectrometer

Manufacturer: Applied Biosystems Co. Ltd

(18) Name: Mass Spectrometer (For 2.6.1)

Model: AB 3200 QTRAP

Manufacturer: Applied Biosystems Co.Ltd.

2.2 Animals

2.2.1 Mice

Male and female 8-week old C57BL/6 mice (50 males and 50 females, weighing 18-22 g) were obtained from Shanghai Institute of Materia Medica (SIMM), Chinese Academy of Science. The animals were kept at standard housing conditions (22±2°C, 50-60% humidity with a range of 40-80% and 12/12 light/dark cycle). They were fed with standard laboratory chow (normal diet: MO2-type Feed, Table 2.1) and water *ad libitum*.

Table 2.1 The nutrient and other content of MO2-type Feed (Lys: lysine, Met: methionine, Cys: cysteine)

MO2	Water	protein	Fat	ash	fiber	Ca	P	Lys	Met&Cys	other	Kcal/g
%	9.7	20.5	4.62	6.2	4.35	1.23	0.91	1.3	0.68	50.51	3.45

2.2.2 Hamsters

Golden hamsters (*Mesocricetus auratus*, 16 weeks old, weighing 80-100g) were obtained from SIMM. The animals were kept at standard housing conditions (22±2°C, 50-60% humidity with a range of 40-80% and 12/12 light/dark cycle). Two dietary regimens were used in this study: hamster fed a standard laboratory chow (normal diet: MO2-type Feed) and water ad libitum, and left to acclimatize before the experiment. During the experiment, selected group of hamsters were fed a high-fat diet (high-fat diet: MO-2type Feed with 20% lard).

2.2.3 Sprague Dawley (SD) Rats

SD Rats (*Rattus norregicus*, 8 weeks old, weighing 200-250g) were obtained from SIMM. The animals were kept at standard housing conditions (22±2°C, 50-60%

humidity with a range of 40-80% and 12/12 light/dark cycle). They were supplied with standard laboratory chow (normal diet: MO2-type Feed, Table 2.1) and water ad libitum.

2.3 Cell culture

2.3.1 HepG2 cell

HepG2 cell cultures (ATCC® HB-8065™) were originally derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma. They are a suitable *in vitro* model system for the study of polarized human hepatocytes. In this study, HepG2 were seeded into either 12-well or 24-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, and 0.1 mM nonessential amino acids (Gibco, Grand Island, NY). Cells were grown for 72 h at 37 Celsius Degree in an atmosphere of 5% CO_2 in a humidified incubator.

2.4 Pharmacological studies of berberine8998

2.4.1 Acute oral toxicity study of berberine8998

2.4.1.1 Preliminary experiment

Berberine8998 powder (99%) was obtained from Youhong Hu of the SIMM group and the preliminary study was aimed at determination of the acute oral toxicity median Lethal Dosage (LD_{50}) for this compound. The value of the median Lethal Dosage (LD_{50}) stands for the acute oral toxicity of test compounds. All the mice were raised to acclimatize for 2 weeks before the experiments. To explore the possible LD_{50} range for berberine8998, 20 mice were randomly divided into 5 groups. Each group contained at least 2 female mice and 2 male mice. The weights of these mice are recorded in Table 2.2.

Table 2.2 Body weight of mice utilized in the preliminary experiment to determine the LD_{50} for berberine8998.

Group No	Female (g)	Female (g)	Male (g)	Male (g)
1	21.5	20	27.8	24.5

2	19.2	19.5	22.9	22.4
3	19.1	19.5	23.6	25.9
4	20.9	19.4	23.6	26.8
5	17.8	18.7	24.7	25.7

Table 2.3 Dosage used for the five groups included in the preliminary experiment.

Group No	Dosage(mg/kg)
1	1000
2	800
3	600
4	500
5	400

After being without dietary exposure for 16 h, intragastric administration of 5 different berberine8998 in CMC-Na formulations were administrated orally using a stainless steel feeding tube. The gavage process was performed under light anesthesia using isoflurane. The dose of 5 groups were randomly set up from 400mg/kg to 1000mg/kg.

2.4.1.2 Further acute toxicity analysis of berberine8998

The preliminary experiment indicated the LD_{50} value to be between the maximum 1.0mg/g and the minimum 0.5mg/g. Subsequently, a second experiment was conducted with 25 male mice and 25 female mice randomly divided into five groups (Group A, B, C, D and E) (n=10, 5 male and 5 female in each group). All mice were fasted overnight (12 h) before treatment. Mice were administered intragastrically (i.g) with 500 mg/kg (Group A), 595 mg/kg (Group B), 707 mg/kg (Group C), 841 mg/kg (Group D) and 1000 mg/kg (Group E), respectively. The dose number was depend on the death number of preliminary experiment. The number of animals dead was recorded after 24 h of treatment. The suspected death reason of mice was intestinal obstruction.

All animal experimental procedures were carried out in accordance with international guidelines for care. The uses of laboratory animals were approved by the Institutional Ethical Committee of Shanghai Institute of Materia Medica. IACUC Number: 2014-08-WYP-18

2.4.2 DiI-LDL uptake by HepG2 cells via Low Density Lipoprotein Receptors (LDLRs)

2.4.2.1 Preparation of Phosphate Buffered Saline (PBS) and Dialysate

PBS is a water-based salt solution containing sodium chloride, sodium phosphate, and, in some formulations, potassium chloride and potassium phosphate are also included. The buffer's phosphate groups help to maintain a constant pH. To prepare PBS solution, 7.9g NaCl, 0.2g KCl, 0.24g KH_2PO_4 and 1.8g K_2HPO_4 were weighted and dissolved in 800ml distilled water. Then the solution was transferred to a 1L volumetric flask. Distilled water and HCl were added to that solution to provide a final volume of 1L and pH of 7.4.The PBS solution (0.01M) was stocked at 4℃.

Tris Dialysate is a solution used to remove ions (NaBr) out of the serum. Serum is placed in a dialysis bag surrounded by dialysate. Diffusion then occurs, whereby the ions move out of the serum into dialysate. To prepare the Tris dialysate, 2.42g Tris, 8.5g NaCl and 0.2g EDTA were weighted and dissolved in 800 ml distilled water. Then the solution was transferred to a 1L volumetric flask. Distilled water and HCl were added dropwise to adjust the pH to 7.4, providing a final volume of 1L. The Tris dialysate (0.02M) was stored at 4℃in a fridge until required.

2.4.2.2 Preparation of Low Density Lipoprotein (LDL) and Lipoprotein Deficient Serum (LPDS)

LDL is one of the five major group of lipoproteins, which in order of size, largest to smallest, are chylomicrons, Very Low Density Lipoprotein (VLDL), Intermediate

Density Lipoproteins (IDL), LDL, and High Density Lipoprotein (HDL), that enable transport of multiple different fat molecules, including cholesterol, by the water around the cells as well as in the water-based bloodstream.

LPDS is usually prepared by ultracentrifugation, all of the lipoproteins are removed and extensively dialyzed. The resultant LPDS is membrane filtered and packaged aseptically. Each fraction is evaluated in Human Skin Fibroblast or other cells in culture with LDL and [I^{125}] LDL (or DiI-LDL and DiO-LDL) to determine the degree of up regulation of LDL receptors. Typical fractions led to a 3 to 4 fold increase in [I^{125}] LDL binding. The LPDS is not diluted and the typical protein concentration for the bovine LPDS is 75 -120 mg/ml and for the human LPDS it is ~50mg/ml.

The human serum was purchased from Shanghai Xuhui Central Hospital. First, it was defrosted from -20℃ to 4℃. The density of the serum was adjusted to 1.019 g/ml by addition of NaBr. It was moved to a centrifugal tube (Hitachi PA40) and centrifuged at 250000g, 4℃ for 4 hours. The top layer of the serum, which contained chylomicron and VLDL was removed. NaBr was added to the rest to adjust the density to 1.063 g/ml. Then the sample was centrifuged at 250000g, at 4℃ for 24 hours. The orange layer of the serum contained LDL.

The density of other serum containing LPDS was adjusted to 1.1g/ml by adding NaBr. To separate the remaining LDL and IDL, the sample was centrifuged at 250000g, at 4℃ for 18 hours and the top layer removed. Then the serum density was raised to 1.21 g/ml and centrifuged at 250000g, at 4℃ for 48 hours. The top layer of the serum was HDL. The bottom layer of the serum contained LPDS.

The LDL serum and the LPDS serum components were separately put into two dialysis bags (5kD pore size). The dialysis bags were placed in a beaker containing Tris dialysate (0.02M) for 48 hours to remove NaBr (the Tris dialysate was changed every 8 hours). Then the dialysis bags were moved to a beaker containing PBS (0.01M) for another 48 hours. After that process, the LDL serum and the LPDS serum were filtered using filter (0.22μm). Finally, they were stored at 4℃ until required.

2.4.2.3 Preparation of Dil-LDL

DiI, also called D282, is a fluorescent lipophilic in docarbocyanine dye, with an 18-carbon-long alkyl hydrocarbon tail, and a 3-carbon bridge between the indolinenuclei. It is used for scientific staining purposes, such as single molecule imaging, fate mapping, and neuronal tracing. Dil was used to label LDL by adding 15 mg/ml Dil suspended in DMSO to LDL in LPDS (volume ratio 1:2) with gentle mixing. The suspension was mixed end-over-end for 18 h at 37℃ in the dark. The density of the suspension was increased by the addition of 1.063g KBr/ml of suspension and centrifuged at 250000g for 24 h at 10℃. DiI-LDL was removed from the top of the tube, dialyzed against PBS (0.01M), and stored at 4℃ in the dark until use.

2.4.2.4 Labeling HepG2 with Dil-LDL

HepG2 cells were obtained from Shanghai Institutes for Biological Sciences (SIBS). These cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 24 hours. To improve the expression of LDLR, the cells were treated with DMEM supplemented with 2% FBS for another 24 hours, and thereafter were incubated with Dil-LDL in DMEM.

2.4.2.5 Determination of Dil-LDL uptake by HepG2 cells

(1) Standard Curve: Using Dil-LDL (Section 2.4) and physiological saline to prepare different concentrations of Dil-LDL (0ng/ml, 100ng/ml, 200ng/ml, 400ng/ml, 800ng/ml, 1200ng/ml, 1600 ng/ml). Using Microplate Reader (Molecular Devices, Spectra Max M2e, Exciting light: 520nm, Emitted light: 570nm) to determine these seven concentration with two replicates to provide the standard curve of Dil-LDL.

(2) Method to determine the of Dil-LDL uptake: The culture medium in a 24-well plate was removed. Cell were left in the 24-well plate. 0.4% BSA (dissolved in PBS w/v) was used to wash the plates three times. Fluorescence microscopy was applied to take a photo of the plates. Then isopropanol (0.5ml each well) was added to extract Dil-LDL taken up by the HepG2 cells. The 24-well plate were placed on an incubating shaker for 20 min., then the isopropanol (containing Dil-LDL) was centrifuged at 1960 g for

15 min. The 200μl of the top layer was recovered and transferred to the fluorescence detector (Exciting light: 520nm, Emitted light: 570nm).

(3) Calculation: First, the total Dil-LDL endocytic content of HepG2 was determined at 37℃ for 2-3h and labeled as A. Then the Dil-LDL bound to the cell membrane of HepG2 cells was determined at 4℃ for 2-3h and labeled as B. The amount of Dil-LDL uptake is equal to A minus B. All the determinations used a fluorescence detector with the methods as described above.

2.4.3 Serum lipid level monitoring in hamsters

2.4.3.1 Preparations of Sodium Carboxyl Methyl Cellulose (CMC-Na) formulations

(1) Fenofibrate formulation (30 mg/ml):A solution of CMC-Na (100ml 0.5%) was mixed with fenofibate powder (3g).

(2) Berberine and berberine derivative formulations:

(60 mg/ml): A solution of CMC-Na (80ml 0.5%) was mixed with 4.8 g berberine powder. The mixture was stirred using a magnetic stirrer for 12 hours.

(30 mg/ml): as above but mixed with 3 g berberine8998 powder.

(15 mg/ml): as above but mixed with 1.5 g berberine8998 powder.

2.4.3.2 Experimental procedure to determine the effect of berberine and berberine8998 on lipid metabolism in hamsters

In the preliminary study, a high-fat diet induced dyslipidemic hamster model was used to study the lipid lowering effects of berberine and berberine8998. Hamsters were randomly divided into six groups with 10 hamsters in each group. These were:

Group 1 (negative control: high-fat diet),

Group 2 (positive control: high-fat diet + 50mg/kg fenofibrate),

Group 3 (berberine8998 50mg: high-fat diet + 50mg/kg berberine8998),

Group 4 (berberine8998 100mg: high-fat diet + 100mg/kg berberine8998),

Group 5 (berberine 200mg: high-fat diet + 200mg/kg berberine) and

Group 6 (control on normal diet).

All groups of hamsters with the exception of Group 6 were fed with a high-fat diet (15% lard, 0.2% cholesterol and MO2 feed) for a total of 4 weeks.

After being fed the high-fat diets for two weeks, fenofibate (30 mg/ml), berberine8998 (15 mg/ml and 30 mg/ml) and berberine (60 mg/ml) were given (i.g.) to the appropriate groups for the following three weeks via intragastric administration. Food and water were changed every day. Blood samples (0.3-0.5 ml) were collected weekly by the retro-orbital bleeding of the hamsters maintained without dietary exposure for 16 h. Blood samples were centrifuged at 3000g for 15 min to collect the serum sample. Total cholesterol, LDL, triglyceride and HDL were analyzed by Automatic Biochemical Analyzer (Hitachi 7020). At the end of the experimental period, the hamsters were sacrificed, their livers were harvested and cut into small pieces (approximately 3 x 3 mm) and then immediately separately frozen at -70℃ for further studies (e.g., Western blot analysis).

After the preliminary study, it was considered necessary (clarification provided in Chapter 5, Section 5.1.1) to modify the arrangement of the hamster groups: Group 1 (model: high-fat diet), Group 2 (berberine8998: high-fat diet + 50mg/kg berberine8998) and Group 3 (berberine: high-fat diet + 50mg/kg berberine). Group 4-6 were replicates of Groups 1-3. However, this was later found to be unnecessary since the same was undertaken for 2 weeks of feeding on high-fat diet in the preliminary experiment. Unless otherwise stated, the other experimental procedures were the same as in the preliminary study detailed above.

All animal experimental procedures were approved by the Ethical Review Committee for the use of laboratory animals of the Shanghai Institute of Materia Medica and carried out in accordance with international guidelines for care.

2.4.4 Western blot analysis

2.4.4.1 Constituents of the buffers

(1) 1x RIPA Buffer: 50 mMTris, 150 mMNaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100 or NP40.

(2) 1x PBS Buffer: 137 mMNaCl, 2.7 mMKCl, 2.7 mM Na_2HPO_4, 2.7 mM KH_2PO_4, pH 7.4

(3) 1.5 M Tris buffer (pH 8.8): 90.68 g Tris-HCl to ddH_2O, adjust pH to 8.8 using HCl and to final volume 500ml

(4) 1.0 M Tris ype of samplebuffer (pH 6.8): 60.58 g Tris-HCl to ddH2O, adjust pH to 6.8 using HCl and to final volume 500ml

(5) 10% APS: 100 mg AP in 1 ml ddH2O. Prepared before use.

(6) 10% SDS: 10 g SDS in 100 ml ddH2O.

(7) 1x Tris-Glycine running buffer: 25 mM Tris, 230 mM Glycine (pH 8.3), 0.1% SDS.

(8) 3x SDS Protein loading buffer: 150 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 30 mM EDTA and 0.2%

(9) 1x TBST: 25 mM Tris (pH 7.5): 0.15 M NaCl, 0.05% Tween-20, 0.001% Thimerosal

(10) 1x Transfer Buffer: 3 g Tris, 14.4 g Glycine and 200 ml methanol, add ddH2O to 1L

2.4.4.2 Sample preparation

(1) Preparation of Hype of epG2 cell samples

Firstly, the supernatant was removed. The cells were then washed with1X PBS to remove residual media. 400 µL-1 ml 1X RIPA buffer/100 mm was added to the cells in a culture dish. The dish was incubated on ice for 5-10 min. Then the cells were scraped completely and transfer to pre-chilled 1.5 ml microtubes on ice before being homogenized (sonicated 15s) thoroughly and centrifuged at 1960g at 4℃for 10-15 min. The supernatant was collected for use. Total protein recovered was stored at -20℃ until needed.

(2) Preparation of hamster liver samples

The livers were chopped into small pieces. 500-600 μL pre-chilled 1x RIPA buffer/100 mg tissue was added. The tissue was homogenized using Pellet pestle motor (Axygen), before being sonicated for 10s and then centrifuged at 1960g at 4℃ for 15-20 min. The supernatant was collected for use. Total protein recovered was stored at -20℃ until needed.

2.4.4.3 Protein quantification

(1) Bicinchonic acid (BCA) assay

The protein concentration within the various extracted samples was determined by the means of the bicinchonic acid assay kit (BCA assay, Beyotime Ltd.). 1-20 μL of the protein samples were used for each measurement in triplicate. Protein standards comprised of bovine serum albumin (BSA) in the range 0-0.5 mg/ml (0 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5mg/ml). Standards and samples were added to a 96 well plate, individually mixed with 200 μL of BCA working solution. The resultant mixtures were incubated at 37℃ for 30 min to allow blue coloration to develop. Absorbance was then measured at 550 nm using a Microplate Reader (Molecular Devices, Spectra Max M2e). Protein concentrations were obtained by deriving the equation of line of best fit from average standard values according to $y=mx+c$.

(2) Preparations of SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Mini-Protean Tetra casting frame, casting stand, and gel cassette (Bio-Rad Laboratories Ltd.) were assembled according to the manufacturer's instructions. The composition of the resolving gels was 6-12% (v/v)43 acrylamide/*bis*acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% (v/v) sodium dodecyl sulphate (SDS), 0.05% (v/v) ammonium persulfate (APS), and 0.02% (v/v) tetramethylethylenediamine (TEMED). Gels were poured, over laid with water and set for 30 mins, before removing the water and stacking gels of composition 4% (v/v) acrylamide/*bis*acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.1% (v/v)

SDS, 0.05% (v/v) ammonium persulfate (APS), and 0.02% TEMED (v/v) were poured on top of the resolving gel. Combs (Bio-Rad Laboratories Ltd.) were inserted and the gels set for 5 mins. The gels were usually used immediately, but occasionally stored for up to 5 days at 4°C wrapped in wet paper towels and Clingfilm.

(3) SDS-PAGE

3x SDS Sample Loading Buffer was added to the samples and these were boiled for 10min. Mini-Protean Tetra Gel Cassette and electrophoresis module (Bio-Rad Laboratories Ltd.) was assembled according to the manufacturer's instructions. 20-40 µL of each denatured protein sample were loaded and resolved at 80V, in 6-12% (w/v) SDS-polyacrylamide gels for 120 min in electrophoresis buffer. 5-10 µL of pre-stained molecular weight markers (Thermo, Ltd.) was resolved alongside the protein samples serving as a reference for approximate protein size determination.

(3) Electrophoretic protein transfer

Resolved protein gels were transferred to polyvinylidene fluoride (PVDF) membranes (Biorad, Ltd). Trans-Blot SD Cell (BioRad Laboratories Ltd.) were assembled. A PVDF membrane was pre-treated in absolute methanol for 5 sec, then washed in 1x transfer buffer for 5 min before use. Both gel and membrane were then sandwiched between buffer-soaked filter papers X2. Proteins were transferred onto the blotting membrane through electrophoretic transfer (conditions: 70 mA) for 1.5hrs using pre-chilled transfer buffer. Following the electrophoretic transfer, the blots were trimmed to size and submerged in blocking buffer (5% non-fat milk dissolved in 1x TBST) for 2 hours at room temperature, prior to probing with antibodies.

(4) Immunoblotting

Primary antibodies were mouse-monoclonal antibodies against LDLR (1:2000), ACOX1 (1:200) and ACSL1 (1:200) (all from Abcam, Cambridge, UK). Antibody was diluted in 1X TBST+5% BSA at the recommended dilution and incubated overnight

with the membrane at 4℃. Primary antibody was recovered and stored at 4℃. Then, membrane was washed with1X TBST 5 times for 10min on a shaker at room temperature. Secondary antibody was anti-mouse IgG (1:2000; Bio-Rad). The membrane was incubated in dilutions secondary antibody solution (diluted in 1X TBST) for 1 hour at RT. It was washed 5 times for 10min in TBST on a shaker at RT. ECL were used for conjugated secondary antibody. Photos were taken by Molecular Imager ChemiDOC^{TM} XRS+ (Bio Rad).

2.5 Proteomics research

2.5.1 Protein extraction and sample preparation.

In this work, fresh liver tissue (3 × 3 mm) was removed from berberine- and berberine8998-treated and untreated control animals and stored at −80°C until required. Samples were cut into small pieces (0.1 g). To eliminate the effect of sample variation in proteomic analyses, five small pieces from each group were ground together in liquid nitrogen to produce 12 test samples (four each for berberine, berberine8998 exposed and control tissues) for protein extraction.

The 12 frozen test 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 10s intervals, followed by three rounds of homogenization using a whirlpool mixer at 10 min intervals. Samples were centrifuged at 1960 g 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 isobaric tags for relative and absolute quantitation (iTRAQ) labeling were carried out according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). Briefly, 150 μg proteins from each sample 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.

2.5.2 Column separation strategy for the recovered peptides

In the present work, mixed peptides were fractionated by strong cation exchange (SCX) chromatography on a 20AD high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) using a 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 KH_2PO_4 in 25% 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 SCX fractions were collected along the gradient, dried, dissolved in Buffer C (5% ACN and 0.1% formic acid [FA]) and analyzed on a model 5600 mass spectrometer (Applied Biosystems, see 2.5.3). Peptides were separated on a Zorbax 300SB-C18 reverse phase (RP) 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.

2.5.3 Conditions for mass detection

In our study, the ESI ion source in series with NanoLC system was chosen. CID/CAD peptide sequencing strategy and DDA fragment spectrum acquisition mode were selected for detection. Survey scans were acquired from 400 to 1800 cm^{-1}, with ≤ four 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 Th; 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.

2.5.4 Data analysis

Protein Pilot v4.5 (Applied Biosystems) was used to identify and quantify iTRAQ-labeled peptides. This software was also used to generate the minimum number of identified peptides by removing redundant hits. MS/MS data were searched with UniProt. The rat database was used since the corresponding hamster database is not available. The precursor and iTRAQ fragment tolerance values were set to 100 ppm and 0.6 Da. The 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 FDR<1% was applied to protein identification. At least one peptide with a confidence interval of 95% was included [29]. Data were considered reliable for P <0.05 and error factor <2. Student's t-test and P-value was used to evaluate the significance of protein expression level change. Fold-change ratios <0.7 (down regulated) or >1.3 (up regulated) were selected as cutoff values for significant changes in protein expression. Gene Ontology (GO, http://www.geneontology.org/) was used to verify the function of proteins that were up- or down-regulated by berberine or berberine8998 treatment. The signaling pathways of proteins were elucidated by searching the Kyoto Encyclopedia of Genes

and Genomes database. MATLAB was used for the mathematical modeling and analyzing the KEGG pathways.

2.6 Proteomics validation

2.6.1 Validation of results from the proteomic analyses

2.6.1.1 Sample preparation

Hamster plasma (40μL) and sulfosalicylic acid (10μL) were mixed in a 1.5 mL centrifugal tube and vortexed for 10s. The mixture was then centrifuged at 1000g for 2 min. Supernatant (10μL) and labeling buffer (40μL) were mixed in a 1.5mL centrifugal tube and vortexed for 10s. The mixture was centrifuged at 1000 g for a further 2 min. 10μL of supernatant was removed for the analysis (denoted as S1).

A bottle of iTRAQ115 regent was thawed to room temperature and then centrifuged at 1000g for 2 min. Isopropanol (70μL) was added to dilute the iTRAQ115 reagent. The mixture was vortexed for 10s and centrifuged at 1000g for 2min. Diluted iTRAQ115 reagent (5μL) was mixed with S1. The mixture was left at room temperature for 30 min for the derivatization reaction. After 30 min, hydroxylamine solution (5μL) was added to stop the derivatization reaction. The sample was vacuum dried at 40°C (denoted as P1).

AA45/32 (1.8mL) internal standard diluted solution was added to dissolve iTRAQ114 powder. The solution was vortexed for 30-60sec. The solution (32μL) was added to dissolve P1 (denoted as S2). The S2 was vortexed for 30 seconds and centrifuge at 1000g for 2 min. S2 (2μL) was analyzed for determination of the amino acids in the serum.

2.6.1.2 LC condition

AAA C18 (150 mm×4.6 mm, 5 μm, AB Sciex) was selected as the column for the analysis. C18 (4.0×3.0mm, 5 μm, Phenomenex) was chosen to be the protection column. Mobile Phase A was water containing 0.01% heptafluorobutyric acid and 0.1% formic acid. Mobile Phase B was acetonitrile containing 0.01% heptafluorobutyric acid and

0.1% formic acid. LC grads and LC time are listed in Table 2.4 Flow rate was set at 0.8ml/min. Column temperature was 50°C. The temperature in auto sampler was maintained at 4°C.

Table 2.4 LC gradient conditions for determination of amino acids

Total Time (min)	Mobile Phase A %	Mobile Phase B %
0	98	2
9.5	76	24
11	20	80
14	20	80
16	98	2
25	98	2

2.6.1.3 Mass spectra condition

ESI ion source was selected for determination. Scan model was set at MRM for positive ion.

Ion source：ESI; Polarity: Positive ion; Scan model: MRM; Ion transitions: See Appendix;

Parameter: GS1 60psi, GS2 60psi, Curtain gas 20psi, Collision gas Medium; Voltage 1500V.

An AB 3200 QTRAP (Applied Biosystems/MDS Sciex, Toronto, Canada) was operated in the positive ionization mode. Ion transitions of amino acid and IS were set as given in the Appendix 1. Highly pure nitrogen was used for mass spectrometry analysis. The source/gas parameters were set as: GS1: 60, GS2: 60, Curtain gas 20, ion spray voltage 1500V. The other analyte parameters are listed in Table 2.5.

Table 2.5 Analyte parameters for amino acid determinations

DP（V）	EP（V）	CE（V）	CXP（V）
30	10	30	5

2.6.2 Serum levels of NEFAs

2.6.2.1 Sample collection and preparation

The serum samples were gathered from 76 hamsters, which were fed for five weeks. For the Normal diet group, the hamsters were always fed with normal diet (MO2 feed) for five weeks. See 2.4.3.2 for animal grouping. Blood samples (0.3-0.5 ml) were collected weekly from the orbit of the hamsters without dietary exposure for 16 h. The blood samples were centrifuged at 3000g for 15 min to prepare the serum sample.

2.6.2.2 Analysis by automatic biochemical analyzer

The model 7020 Hitachi's one of the smallest automatic analyzer yet. It brings great flexibility in automatic clinical analysis. Despite its compactness, it offers a performance of up to 36 test items simultaneously and up to 200 tests per hour. And STAT is achieved through very easy operation.

Automatic biochemical analyzer was applied with NEFA-HA Test kit to validate the proteomic results on protein pathway by testing the 76 hamster serum samples (above section 2.6.2.1). There are the four reagents in the NEFA-HA Test kit. (1) Color Reagent A: 0.53 U/mL ACS, 0.31 mM CoA, 4.3 mM ATP 4-aminoantipyrine, 2.6 U/mL ascorbate oxidase and 0.062% sodium azide (2) Solvent A: 50 mM phosphate buffer PH7.0 and 0.05% sodium azide (3); Color Reagent B: 12 U/mL ACOD and 14 U/mL POD. (4) Solvent B: 2.4 mM MEHA. All stored between 2-10℃. Before testing, one bottle of Solvent A was added to one vial of Color Reagent A to prepare Color Reagent A Solution. One bottle of Solvent B was added to one vial of Color Reagent B to prepare Color Reagent B Solution. These two Color Reagent Solutions were placed in the automatic biochemical analyzer along with the 76 hamster serum samples from the four groups (Normal diet group, High-fat diet group, High-fat diet with Berberine8998 50 mg group, and High-fat diet with Berberine 50mg group). Data were collected and processed by Zhifang Software.

2.6.3 Western blots validation of differently expressed proteins

2.6.3.1 Preparation of buffers

See section 2.4.5.1

2.6.3.2 Liver sample preparation

See section 2.4.5.2

2.6.3.3 Protein quantification

See section 2.4.5.3

2.7 Formulation of berberine8998

2.7.1 Methods for determination of berberine levels

(1) HPLC conditions

Analytic column: CAPCELL PAK C18，2.0lytimm，5μm

Protect column: C18（4.0×3.0mm，5μm）

Mobile phase: Mobile phase A 0.1% formic acid in water,

Mobile phase B acetonitrile

Elution program: A: B=69:31, isocratic elution

Flow rate: 0.3mL/min

Column temperature: room temperature

Autosampler temperature: 4℃

Sample size: 5μL

The chromatographic separation of berberine and carvedilol (internal standard) was carried out on Shimadzu LC-20AD HPLC system (Kyoto, Japan) equipped with two LC-20AD pumps, a HIL-HTc auto-sampler and an online DGU-20A3 vacuum degasser.

The analytical column was CAPCELL PAK C18 (2.0to-smm 5μm) coupled with a C18 protect column (4.0 mm × 3.0 mm, 5 μm). The mobile phase consisted of phase A (0.1% formic acid in water) and phase B (acetonitrile). Berberine was eluted at a flow rate of 0.3 ml/min with an isocratic elution program of A/B at 69:31 (v/v). Aliquots of 5 μl was injected for the HPLC analysis.

(2) Conditions for determination of the molecular mass conditions

Ion source：ESI

Polarity: Positive ion

Scan model: MRM

Ion transitions: Berberine 336.2→320.0amu, IS：407.3 →100.1amu

Parameter: GS1 60psi, GS2 60psi, Curtain gas 20psi,

Voltage 5500V, Temperature 550℃

An API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a Turbo Ionspray source was operated in positive ionization mode. Ion transitions of *m*/*z* 336.2/320.0 were used for quantitative detection of berberine, respectively and *m*/*z* 407.3/100.1 for IS detection. Highly pure nitrogen was used for the mass spectrometry analysis. The source/gas parameters were set as: GS1: 60, GS2: 60, Curtain gas 20, ion spray voltage 5500V and the source temperature 550 °C. Other mass spectrometry analysed parameters are listed in Table 2.6.

Table 2.6 Mass spectrometry analysis parameters for berberine and internal standard.

<table>
<tr><th>Analyte</th><th>DP（V）</th><th>EP（V）</th><th>CE（V）</th><th>CXP（V）</th></tr>
<tr><td>Berberine</td><td>65</td><td rowspan="2">5</td><td rowspan="2">45</td><td>20</td></tr>
<tr><td>IS</td><td>60</td><td>10</td></tr>
</table>

2.4.2.2 Solution preparation

2.4.2.2.1 Preparation of stock and working solutions

(1) Berberine stock solution: Berberine 3.23mg was weighed and dissolved in 50%

acetonitrile water solution to form 291μg/mL berberine stock solution.

(2) Berberine working solution: Berberine stock solution was transferred into 50% acetonitrile water solution.

(3) Internal standard stock solution: Carvedilol 3.41mg was dissolved in 50% acetonitrile water solution to provide 308μg/mL carvedilol stock solution.

(4) Internal standard work solution: Appropriate berberine stock solution was transfered into 50% acetonitrile water solution.

Stock solutions were stored at 4 ℃ and kept stable for 4 weeks.

The concentration of stock solution was test by LC/MS/MS during four weeks.

2.4.2.2.2 Mobile phase preparation

Mobile phase A (contain 0.1% formic acid water solution): Formic acid 1mL was removed into 1000ml water, mix for 10s.

Mobile phase B was acetonitrile

2.4.2.3 Preparation of standard curve samples and quality control (QC) sample

2.4.2.3.1 Standard curve annex solution

To prepare 5ng/ml, 10ng/ml, 20ng/ml, 40ng/ml, 80ng/ml, 150ng/ml, 300ng/ml standard curve solution, 5μl standard curve annex solution was added into 50μl blank serum. For equal matrix effect for all the test samples, the concentration here stands for the compounds concentration in the matrix (pure serum).

2.4.2.3.2 Solutions for a standard curve

The highest concentration of stock solution (3000ng/ml) was prepared by means of a closed system transfer device (CSTD7-3000 ng/mL). The preparation of other standard curve annex solution (CSTD-1500ng/mL, CSTD-800ng/mL, CSTD-400ng/mL, CSTD-200ng/mL, CSTD-100ng/mL, CSTD-50ng/mL) was accomplished by adding 50%-acetonitrile water solution, as shown in Table 2.7.

Table 2.7 Preparation of solutions for derivation of the standard curve.

Solution type	Solution code	Transferred solution（μL）	50% acetonitrile water solution（μL）	Concentration（ng/mL）
Standard solution	CSTD7	Suitable working solution	Suitable	3000
	CSTD6	CSTD7：400	400	1500
	CSTD5	CSTD6：480	420	800
	CSTD4	CSTD5：400	400	400
	CSTD3	CSTD4：400	400	200
	CSTD2	CSTD3：400	400	100
	CSTD1	CSTD2：400	400	50

2.4.2.3.3 Quality control annex solution

To prepare highest concentration quality control solution (QC3-2000 ng/mL), the appropriate stock solution was removed into acetonitrile:water 50:50 solution. The preparation of other standard curve annex solutions (QC2-500ng/mL, QC1-150ng/mL, QC0-50ng/mL) was adding the required amount of the higher concentration standard curve annex solution into 50% acetonitrile water solution as given in Table 2.8.

Table 2.8 Preparation of quality control annex solutions

Solution type	Solution code	Removed solution（μL）	50% acetonitrile water solution（μL）	Concentration（ng/mL）
QC	QC3	Appropriate working solution	Appropriate	2000
	QC2	QC3：250	750	500
	QC1	QC2：300	700	150
	QC0	QC2：300	600	50

2.4.2.3.4 Quality control solutions

To prepare 5ng/mL (LLOQ, Lower Limit of Quantification), 15ng/mL (LQC, Low QC), 150ng/mL (MQC, Middle QC), 200ng/mL (HQC, High QC) solutions, removed 5μl quality control annex solution into 50μl blank serum. For equal matrix effect for all the test samples, the concentration here represents the compounds concentration in the matrix (pure serum).

2.3.2.4 Serum sample preparation (mice, hamster and rat)

2.3.2.4.1 Sample preparation for standard curve and quality control analysis

The standard curve solution (55μl) and the quality control solution (5μl) were added to acetonitrile (100μl). The mixture was vortex for 30s, then centrifuged at 1960 g (4℃) for 10min. The supernatant was recovered and retained for analysis.

2.3.2.4.2 Sample preparation of quality control solution

The blank serum (50μl), 50% acetonitrile water solution (5μl) and internal standard curve solution/quality control solution (5μl) was removed into 100μl acetonitrile solution. The mixture was vortex for 30s, then centrifuged at 1960 g (4 °C) for 10min. The supernatant was recovered and retained for analysis.

2.3.2.4.3 Sample preparation of blank serum

The blank serum (50μl) and 50% acetonitrile water solution (10μl) was removed into 100μl acetonitrile solution. Mixture was vortexed for 30s, then centrifuged with 1960g in g (4°C) for 10min. The supernatant was recovered for analysis.

2.3.2.5 Data analysis

Data from the berberine analyses were collected and processed by Analyst1.4.2 software (AB SCIEXInc). Individual pharmacokinetic parameters were assessed by a noncompartmental method using DAS version 2.0 (Drug And Statistics). C_{max} and T_{max} were obtained by direct inspection of individual plasma concentration– time profiles. The AUC_{0-t} was calculated using the linear-up/log-down trapezoidal method. The terminal elimination constant (λz) was estimated from the natural logarithm–transformed plasma concentration–time curve using linear regression, and the $t_{1/2}$ was calculated as ln2/λz. The $AUC_{0-\infty}$ was calculated as AUC_{0-t} + Clast/λz. Data were presented as Mean±SD.

2.7.2 Preparation of berberine8998 formulation

Formulation A (berberine8998 microcrystal suspensions): 0.714g of berberine8998 was dissolved in dimethyl sulfoxide (DMSO). This was used to prepare berberine8998 microcrystal suspension (35 mg/ml, 40ml).
Formulation R (berberine8998 CMC-Na solution): A solution of CMC-Na (100ml 0.5%) was mixed with 1.072 g berberine8998 powder to prepare berberine8998 CMC-Na solution (17.8mg/ml, 30ml). The mixture was stirred using a magnetic stirrer for 12 hours.

2.7.3 Experimental procedure to compare the pharmacokinetic (PK) characteristics of formulation 1 with that of formulation 2

Male Sprague-Dawley (SD) rats (weighing 180-200g, n=10, provided by the Animal Center of Shanghai Institute of Materia Medica) were randomly divided into two groups. Their weights were recorded before the experiment at Week 1 and Week 2 in Table 2.9. After twelve hours of food deprivation, the rats were administered 35mg of berberine or berberine8998 intragastrically (i.g.). The washout interval between the two treatment periods was 7 days. After the washout period, the rats were given the alternating compound in the second period.

Table 2.9 Weights of rats used for the pharmacokinetic study

Cage No	Rat N	Weight（g）	
		Week1	Week2
1	1	302	327
	2	291.5	326
	3	282.5	309
	4	280	318.5

	5	290	330.5
	1	284.5	315
	2	345.5	376
2	3	330	358
	4	322	345
	5	298	331.5

Sample collection protocol was identical in the two treatment periods. The serum preparation method is described as follows. For all the rats, blood samples (0.5 mL) were collected pre-dose and at 0 h, 0.5h, 1h, 2h, 3h, 4h, 6h, 8h, 12h, 24h, and 48h after dosing in each treatment period. The collected blood samples were centrifuged at 3 000g for 15 min at 4°C within 30 min of the collection time. Serum samples were stored at -70°C before analysis.

3 Acute oral toxicity study of berberine8998

3.1 Introduction

Inbred mouse strains are generated by sister-brother mating for 20 or more consecutive generations (Green et al., 1963). The most well-known inbred lines include C57BL/6, C57BL/10, C3H, CBA and BALB/c strains which are widely used in pharmacological research by allowing independent researchers from different labs to perform reproducible experiments on the same genetic material (Proetzel and Wiles, 2010). The C57BL/6, often referred to as "C57 black 6" or just "black 6" (standard abbreviation: B6) is a common inbred strain of laboratory mice. It is the most widely used "genetic background" for genetically modified mice for use as models of human disease. They are the best-selling inbred mouse strain, due to the availability of congenic strains, easy breeding, and robustness (Mekada et al., 2009).

The value of lethal dosage 50 (LD_{50}) is usually applied to indicate the acute toxicity of a test compound (1956). In toxicology, LD_{50} means the dose required to kill 50% of tested animals after specified test duration. This toxicity tenet of LD_{50} was created by J.W. Trevan in 1927 and provides a useful means for comparing toxic properties. It is

usually determined by test on animals, like mice.

Berberine has potential lipid-lowering activity. However, its low bioavailability (poor absorption) limits its widespread clinical application. Berberine is a low acute toxicity compound and reveals therapeutic effect on acute and chronic hepatic damages (Feng et al., 2011). Therefore, in order to overcome the problem of limited absorption of berberine, a derivative berberine8998 was synthesized. To test the acute oral toxicity of berberine8998, the mice model (C57BL/6) was selected for evaluating the effects of oral administration of appropriate berberine8998 formulations (CMC-Na, 0.5%). The death numbers were counted for calculating the LD_{50} of berberine8998.

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3.2 Results

3.2.1 Formulation preparation

A reliable berberine8998 liquid formulation was required. Berberine8998 does not readily dissolve in water. Sodium carboxyl methyl cellulose (CMC-Na) suspension (0.5%) was selected for preparing the suspensions. To ensure the administration accuracy, individual formulations were prepared for each group. The final oral amount administrated was determined by Weight ×Dosage÷ Concentration.

3.2.2 Establishment of the dose range for berberine8998

The purpose of the preliminary experiment was to look for the dose range for the subsequent main experiment to examine LD_{50} 24 hours after intragastric (i.g.), the number of dead mice in each group were counted. These data were calculated, recorded and is presented in Table 3.1. These results suggest that the dose range is from 500mg/kg to 1000mg/kg and should be selected to process the main experiment.

Table 3.1 Establishment of the dose range for berberine8998

Group No	Dosage（mg/kg）	Number of animals	Number of animals that died	Death rate of the animals（%）
1	1000	4	3	75
2	800	4	3	75
3	600	4	1	25
4	500	4	1	25
5	400	4	0	0

3.2.3 LD_{50} analysis

The value of lethal dosage 50(LD_{50}) illustrates the acute oral toxicity of the test compound. To obtain the LD_{50} of berberine8998, a single dose of berberine8998 was administered to the mice in different dosage groups: 500 mg/kg (Group A), 595 mg/kg (Group B), 707 mg/kg (Group C), 841 mg/kg (Group D) and 1000 mg/kg (Group E). After 24 hours, the numbers of dead mice in each group were counted and recorded in Table 3.2. SPSS probit analysis was used to obtain the LD_{50} values and the 95% confidence limits. As shown in Tables 14 and 15, the LD_{50} (Probit .500) of berberine8998 was 870mg/kg (SPSS Probit analysis).

Table 3.2 LD_{50} analysis for berberine8998

Group	Dosage（mg/kg）	Number of animals	Number of animals that died
1	1000	10	7
2	841	10	4
3	707	10	3
4	595	10	1
5	500	10	1

3.3 Discussion

The main aim of the LD_{50} experiment was designed to test the acute oral toxicity of berberine8998, and it is most important to ensure the accuracy of the amount of the orally administered berberine8998. Previous study has demonstrated higher bioavailability of berberine8998 in SD rats.

SPSS Probit analysis was used in this experiment. It provides fitting probit and logit sigmoid dose/stimulus response curves as well as calculating confidence intervals for dose-response quantiles such as LD_{50}. The fitted model is assessed by statistics for heterogeneity which follows a chi-square distribution. For most systems the probit (normal sigmoid) and logit (logistic sigmoid) give the best fitting result. Logistic methods are useful in biological assays because the odds ratios can be determined easily from the differences between the fitted logits (Finney, 1964, Finney, 1971).

Existing research has reported 30% death rate following intragastric (i.g.) of 4160mg/kg berberine in ICR mice (Kheir et al., 2010). The LD_{50} of berberine8998 in mice was 870mg/kg. Therefore, berberine8998 is significantly more toxic than berberine. However, compared with the lipid lowering drugs it is a safe candidate and has a good potential therapeutic window (Maji et al., 2013). Side effects such as proteinuria and hematuria occurred in over 1% of total patients taking statins. Serious side effects such as myopathy and rhabdomyolysis, though in low occurance rate, was life threatening. The withdrawal of cerivastatin was due to this side effects. Compared with the side effects mentioned above, berberine was relatively safe in a long history of clinical application (Kheir et al., 2010). One can thus conclude that berberine8998 has relatively low toxicity and has the potential of being a good lipid-lowering drug.

4 Monitoring lipid levels in hamster serum

4.1 Introduction

Hamster (*Mesocricetus Auratus*) is a small rodent of Middle Eastern origin that is excellent for assessment of drugs with combined efficacy on atherosclerosis, weight loss, hypertriglyceridemia and hypercholesterolemia (Dalboge et al., 2015). The hamster carries a significant portion of its plasma cholesterol in the LDL lipoprotein fraction. In this respect, it is closer to humans than to the rodents (Lock et al., 2005). Hamsters have an atherogenic lipoprotein profile with a large proportion of the circulating lipoproteins, they possess cholesteryl ester transport protein (CETP), receptor mediated uptake of LDL lipoproteins via the LDLR, exclusively hepatic production of apolipoprotein (apo) B-100 and intestinal production of apo B-48 (Tsutsumi et al., 2001, Bishop, 1992, Liu et al., 1991). When fed with high-fat diet, hamsters quickly develop hypercholesterolemia and hypertriglyceridemia. Therefore, it has been suggested that hamsters fed with high cholesterol and saturated fat diets responded similarly to humans in terms of lipoprotein metabolism (Dalboge et al., 2015). The concentration of LDL in hamster plasma is strongly influenced by the amount and the type of lipid in the diet. Previous studies showed that dietary lipids alter the plasma LDL concentration in the hamster (Lock et al., 2005, Tsutsumi et al., 2001, Nistor et al., 1987). These studies indicated that the high-fat diet-induced dyslipidemic hamster model can be applied for lipid lowering drug evaluation.

The hypothesis that atherosclerosis disease is due to hyperlipidemia has been a powerful concept that has fostered the development of lipid-lowering therapeutics (Davignon, 2004). Serum LDL levels are commonly considered as the most important modulators and biomarkers of atherosclerotic processes (Bahiru et al., 2017, Tsai et al., 2009). Studies using hamsters to evaluate the effects of diet cholesterol and fat on the kinetics of hepatic LDLR-mediated LDL clearance have been well characterized (Horton et al., 1993, Ugawa et al., 2002). Hence, as a part of this work and an extension of the existing studies, berberine and berberine8998 were analysed to see if these drugs

can reduce the LDL levels in hamsters fed with a high-fat diet.

4.2 Results

4.2.1 Results of a preliminary pharmacodynamic study

The lipid metabolic profile was significantly improved through lipid-lowering effects, as observed from the changes in the slope of the LDL level (Fig 4.1). The dose range was set up based on the data of previous berberine research (Kong et al., 2004). The berberine at 200mg/kg dose showed the expected positive effects. However, the hamsters in this group (Group 5) became extremely lean and weak. Yellow spots were also found in the anuses of these hamsters. Two of these animals died in the weeks three and four of the treatment. These experimental results indicated that the oral dose of 200mg/kg of berberine is too high and can be toxic. The dose was therefore lowered in the later experiments.

Thereafter, it was found that at lower dose both berberine and berberin8998 have beneficial effects. As judged by their body weight and hair color, all the hamsters in the berberine8998 groups were healthy throughout the experiment. However, there was a significant difference ($p<0.05$) between the treated and control groups. The levels of LDL in the 50 mg/kg berberine8998 group reduced by 44.53% (Fig 4.1 Group 3) and LDL levels in the 100mg/kg berberine8998 group by 51.07% (Fig 4.1 Group 4). These reductions in the LDL levels showed trends (down regulation of the slope) that can be considered as an improvement of the metabolic syndrome. The results suggested that the given berberine8998 dose is safe and that it has a dose dependent lipid-lowering effect.

Group 1 (negative control: high-fat diet)

Group 2 (positive fenofibrate control: high-fat diet + 50mg/kg fenofibrate)

Group 3 (berberine8998 50mg: high-fat diet + 50mg/kg berberine8998)

Group 4 (berberine8998 100mg: high-fat diet + 100mg/kg berberine8998)

Group 5 (berberine 200mg: high-fat diet + 200mg/kg berberine)

Group 6 (control on normal diet)

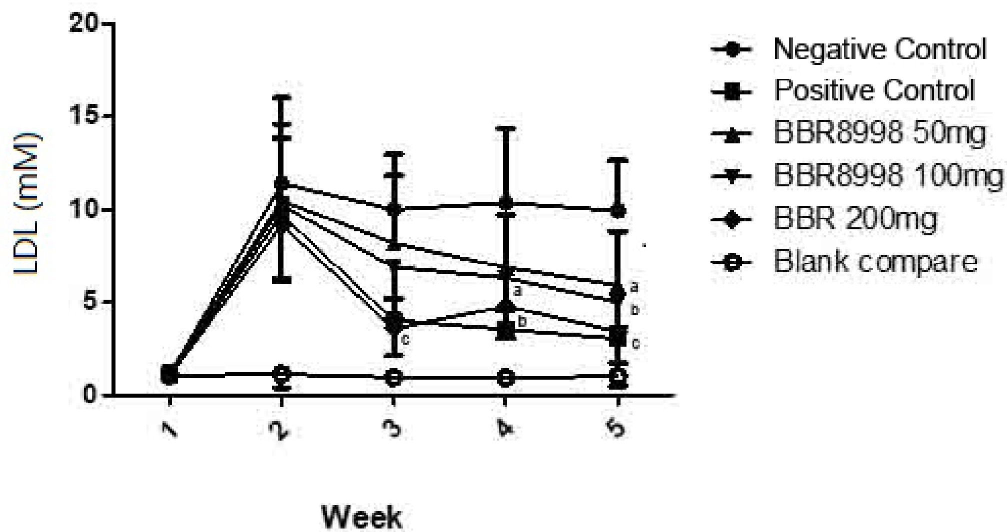

Figure 4.1 LDL levels in treated hamster plasma from a preliminary pharmacodynamic study, for the groups 1-6, as described above. N=12 The data were analyzed by two-way ANOVA with post-hoc testing by compared with high fat diet group. [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$.

4.2.2 Results of the second pharmacodynamics study

Based on the results of the preliminary study, several experimental conditions were modified. The lipid lowering trends presented in Fig. 4.2 suggested that the total number of experimental groups can be limited to three. To prevent animals dying from too-high dosage of berberine, the doses of both berberine and berbeine8998 were set at 50 mg/kg. This dose was well below the LD_{50} of 870 mg/kg established in the previous experiment.

To evaluate the lipid-lowering effects of berberine and berberine8998, the weight as well as serum LDL TC and TG levels were measured in high-fat diet hamsters treated

with berberine, berberine8998, or left untreated. The weights of hamsters in the berberine and berberine8998 groups showed no significant difference (Fig 4.2 D). Hypercholesterolemia developed slowly in the normal diet group (Fig 4.2A-4.2C), and serum lipid levels in the high-fat diet (HFD) group increased significantly (Fig 4.2A-4,2C). Notably, berberine8998 treatment significantly reduced total cholesterol (Fig 4.2A) and LDL-c (Fig 4.2B) 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 (Fig 4.2B). Mean LDL-c levels at week 2 and week 3 were 10.30±3.63 and 13.15±7.34 mM, respectively for the HFD group and 6.00±1.85 and 8.55±2.59 mM, respectively, for the berberine8998 group (Fig 4.2B).

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% (Fig 4.2C). Triglyceride levels were significantly attenuated by week 3 in the berberine ($P < 0.05$) and berberine8998 groups ($P < 0.01$) (Fig 4.2C). Body weight was not significantly different between the treatment, control and normal diet groups, as shown in Fig 4.2D.

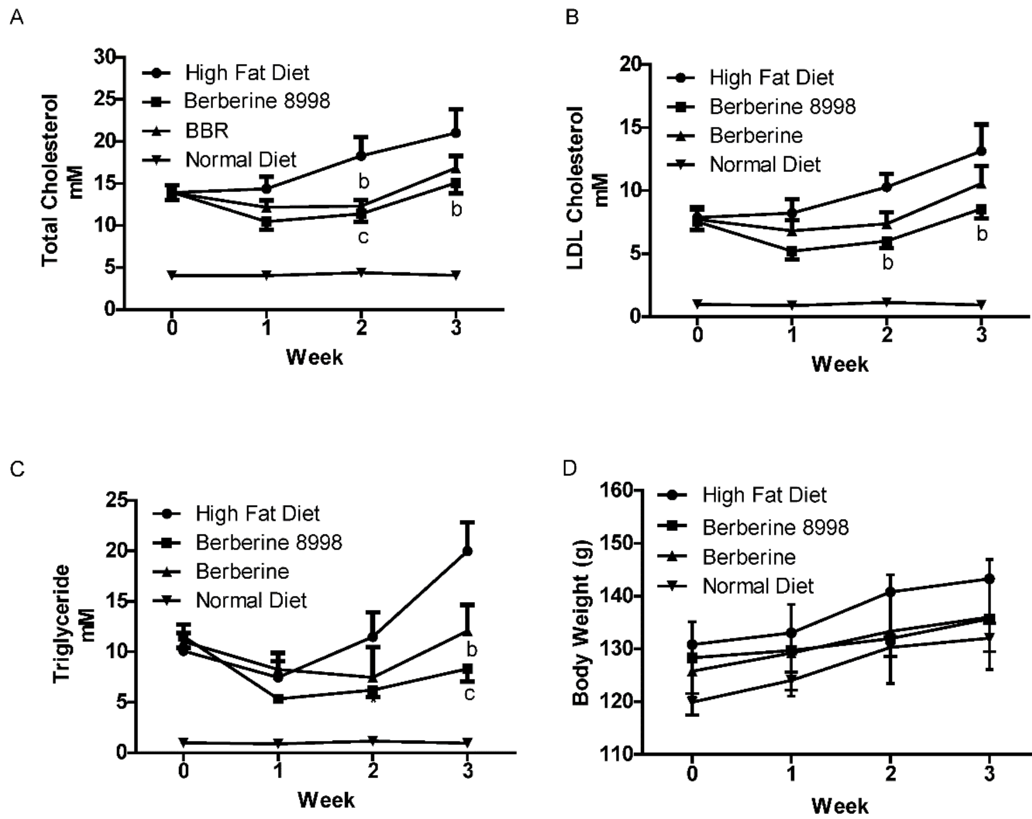

Figure 4.2 Analysis of (A) LDL and serum (B) TC (C) TG and (D) Body Weight levels in hamsters treated with berberine or berberine8998 or left untreated over 3 weeks. The data were analyzed by two-way ANOVA with post-hoc testing by compared with high fat diet model group. $^{b}p<0.05$ and $^{c}p<0.01$ by Two-way ANOVA compared with model group.

4.3 Discussion

For seasonally bred rodents such as the golden hamster, photoperiod tightly regulates the timing of the onset and cessation of testicular functions characterized by dramatic alterations in steroidogenesis and spermatogenesis (Mukherjee and Haldar, 2016). Exposure to long-day photoperiod elevates testicular spermatogenesis and steroidogenesis, whereas short-day exposure inhibits testicular functions in photoperiodic rodents, thereby fine tuning the seasonal cycle of male fertility (Frungieri

et al., 2005). Photoperiod also plays a critical role in the modulation of metabolic stress-induced variation in steroidogenesis and antioxidant status in hamsters (Mukherjee and Haldar, 2015). The impact of energy availability in regulation of testicular steroidogenesis and antioxidant status has not been investigated for any seasonal breeder in general, especially for golden hamster. High fat-diet induced obesity leads to liver damage, so that the liver of high-fat diet hamsters is vulnerable (Huang et al., 2015). Thus, the temperature and light/dark cycle needed to be strictly controlled in this research.

Previous studies on feeding hamsters diets supplemented with conjugated coconut oil, linoleic acid, macademia oil, canola oil, sunflower oil, palm oil, and olive oil were used to determine if different oils alter plasma lipids levels (Wilson et al., 2006, Matthan et al., 2009, Lecker et al., 2010). However these liquid oils are difficult to make into solid fodder. To establish a high-fat diet hamster model with high LDL levels, 10% of lard was used in the high-fat diet group in this study.

The LDL and TC levels in high fat model groups were significantly elevated (**P=0.004<0.005) (9 *vs* 2 mM, 17 *vs* 7 mM). Berberine and berberine8998 significantly attenuated the elevation of lipid profiles, especially after three weeks' treatment. But the lipid-lowering effect was not different between the two compounds. However, the LDL results in week 5 did not show any statistical difference. This can be attributed to the threshold effect. Some hamsters might have reached the treatment threshold value in week 5. The weights of the hamsters in the berberine and berberine8998 groups also showed no statistical difference, indicating that berberine8998 is as safe as berberine with respect to the doses used in this part of the study. Treatment with either compound decreased the speed (see Fig 4.2) of serum TC and LDL level elevation, with berberine8898 showing a greater effect. Therefore, the corresponding mechanisms of lipid lowering effect of berberine8998 were thoroughly elucidated.

5. DiI-LDL uptake by HepG2 cells

5.1 Introduction

Majority of the LDL is removed from the body via the liver (Libby et al., 2002). An elevated level of LDL in the plasma is one of the major causative factors of hypercholesterolemia, atherosclerosis, and coronary heart diseases. Lowering LDL by means of a therapeutic drug is an effective and safer approach for treating vascular disease (Bulbulia et al., 2011). More than 70% of circulating LDL is taken up from the blood via hepatic LDL receptor (LDLR) mediated endocytosis. The uptake of LDL by LDLR plays a major part in the elimination of LDL. Loss of function mutations in the LDLR gene has been identified as the major cause of familial hypercholesterolemia (FH), which is characterized by elevated levels of plasma LDL-cholesterol (LDL-c) and an increased risk of cardiovascular disease (Kong et al., 2006). Enhancement of LDLR activity is therefore an effective method for down-regulation the serum cholesterol levels.

Human hepatoma cells (HepG2) are recognized as a good surrogate for adult hepatocytes. They are able to reproduce most of the hepatocytic functions (Javitt, 1990). The HepG2 cell line has been widely used as a functional model for studying the pharmacology of berberine in the last decade. It has been reported that berberine increased LDLR expression and LDLR mRNA stability via the SREBP pathway in HepG2 cells (Kong et al., 2004). Berberine can also reduce the level of PCSK9 mRNA, leading to reduction in the amount of PCSK9 protein secreted into the media in the HepG2 cells (Cameron et al., 2008).

Methods which involve labeling LDL complexes (ep: Dil-LDL) are useful for studying the function of LDLR. This labeling renders individual molecules visible by their fluorescence and enables the complexes to be used in dynamic studies of LDLR motion on living fibroblasts by standard fluorescence techniques. 125I-labeled LDL has primarily been the method of choice for rapid quantitation of receptor-mediated LDL metabolism since it was first introduced by Brown and Goldstein (Goldstein and Brown,

1974). Compared with the 125I-labeled LDL method, which is reliable and highly sensitive, the Dil-LDL method is safer (no radiation) and more cost-effective (Stephan and Yurachek, 1993).

The Dil-LDL method is simple, rapid and nonradioactive, and has a potential of being adapted to a high through-put screen of compounds modulating LDLR activity in cells. The purpose of this study is to see if berberine8998 is exerting its lipid-lowering effect via the Dil-LDL uptake regulation on the HepG2 cell line.

5.2 Results

5.2.1 DiI-LDL uptake by HepG2 cells via LDLRs

One study has reported that hamsters that were orally given 100mg/kg berberine could convert it to 15μg/ml (44.73μM) for a cell-based experiment (Kong et al., 2004). Consequently, two dosages (20μM and 40μM) of berberine8998 were selected in this experiment for monitoring its uptake. The results reported in the previous chapter indicate that berberine8998 treatment lowered serum LDL levels. To further unveil related mechanisms, HepG2 cells were incubated with the test compounds (vehicle control, pravastatin, berberine, berberine8998) and DiI-LDL. A fluorescent detector was used to scan the intensity of the Dil-LDL uptake. Cells treated with the cholesterol-lowering agent pravastatin served as a positive control. The results were encouraging and berberine8998 (40μM, 2.26 absorption fold of control) was relatively more effective for stimulating LDL uptake than berberine (40μM, 1.22 absorption fold of control) and pravastatin (5μM, 2.20 absorption fold of control), an effect that was dose-dependent (Fig 5.1). The images from the fluorescence microscope (Fig 5.2) confirmed the findings.

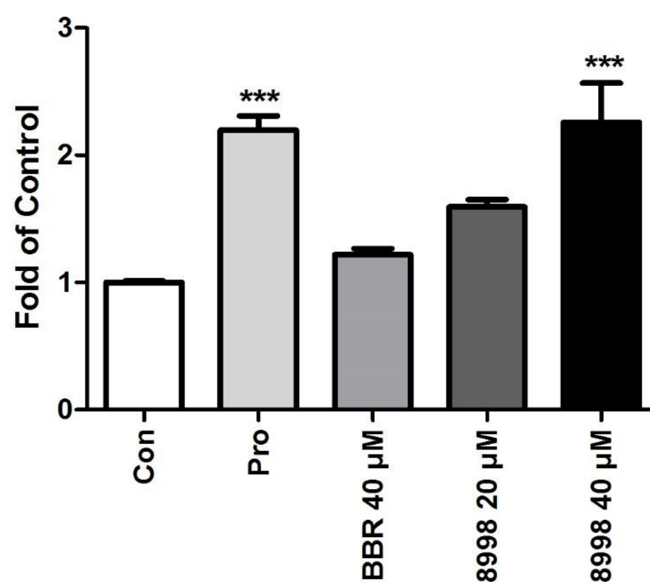

Fig 5.1 Average uptake amount of Dil-LDL in HepG2 cell incubated with test compounds: Con, control; Pro, pravastatin; BBR, berberine; 8998 berberine8998. N=3 ***p<0.001, compared with control group by Dunnet's test after one way ANOVA

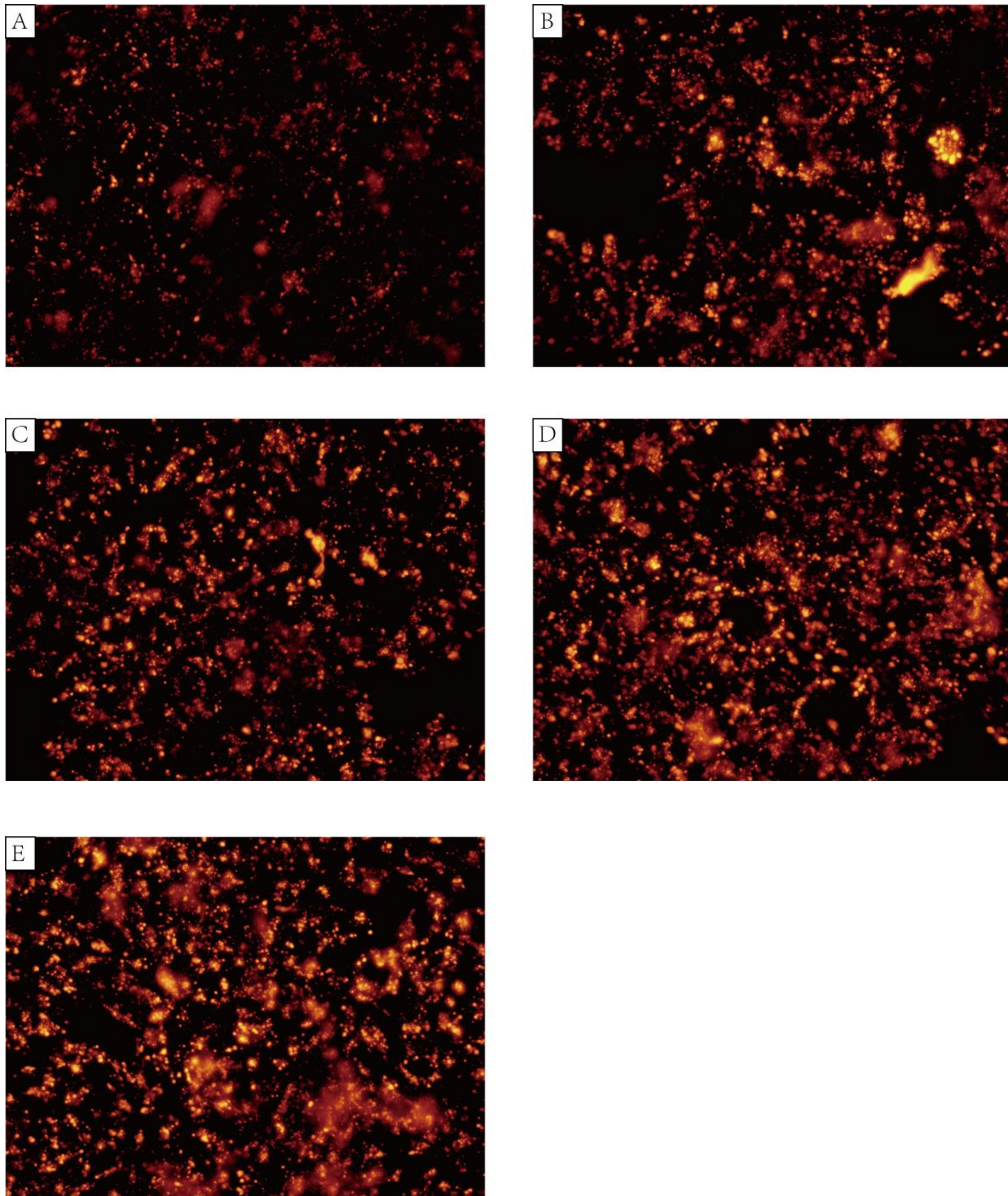

Fig. 5.2 Fluorescence images illustrating the uptake amount of Dil-LDL in HepG2 cells with test compounds. (A) Control group, HepG2 cells treated with Dil-LDL. (B) Pravastatin group, HepG2 cells treated with Dil-LDL and 5 μM pravastatin. (C) Berberine 40 μM group, HepG2 cells treated with Dil-LDL and 40 μM berberine. (D) Berberine8998 20 μM group, HepG2 cells treated with Dil-LDL and 20 μM berberine8998. (E) Berberine8998 40 μM group, HepG2 cells treated with Dil-LDL and 40 μM berberine8998. Magnifcation:10X

5.3 Discussion

The Dil-LDL study was designed to verify the hypothesis that berberine8998 is able to promote LDL uptake *in vitro*. To confirm the hypothesis that berberine and berberine8998 both increase the uptake amount of LDL, HepG2 cell line was incubated with the test compounds and Dil-LDL. A fluorescent detector was used to scan the uptake amount of Dil-LDL.

The Dil-labeled lipoproteins showed specific high affinity to human fibroblasts via the LDL (apo-B, -E) receptors (Pitas et al., 1981). LDL bound at 37°C was rapidly internalized by the cell in specific membrane areas called coated pits (Orci et al., 1978). Dil-LDL is internalized rapidly at 37°C by normal fibroblasts and it depresses the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) in a manner similar to that of LDL (Barak and Webb, 1981). Lipoproteins labeled with Dil were suitable for the direct viewing of lipid uptake by cells *in vitro*.

Cells treated with the cholesterol-lowering agent pravastatin served as a positive control. The results were encouraging and berberine8998 (40μM, 2.26 fold) was relatively more effective (***p<0.001) for stimulating LDL uptake than berberine (40μM, 1.22 fold) and pravastatin (5μM, 2.20 fold), an effect that was dose-dependent.

The use of fluorescent labeled lipid provides us a tool to quantify the amount of lipid uptake. By using Dil-LDL as a marker, both berberine-8898 and berberine treated cells stimulated the up-take of LDL. The intensity (a.u.) of Dil-LDL in HepG2 cells was then screened to compare the amount of lipid uptake by the positive control group (Pravastatin), and the berberine and berberine8998 groups. In this experiment, berberine8998 (40μM) exerted more LDL uptake in HepG2 cells than berberine and positive control pravastatin (2.26 vs 1.22, 2.20, p<0.001). This improvement may contribute to the lipid lowering effect of berberine8998. In general, the exploration here suggests that berberine, and to a greater extent berberine8898, stimulated the uptake of LDL in the HepG2 cells, similar to the cholesterol-lowering agent pravastatin.

6. Analysis of LDLR and PCSK9 levels

6.1 Introduction

Liver low-density lipoprotein receptor (LDLR) plays a central role in manipulating cholesterol homeostasis by mediating LDL clearance from the bloodstream. LDLR is a membrane-spanning glycoprotein with a highly conserved structure in human and other animals. Since LDLR was discovered by Goldstein and Brown in 1974 on cultured human skin fibroblasts, extensive study has been undertaken to reveal its structure, function, mutation, and physiological as well as pharmacological modulations (Kong et al., 2006). The mature form of the human LDLR protein contains 839 amino acid residues with a molecular mass of 160 kDa (Tolleshaug et al., 1982). It is made up of six functional domains; the signal sequence, ligand binding domain, EGF precursor-like domain, *O*-linked sugar domain, transmembrane domain and the cytoplasmic domain (Sudhof et al., 1985). The gene is located on chromosome 19p13.1 and the cytoplasm, ligand binding domain, EGF precursor-like domain, mediating LDL clearance from the blood (Sudhof et al., 1985, Tolleshaug et al., 1982). Mutations in the LDLR give rise to familial hypercholesterolaemia (Heath et al., 2001). Up-regulation of liver LDLR expression has been proven to be one of the most effective ways to lower plasma cholesterol level (Bays and Stein, 2003).

Proprotein convertase subtilisin/kexin type 9 (PCSK9) was discovered in the past decade as a novel therapeutic target for the management of patients with hypercholesterolemia. This protein can bind to the epidermal growth factor-like repeat A site of LDLRs at the hepatocyte surface, leading to their degradation in the liver, thereby promoting their lysosomal degradation (Zhang et al., 2007). Inhibition of PCSK9 leads to an increase in LDLR and consequently results in a reduction of LDL cholesterol in blood. Berberine has been reported to decrease the level of PCSK9 and thus has a positive effect on LDL levels (Cameron et al., 2008).

Western blotting is an important technique used in analysis of cell and molecular

biology (Mahmood and Yang, 2012). It is a protein analysis technique which combines protein separation in an electric field with immunochemical methods of protein detection. It is widely used to detect, characterize, quantify, and/or isolate specific proteins or antibodies in complex biological samples. The technique initially involves gel electrophoresis and is used to separate native proteins based on the 3D structure of proteins and the molecular size of the polypeptide. This is followed by the electrophoretic transfer of the proteins out of the gel to a two-dimensional, protein-binding membrane (typically PVDF) (Gibbons, 2014). The membrane is then incubated with labeled antibodies specific to the protein of interest. The unbounded antibody is washed off, leaving only the binding protein-specific antibody or ligands. Finally, the bounded antibodies are visualized by developing the film. The LDLR and PCSK9 expression levels of tissue and cell samples were analysed by Western blot to evaluate the lipid lowering effect of berberine8998.

6.2 Results

6.2.1 Western blot analysis results of LDLR levels in hamster liver samples

Western blots analysis were applied to analyze the LDLR level in the liver tissue of hamsters in 5 groups after 3 weeks of high-fat diet (preliminary pharmacodynamics study). Fig 7.1, showed that LDLR levels were low in the negative control group. The LDLR level promoting effect of berberine8998 was clearly dose dependent (50mg, 100mg). The LDLR level promoting effect of berberine8998 (100mg) was higher (as exhibited by the darker shade) than that of berberine (200mg) *in vivo*.

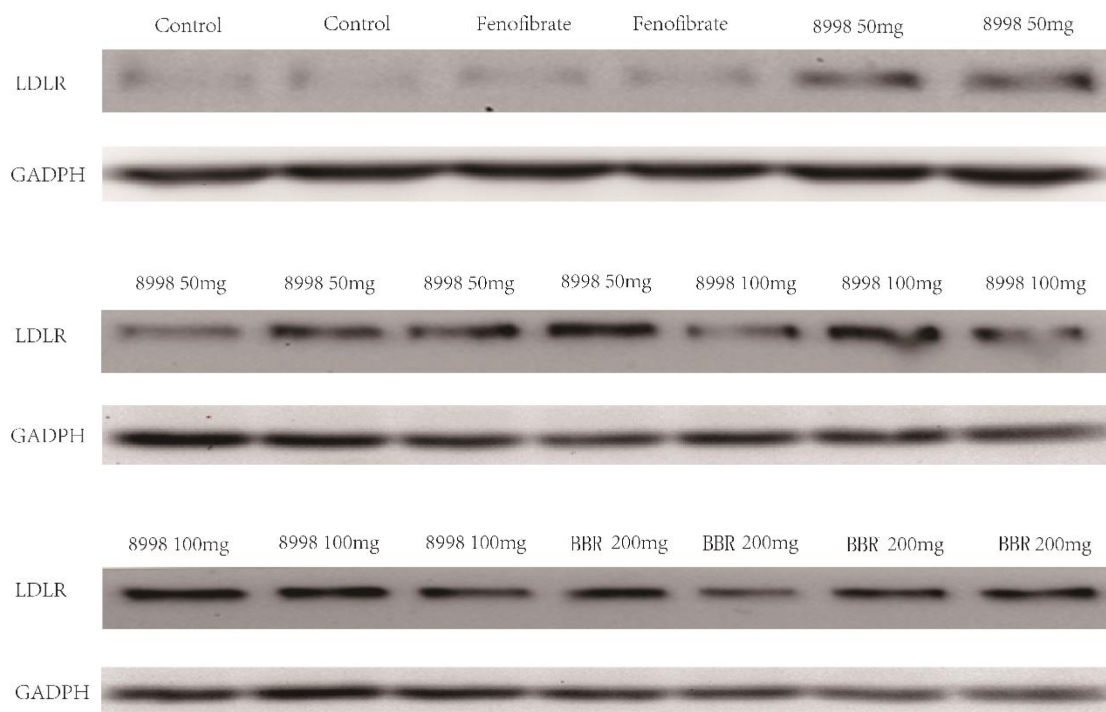

Fig 6.1 Western blot analysis of LDLR expression level of hamster liver samples (given berberine8998 and berberine for three weeks). Control: n=2, Fenobirate: n=2, berberine8998-50 mg/kg n=6; berberine8998-100 mg/kg n=6; berberine-200 mg/kg n=4

6.2.2 Western blot analysis of LDLR in HepG2 cells

LDLR expression in the HepG2 cells was screened by Western blotting. LDLR was up regulated dose-dependently upon treatment with berberine and berberine8998 (Fig 7.2), with the latter inducing a greater effect. These results confirm that berberine8998 promotes the uptake of LDL by increasing the expression of LDLR *in vitro*.

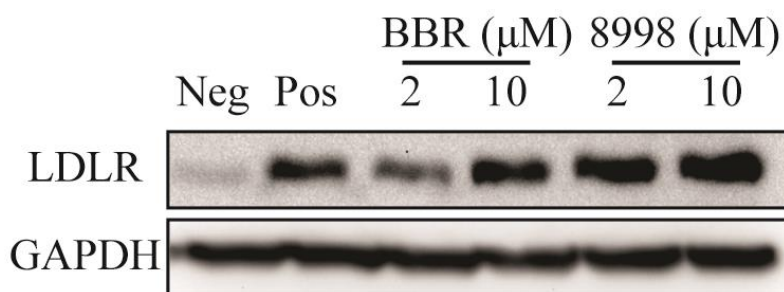

Figure 6.2 Western blot analysis of LDLR expression level of HepG2 samples. Neg, blank; Pos, pravastatin; BBR, berberine; 8998, berberine8998.

6.2.3 Western blot result for PCSK9 in HepG2 cells

The PCSK9 lowering effect of berberine8998 on HepG2 cell was tested by the Western blot method. Berberine was used as positive control. The results showed that berberine8998 has a dose dependent down-regulation of PCSK9 (Fig 7.3). However, the PCSK9 down-regulation effect of berberine8998 is more pronounced than that of berberine.

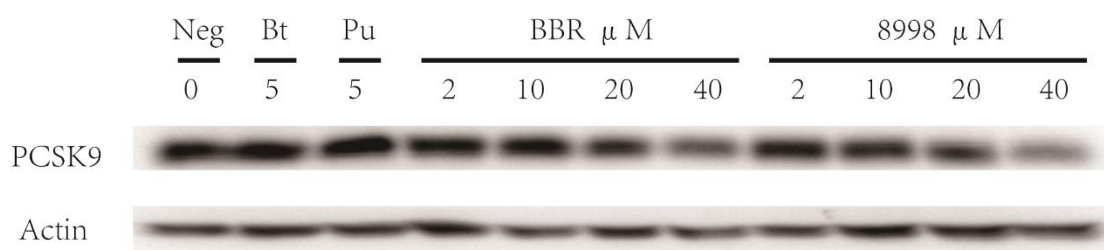

Figure 6.3 Western blot analysis of PCSK9 expression level of HepG2 samples. Neg, blank; Bt, fenofibrate; Pu, pravastatin; BBR, berberine; 8998, berberine8998.

6.3 Discussion.

Fenofibrate is the most frequently prescribed agent in the treatment of hyperlipidemia (Brown, 1987, Tarantino et al., 2017). It is a Peroxisomal Proliferator-Activated Receptor (PPARα) agonist. PPARα is a nuclear receptor belonging to the PPAR family

of ligand-activated transcription factors (Willson et al., 2000). Fenofibrate is an effective lipid lowering agent that can be used safely for hamster experiments. However, it cannot influence and regulate liver LDLR levels. This is indicated by the absence of any difference in the expression of the LDLR level of the fenofibrate control group and the negative control group (Fig. 6.1).

The Food and Drug Administration (FDA) approved pravastatin in 2006 primarily for use in the treatment of dyslipidemia and prevention of cardiovascular disease. Pravastatin is in a class of medications called HMG-CoA reductase inhibitors, better known as statins. These drugs induce plasma cholesterol lowering by increasing hepatic intake of LDL particles, which results from the up-regulation of hepatic LDLR (Mach et al., 2014, Sahebkar et al., 2016). Hence pravastatin was chosen as the positive control in the HepG2 cell experiment on LDLR.

LDLR is the most important membrane protein media for lipid uptake. The Western blot analysis showed that the up-regulation of LDLR expression was greater in hamster liver samples feed with berberine-8898 and in HepG2 cells treated with berberine-8898 in comparison to berberine, indicating that berberine-8898 has superior lipid-lowering effects than the parent molecule *in vivo* and *in vitro*. The results also demonstrated that berberine8998 can down-regulate PSCK9, another atherosclerotic therapeutic target. Both fenofibrate and pravastatin showed no effect on this target (Fig 6.3). This suggested that berberine8998 could be improving lipid uptake via a yet unknown pathway.

7. Proteomic research using iTRAQ labeled analysis

7.1 Introduction

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 (Kristensen et al., 2014, Ray et al., 2011, Qin et al., 2013, Hou et al., 2013). It is a stable isobaric labeling multiplexed protein quantization strategy launched by Applied

Biosystems (USA) in 2004. It provides relative and absolute measurements of proteins in complex mixtures.

The 2D LC-MS/MS is usually performed using a SCX column in series with a RP column. In the course of analysis, tryptic peptides are eluted stepwise by injecting salt plugs of increasing ionic strength from the SCX column in the first dimension. In the second dimension these peptides are first trapped on a RP enrichment column and finally separated on an analytical RP column (Davis et al., 2001). By combining two different LC techniques with individual peak capacities between 50 and 100, it is theoretically possible to achieve total capacities of 2500–10,000 peaks (Nagele et al., 2004). Data (MS1 and MS2) is acquired using a mass spectrometry that generates high quality spectrums useful for further database searching of peptide and protein quantization. This method can be a high throughput quantitative technique and an efficient way to screen lipid lowering proteins (Kristensen et al., 2014). 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 the iTRAQ method was coupled with 2D LC-MS/MS (TripleTOF 5600) for investigating the operation mechanisms of berberine and berberine8998.

7.2 Results

7.2.1 Protein identification and quantitation

To clarify the mechanism of berberine and berberine8998, hamster liver protein expression levels were evaluated by iTRAQ analysis. Hamster liver samples from the berberine, berberine8998 and high-fat diet model groups were analyzed using iTRAQ shot-gun 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. 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.

Untreated, berberine8998 treated and berberine treated hamsters were labeled with iTRAQ reagent 113/114, 115/116 and 117/118, respectively. A total of 12 groups of hamsters and two iTRAQ labeling experiments were carried out. Thus for each treatment, there were 4 biological replicates. Student's t-test and P-value were used to evaluate the significance of protein expression level change among the liver tissue of differently treated hamster groups in iTRAQ, and the median of the ratio between each the two groups were calculated. The untreated model was taken as a reference. To filter the non-significant changes in liver protein expression levels between sample groups, 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 7.1

and Table 7.2.

Table 7.1. 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.

Table 7.2. 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-	Mink1	2.23×10^{-5}	0.68 ↓	Serine/threonine kinase which acts as a negative

Protein Name	Gene name	P-value	Fold of change	Protein Function
like kinase 1				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.

7.2.2 Gene Ontology (GO) analysis

A Gene Ontology (GO) analysis was carried out for the differently expressed proteins (Fig. 7.1). The top five enriched molecular functions of upregulated proteins in berberine and berberine8998 treated hamsters were metabolic processes, cellular metabolic processes, regulation of biological processes, and responses to chemical

stimulus. A larger number of proteins in the berberine8898 treated group showed altered expressions (Fig 7.1A). Most upregulated proteins were involved in FA metabolism and the degradation of the branched-chain amino acids valine/leucine/isoleucine (Fig 7.1B).

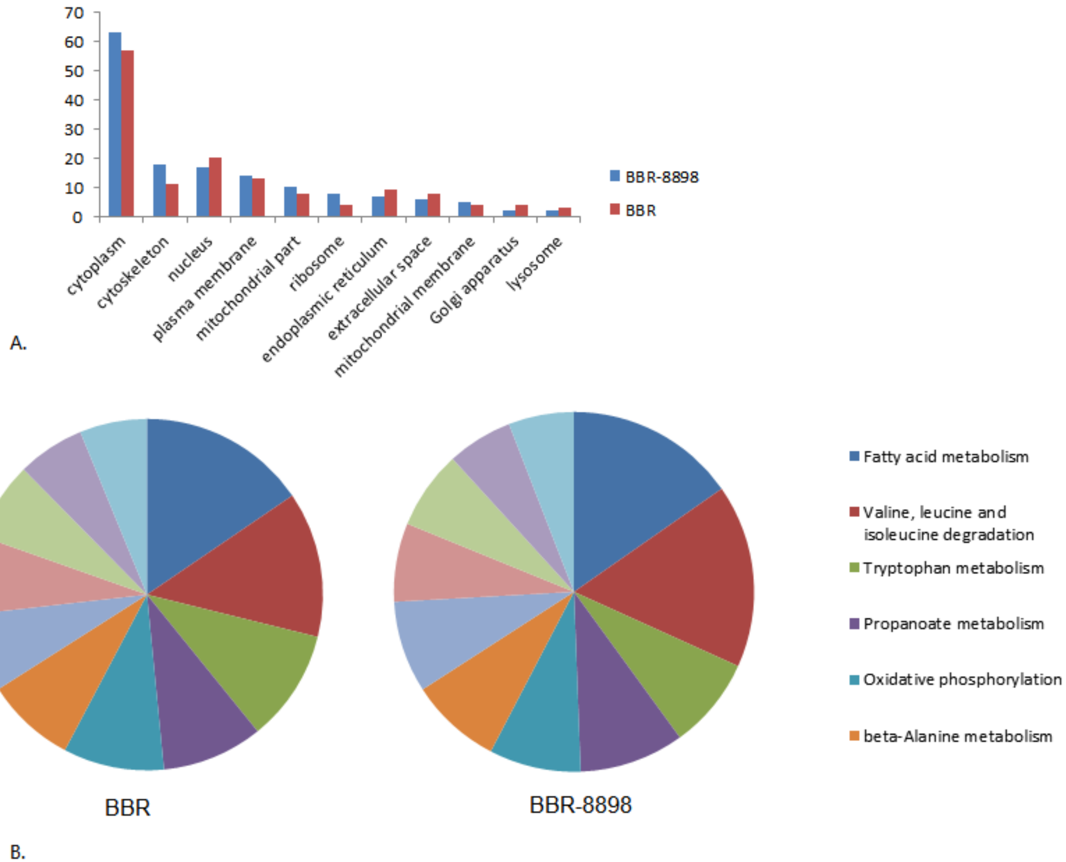

Figure 7.1 GO analysis of cellular components (A) and biological processes (B) associated with differently expressed proteins in berberine- and berberine8998-treated hamsters.

7.2.3 Ingenuity pathway analysis (IPA)

Differently expressed liver-based proteins of berberine and berberine8998 treated hamster were further analyzed by Ingenuity Pathway Analysis (IPA). The most enriched canonical pathway of the two group of proteins is LXR/RXR Activation pathway, which is involved in the regulation of lipid metabolism, inflammation, and cholesterol to bile acid catabolism (Fig. 7.2). ALB, APOA4, APOB and TF were involved in the

pathway, and down regulated in both treatment groups. Disease and biological function of the proteins is conversion of lipid (Fig. 7.3). ACOX1, ALB, APOB and CROT are downregulated in the berberine8898 treated hamsters, and ACOX1, ABHD5, ALB and TTPA which were differently expressed in the berberine treated hamsters. ACOX1 was chosen for further validation by western blot analysis.

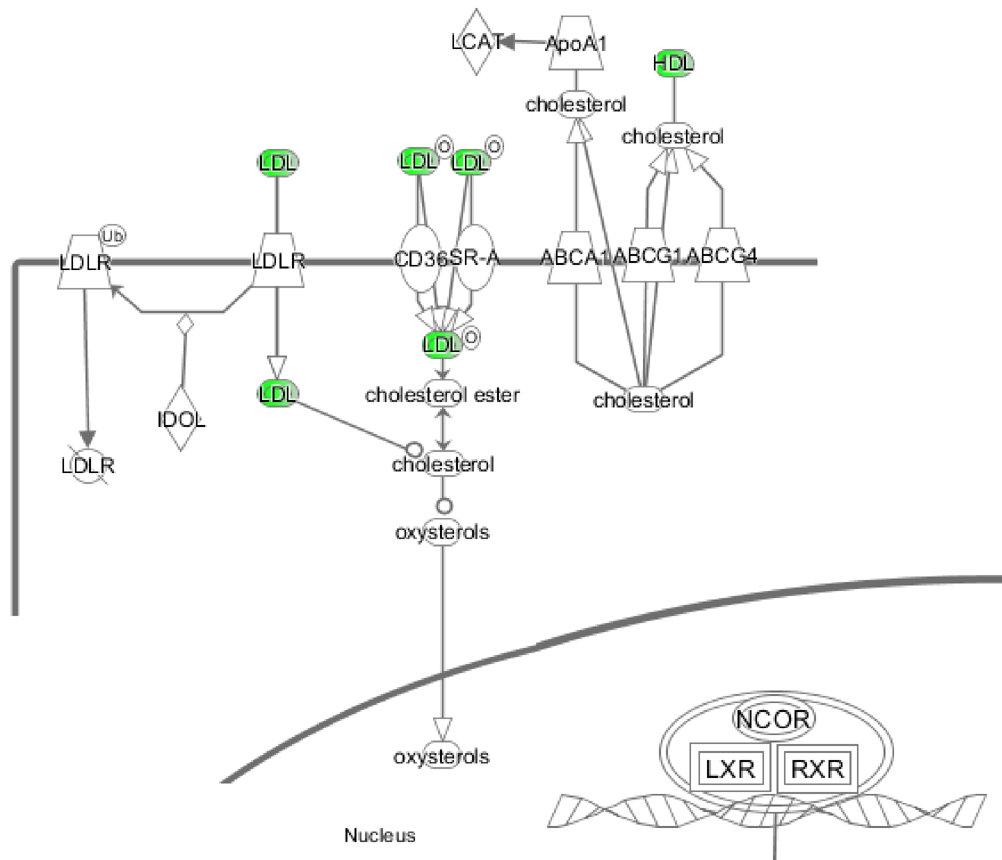

Figure 7.2 LXR/RXR activation pathway. Green labeled molecules were downregulated in this work.

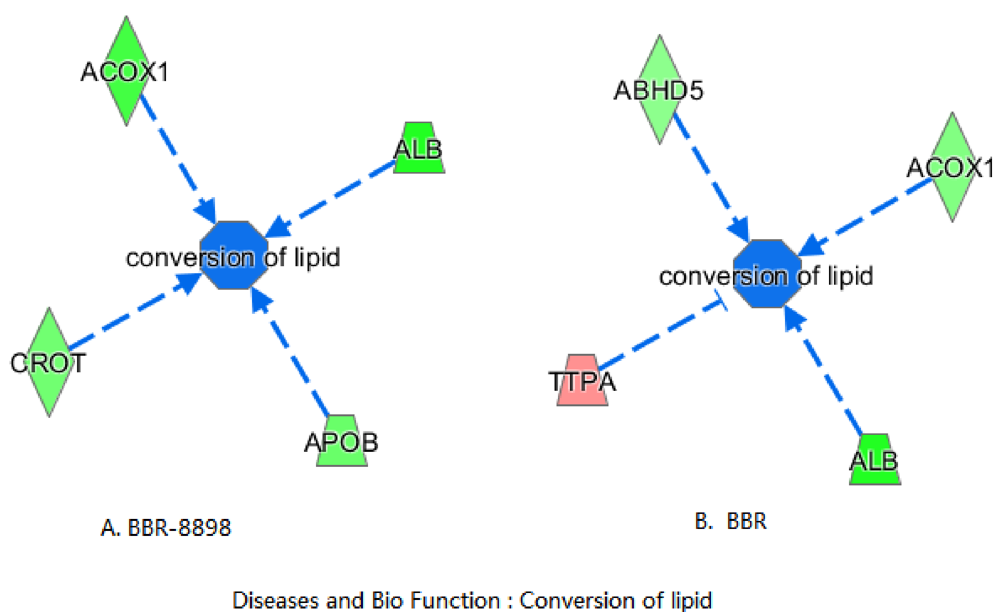

Figure 7.3 The disease and biological function pathway analysis of conversion of lipid. Green labeled proteins were down regulated and red labeled proteins were up regulated in berberine-8898 (left) and berberine (right) treated hamsters.

7.2.4 Heatmap analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

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 Fig 7.4, 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. The major KEGG pathways were FA degradation, FA metabolism, and degradation of branched-chain amino acids that were related to lipid metabolism as well.

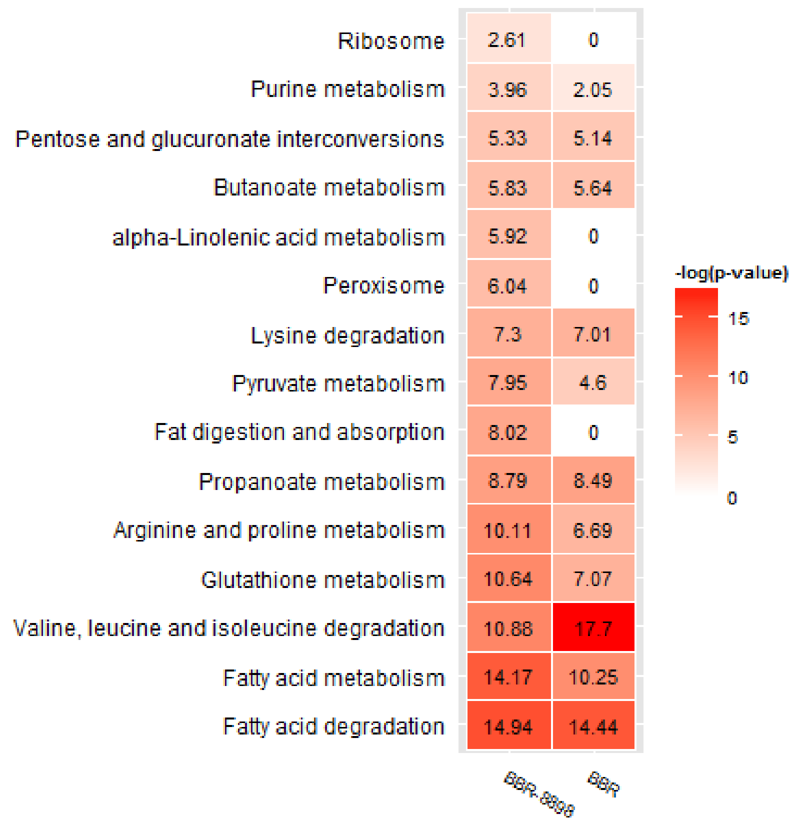

Figure 7.4 Protein KEGG pathways identified by heat map analysis. Proteins associated with FA metabolism and branched-chain amino acid degradation are in the red boxes. The colour bar is for the –log(p-value).

7.3 Discussion

Over the last decade, research in this area has been centered on understanding the behavior of proteins. The functions, interplays and changes in the abundance of proteins are in response to internal and external cues (Ray et al., 2011). So the iTRAQ method combined with 2D 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 beberine8898 in order to evaluate the effect of the two drugs. This is among the most powerful methodologies in quantitative proteomics (Kristensen et al., 2014, Qin et al., 2013, Hou et al., 2013) employing a stable isobaric labeling multiplexed protein quantization

strategy to provide relative and absolute measurements of proteins in complex mixtures. Using the iTRAQ method, 85 proteins were identified that were expressed differently between the treatment and control groups of which 19 were common to both the berberine and berberine8998 groups. These 19 proteins have the potential to be the target of berberine and berberine8998 therapy.

Functional similarity of proteins or genes can be estimated by controlled biological vocabularies, such as Gene Ontology (GO) (2008). Differently expressed proteins were annotated by GO using the DAVID software. The GO terms with computed P-values less than 0.05 were considered as significantly enriched. The pathway analysis of the protein expression pattern was performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems Inc.). The pathways were identified with the help of Ingenuity Knowledge Base which is comprised of known molecular interactions, functions, and most promising and relevant biomarker candidates within experimental datasets. The GO analysis and IPA analysis showed that proteins that were expressed differently in the treatment relative to the control group had similar functions. The results of the disease and bio function pathway analysis of conversion of lipid showed that ACOX1 and ACSL1 were significantly down regulated by both berberine and berberine8998.

KEGG is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks (Kanehisa et al., 2014). It was widely used for biological interpretation of genome sequences and other high-throughput data. The KEGG pathway enrichment heat map suggested that branched-chain amino acid degradation and FA metabolism pathways were specifically activated by the treatment, and the detailed mechanisms were analyzed in LC-MS/MS, western blot and NEFA assay.

8. Serum levels of branched-chain amino acid

8.1 Introduction

Amino acids, as the basic units of protein molecules, are closely correlated with biological activities. Hence, quantitative plasma amino acid analysis is an essential tool for the investigating metabolic disorders linked to these compounds. The use of amino acid analysis in neonatal screening programs has allowed the rapid diagnosis of several inborn errors of metabolism (IEMs), notable examples of which are phenylketonuria (PKU) and maple syrup urine disease (MSUD) (Wojnicz et al., 2016, Woontner and Goodman, 2006, Chace et al., 1993, Chace et al., 1995). In addition amino acid analysis has been widely applied in the study of a growing number of pathologies including hepatic dysfunction, inflammatory bowel disease, cancer, muscle catabolism , neurotransmitter disorders, cardiovascular disease and insulin resistance (Peake et al., 2013).

Great effort has been put forth in the past 30 years to develop a reliable, rapid, and accurate method for the analysis of amino acids. Amino acid analysis methods rely on the derivatization procedures. Post-column derivatization techniques based on ion exchange separation of the underivatized amino acids are most popular. For optical detection, *O*-phthalaldehyde (OPA), dansyl chloride, 2,4-dinitroflurobenzene (DNFB), phenylisothiocyanate (PITC), fluorescein and isothiocyanate (FITC) are used as derivatization agent (Sherwood, 2000, Krok and Seaver, 1991). Optical detection of amino acids could achieve high sensitivity. However, the specificity and selectivity of optical methods are poor. For mass detection, *N,O*- bis (trimethylsilyl) trifluoroacetamide (BSTFA) and *N*-Methyl-*N*-*tert* (butyldimethylsilyl) tri-fluoroacetamide (MTBSTFA) are usually employed as derivatization agent (Starke et al., 2001, Halama et al., 2016).

An increase in plasma branched chain amino acid (BCAA) concentrations is a hallmark of adult obese individuals (Halama et al., 2016). BCCA related metabolic signature was found to differentiate obese and lean humans (Newgard et al., 2009). BCAAs have also

been shown to contribute to obesity-associated insulin resistance. Moreover, studies with obese children and children with type 2 diabetes compared to healthy lean subjects has indicated that plasma BCAA levels are related with these diseases (Mihalik et al., 2012, Wahl et al., 2012).

In the present investigation, improved analyses of aTRAQ Reagent 115 labeled amino acids were developed, permitting the study of 42 amino acids from serum samples in a single run. BCAA metabolism is altered in obesity, however, how it is regulated and cross-linked to other pathways is still not fully understood.

8.2 Results

Thirty-six hamsters were maintained on a high-fat diet, divided to three groups (12 in each group), one group received no treatment, and the other two groups were treated with either berberine or berberine8998 (50 mg/kg each). The hamster serum level of Val, Ile and Leu were determined by aTRAQ amino acid test kit. The results are shown in Fig 9.1. The serum level of Val, Ile and Leu of both the berberine8998 group and the berberine group were not significant higher than that of control group. The results also showed no statistical difference (one way ANOVA) between the berberine8998 group and the berberine group (Appendix Table 1). It can be concluded that these two compounds had similar effect on BCCA.

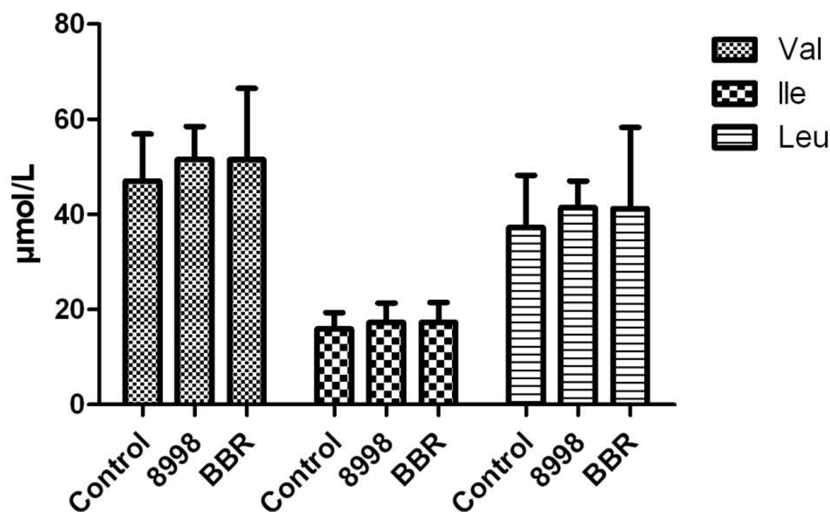

Figure 8.1 A comparison of serum levels of Val, Ile and Leu after treatment of hamsters with BBR8998 and BBR against control. N=12, Mean ±SD

8.3 Discussion

A critical step for amino-acid testing is the sample preparation. The key task of sample preparation is to remove the proteins that are in high abundance. Organic solvents such as acetone and methanol were first applied to remove high abundance proteins (Arola et al., 1977, Lavi et al., 1986). However, organic solvents depressed the extraction recovery and decoction sensitivity (Grossie et al., 1993). Ultracentrifugation could be another option for removing the high-abundant proteins, but that method is inefficient and time consuming (Hubbard et al., 1988). In this study, salicylsulfonic acid was selected for recovering the proteins and it proved to be efficient for high extraction recovery.

This LC-MS/MS method (Section 2.6.1) determines the concentration of 42 amino acids (branched-chain amino acid, including Val, Ile and Leu) in the hamster serum sample. The system suitability test is performed using a standard mixture of aTRAQ Reagent 115 to label the amino acids. The retention times of key amino acids was kept to within 2 to 11 minutes (Fig 8.2). The total gradient elution time (Section 2.6.1.2) was

set at 13 minutes for each run. Both berberine and berberine8998 were observed to have slight increases in the serum BCCA level. However, such increase is not significant. These results indicate that berberine8998 had similar limited promotion effects on BCCA as berberine.

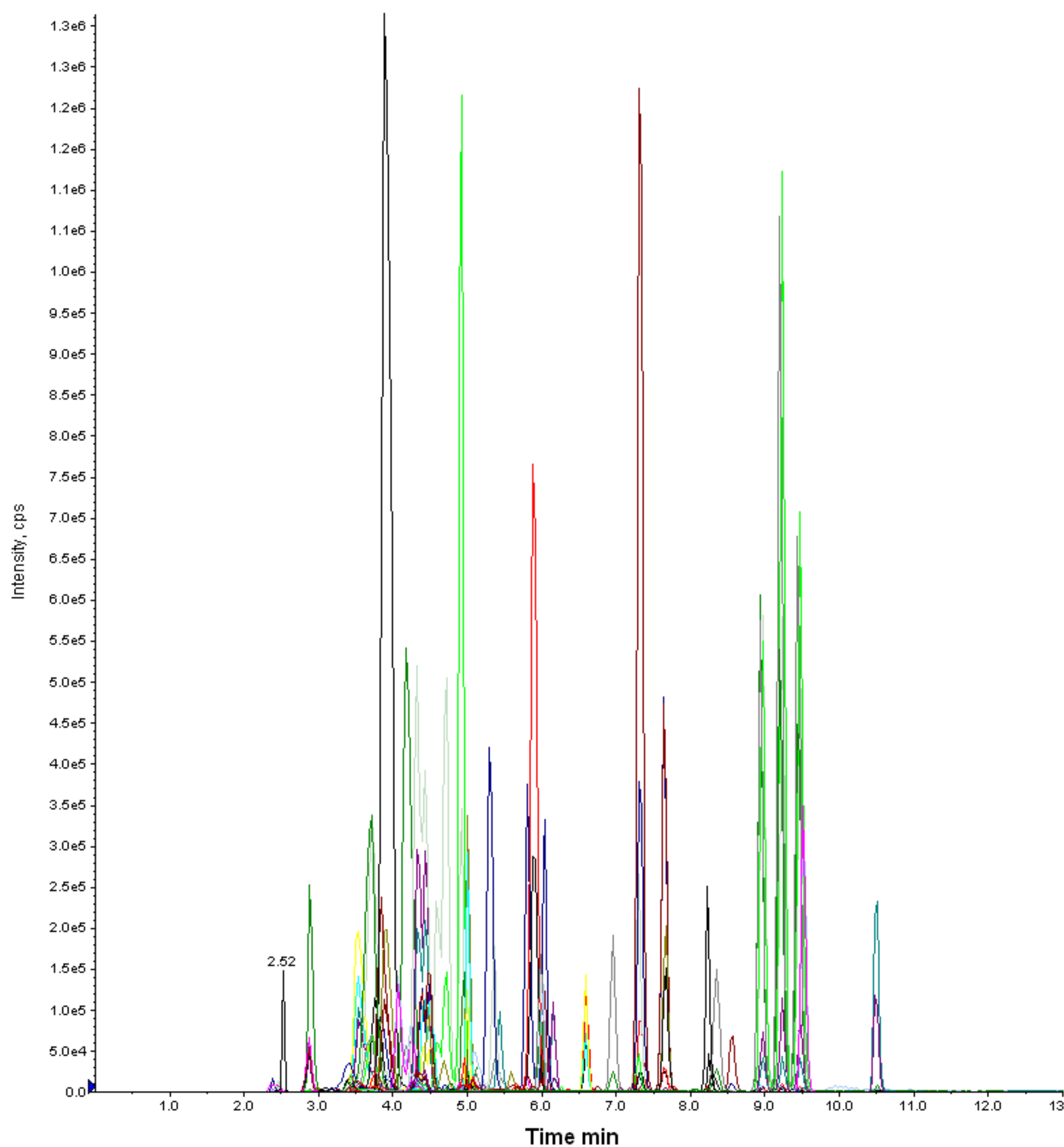

Figure 8.2 Retention times of key amino acids, (each colour represents an amino acid ion pair)

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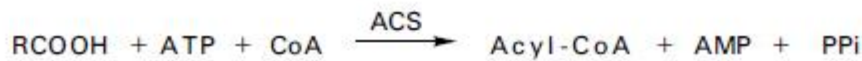
9. Hamster serum levels of NEFAs and validation of relevant protein expressions

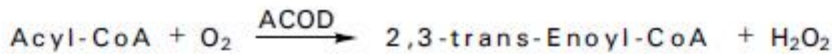
9.1 Introduction

NEFA (Non-esterified Fatty Acid, Free Fatty Acid) are defined as fatty acids that are not bound to any lipid fractions present in biological fluids (*e.g.* triglycerides, phospholipids, cholesterols). These play a pivotal role in the development of non-alcoholic fatty liver disease (Neuschwander-Tetri, 2010). They are also associated with coronary heart disease, recurrent ischemic stroke, and carotid atherosclerosis (Yang et al., 2014, Jickling and Spence, 2014, Taniguchi et al., 2002).

Fatty acids synthesized by the liver are converted to triglycerides and transported to the blood as very-low-density lipoprotein (VLDL). In the peripheral tissues, lipoprotein lipase digests part of the VLDL into LDL and NEFA, which are taken up for metabolism. LDL can be absorbed via LDLR, providing a mechanism for uptake of LDL into the liver.

Extraction methods are widely used for the colorimetric determination of NEFA. NEFA are converted to their cropper salts that are then extracted into an organic solvent. The salts are then made to complex with a dye for the purpose of colorimetric measurement (Duncombe, 1964, Itaya and Ui, 1965, Novak, 1965). These approaches are time consuming, hazardous and not easily automated. The choice of the NEFA determinations method is NEFA-HA Test kit: acyl-CoA synthetase (ACS) and acyl-CoA oxidzase (ACOD) method. This method relies on the production of CoA by the fatty acids in the presence of added ACS (Fig 10.1). The acyl-CoA thus produced is oxidized by the added ACOD with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD), permits the oxidative condensation of MEHA with 4-aminoantipyrine to form a purple coloured product which can be measured at 550nm.





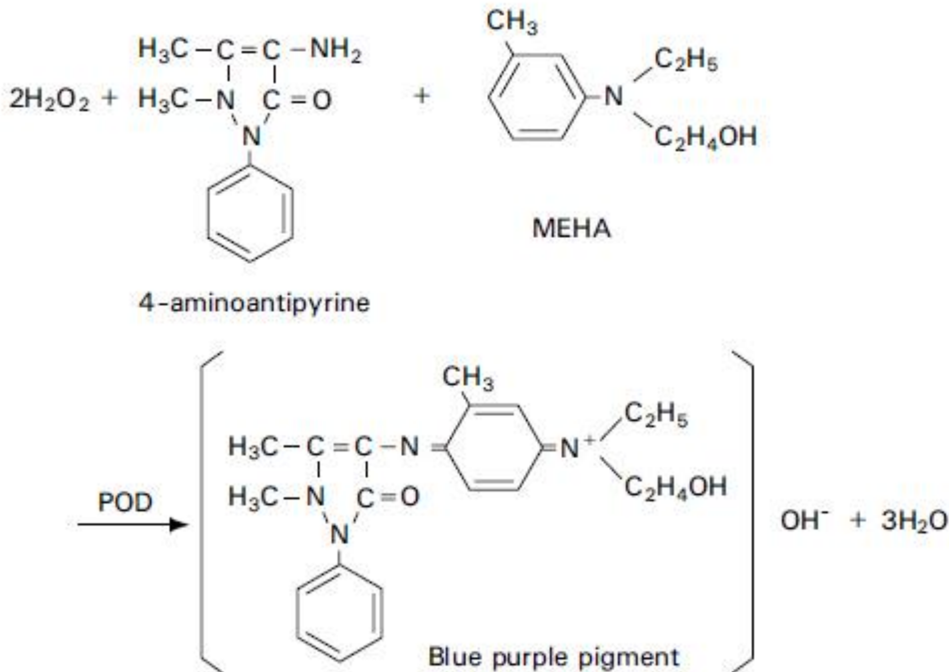


Figure 9.1 The principle of NEFA determination

9.2 Results

9.2.1 Serum levels of NEFAs

Serum levels of NEFAs were evaluated by means of biochemical analysis. These were higher in the high-fat diet group than in the berberine8998 treatment group (4.93 vs. 4.07 mM; $P < 0.05$, compared with High Fat Diet Group by Dunnet's test after one way ANOVA; Fig 9.2) and was lower in animals maintained on a normal as compared to a high-fat diet (1.45 vs. 4.93 mM; $P < 0.001$, compared with High Fat Diet Group by Dunnet's test after one way ANOVA). These results suggest that berberine8998 reverses the increase in serum NEFAs levels induced by a high-fat diet.

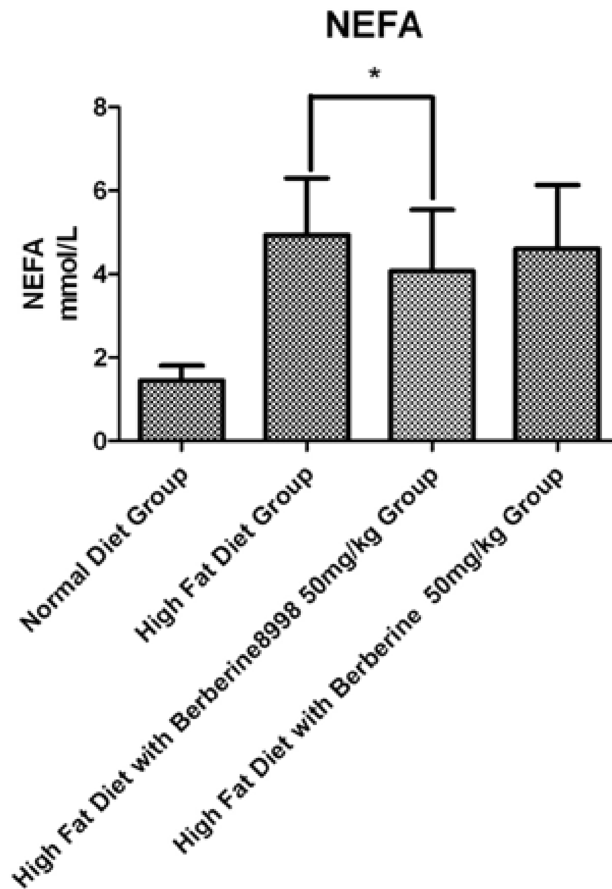


Figure 9.2 Serum levels of NEFAs. Hamsters were maintained on a normal or high-fat diet. In high-fat diet hamsters, one group received no treatment, while the other two groups were treated with either berberine (50 mg/kg, n=12) or berberine8998 (50 mg/kg, n=12). *p<0.05, compared with High Fat Diet Group by Dunnet's test after one way ANOVA

9.2.2 Validation of differently expressed proteins

Although the major metabolic pathways were similar in berberine and berberine8998 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. 9.3).

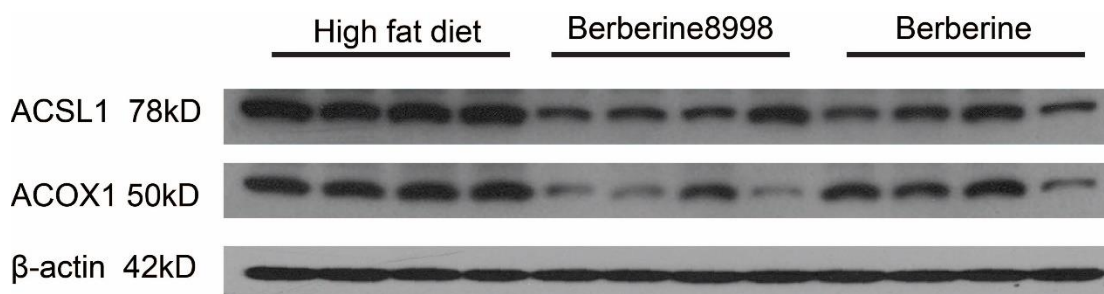


Figure 9.3 Western blot analysis of ACOX1 and ACSL1 expression in hamsters after being treated with berberine or berberine8998 vs. untreated controls fed a high-fat diet.

9.3 Discussion

Serum levels of NEFA play an important role in the development of non-alcoholic fatty liver disease (Novak, 1965) and are linked to coronary heart disease, recurrent ischemic stroke, and carotid atherosclerosis (Jickling and Spence, 2014, Varin et al., 2015, Yang et al., 2014). NEFA levels can be determined by colorimetric methods that induce their conversion to copper salts, which are then extracted into an organic solvent and undergo complex formation with a dye whose absorbance can be measured (Elphick, 1968, Itaya and Ui, 1965, Tinnikov and Boonstra, 1999). Serum levels of NEFAs were decreased after treatment with berberine8998. The majority of the differently expressed proteins that were found to be associated with the FA metabolism pathway were common to both berberine and berberine8998 treatment groups, suggesting that the two compounds have similar functional mechanisms.

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 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.

A major function of peroxisomes is β-oxidation of FAs. For this purpose, peroxisomes have two forms of ACOX (1 and 2)—which catalyze the second and third steps of β-oxidation, respectively—as well as two 3-oxoacyl-CoA thiolases (Wanders, 2004). The two isoforms of ACOX1 isoforms are distinct with respect to their biochemical functions (Oaxaca-Castillo et al., 2007): isoform 1a has the highest activity against medium-chain fatty acyl-CoAs, which decreases with increasing chain length, and isoform 1b has a broader range of substrates, including long-chain acyl-CoAs (Ferdinandusse et al., 2007). We confirmed via Western blotting analysis that the expression of ACOX1 was downregulated by treatment with berberine and berberine8998, suggesting that these agents can reduce serum levels of NEFAs by modulating ACOX1 expression. Since high levels of ACOX1 are associated with hepatocellular carcinoma and hepatomegaly (Oaxaca-Castillo et al., 2007, Ferdinandusse et al., 2007), these findings highlight the possibility of therapeutic applications for berberine8998.

10. Method validation of berberine in SD rats by LC-MS/MS

10.1 Introduction

The triple quadrupole mass coupled with liquid chromatography (QqQ, LC-MS/MS) is the most frequently used highly sensitive and selective spectrometric method for the determination of drug levels in biological fluids (Hurtado-Gaitan et al., 2017, Schuhmacher et al., 2008). It consists of the first quadrupole (Q1), collision cell (Q2) and the third quadrupole (Q3). Therefore, a QqQ mass spectrometer can be operated in four different modes with collision-induced dissociation: multiple-reaction monitoring (MRM/SRM), product-ion scan, precursor-ion scan, and constant neutral loss. MRM is the most common function utilized for quantification and confirmation. In general, a product-ion spectrum is first acquired with a reference standard, where MRM transitions are defined and selected to perform LC/MS/MS analysis. The newest instruments enable us to set short MRM dwell times without loss of sensitivity, making multi-target methods possible (Draisci et al., 2001, Zi-Min et al., 2017). LC-MS/MS with MRM scan has now been applied by many investigators to identify trace levels in biological fluids as a gold standard method. Compared with enzyme-label immunoassay, which is already highly sensitive and specific, it has not only better sensitivity and specificity but also good accuracy and reproducibility.

To determinate the berberine levels, multiple detection methods have been developed, such as high performance liquid chromatography-fluorescence detection (HPLC-FD) (Akao et al., 2000), field desorption mass spectrometry (FDMS) (Lee et al., 2003), gas chromatography chemical ionization mass-spectrometry (GC-CIMS) (Miyazaki et al., 1978) and high performance liquid chromatography–mass spectrometry (HPLC-MS) (Lu et al., 2006, Liu et al., 2010).

However, although these methods have good sensitivity and selectivity, they do not provide evidence and procedure for method validation. For this reason, a sensitive and reliable liquid chromatography tandem mass spectrometry (LC-MS/MS) method with liquid–liquid extraction procedure was developed. The determination of berberine in

plasma was undertaken using MRM with carvedilol (It's characteristic of separation is similar to berberine) as internal standard. The methods were validated by verifying linearity, lower limit of quantification, intra- and inter-assay precision and accuracy, specificity, quality control and stability for berberine in human serum. It was carried out according to the Guidance for Industry Bioanalytical Method Validation recommended by Food and Drug Administration (Guidance).

The structures of berberine and berberine8998 (Fig.1.5) are similar. After oral administration of berberine8998, it is transformed to berberine (ratio 1:1) in the plasma, thus it might work as a prodrug. Hence the method developed here (Chapter2.4.2) could also be applied to analyze plasma samples after administration of berberine8998. This method was successfully applied to the pharmacokinetic study of berberine for evaluating and comparing the formulation of berberine8998.

10.2 Results

10.2.1 Standard curve analysis

To verify linearity, a standard curve was established on each validation day. The calculated peak area ratios of berberine to the internal standard (IS) versus the nominal concentration in rat plasma showed a good linear relationship in the concentration range from 5 to 300ng/ml. A typical standard curve was $Y= 0.0112X+0.00829$ ($R=0.9984$, Fig 10.1), where Y represents the ratio of berberine peak area to that of the IS, and X represents the plasma concentration of berberine. For each round, different standard curves were carried out (Table 10.1). The mean correlation coefficients (r) of all of the curves were all above 0.99, which means that Y and X have strong linear positive correlation. Thus, the calibration curves exhibited good linearity within the given range.

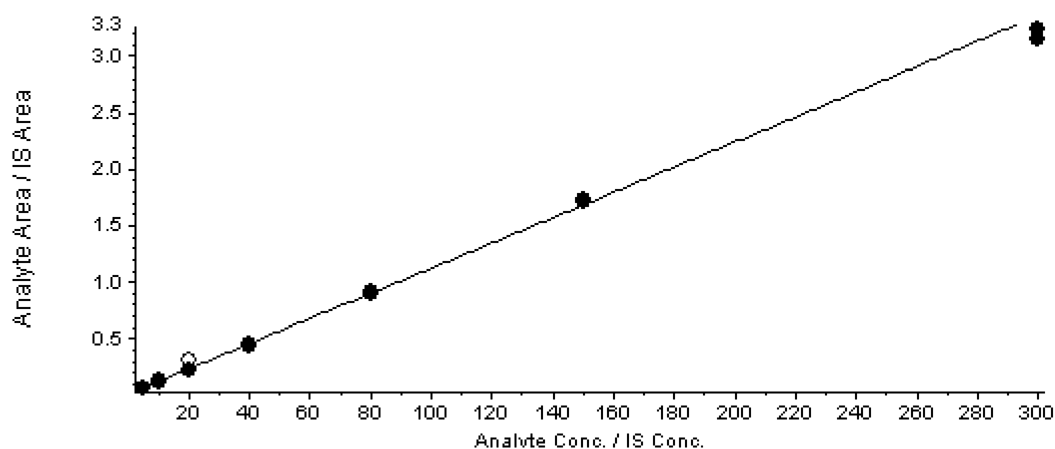


Fig 10.1 A standard curve of berberine

Table 10.1 Standard curves values for the berberine analysis n=6

Analyte Conc.（ng/mL）	**Calculated Conc.（ng/mL）**					
5	4.92	4.79	4.67	5.18	4.8	5.12
10	11.2	10.1	9.81	10.5	9.91	10.3
20	26.8	19.4	19.9	20.4	19.7	20.2
40	40.6	39	39.2	43.5	41	40.4
80	80.6	80.1	79.9	83	88.9	75.5
150	153	155	152	149	156	148
300	282	290	243*	260	291	275
formula	y=0.0112x+0.00829		y=0.0101x+0.00632		y=0.0101x+0.02	
R	0.9984		0.9974		0.9982	
5	4.53	5.05	5.17	4.74	5.23	4.28
10	10.7	10.4	9.22	10.5	11.3	9.5
20	20	22.3	20.7	22.3	22.3	22.5
40	39.5	41.8	36	42.9	39.4	41.9
80	74.9	87	78.9	79.6	73.9	81.9
150	137	150	143	156	152	152
300	248*	268	230*	283	264	267
formula	y=0.0123x+0.0153		y=0.00785x+0.00257		y=0.00775x+0.00989	
R	0.9957		0.9966		0.9935	

10.2.2 Lower Limit of quantitation (LLOQ)

The lower limit of quantification (LLOQ), which reflects the signal-to-noise ratio (S/N), was more than 6 at 5 ng/ml for berberine (Fig 10.2). The LLOQ, which was set at 5ng/ml, was sensitive enough for investigating the lowest dose of berberine in the serum samples. Acceptable precision and accuracy for the LLOQ were obtained. The average accuracy of these data was in the range 94.2%-109% (Table 10.2). The precision (RSD) of these data were all lower than 7% (Table 10.3).

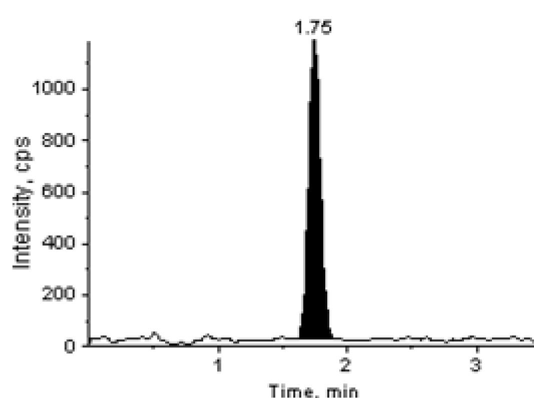


Fig 10.2 LLOQ result for berberine (5ng/ml)

Table 10.2 Results for accuracy of LLOQ for determination of berberine

Replicates		%Accuracy 5（ng/mL）		
1		99.2	103	94.2
2		95.8	108	97.6
3		103	114	111
4		102	110	110
5		109	106	110
6		103	112	107
Within-run	Mean	102	109	105
	N	6	6	6
Between-run	Mean	105		
	N	18		

Table 10.3 Results for precision of LLOQ for determination of berberine

Replicates		Calculated Conc.（ng/mL）		
		5		
		1	2	3
1		4.96	5.13	4.71
2		4.79	5.39	4.88
3		5.13	5.71	5.53
4		5.09	5.49	5.49
5		5.45	5.31	5.52
6		5.16	5.61	5.33
Within-run	Mean	5.1	5.44	5.24
	SD	0.22	0.21	0.359
	RSD (%)	4.32	3.85	6.84
	N	6	6	6
Between-run	Mean	5.26		
	SD	0.293		
	RSD (%)	5.58		
	N	18		

10.2.3 Intra- and inter-assay precision and accuracy

The within-run (intra-assay) and between-run (inter-assay) precision and accuracy for assays were characterized by the four levels of QCs run on three sequential batches in six replicates. All QC samples were randomized daily, processed and analyzed, together with calibration samples. The precision was expressed as a relative standard deviation (RSD) by calculating the standard deviation as the percentage of the mean calculated concentration, while the accuracy of the assay was determined as the percentage of the mean with reference to the nominal concentration. The results demonstrated that the precision (Table 10.4) and accuracy (Table 10.5) of this assay are within the acceptable range, and the method is accurate and precise.

Table 10.4 The precision of results of both the within-run and between-run for quantification of berberine in serum samples

Calculated Conc.（ng/mL）							
		5（ng/mL）			15（ng/mL）		
Within-run	Mean	5.1	5.44	5.24	14.5	13.1	14.2
	SD	0.22	0.21	0.359	0.683	0.655	0.898
	RSD (%)	4.32	3.85	6.84	4.7	5.01	6.33
	N	6	6	6	6	6	6
Between-run	Mean	5.26			13.9		
	SD	0.293			0.951		
	RSD(%)	5.58			6.83		
	N	18			18		
		50（ng/mL）			200（ng/mL）		
Within-run	Mean	54.5	50.5	51.1	203	182	201
	SD	1.64	2.08	1.67	6.05	2.42	4.46
	RSD(%)	3	4.12	3.27	2.98	1.33	2.21
	N	6	6	6	6	6	6
Between-run	Mean	52			195		
	SD	2.47			10.8		
	RSD(%)	4.76			5.53		
	N	18			18		

Table 10.5 The percentage accuracy of the results for quantification of berberine in serum samples for both the within-run and between-run.

%Accuracy							
		5（ng/mL）			15（ng/mL）		
Within-run	Mean	102	109	105	97.1	87.2	94.6
	N	6	6	6	6	6	6
Between-run	Mean	105			92.9		
	N	18			18		
		50（ng/mL）			200（ng/mL）		
Within-run	Mean	109	101	102	102	90.9	101
	N	6	6	6	6	6	6
Between-run	Mean	104			97.7		
	N	18			18		

10.2.4 Interference

Six different source drug-free serums (rat) were used to prepare blank samples and LLOQ samples. The interference peak areas of these samples were all lower than 15% of that of LLOQ samples (Table 10.6). It can be concluded that the internal interference was acceptable.

Table 10.6 Results of internal interference with determination of berberine in serum

Replicates	Peak Area (BBR)											
	Blank1		Blank2		Blank3		Blank4		Blank5		Blank6	
	blank	LLOQ	blank	LLOQ	blank	LLOQ	blank	LLOQ	blank	LLOQ	blank	LLOQ
1	718	6970	180	9240	257	8510	468	6610	262	5870	357	5100
2	427	6470	435	8760	253	7440	738	8730	330	3940	74.7	7390
3	886	11900	3380	8690	459	8630	1170	7410	628	7260	871	13900
Mean	677	8447	1332	8897	323	8193	792	7583	407	5690	434	8797
%Interference	8.01		15.0		3.94		10.4		7.15		4.94	

10.2.5 Quality control

The entire QC samples in three round of analysis were found to be acceptable. The accuracies of the QC samples were in a range 86.6% to 107% (Table 10.7). It can be concluded that the quality of the measure in all three rounds were good.

Table 10.7 Quality control of berberine determination

Replicates		Calculated Conc.（ng/mL）			%Accuracy		
		15	50	200	15ng/mL	50ng/mL	200ng/mL
Round1	QC1	14.3	43.3	196	95.5	86.6	97.8
	QC2	14.9	51.1	210	99.1	102	105
	QC3	14.9	51.8	180	99.6	104	90.1
Unacceptable Quant./ Total Quant.		0 / 9					
Round2	QC1	14.5	44.1	187	96.9	88.1	93.4
	QC2	15.4	53.1	184	103	106	92.1
	QC3	16.0	50.2	190	107	100	95.1

Unacceptable Quant./ Total Quant.		0 /9					
Round3	QC1	15.1	46.8	181	101	93.6	90.7
	QC2	14.8	49.8	187	98.4	99.7	93.4
	QC3	14.7	49.5	183	98.2	99.1	91.3
Unacceptable Quant./ Total Quant.		0 /9					

10.2.6 Stability

The auto-sampler stability was assessed by analyzing the extracted QC samples in the auto-sampler at room temperature for 0, 2, and 4 hours. The relative standard deviation (RSD) was expressed by calculating the standard deviation as the percentage of the mean calculated concentration at the time intervals investigated. These results are outlined in Table 10.8. The RSD values of these samples (three different concentrations of QC samples) were within 5%. The analytes remained considerably stable under the above conditions at all-time intervals investigated. The good stability of the berberine simplified the precautions needed for laboratory manipulations during the sample preparation.

Table 10.8 Results from stability analysis for determination of berberine

Analyte Conc. （ng/mL）	Bench-Top Time （h）	Calculated Conc. （ng/mL）			Mean	SD	RSD(%)	RE
15	0	14.6	14.8	14.8	14.7	0.115	0.784	/
	2	14.5	14.1	13.8	14.1	0.351	2.48	-4.07
	4	14.5	14.2	14.7	14.5	0.252	1.74	-1.81
50	0	49.9	49.7	50.2	49.9	0.252	0.504	/
	2	51.2	50.3	51.6	51.0	0.666	1.30	2.20
	4	51.5	49.4	46.8	49.2	2.35	4.78	-1.40
200	0	178	179	173	177	3.21	1.82	/
	2	189	182	183	185	3.79	2.05	4.53
	4	191	185	176	184	7.55	4.10	4.15

10.2.7 Extraction recoveries and matrix effects

In this study, four organic precipitation solvents were tried: acetonitrile, methanol, acetonitrile and water (1:1,v/v), methanol and water (1:1,v/v). Among them the acetonitrile:water solution yielded a good clean-up of the plasma samples and adequate recovery of the berberine. Mean results of extraction recoveries of berberine at 5, 15, 50 and 200 ng/ml were 80.5%, 84.2%,78.6% and 86.3%, respectively (n = 6, Table 10.9). The extraction recoveries is over 75%. It can be concluded that the extraction recoveries by this method were acceptable.

Mean results of matrix effects of berberine at 5, 15, 50 and 200 ng/ml were 94.4%, 97.1%,96.1% and 97.6 %, respectively (n = 6, Table 10.9). It indicated that the suppression of matrix effects in this method were not significant.

Table 10.9 Extraction recoveries and matrix effects on determination of berberine

Conc. (ng/ml)		5	15	50	200
Berberine	ER (%)	80.5	84.2	78.6	86.3
	ME (%)	94.4	97.1	96.1	97.6

10.3 Discussion

Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) have evolved as standard techniques for the ionisation of most small molecules in biological fluids analyses. In addition, atmospheric pressure photoionisation (APPI) which has the similar ionization range as APCI has been developed to increase ionisation efficiencies of non-polar compounds (Robb et al., 2000). APPI has been shown to be especially advantageous with respect to minimizing matrix effects. In this study, ESI which is applicable to polar and medium non-polar analytes that cover a very broad mass range was selected. Berberine is an alkaloid and hence a nitrogen atom-containing compound. To avoid ion-pair effect, hydrophobic ion-pair regents such as heptafluorobutyric acid (HFBA), trifluoroacetic acid (TFA), and non-afluoropentanoic

acid (NFPA) should be avoided in mobile phase preparation as well as in sample preparation (Gustavsson et al., 2001, Codony et al., 2002).

Internal standard is a known amount of chemical added to an analytical sample, which allows calibration of laboratory instruments. It is always used when performing MS quantization as quality control for extraction of a compound, HPLC injection and ionization variables. The internal standard could be a compound that is very similar, but not identical to the chemical species of interest in the samples. In this research, carvedilol which has the similar molecule weight to berberine, was selected as the internal standard (Fig 10.3).

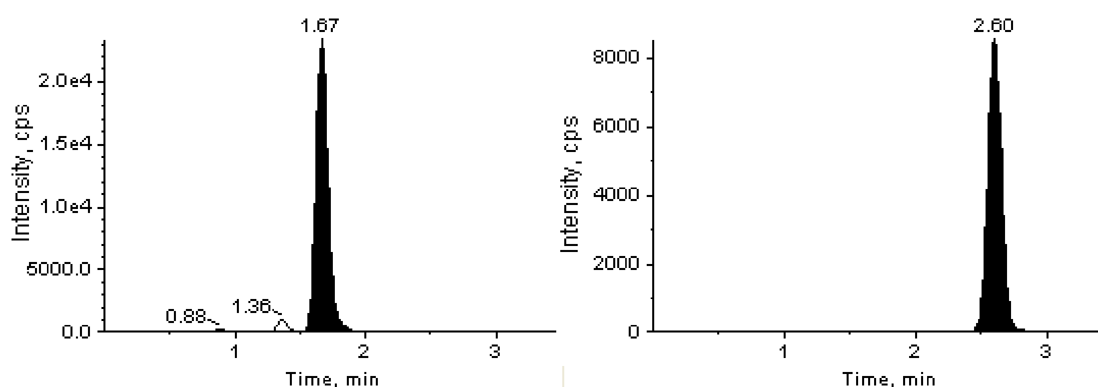


Fig 10.3 The results of sample (Left 198 ng/mL) and internal standard (Right 4.29μg/mL) from LC-MS/MS analyses of berberine determination

Sample preparation is a critical step for accurate and reliable LC–MS/MS assays. The high selectivity of MS instruments has initially led to the assumption that less effort is needed for sample preparation and chromatographic separation prior to MS detection. Precipitation is a widely adopted method and often achieves satisfactory extraction recoveries of an analytes from biological samples. Compared with liquid-liquid extraction and solid phase extraction, precipitation can provide a saving for both time and cost. Hence, precipitation was chosen to prepare the samples in this study.

The matrix effect is a challenge in LC-MS/MS quantitative analyses, especially when ESI is used as the interface. Eluting matrix components limits the accuracy of quantitative analytical methods by ion suppression/enhancement in the MS ion source

through competition for the electrical charges or through affecting the evaporation of the ESI droplets and the analyte transfer to the gas phase (Panuwet et al., 2016). The matrix effects can be estimated or determined by comparing the responses of analytes in solvent or buffer to those in the presence of matrices (Barrett et al., 2005). More specifically, the matrix effect was assessed by comparing the percentage of peak areas of berberine in neat QC chemical standards to blank serum samples after extraction.

The current study described, developed and validated an accurate cost-effective sensitive and specific LC/MS/MS method to determine berberine. Therefore, this method can also be applied in the pharmacokinetic study of berberine8998 to evaluate the absorption and quantification of berberine8998 in different formulations

11 Formulation of berberine8998

11.1 Introduction

Berberine, an isoquinoline alkaloid, has wide application in many research areas in recent years because of its many novel pharmacological functions, such as antioxidant, cardiovascular protection, anti-depressant, neuroprotective, anti-diabetic, anti-obesity, hepatoprotective, anti-rheumatic, antiangiogenic, anti-clastogenic and anti-cancer activities (Wang et al., 2017, Doggrell, 2005, Sun et al., 2009, Tang et al., 2009). However berberine's low oral bioavailability restricts its wide application since it is a lipophobic compound, it is restrained from passing through the membranes of intestinal cells (Zhang et al., 2012).

There are several reagents that can be combined with lipophobic drugs to enhance their uptake from the gastrointestinal tract. D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS) is a water-soluble derivative of natural vitamin E, which is formed by esterification of Vitamin E succinate with polyethylene glycol (PEG) 1000. TPGS can function as a P-gp inhibitor, solubilizer/absorption and permeation enhancer in drug delivery and TPGS-related formulations for nanocrystals, nanosuspensions, tablets/solid dispersions, as an adjuvant in vaccine systems, nutrition supplements, plasticizer films, anticancer reagents, etc. (Guo et al., 2013). It has been reported that TPGS at a concentration of 2.5% can improve the peak concentration (C_{max}) and the area under the curve (AUC) for berberine by 2.9 and 1.9 times, respectively (Chen et al., 2011).

Chitosan is an attractive polymer for biomedical applications due to its biocompatibility, biodegradability, natural origin, and low cost. It is a linear polysaccharide with a molecular weight up to ca. 2000 kDa, obtained by partial *N*-deacetylation of chitin from crustacean shells or mushrooms (Younes and Rinaudo, 2015). The mechanism by which chitosan enhances mucosal absorption is complicated. The most important factor is chitosan's positive charge which can interact electrostatically with the negatively charged sialic acid residues on mucin (Deacon et al., 2000). Formulations containing

0.5%, 1.5%, and 3.0% chitosan have resulted in improvement of AUC_{0-36} values of berberine by 1.9, 2.2, 2.5 times (Chen et al., 2012).

Sodium caprate, is the sodium salt of the aliphatic saturated 10-carbon MCFA. It is a compound already approved as a food additive. Rectal formulations containing sodium caprate have been shown to increase the absorption of a range of β-lactam antibiotics (Murakami et al., 1993). It increases the flux of many different types of poorly permeable agents across intestinal epithelia *in vitro* (Cho et al., 2002). The AUC values of berberine were increased by 28% compared to in the absence of sodium caprate (Lv et al., 2010). The pharmacokinetic study was applied to evaluate the AUC of the novel berberine8998 formulation.

11.2 Results

Method validation results for determination of berberine by LC-MS/MS were shown in section 4.2. In order to find out the pharmacokinetic differences between the two formulations, a study with 10 rats (two-way crossover) was undertaken. The tolerable daily intake limit (TDI) of the two formulations was both set at 100mg/kg according to the earlier hamster experiment (Section 4). Pharmacokinetic parameter could reflect the absorption of two formulations (administered Formulation R and Formulation A at same time). The mean serum concentration *versus* time profile obtained is presented in Fig 11.1. The principal pharmacokinetic parameters of Formulation R and Formulation A are summarized in table 11.1 and table 11.2. The maximum concentration of Formulation R and Formulation A in serum (Cmax) was 90.8ng/mL and 155.02ng/mL, respectively. The half-life of Formulation R and Formulation A in serum ($t_{1/2}$) was 3.42 h and 3.92 h, respectively. The area under the curve (AUC_{0-48}) of Formulation R was 727.789 h*ng/mL, and the AUC_{0-48} of Formulation A was 1702.162 h*ng/mL.

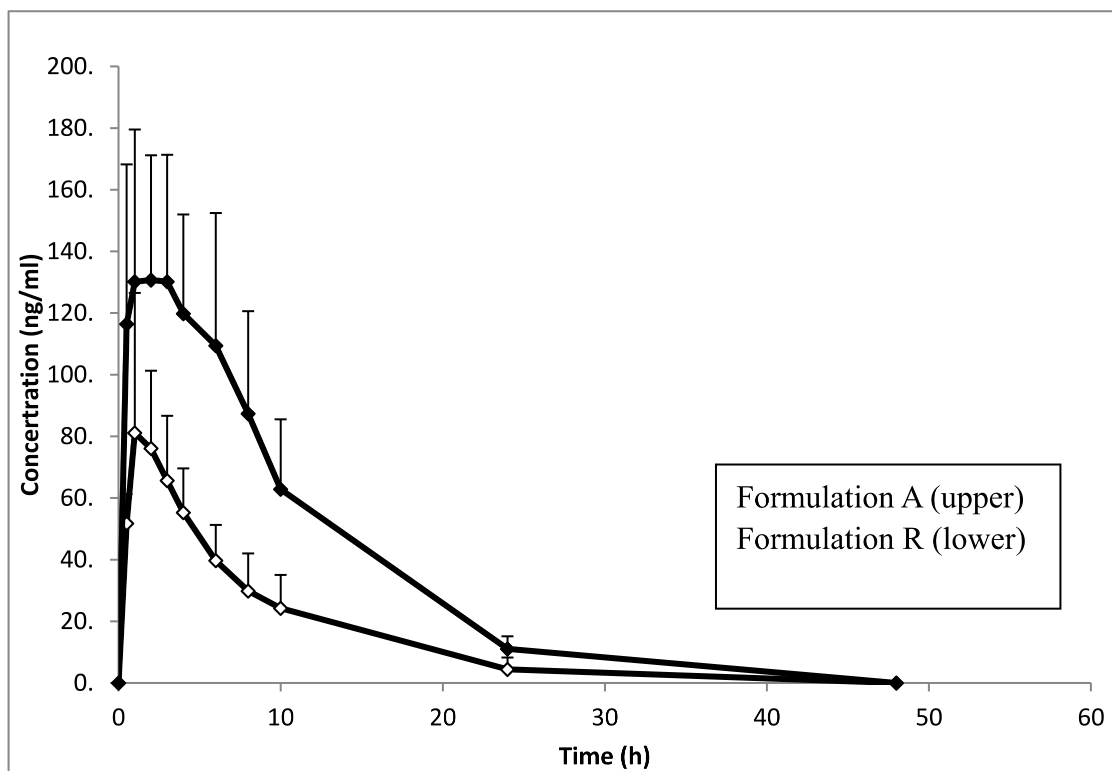


Figure 11.1 The mean concentration–time profile of Formulation R and Formulation A following a single oral administration of 100 mg/kg berberine8998 to 10 rats (dual crossing). N=10, Mean±SD

Table 11.1 The plasma pharmacokinetics parameters of Formulation R following a single oral administration of 100mg/kg berberine8998 to 10 rats (dual crossing).

Pharmacokinetic parameters (R)					
Rat No	$T_{1/2}$ H	C_{max} ng/ml	T_{max} h	$AUC_{(0-t)}$ ng/ml*h	$AUC_{(0-\infty)}$ ng/ml*h
1	2.754	69.3	2	681.415	681.419
2	2.802	60.8	1	607.39	607.394
3	2.92	50.4	1	358.55	358.554
4	2.84	198	1	703.44	703.444
5	6.192	136	2	1001.98	1006.04
6	2.643	92.6	3	875.75	875.754
7	2.523	72.5	3	840.7	840.704
8	2.66	78.7	2	627.7	627.704
9	6.202	58.5	2	798.36	802.519
10	2.696	91.2	2	782.6	782.604
Mean	3.423	90.8	1.9	727.789	728.614
SD	1.466	44.792	0.738	176.824	177.713
RSD(%)	42.8	49.3	38.8	24.3	24.4

Table 11.2 The plasma pharmacokinetics parameters of Formulation A following a single oral administration of 100mg/kg berberine8998 to 10 rats (dual crossing).

Pharmacokinetic parameters (A)					
Rat No	$T_{1/2}$ H	C_{max} ng/ml	T_{max} h	$AUC_{(0-t)}$ ng/ml*h	$AUC_{(0-\infty)}$ ng/ml*h
1	2.375	203	2	2203.5	2203.503
2	2.349	174	6	1971.3	1971.303
3	4.168	185	6	2440.21	2441.249
4	2.542	223	0.5	1854.5	1854.504
5	2.449	129	6	1841.95	1841.954
6	5.283	112	2	1592.7	1596.097
7	8.836	85.2	3	1330.875	1362.831
8	6.134	143	0.5	1114.8	1119.18
9	2.508	147	0.5	1464.26	1464.264
10	2.6	149	1	1212.52	1212.524
Mean	3.924	155.02	2.75	1702.662	1706.741
SD	2.202	41.939	2.383	434.922	431.428
RSD(%)	56.1	27.1	86.7	25.5	25.3

11.3 Discussion

Formulation A (berberine8998 microcrystal suspensions) was prepared by another

research group, they did not want to provide their preparation method. Highest dose for hamster experiment (in section 4) was converted to TDI to a rat value in this study, because the conversion coefficient of body surface area (BSA) for hamster and rat are very close (hamster 9.1 and rat 9.0) (Ohwada, 1992). Thus, 100mg/kg dose was selected. At this dose of berberine8998, good tolerability was observed in all the rats. No related adverse effects were observed and no significant decrease in weight was found between the week 1 and week 2.

Pharmacokinetics has been proposed for studying the absorption, distribution, biotransformation and elimination of drugs in human and animals (Urso et al., 2002). Triple quadrupole tandem mass spectrometer was applied for analysis of the serum concentration of berberine and berberine8998. These two compound shared the ion pair in MRM scan model. As the chemical structures of berberine and berberine8998 are very similar, it is feasible that berberine8998 might be converted to berberine after absorption, functioning like a prodrug (might be). Hence the same method as described in Chapter 4 was used to determine the serum concentration of berberine8998. The serum mass test can prove berberine8998 convert to berberine after taken by hamster and rat. However, the place berberine8998 convert to berberine was not found in this research. The AUC of Formulation R was 727.789, and the AUC of Formulation A was 1702.662. In this part of the study, The modified Formulation A greatly improved the absorption of berberine8998 by 2.34 times (Formulation A AUC_{0-48} of berberine8998 / Formulation R AUC_{0-48} of berberine8998).

12. General Discussion

Berberine8998 is a novel berberine derivative with Chinese patent accreditation. This study aims was to elucidate the pharmacological effects of berberine8998 on lipid profiles and associated mechanisms via proteomic approaches. Tentative toxicological, pharmacokinetic and pharmaceutical optimization was also included in this study.

Toxicological profiles were of high importance in drug development. Therefore, acute toxicity was conducted. The results of the previous study showed that the LD50 of berberine from intravenous injection and intrapetoneal injections is 9.0386 and 57.6103 mg/kg, respectively; but no LD50 was found in the intragastric administration i.g. (Kheir et al., 2010). The results of the present study showed that the LD50 for berberine8998 on C57BL/6 mice (i.g.) was 870mg/kg. Acute toxicity can result from interference with signal transduction systems and through chemical reactions that damage the organism caused by the administered compound or reactive metabolites formed through its metabolism. There is reason to believe that the same pharmacological effects of drugs that cause the therapeutic effect can also account for much of their acute toxic effects (Svennebring, 2016).

The pharmacological studies were conducted in this study. The results showed for the first time that berberine8998 lowered the lipid profiles to a greater extent. In vivo studies showed that berberine8998 significantly lowered the total cholesterol (25%, $P<0.05$), low-density-lipoprotein (51%, $P<0.05$) and triglyceride levels (60%, $P<0.01$) comparing with the hamster model group, while berberine only lowered total cholesterol and triglyceride levels (Fig 4.2, section 4.2.2). Further mechanism studies revealed that berberine8998 enhanced hepatic LDL uptake (Fig 5.1) by increasing LDLR expression (Fig 6.1 and Fig 6.2). These results explained the LDL-lowering effect of berberine8998, but they were unable to unveil its beneficial effect on TG. Therefore, iTRAQ method coupled with 2D LC-MS/MS, was conducted.

The lipid lowering effect of berberine was widely reported (Kumar, A, et al. 2015). 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 LDL by 42% in hyperlipidemic hamsters (Kong et al., 2006). Compared with the effect of berberine, berberine8998 improved the lipid lowering effect to a greater extent (51% vs 42%), though not significant in statistics. Besides, triglyceride levels (60%, P<0.01) were significantly reduced comparing with the hamster model group, while berberine only lowered total cholesterol and triglyceride levels (Fig 4.2, section 4.2.2). Therefore, extensive studies were conducted to unveil the associated mechanisms.

In terms of LDL-c reduction, DiI-LDL and western blot were conducted on HepG2 cells and hamster liver tissues. Consistent with the findings in serum LDL-c, berberine8998, to a greater extent, stimulated the uptake of LDL in HepG2 cells, which is similar to the cholesterol-lowering agent pravastatin (Fig 5.1, 2.26 vs 1.22 fold of control). The intensity (a.u.) of Dil-LDL in HepG2 cells was screened to compare the lipid uptake amount of the positive control group (Pravastatin), the berberine group and the berberine8998 group. The western blot analysis showed that the upregulation of LDLR expression was greater in cells treated with berberine8998 as compared with that of berberine (Fig 6.2), indicating that berberine8998 induces higher LDLR expression. These results illustrated that the berberine8998 lead LDL-c reduction was induced by LDLR and PCSK9 pathway.

For the TG reduction, extensive studies were conducted. 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. In-depth iTRAQ proteomics revealed that ACOX1 and ACSL1, proteins involved in fatty acid metabolism, were expressed differently in the berberine8998 group compared to the control group (Table 7.1 and Table 7.2). Further studies showed that berberine8998 significantly lowered non-esterified fatty acid (NEFA) levels (Fig 9.2), which may lead to a reduction in TG levels in berberine8998 treatment groups. All these studies show that berberine8998 lowered TG via induction of fatty acid metabolism which is a newly defined mechanism of berberine8998.

It was found that berberine8998 can reduce TG and NEFA levels, but berberine can only lower TG. Serum FA includes triglycerides, phospholipids, and cholesterol that are not bound to lipid fractions of biological fluids. These play an important role in the development of non-alcoholic fatty liver disease (Neuschwander-Tetri, 2010, Tabeshpour, J, 2017). The lipid lowering effect of berberine on NEFA was not significant. That result suggested that berberine8998 lowered NEFA through a unique mechanism. TG reduction was observed in some most important LDL lowering drug. For example, Statins lower TG by endocytosing TG-rich apoB particles, the TG themselves are simply re-compartmentalized by going from plasma into the liver (Maron et al., 2000). The TG lowering effect of berberine8998 insure it's potentail therapeutical effect in future study. The observed pharmacological differences between berberine and berberine8998 may stem from the polarity variance of the two molecules: berberine is a quaternary ammonium salt and berberine8998 is expected to be less polar due to its lack of this molecular feature.

In order to elucidate the possible mechanisms involved, the function, the interplay and the changes in abundance of proteins in response to internal and external cues should be considered (Ray et al., 2011). Hence, the iTRAQ method was combined with two-dimensional LC–MS/MS to analyze protein expressions in the liver tissue of mice maintained on a high-fat diet and treated with either berberine or berberine8998 in order to evaluate the effect of the two compounds. This is among the most powerful methodologies in quantitative proteomics (Hou et al., 2013). Using this iTRAQ method, 85 identified proteins among the treatment and control groups were differently expressed, of which 19 proteins were common in the berberine and berberine8998 groups. The GO and IPA analyses showed that proteins that were differently (relative to the control group) expressed in the treatment group had similar functions. The KEGG pathway enrichment heat map suggested that FA metabolism and branched-chain amino acid degradation pathways were specifically activated within the treatment group. It is found that ACOX1 and ACSL1 were significantly lower in the berberine8998 group,

suggesting that these agents can reduce serum NEFA levels by modulating ACOX1 and ACSL1 expression.

The mass quantitative methods were validated by verifying the standard curve, LLOQ, intra- and inter-assay precision and accuracy, specificity, matrix interference, quality control, stability matrix effect and extraction recovery for berberine in mouse and rat serum. It was carried out according to the Guidance for Industry Bioanalytical Method Validation recommended by the FDA (FDA Guidance). Berberine8998 (not a quaternary ammonium salt) may be transformed into berberine after absorption. Therefore, this quantitative method (section 10) could be applied to determine berberine8998 in the SD rat pharmacokinetic study.

Pharmacokinetic study was used to evaluate the two formulations of berberine8998. Both formulation R and formulation A were designated for future studies. The AUC_{0-48h} was the main value reflecting the quality of the formulation. The natural product berberine has become a potential drug in the treatment of diabetes, hyperlipidemia, and cancer. However, the oral delivery of berberine is challenged by its poor bioavailability. It is necessary to improve the oral bioavailability of berberine before it can be used in many clinical applications. Understanding the pharmacokinetic characteristics of berberine enables the development of suitable formulation that have improved oral bioavailability. The key considerations for berberine are how to enhance the drug absorption. (Chang-shun L.et, al, 2016)

In summary, in the current mechanism study, the lipid lowering effect of a berberine derivative—berberine8998 was explored. Results from the high-fat diet hamster model have shown that berberine8998 significantly lowers the LDL, total TC and TG levels in hamsters. Further mechanism studies revealed that berberine8998 can enhance hepatic LDL uptake by increasing LDLR expression. NEFA was down-regulated by berberine8998. From iTRAQ proteomic analysis, 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 novel mechanism of

berberine8998 in its lipid lowering effect. Consistent with the lipid lowering effect, berberine8998 up-regulated (compared with berberine) the bioavailability in SD rats.

13. Future Studies

Many aspects of the current research for the potential involvement of the berberine and berberine8998 are currently speculative, based partly on findings presented here and partly on existing recorded evidence. Together with important clinical therapeutic concerns associated with berberine and berberine8998, future studies should include:
Since acute oral toxicity study has been completed, animals such as rats, hamsters and beagles can be used to explore the long-term toxicity. In the case of rodents, each group should consist of at least ten males and ten females. In the case of non-rodents, each group should consist of at least three males and three females. Large animals will consume more API of berberine8998. Long-term repeated administration should be for more than six months. For rodents, blood samples should be taken before the autopsy. For non-rodents, blood samples should be taken before the start of drug administration, at least once during the administration period (for studies of longer than one month), and before autopsy. For both haematological and blood chemistry examinations, it is desirable to include as many parameters as possible. Since liver and kidney are the organs of metabolism and excretion, they are easily affected by potentially toxic agents, so that their functions should be monitored in long term toxicity studies. These tests will provide more specific information about the toxicity of berberine8998.

In this work, the systemic exposure of berberine8998 was 7.76 fold of that of berberine in SD rats but the mechanisms are still not fully clear. Caco-2 cell model can be applied. The objective of the future study is to use caco-2 cell to study the permeability of berberine8998. When cultured under specific conditions, the Caco-2 cell become differentiated and polarized, such that their phenotype, morphologically and functionally, resemble the enterocytes lining the small intestine (Hidalgo et al., 1989).

Thus, we can develop a rapid *in vitro* digestion/Caco-2 cell culture model for assessing the relative bioavailabilities of berberine and berberine8998 (Yun et al., 2004). Since berberine8998 is not soluble in water, there are three possible way to handle the problem: 1) the Microemulsion method, 2) the HP-β-CD method and 3) nanosized powder.

The distribution of berberine8998 may be analyzed in rats to understand the target organ for this compound. The metabolism and elimination studies may be conducted to characterize the major metabolic pathway and elimination route of berberine8998.

Nonalcoholic steatohepatitis (NASH) is the most extreme form of NAFLD, and has been regarded as a major cause of cirrhosis of the liver. Most people can be treated if the condition is caught in its early stages. About 12 to 25% of the people in the United States have NAFLD and between 2 to 5% have NASH. It is expected that NASH will become the major cause of cirrhosis in China in 2030. Previous study (Hong-Mei et al. 2015) has revealed that berberine, with its lipid lowering effect, attenuated NAFLD in the mouse model. One can thus expect that berberine8998 can be effective on NAFLD and NASH. Pre-clinical studies may be conducted to evaluate its efficacy. The NASH model includes methionine-choline deficient (MCD) diet model, high-fat diet model in mice, etc.

MS-based quantitative proteomics is an essential tool for elucidating the complex and dynamic nature of proteomes, enabling in-depth characterization of changes in protein expressions. For further validation and verification of novel proteins discovered in comparative proteomics, targeted approaches such as selected reaction monitoring (SRM; or multiple reaction monitoring, MRM, as commonly known) offer much higher sensitivity and much greater speed of analysis. MRM can precisely quantitate targeted proteins with known fragmentation properties in complex backgrounds. Integration of the chromatographic peaks for each transition of peptides supports the relative or, if suitable heavy isotope–labeled reference standards (SIS) are used, absolute quantification of the targeted peptide(s) across different samples robustly with high throughput (Addona et al., 2009, Ahn et al., 2015). Developing and validating SRM

assays is an extensive process. Once generated for a particular protein, this mass spectrometric assay can be deployed for accurate identification and quantification of that protein in biological samples.

14. References

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Postgraduate Research Student (PGRS) Information			**Student ID:**	UP514599
PGRS Name:	Chengyin Yu			
Department:	School of Pharmacy and Biomedical Sciences	**First Supervisor:**	Asmita Sautreau	
Start Date: (or progression date for Prof Doc students)		**02/2012**		
Study Mode and Route:	Part-time ☐ Full-time ☐	MPhil ☐ PhD ☒	MD ☐ Professional Doctorate ☐	

Title of Thesis:	Berberine8998, a new derivative of berberine, improves hyperlipidemia through additional mechanisms
Thesis Word Count: (excluding ancillary data)	32551

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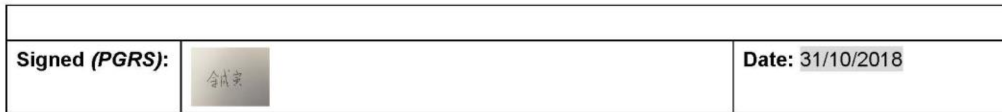

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