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LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet].

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Berberine

Last Update: October 6, 2020.

OVERVIEW

Introduction

Berberine is a quaternary ammonia compound found in many botanical products, including goldenseal, barberry and Oregon grape, which is used for its purported antioxidant and antimicrobial properties for a host of conditions, including obesity, diabetes, hyperlipidemia, heart failure, H. pylori infection and colonic adenoma prevention. Berberine has not been linked to serum aminotransferase elevations during therapy nor to instances of clinically apparent liver injury.

Background

Berberine is an isoquinoline alkaloid that is found in many plants and is purported to have antioxidant, antimicrobial, antiinflammatory and antineoplastic activities. It has been used in Ayurvedic as well as traditional Chinese medicine for a wide variety of conditions, including

infections, diarrhea, bronchitis and digestive complaints. Berberine is found in many plants and in particularly high concentrations in Hydrastis canadensis (goldenseal), Coptis chinensis (coptis or goldenthread), Berberis aquifolium (Oregon grape), Berberis vulgaris (barberry) and Berberis aristata (tree turmeric). Berberine has been extensively evaluated in vitro and in vivo and demonstrates antioxidant, antiproliferative, and antimicrobial activities. It has been reported to be beneficial in alleviating symptoms of arthritis, in improving glucose control and insulin resistance, in improving hyperlipidemia and lowering blood pressure. Nevertheless, berberine has not been approved for any of these uses in the United States. Berberine is widely available over-the-counter in varying concentrations alone and combined with other herbs and nutritional substances in many multiingredient dietary supplements. The usual recommended dose of the herbal product is 250 to 500 mg two or three times daily. Side effects are few and largely mild and transient gastrointestinal symptoms of nausea, abdominal bloating, diarrhea or constipation. In most controlled studies, adverse events were no more frequent with berberine than with placebo and serious adverse events have been rare.

Hepatotoxicity

Berberine has not been linked to serum enzyme elevations during therapy, although there have been few prospective studies in humans that have reported on its effects on laboratory test results in any detail. In published trials, berberine has appeared to be well tolerated with only minor and few adverse effects which have been similar in frequency among persons receiving placebo. Despite wide scale use as an herbal supplement, berberine has not been linked to published instances of clinically apparent liver injury. The frequency of hypersensitivity reactions to berberine is also not known.

Likelihood score: E (unlikely cause of clinically apparent liver injury).

Other Names: Goldenseal, Oregon grape, Tree turmeric.

Drug Class: Herbal and Dietary Supplements

PRODUCT INFORMATION

REPRESENTATIVE TRADE NAMES

Berberine – Generic

DRUG CLASS

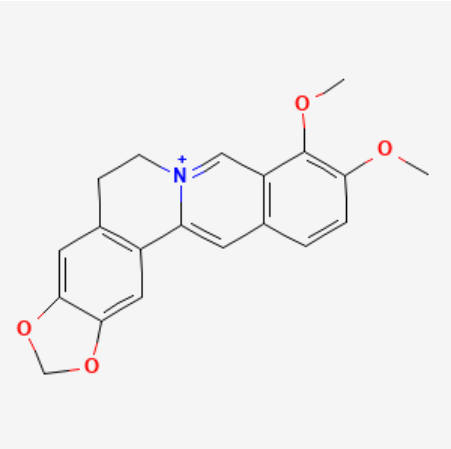
Herbal and Dietary Supplements

SUMMARY INFORMATION

Fact Sheet at MedlinePlus, NLM

Fact Sheet at National Center for Complementary and Integrative Health, NIH [Goldenseal]

CHEMICAL FORMULA AND STRUCTURE

DRUG	CAS REGISTRY NUMBER	MOLECULAR FORMULA	STRUCTURE
Berberine	2086-83-1	C20-H18-N-O4	

ANNOTATED BIBLIOGRAPHY

References updated: 06 October 2020

Abbreviations: HDS, herbal and dietary supplements.

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(Expert review of hepatotoxicity published in 1999; several herbal medications linked to liver injury are discussed, but berberine is not mentioned).

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(Review of hepatotoxicity of herbals does not mention berberine or goldenseal).

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  (Among ~50,000 liver transplants reported to UNOS between 1990 and 2002, 270 [0.5%] were done for drug induced acute liver failure, including 7 [5%] for herbal medications, none were specifically attributed to a product containing berberine or goldenseal).

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(Metaanalysis of 27 randomized controlled trials of berberine for type 2 diabetes and hypertension in 2569 patients found that its effects were similar to those of oral hypoglycemic agents and superior to placebo and that there were no serious adverse events, minor side effects being nausea, diarrhea, constipation, abdominal bloating and pain that were usually transient and did not require dose adjustment or discontinuation; no mention of hepatotoxicity).

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(Review of the problems of liver injury and HDS products, mentions that multiingredient dietary supplements account for the major of cases, but does not mention a product with berberine as a component).

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(Among 891 Chinese patients with adenomatous polyps on colonoscopy treated with either berberine or placebo for 2 years, recurrence of adenoma was less frequent with berberine [36% vs 47%] while there were no serious adverse events; no mention of ALT elevations or hepatotoxicity).

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(Review of the chemical ingredients, pharmacologic activities, clinical efficacy and safety of goldenseal extracts in the treatment of many conditions, including obesity, hyperlipidemia, diabetes, hypertension, bacterial, fungal and viral infections, cancer, arthritis, Alzheimer disease, renal and liver disease with short discussions of toxicity, which were largely limited to in vitro and in vivo studies; no mention of ALT elevations or hepatotoxicity in humans).

Absorption Kinetics of Berberine and Dihydroberberine and Their Impact on Glycemia: A Randomized, Controlled, Crossover Pilot Trial

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PMCID: PMC8746601 PMID: 35010998

Abstract

Berberine is a natural alkaloid used to improve glycemia but displays poor bioavailability and increased rates of gastrointestinal distress at higher doses. Recently, dihydroberberine has been developed to combat these challenges. This study was designed to determine the rate and extent to which berberine appeared in human plasma after oral ingestion of a 500 mg dose of berberine (B500) or 100 mg and 200 mg doses of dihydroberberine (D100 and D200). In a randomized, double-blind, crossover fashion, five males (26 ± 2.6 years; 184.2 ± 11.6 cm; 91.8 ± 10.1 kg; 17.1 ± 3.5% fat) completed a four-dose supplementation protocol of placebo (PLA), B500, D100, and D200. The day prior to their scheduled visit, participants ingested three separate doses with breakfast, lunch, and dinner. Participants fasted overnight (8–10 h) and consumed their fourth dose with a standardized test meal (30 g glucose solution, 3 slices white bread) after arrival. Venous blood samples were collected 0, 20, 40, 60, 90, and 120 minutes (min) after ingestion and analyzed for BBR, glucose, and insulin. Peak concentration (C_{Max}) and area under the curve (AUC) were calculated for all variables. Baseline berberine levels were different between groups ($p = 0.006$), with pairwise comparisons indicating that baseline levels of PLA and B500 were different than D100. Berberine C_{Max} tended to be different ($p = 0.06$) between all conditions. Specifically, the observed C_{Max} for D100 (3.76 ± 1.4 ng/mL) was different than PLA (0.22 ± 0.18 ng/mL, $p = 0.005$) and B500 (0.4 ± 0.17 ng/mL, $p = 0.005$). C_{Max} for D200 (12.0 ± 10.1 ng/mL) tended ($p = 0.06$) to be different than B500. No difference in C_{Max} was found between D100 and D200 ($p = 0.11$). Significant differences in berberine AUC were found between D100 (284.4 ± 115.9 ng/mL × 120 min) and PLA (20.2 ± 16.2 ng/mL × 120 min, $p = 0.007$) and between D100 and B500 (42.3 ± 17.6 ng/mL × 120 min, $p = 0.04$). Significant differences in D100 BBR AUC (284.4 ± 115.9 ng/mL×120 min) were found between PLA (20.2 ± 16.2 ng/mL × 120 min, $p = 0.042$) and B500 (42.3 ± 17.6 ng/mL × 120 min, $p = 0.045$). Berberine AUC values between D100 and D200 tended ($p = 0.073$) to be different. No significant differences in the levels of glucose ($p = 0.97$) and insulin ($p = 0.24$) were observed across the study protocol. These results provide preliminary evidence that four doses of a 100 mg dose of dihydroberberine and 200 mg dose

of dihydroberberine produce significantly greater concentrations of plasma berberine across of two-hour measurement window when compared to a 500 mg dose of berberine or a placebo. The lack of observed changes in glucose and insulin were likely due to the short duration of supplementation and insulin responsive nature of study participants. Follow-up efficacy studies on glucose and insulin changes should be completed to assess the impact of berberine and dihydroberberine supplementation in overweight, glucose intolerant populations.

Keywords: absorption, safety, plants, glucose, insulin

1. Introduction

Berberine is an isoquinoline derivative alkaloid present in various parts (root, stem, fruit, bark) of multiple plants including, in particular, species found in the Coptis, Hydrastis, and Berberis genus [1]. Various animal and culture models have reported on the ability of berberine to exert lipid and glucose-lowering effects in addition to helping with weight loss and improving glucose tolerance [2,3]. For this reason, interest in berberine has suggested it may be beneficial in treating diabetes and obesity. While still being fully characterized, mechanistic actions of berberine consist of it reducing blood lipid levels via action on hepatocyte nuclear factor 1 alpha (HNF-1 alpha) and hepatic LDL receptors, intestinal absorption of cholesterol [4], and glucose levels while increasing insulin sensitivity.

Moreover, berberine is a known activator of AMP Kinase [1,5], which contributes to increases in fatty acid oxidation and a reduction of lipogenic gene expression.

Yin and colleagues [6] published results from two separate studies that both examined the impact of berberine at improving various biomarkers associated with glucose homeostasis. Throughout the first study, berberine (500 mg/dose, 3 doses/day) or metformin was administered over a 3-month period to 36 adults who were recently diagnosed with type 2 diabetes. Levels of hemoglobin A1C, fasting blood glucose, postprandial blood glucose, and triglycerides were all significantly improved in comparison to pre-treatment, and, importantly, the magnitude of changes was similar to what was observed with metformin. An additional investigation was reported where berberine was added to the established treatment plan 48 type 2 diabetes patients. Over a 13-week period, blood glucose (both fasting and postprandial), hemoglobin A1C, fasting, insulin, and HOMA-IR were all improved when berberine was added. These results align with the Perez-Rubio et al. [7] investigation that reported improvements in waist circumference, blood pressure, triglycerides, and area under the curve values for both glucose and insulin (after a 75-g glucose load over a two-hour period) in 24 patients with metabolic syndrome. In this study, berberine was randomly assigned in a double-blind, placebo-controlled fashion at a daily dosage of 1500 mg/day (500

mg/dose, 3 doses/day with meals) for three months. Similar outcomes using berberine have been reported by Rao et al. [8] after completing a randomized, open-label study on 41 diabetic patients where various indicators of glucose and insulin metabolism were improved over the three-month study period.

One challenge associated with oral ingestion of berberine has been its low bioavailability (<1%) reported in both animal [9,10] and human models largely due to poor intestinal absorption and high levels of first-pass removal in the intestines and liver [5]. To overcome this limitation, higher doses of berberine are commonly administered, which has led to a consistent reporting of gastrointestinal adverse events. For example, Yin et al. [6] reported that gastrointestinal adverse events were 34.5% during a 13-week study which provided berberine hydrochloride as a monotherapy or in combination with other treatments. Interestingly all adverse events were reported when berberine was combined with other treatments (metformin, insulin, etc.) and all incidents occurred during the first four weeks of treatment.

For this reason, alternative approaches have been explored to help improve its poor bioavailability. For example, techniques such as encapsulation [11,12], packaging with other nutrients and compounds [13], and spray drying [14] have all been reported on in the literature. In addition, the reduced derivative of berberine, dihydroberberine, has been suggested as a natural alternative to improve bioavailability [15]. Buchanan and investigators [16] examined the ability of transdermal application of dihydroberberine in 40 Sprague-Dawley rats to improve bioavailability, improve glucose homeostasis parameters, and reduce adverse events in comparison to oral berberine gavage and transdermal applications of berberine. Results from both acute and chronic (14 days) pharmacokinetic approaches indicated that transdermal dihydroberberine achieved higher bioavailability than both transdermal and oral berberine administration. Oral administration of dihydroberberine has yet to be reported in human literature, thus initial studies should provide some level of insight into its impact on absorption kinetics, preliminary safety indicators, and adverse events associated with its ingestion. Consequently, the purpose of this study was to conduct a randomized, double-blind, placebo-controlled investigation to examine the dose-dependent impact on absorption kinetics, clinical safety parameters, and adverse events of oral dihydroberberine ingestion in humans.

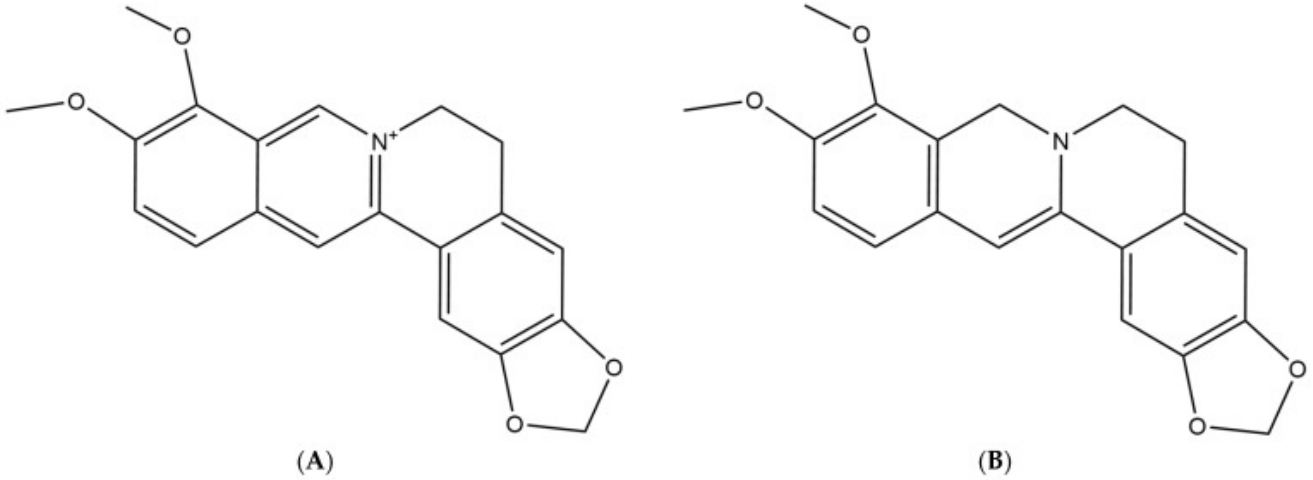
2. Methods

2.1. Overview of Research Design

This study was completed using a randomized, double-blind, crossover study design. Prior to beginning, all participants signed an IRB-approved informed consent document (Lindenwood

University: IRB-20-173, approval date: 22 June 2020) and completed a healthy history questionnaire to determine study eligibility. A power analysis was not computed as this study was considered a proof of concept, pilot investigation. This study protocol and design was retrospectively registered on Clinicaltrials.gov on 25 August 2021 as NCT05021341 (https://clinicaltrials.gov/ct2/show/NCT05021341 accessed on 24 December 2021). All four supplementation conditions were completed by each participant in a crossover fashion with a minimum washout of 72 h. For each supplementation condition, four total doses were consumed with three doses being consumed the day before testing (with breakfast, lunch, and dinner) and the fourth dose being consumed the morning of testing with a standardized test meal after observing an overnight (8 to 10 h) fast (Figure 1). Participants were scheduled to begin each visit at approximately the same times and reported to the laboratory between 600 and 1000 h. Using a randomized, double-blind, placebo-controlled fashion, study participants consumed either a placebo (resistance dextrin) (PLA), 500 mg of berberine (B500), 100 mg dihydroberberine (D100), or 200 mg dihydroberberine (D200). To minimize any order effects from testing, participants were randomized using an online randomization software program (www.random.org accessed on 1 July 2020). Upon arrival for each study visit, participants had their height, body mass, body composition, and hemodynamics (resting heart rate and blood pressure) measured along with an assessment of capillary glucose levels approximately 30 min prior to supplementation ingestion to evaluate fasting status. An indwelling catheter was then implanted into a forearm vein and flushed with saline to remain patent. The fourth and final dose of their assigned supplement was then consumed with 240 milliliters of cold tap water. After supplement ingestion, participants consumed a standardized test meal consisting of a 30-g glucose solution and four slices of white bread. Immediately after ingestion of the test meal, venous blood samples were collected 20, 40, 60, 90, and 120 min (Figure 2). Participants were provided 200 mL of cold water to ingest after each blood collection. Upon processing, all blood samples were stored at −80 °C. Throughout each visit, study participants were asked to report the occurrence of any adverse events (dizziness, headache, nausea, upset stomach, cramping, diarrhea, etc.) that occur throughout completion of the study protocol. All subsequent study visits were completed in an identical fashion and scheduled with at least 72 h between visits.

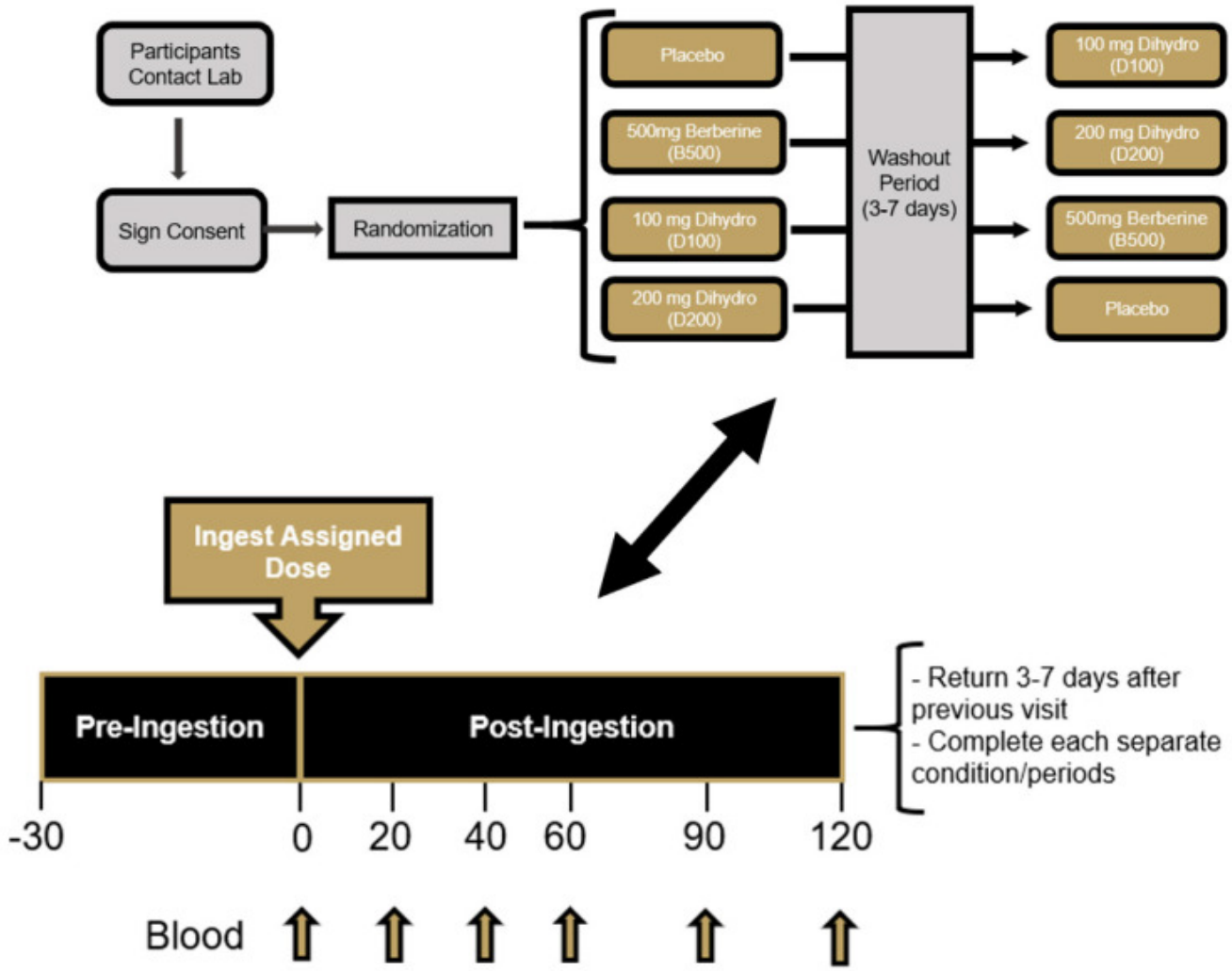
Figure 1.



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(**A**) Chemical structure of berberine. (**B**) Chemical structure of dihydroberberine.

Figure 2.



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Overview of research design.

2.2. Study Participants

Prior to participation, all recruited individuals provided signed informed consent using an IRB-approved consent form (Lindenwood University: IRB-20-173, approval date: 22 June 2020). Five healthy males (26.0 ± 2.6 years, 184.2 ± 11.6 cm, 91.8 ± 10.1 kg, 17.1 ± 3.5% fat) successfully completed all study visits (see Table 1 for full participant characteristics). Inclusion criteria included age (18–45 years), healthy and free of disease (as reported by the health screening questionnaire), and physically active (reported at least 30 min of moderate exercise three days a week). Any individual diagnosed with or being treated for cardiac, respiratory, circulatory, musculoskeletal, metabolic, obesity (defined as body mass index > 30 kg/m^2 and body fat greater than 30%), immune, autoimmune, psychiatric, hematological, neurological, or endocrinological disorder or disease were not allowed to participate in the

current study. Any participant taking any dietary supplement besides a multi-vitamin/mineral or a protein supplement were required to abstain from consuming any further doses for 30 days prior to beginning the study protocol.

Table 1.

Baseline age, gender, height (cm), weight (kg), body mass index, % fat, heart rate, systolic blood pressure, diastolic blood press, energy, carbohydrates, proteins, and fat intake.

	Mean	SD	Minimum	Maximum
Age	26.0	2.6	23	30
Height (cm)	184.2	11.6	171	202
Weight (kg)	91.8	10.1	82.4	108.5
Body mass index (kg/m^2)	27.1	3.9	22.6	33.1
% fat	17.1	3.5	12.5	20.9
Heart rate (beats/minute)	61.0	12.0	41	70
Systolic blood pressure (mm Hg)	122.3	11.4	111	138
Diastolic blood pressure (mm Hg)	72.4	8.8	64	70
Energy intake (kcals/day)	2433	810	1503	3695
Carbohydrate intake (grams/day)	217	62	144	312
Protein intake (grams/day)	121	34	66	153
Fat intake (grams/day)	112	45	75	186

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2.3. Supplementation Protocol

Using a randomized, double-blind, placebo-controlled fashion, study participants consumed either a placebo (resistance dextrin) (PLA), 500 mg of berberine (B500), 100 mg dihydroberberine (D100), or 200 mg dihydroberberine (D200). The dihydroberberine was manufactured by NNB Nutrition (Nanjing, China) and is marketed as Glucovantage®.

Berberine (≥99%) was extracted from *Berberis Aristata* in the hydrochloride form and was used as raw material. Dihydroberberine was obtained by a patented preparation method (CN108997332A). Neither molecule contains any crystalline water. Study participants supplemented consumed four total doses prior to having the absorption kinetics determined. This regimen started the morning before their scheduled visit with participants consuming one dose each with breakfast, lunch, and dinner. In addition to consuming with a meal, all doses were consumed with at least eight fluid ounces of cold tap water. Participants were then required to follow an overnight fast (approximately 8 to 10 h). Upon arrival, capillary glucose levels were determined from a finger-stick blood sample and a handheld glucose monitor (CVS Health Advanced Glucose Monitor, Agamatrix, Inc., Salem, NH, USA). After fasting status was confirmed, the fourth and final dose of their assigned supplement was consumed in front of a study team member with approximately eight fluid ounces of cold tap water. A catheter was then inserted into a forearm vein and approximately 30 min after consuming their final dose a standardized test meal was ingested which consisted of four slices of white bread (approximately 264 kcals, 3.2 g of fat, 50.6 g of carbohydrates and 7.6 g of protein) and 30 g of glucose powder that was mixed with approximately 8–12 fluid ounces of water. The test meal was ingested within five minutes. The ingestion of the test meal was considered to be the 0-min time point and all subsequent measurements occurred as outlined.

All participants were required to complete a supplementation log to document when each dose was consumed. No doses were reported being missed by any participant throughout the study protocol. All study materials were prepared into gelatin capsules of identical size, color, shape, and transparency. All doses were prepared into a single capsule which required each participant to consume a single capsule for each required dose. A resistant dextrin was used as the placebo. All doses were placed into individually labeled bags for each condition for each participant throughout the entire study protocol.

2.4. Procedures

2.4.1. Baseline Demographics and Hemodynamics

During the initial visit, after providing written consent, participants were instructed to rest quietly for approximately 10 min before measuring resting heart rate and blood pressure. Resting heart rate was measured by palpating the radial artery for a period of 60 s. While still resting, blood pressure (Omron BP785, Omron Corporation, Kyoto, Japan) measurements were taken before participants resumed normal activity. Participants then had their body mass determined (Tanita BWB-627A, Tokyo, Japan) and recorded to the nearest ±0.1 kg upon arrival prior to each study visit. All recorded body masses were compared to ensure the participant was weight stable. Any participant whose body mass changed by more than 2% between consecutive study visits was excluded from participation. Following body mass

measurements, height was measured using a standard wall-mounted stadiometer (Tanita, HR-200, Tokyo, Japan) and recorded to the nearest ±0.5 cm (cm). Fat and fat-free mass was determined using a bioelectrical impedance analyzer (InBody 570, Beverly Hills, CA, USA). Participants were required to observe an overnight fast to ensure an accurate determination of body composition. Body composition analysis occurred between 600 and 1000 h by trained research personnel. All assessments were completed according to device specifications.

2.4.2. Dietary Monitoring

Prior to their baseline visit, study participants completed a hand-written four-day food record (three weekdays and one weekend day). The four-day food log was provided to allow participants to replicate their diets. In addition to the four-day food log, participants were instructed on how to complete the ASA24 online dietary assessment tool (https://epi.grants.cancer.gov/asa24/ accessed on 23 December 2021), for determination of baseline caloric and macronutrient intake. From this information, study participants were asked to replicate their dietary intake prior to each subsequent testing visit. Study participants reported 100% compliance to this protocol.

2.4.3. Venous Blood Collection and Processing

To confirm a fasting state prior to catheter insertion and supplement administration, capillary blood was collected from a finger on their non-dominant hand. The collected blood was analyzed using a hand-held glucose analyzer (CVS Health Advanced Glucose Monitor, Agamatrix, Inc., Salem, NH, USA). Fasting was confirmed if glucose was <110 mg/dL. Within each supplementation condition, study participants had their venous blood collected on six different occasions: 0, 20, 40, 60, 90, and 120 min after ingestion of their assigned study agent. Blood was collected via a forearm vein using standard phlebotomy techniques into serum and ethylenediaminetetraacetic acid EDTA-coated Vacutainer™ tubes. To establish safety, whole blood samples were collected into EDTA and serum tubes for assessment of complete blood count and comprehensive metabolic count, respectively. Blood samples collected into EDTA were gently inverted ten times while blood samples collected into serum tubes were gently inverted ten times and allowed to clot at room temperature. For same day analysis, these samples were placed into a chilled container and sent to a commercial diagnostic laboratory (Quest Diagnostics). Plasma samples used for berberine determination were collected into EDTA tubes, gently inverted ten times and centrifuged at 3000 revolutions per minute (rpm) (MegaFuge XFR, Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C for 20 min. After centrifugation, 400 µL aliquots of plasma were removed and frozen at −80 °C within four hours of collection.

2.4.4. Complete Blood Count

To assess adverse events reported in response to the assigned supplementation, whole blood samples were collected in EDTA tubes and analyzed at a commercial diagnostic laboratory (Quest Diagnostics) for changes in red blood cell count, white blood cell count, platelet count, hemoglobin, hematocrit, red blood cell dimension width (RDW), mean corpuscle volume (MCV), mean corpuscle hemoglobin (MCH), mean corpuscle hemoglobin content (MCHC), neutrophil % and cell count, lymphocytes % and cell count, monocytes % and cell count, eosinophils % and cell count, and basophils % and cell count (granulocytes → neutrophils, eosinophils, basophils). Complete blood counts were only assessed at the 0 and 120-min time points.

2.4.5. Comprehensive Metabolic Panel

To assess adverse events in response to the assigned supplementation, serum samples were assayed at a commercial diagnostic laboratory (Quest Diagnostics) for a comprehensive metabolic panel (albumin, albumin/globulin ratio (calculated), alkaline phosphatase, ALT, AST, BUN/creatinine ratio (calculated), calcium, carbon dioxide, chloride, creatinine with estimated GFR, globulin, glucose, potassium, sodium, total bilirubin, total protein, and urea nitrogen). Comprehensive metabolic panels were only assessed at the 0 and 120-min time points.

2.4.6. Glucose and Insulin Determination

Each plasma sample was analyzed in duplicate for glucose and insulin concentration. Plasma glucose was analyzed at a commercial diagnostic laboratory (Quest Diagnostics) using automated clinical analyzers while insulin concentrations were assessed using a standard ELISA technique (DRG International, EIA2935, Springfield, NJ, USA). Briefly, all standards, samples, and controls were analyzed in duplicate across a range of 1.76–100 µIU/mL. Plate to plate controls were employed between all analyzed plates and exhibited a 6.3% coefficient of variation between the plates.

2.4.7. *Plasma Berberine Determination*

Berberine analysis was performed in plasma samples by Heartland Assays (Iowa State University Research Park, Ames, IA, USA) using coupled liquid chromatography-mass spectrometry-mass spectrometry technology (LC-MS-MS) following the procedures of Buchanan et al. [16]. Briefly, BBR and d_6-BBR (Sigma-Aldrich, St. Louis, MO, USA and Toronto Research Chemicals, Toronto, Canada, respectively) standards were prepared using gravimetric determination and were dissolved in 50:50 methanol: water. As previously published, plasma samples and controls were removed from freezer storage and allowed to thaw at room temperature. An amount of 100 µL of plasma was transferred to a 13 × 100

glass test tube followed by the addition of 15.0 µL of d_6-BBR internal standard (ISTD, 0.5 ng/µL). Next, samples were treated with 400 µL of acetonitrile containing 3% acetic acid, vortexed and centrifuged. The supernatant was transferred to 12 × 75 glass test tube and dried in a vacuum dryer. A standard curve (0.3–1000.0 ng/mL) was prepared and dried. Samples, controls, and standard curve were reconstituted in 50 µL of LC/MS/MS organic and 50 µL aqueous mobile phase solutions (Mobile phase B, LCMS-grade methanol and Mobile phase A, LCMS-grade water, both containing 0.2% formic acid and 0.1% 10 M ammonium formate solution) vortexed and transferred into LC/MS/MS vials. Chromatographic separation of BBR and d_6-BBR was conducted on an Agilent Poroshell C18 column (2.1 × 50 mm, 2.7 µm) using an Agilent 6460 triple quadrupole LC/MS/MS system (Santa Clara, CA, USA). The initial gradient conditions were 45% B from 0 to 2 min and advanced to 95% B over 1 min with a flow rate of 0.40 mL/minute. BBR and d_6-BBR data were collected using the Multiple Reaction Monitoring (MRM) mode with Mass Hunter acquisition software (Agilent, Santa Clara, CA, USA). The MRM pairs used for BBR were 337.1 → 322.1 and 337.1 → 293.1 and 343.1 → 325.2 and 343.1 → 307.1 for d_6-BBR. Mass Hunter Quantitation software was used to quantitate the unknown plasma samples based on best fit standard curves. Quality control samples were run at two levels in each assay (4.0 and 100.0 ng/mL). Average recovery was 102% and ranged from 95% to 108%. The average accuracy (bias from nominal) was 1.0%. The average precision (CV) was 2.1% and 1.5% for intra- and inter-assay precision, respectively.

2.5. Statistical Analysis

The primary outcome for this study protocol was berberine AUC while secondary outcomes were considered to the maximum berberine concentration (C_{Max}) as well as the AUC and C_{Max} for glucose and insulin. Area under the curve (AUC) for berberine, glucose, and insulin were calculated using the trapezoidal method (Equation (1)), where C_0 is the unknown concentration at the first time point of interest, C_1 is the unknown concentration at the second time point of interest, and $Time_{1-0}$ is the total time interval between the two time points when the unknown was measured. These individual AUC values for each time interval were then summed to determine the total AUC for berberine, glucose, and insulin (Equation (2)).

(1) (2)

All analyses were completed using Microsoft Excel and the Statistical Package for the Social Sciences (v26; SPSS Inc., Chicago, IL, USA) while provided figures were developed using GraphPad (La Jolla, CA, USA). For all dependent measures, descriptive statistics are presented herein as mean ± standard deviations. Before any statistical tests were completed, normality was assessed using a Shapiro-Wilk test for all dependent variables. All non-normal

data were transformed using natural logarithms, cubed, and then square root transformation approaches and was retested for normality. Data were then analyzed using both parametric and non-parametric approaches. In all such situations, the final statistical decision was identical whether parametric or non-parametric approaches were completed. All reported *p*-values are computed using parametric approaches. Blood markers were analyzed with a mixed factorial ANOVA, and if a significant interaction was observed then follow-up simple effects testing was performed using a repeated measures ANOVA followed by post-hoc testing consisting of paired samples *t*-tests. Body mass, blood pressure, AUC, and C_{Max} variables were analyzed with a repeated measures ANOVA followed by post-hoc testing consisting of paired samples t-tests. For all statistical tests, data were considered statistically significant when the probability of type I error was 0.05 or less. Trends were highlighted and discussed when *p*-values were between 0.05 and 0.10.

3. Results

3.1. Pre-Supplementation Status

Table 2 outlines the pre-supplementation values for body mass and hemodynamics. Body mass was significantly different between groups ($p = 0.045$), and post-hoc testing revealed differences between PLA (90.6 ± 10.1 kg) and D200 (91.4 ± 9.7 kg). Resting heart rate ($p = 0.21$), systolic blood pressure ($p = 0.07$), and diastolic blood pressure ($p = 0.87$) were not different.

Table 2.

Baseline body mass, hemodynamic, glucose, insulin, and berberine.

Raw Data	Group	Pre	*p*-Value
Body Mass (kg)	PLA	90.6 ± 10.1 ‡	0.043
	D100	91.6 ± 10.3	
	D200	91.4 ± 9.7	
	B500	90.8 ± 10.0	

Raw Data	Group	Pre	*p*-Value
Resting Heart Rate (beats/minute)	PLA	64 ± 10	0.21
	D100	62 ± 6	
	D200	60 ± 5	
	B500	57 ± 9	
Systolic Blood Pressure (mm Hg)	PLA	120 ± 8	0.07
	D100	115 ± 10	
	D200	118 ± 11	
	B500	124 ± 10	
Diastolic Blood Pressure (mm Hg)	PLA	72 ± 5	0.87
	D100	70 ± 5	
	D200	70 ± 10	
	B500	72 ± 7	
Glucose (mg/dL)	PLA	91.8 ± 7.6	0.59
	D100	92.0 ± 8.5	
	D200	90.6 ± 3.4	
	B500	89.8 ± 1.8	
Berberine (ng/mL)	PLA	0.14 ± 0.09 †,‡	0.006
	D100	2.60 ± 1.06	
	D200	4.54 ± 2.24	
	B500	0.30 ± 0.12 †,‡	

Raw Data	Group	Pre	*p*-Value
Insulin (μIU/mL)	PLA	15.4 ± 5.4	0.20
	D100	16.7 ± 9.1	
	D200	14.8 ± 4.4	
	B500	18.4 ± 6.3	

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† = Different than D100 ($p < 0.05$); ‡ = Different than D200 ($p < 0.05$).

3.2. Protocol Compliance

Compliance to the supplementation regimen was 100% with all participants reporting that all supplements were ingested as instructed with the final dose being ingested in front of investigators. All study participants were asked to record their food and fluid intake for two days. Compliance was reported at 87.5% as one participant did not record their food and fluid intake on one of the days. Dietary intake is provided in Table 1.

3.3. Adverse Events

A total of 11 adverse events were reported throughout the study protocol by two or 40% of the study participants. The largest number of adverse events ($n = 6$) were reported in D100, $n = 3$ were reported in PLA, $n = 1$ for D200, and $n = 1$ for B500. All but one reported adverse event (10/11 or 90.9%) was deemed by study participants to be mild in severity and one being moderate severity (See Table 3).

Table 3.

Adverse event table.

	PLA	D100	D200	B500
n	5	5	5	5
# of AE's Reported				
Mild	3	5	1	1
Moderate	0	1	0	0

	PLA	D100	D200	B500
Severe	0	0	0	0
	Adverse Events Breakdown			
Gastrointestinal				
Diarrhea	0	0	0	0
Nausea	0	2	0	0
Upset stomach	0	0	1	0
Stomach cramping	2	0	0	0
Pain				
Headache	0	2	0	1
Cardiovascular				
Abnormal heart rhythm	1	0	0	0
Dizziness	0	2	0	0
Constitutional Symptoms				
Nervousness	0	0	0	0
Blurred vision	0	0	0	0
Summary				
Total Adverse Events	3	6	1	1
# of Subjects Reporting AE	2	2	1	1
% of Subjects Reporting AE	40%	40%	20%	20%

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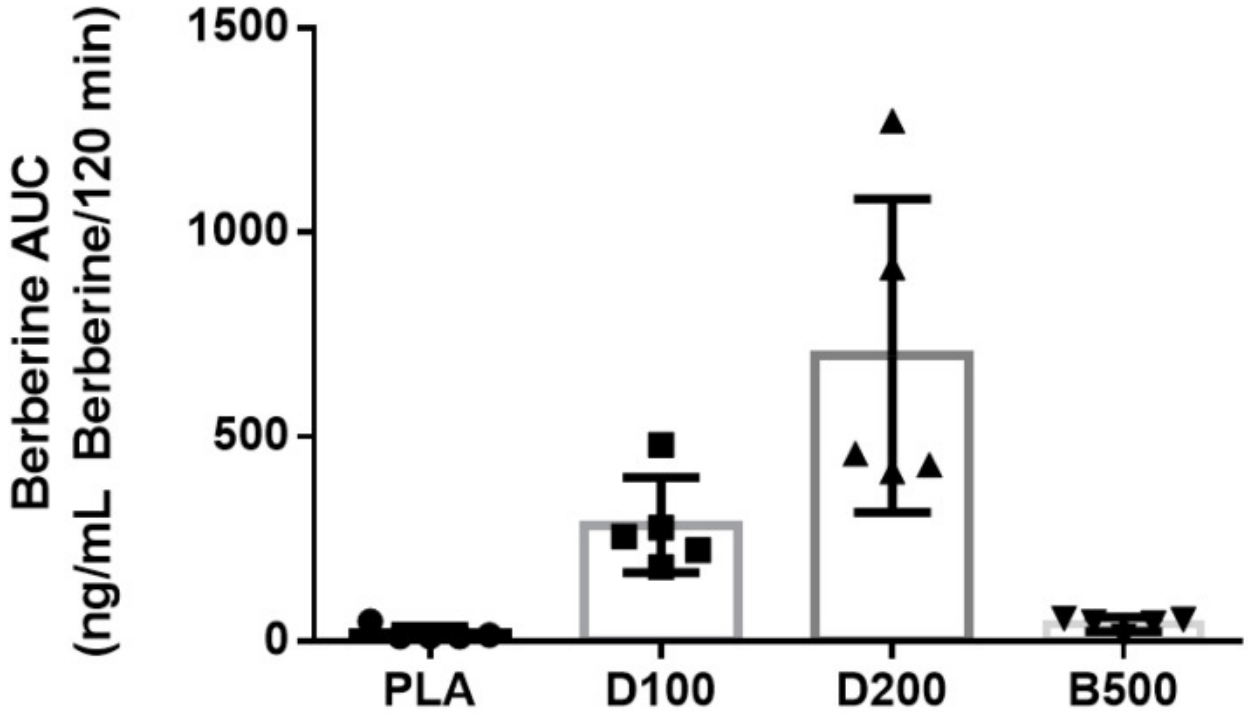
PLA = Placebo; D100 = 100 mg dose of dihydroberberine; D200 = 200 mg dose of dihydroberberine; B500 = 500 mg dose of berberine.

3.4. Berberine

Berberine data were not-normally distributed. The data were transformed using the aforementioned methods and reassessed for normality, which was unable to be achieved due to two high values in the D200 condition at the 60-min timepoint. Subsequently, all berberine data were analyzed using parametric approaches. A significant group x time interaction was revealed for berberine ($p = 0.05$). Simple main effects tests revealed no changes over time within each condition ($p > 0.05$), but significant changes between conditions at the following time points: baseline ($p = 0.006$), 20-min ($p < 0.001$), and 40-min ($p = 0.039$). At baseline, both D100 (2.60 ± 1.06 ng/mL) and D200 (4.54 ± 2.24 ng/mL) conditions had higher berberine values compared to placebo (0.14 ± 0.09 ng/mL; D100, $p = 0.007$; D200, $p = 0.011$) and B500 (0.30 ± 0.12 ng/mL; D100, $p = 0.006$; D200, $p = 0.013$). At the 20-min time point, both D100 (3.00 ± 1.85 ng/mL) and D200 (6.06 ± 2.50 ng/mL) conditions had higher berberine values compared to placebo (0.18 ± 0.18 ng/mL; D100, $p = 0.030$; D200, $p = 0.005$) and B500 (0.32 ± 0.20 ng/mL; D100, $p = 0.008$; D200, $p = 0.029$), but D200 had higher berberine values compared to D100 ($p = 0.050$). Lastly, at 40 min both D100 (2.32 ± 1.27 ng/mL) and D200 (6.90 ± 4.94 ng/mL) conditions had higher berberine values compared to placebo (0.20 ± 0.14 ng/mL; D100, $p = 0.018$; D200, $p = 0.037$) and B500 (0.36 ± 0.15 ng/mL; D100, $p = 0.022$; D200, $p = 0.040$), but D200 had higher berberine values compared to D100 ($p = 0.050$). There was a significant main effect for condition ($p = 0.04$), but no significant main effect for time ($p = 0.18$) was observed.

Berberine AUC values (Figure 3) were significantly difference between conditions ($p = 0.045$). Follow-up analysis indicated that higher berberine AUC values were identified in D200 (929 ± 694 ng/mL berberine/120 min) when compared to PLA (20.2 ± 16.2 ng/mL Berberine/120 min, $p = 0.042$) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min, $p = 0.045$). Significantly higher berberine AUC values were identified for D100 (284.2 ± 115.9 ng/mL Berberine/120 min) when compared to placebo (20.2 ± 16.2 ng/mL Berberine/120 min, $p = 0.007$) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min, $p = 0.007$). Berberine AUC values between D100 (284.2 ± 115.9) and D200 (929 ± 694 ng/mL Berberine/120 min) approached statistical significance ($p = 0.07$). Berberine AUC values between PLA (20.2 ± 16.2 ng/mL Berberine/120 min) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min) tended ($p = 0.096$) to be different. Berberine C_{Max} values tended to be different between conditions ($p = 0.064$). Further, differences in C_{Max} between D100 and PLA were significant $p = 0.005$) while differences between PLA and D200 ($p = 0.06$) as well as PLA and B500 ($p = 0.09$) tended to be different.

Figure 3.



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Individual and aggregated mean values for berberine AUC by condition.

Each individual symbol represents the individual AUC response for one study participant. For all graphs below, *n* = 5 participants completed each condition. PLA = Placebo; D100 = 100 mg dose of dihydroberberine; D200 = 200 mg dose of dihydroberberine; B500 = 500 mg dose of berberine. Data are presented as mean ± SD.

3.5. Glucose

There was no significant group x time interaction ($p = 0.97$) for glucose measures. However, a significant time effect ($p < 0.001$) was observed where glucose concentrations were significantly elevated at the 40-min timepoint (129.25 ± 27.43 mg/dL) compared to the baseline timepoint (91.05 ± 5.61 mg/dL, $p < 0.022$). Furthermore, both the 20-min timepoint (119.5 ± 21.27 mg/dL) and the 40-min timepoint (129.25 ± 27.43 mg/dL) had elevated glucose concentrations compared to the 90-min timepoint (85.40 ± 18.15 mg/dL; 20 min, $p = 0.045$; 40 min, $p = 0.007$), and the 120-min timepoint (69.6 ± 10.19 mg/dL; 20 min, $p = 0.044$; 40 min, $p < 0.001$). The 60-min timepoint (105.45 ± 23.55 mg/dL) had elevated glucose concentrations compared to the 120-min timepoint (69.60 ± 13.59 mg/dL, $p = 0.033$). Both AUC and C_{Max} values were calculated for all four conditions. Oneway ANOVA indicated no significant differences were observed for AUC ($p = 0.92$) and C_{Max} ($p = 0.77$) values (See Table 4 and Table 5).

Table 4.

Berberine (ng/mL), glucose (mg/dL), and insulin (μIU/mL) data.

Berberine (ng/mL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	0.14 ± 0.09	0.18 ± 0.18	0.20 ± 0.14	0.16 ± 0.13	0.16 ± 0.13	0.16 ± 0.13	Group	0.002
B500	0.30 ± 0.12	0.32 ± 0.20	0.36 ± 0.15	0.38 ± 0.16	0.39 ± 0.16	0.32 ± 0.13	Time	0.18
D100	2.6 ± 1.1 †,‡	3.0 ± 1.9 †,‡	2.3 ± 1.3 †,‡	2.5 ± 1.0	2.1 ± 0.8	1.9 ± 0.9	G x T	0.050
D200	4.5 ± 2.2 †,‡	6.1 ± 2.5 †,‡,*	6.9 ± 4.9 †,‡,*	11.4 ± 10.7	7.8 ± 6.5	7.2 ± 6.5		
Glucose (mg/dL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	91.8 ± 7.6	120.4 ± 25.4	129.6 ± 33.0	102.8 ± 27.6	92.0 ± 23.9	63.4 ± 9.3	Group	0.92
B500	89.8 ± 1.8	116.4 ± 30.1	125.6 ± 35.7	101.8 ± 30.9	84.0 ± 25.1	73.0 ± 13.7	Time	<0.001
D100	92.0 ± 8.5	121.8 ± 20.9	126.8 ± 24.5	106.4 ± 14.9	81.2 ± 10.1	69.4 ± 7.9	G x T	0.97
D200	90.6 ± 3.4	119.2 ± 11.8	135.0 ± 23.3	110.8 ± 25.2	84.4 ± 13.4	72.6 ± 21.6		
Insulin (μIU/mL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	15.4 ± 5.4	47.9 ± 31.3	106.9 ± 26.8	85.0 ± 21.0	78.5 ± 23.5	35.4 ± 19.2	Group	0.43
B500	18.4 ± 6.3	38.4 ± 26.9	87.1 ± 24.5	78.1 ± 23.0	58.0 ± 23.5	58.0 ± 23.5	Time	<0.001

Berberine (ng/mL)	0 min	20 min	40 min	60 min	90 min	120 min		*p*
D100	16.7 ± 9.1	47.2 ± 17.6	93.0 ± 35.2	84.5 ± 33.2	60.9 ± 23.1	34.6 ± 14.8	G x T	0.10
D200	14.8 ± 4.4	69.6 ± 28.6	85.0 ± 9.0	83.9 ± 27.4	78.9 ± 12.6	53.3 ± 29.6		

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† = Different than placebo ($p < 0.05$); ‡ = Different than B500 ($p < 0.05$); * = Different than D100 ($p < 0.05$).

Table 5.

Area under the curve (AUC) and concentration max (C_{Max}) for berberine, insulin, and glucose.

Berberine	AUC(ng/mL × 120 min)	C_{Max}(ng/mL)
PLA	20.2 ± 16.2	0.22 ± 0.18
B500	42.3 ± 17.6	0.40 ± 0.17
D100	284.2 ± 115.9	3.8 ± 1.4
D200	929 ± 694	12.0 ± 10.1
p	0.045	0.06
Insulin	**AUC (μIU/mL × 120 min)**	**C_{Max} (μIU/mL)**
PLA	8260 ± 420	110.6 ± 24.3
B500	7429 ± 1519	99.1 ± 6.6
D100	8502 ± 717	107.2 ± 21.4
D200	7242 ± 1263	98.1 ± 3.4
p	0.22	0.36

Berberine	AUC(ng/mL × 120 min)	C_{Max}(ng/mL)
Glucose	**AUC (mg/dL × 120 min)**	**C_{Max} (mg/dL)**
PLA	12,199 ± 1602	146.2 ± 13.9
B500	12,029 ± 194	139.4 ± 32.8
D100	12,381 ± 1128	140.8 ± 8.8
D200	11,898 ± 2058	139.0 ± 16.6
p	0.92	0.77

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3.6. Insulin

There was no significant group x time interaction ($p = 0.095$) for insulin measures. A significant time effect ($p < 0.001$) was observed where insulin concentrations were significantly elevated at 20 min (50.8 ± 20.7 µIU/mL, $p = 0.02$), 40 min (93.0 ± 18.2 µIU/mL, $p = 0.02$, and 60 min (82.9 ± 13.6 µIU/mL, $p = 0.003$) in comparison to baseline (16.3 ± 6.1 µIU/mL). Additionally, insulin concentrations were significantly higher at 40 min (93.0 ± 18.2 µIU/mL) and 60 min (82.9 ± 13.6 µIU/mL) compared to 20 min (50.8 ± 20.7 µIU/mL; 40 min, $p = 0.002$; 60 min, $p = 0.018$) and 120 min (45.1 ± 24.5 µIU/mL; 40 min, $p = 0.002$; 60 min, $p = 0.013$). No significant condition effect ($p > 0.05$) was identified. No significant differences were observed for AUC ($p = 0.22$) and C_{Max} ($p = 0.36$) insulin values (See Table 4 and Table 5).

3.7. Whole Blood and Serum Markers

Complete blood count analysis was completed on collected whole-blood samples to assess safety and presence of any adverse responses to supplementation. As seen in Table 6, absolute lymphocytes analysis revealed a significant main effect of condition ($p = 0.011$) where D100 had elevated concentrations compared to D200 ($p = 0.032$) but were in clinically accepted norms. Absolute monocytes analysis revealed a significant group × time interaction ($p = 0.047$) and follow-up simple main effects testing revealed significant changes over time within the Placebo ($p = 0.042$) and D100 ($p = 0.029$) conditions. Specifically, at 120 min within the Placebo and D100 conditions absolute monocytes were higher than baseline albeit they were within clinically accepted norms. Furthermore, no significant changes between conditions were revealed at any time point for absolute monocytes ($p > 0.05$). All other

variables for complete blood count did not change over time, between conditions, or reveal any interactions ($p > 0.05$).

Table 6.

Complete blood count.

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)*(p)*	G x T(*p*)
White Blood Cell Count (10^3 × cells/μL)	PLA	5.6 ± 1.0	5.7 ± 1.3	0.74	0.45	0.20
	D100	5.7 ± 1.1	5.9 ± 1.3			
	D200	6.6 ± 2.4	6.2 ± 2.1			
	B500	5.8 ± 1.2	5.3 ± 1.2			
Red Blood Cell Count (10^3 × cells/μL)	PLA	4.8 ± 0.2	4.8 ± 0.1	0.10	0.54	0.30
	D100	4.7 ±0.3	4.7 ± 0.3			
	D200	4.7 ± 0.3	4.7 ± 0.2			
	B500	5.0 ± 0.3	4.8 ± 0.4			
Hemoglobin (g/dL)	PLA	14.8 ± 0.5	14.8 ± 0.7	0.15	0.47	0.20
	D100	14.3 ± 0.4	14.5 ± 0.5			
	D200	14.3 ± 0.6	14.2 ± 0.4			
	B500	15.2 ± 1.0	14.8 ± 0.8			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)*(p)*	G x T(*p*)
Hematocrit (%)	PLA	43.2 ± 0.8	43.4 ± 1.4	0.20	0.73	0.19
	D100	42.2 ± 0.7	42.6 ± 1.4			
	D200	42.1 ± 1.3	42.0 ± 0.4			
	B500	44.6 ± 2.8	43.7 ± 3.0			
Mean Corpuscular Volume	PLA	89.4 ± 3.5	90.2 ± 3.8	0.87	0.44	0.71
	D100	90.1 ±4.7	90.0 ± 4.3			
	D200	90.4 ± 3.2	90.4 ± 3.8			
	B500	90.2 ± 2.8	90.5 ± 3.7			
Mean Corpuscular Hemoglobin	PLA	30.6 ± 1.6	30.7 ± 1.5	0.86	0.88	0.81
	D100	30.6 ± 1.6	30.5 ± 1.6			
	D200	30.7 ± 1.3	30.6 ± 1.6			
	B500	30.6 ± 1.6	30.7 ± 1.4			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Mean Corpuscular Hemoglobin Content	PLA	34.2 ± 0.6	34.1 ± 0.8	0.91	0.43	0.93
	D100	34.0 ± 0.6	34.0 ± 0.5			
	D200	34.0 ± 0.8	33.8 ± 0.7			
	B500	34.0 ± 0.9	33.9 ± 0.7			
Red Cell Distribution Width (%)	PLA	12.1 ± 0.4	12.0 ± 0.5	0.93	0.08	0.55
	D100	12.1 ± 0.4	12.0 ± 0.5			
	D200	12.1 ±0.4	12.1 ± 0.4			
	B500	12.0 ± 0.4	12.0 ± 0.4			
Platelet Count	PLA	242.2 ± 32.6	239.0 ± 37.3	0.85	0.91	0.55
	D100	243.4 ± 26.7	250.2 ± 26.5			
	D200	242.8 ± 23.9	242.0 ± 19.1			
	B500	247.4 ± 36.1	245.8 ± 30.9			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Abs Neutrophils (cells/uL)	PLA	2790 ± 1318	3046 ± 1295	0.45	0.88	0.38
	D100	2902 ± 719	2962 ± 949			
	D200	3926 ± 2324	3723 ± 2020			
	B500	2743 ± 737	2675 ± 702			
Abs Lymphocytes (cells/uL)	PLA	1893 ± 435	1805 ± 297	0.01	0.31	0.28
	D100	2068 ± 719	2173 ± 253			
	D200	1852 ± 528	1721 ± 149			
	B500	2268 ± 524	1899 ± 423			
Abs Monocytes (cells/µL)	PLA	477 ± 134	549 ± 168	0.92	0.04	0.007
	D100	494 ± 159	597 ± 225			
	D200	549 ± 152	504 ± 96			
	B500	545 ± 178	530 ± 205			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Abs Eosinophils (cells/μL)	PLA	218 ± 162	214 ± 162	0.17	0.09	0.62
	D100	179 ± 116	165 ± 96			
	D200	225 ± 148	188 ± 106			
	B500	183 ± 119	179 ± 161			
Abs Basophils (cells/μL)	PLA	42.8 ± 12.4	45.6 ± 10.9	0.47	0.94	0.67
	D100	38.4 ± 7.6	43.8 ± 12.2			
	D200	49.0 ± 18.5	44.6 ± 9.3			
	B500	41.2 ± 20.7	36.8 ± 11.6			
Neutrophils (%)	PLA	52.1 ± 13.2	52.2 ± 11.7	0.24	0.42	0.29
	D100	50.7 ± 7.3	49.1 ± 6.7			
	D200	56.7 ±14.4	57.3 ± 12.0			
	B500	47.5 ± 7.9	50.4 ± 7.8			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Lymphocytes (%)	PLA	34.7 ± 9.9	33.1 ± 7.8	0.17	0.26	0.32
	D100	36.7 ± 6.2	37.4 ± 5.2			
	D200	30.0 ± 9.5	29.8 ± 7.6			
	B500	39.5 ± 7.1	36.0 ± 4.7			
Monocytes (%)	PLA	8.5 ± 2.1	10.0 ± 3.1	0.12	0.15	0.60
	D100	9.0 ± 1.1	10.0 ± 2.9			
	D200	8.9 ± 2.7	8.8 ± 2.7			
	B500	9.3 ± 1.8	9.8 ± 2.4			
Eosinophils (%)	PLA	4.0 ± 3.0	3.9 ± 3.1	0.30	0.25	0.72
	D100	3.0 ± 1.4	2.7 ± 1.3			
	D200	3.6 ± 2.8	3.3 ± 2.5			
	B500	3.0 ± 1.5	3.1 ± 2.3			
Basophils (%)	PLA	0.8 ± 0.3	0.8 ± 0.3	0.37	0.43	0.95
	D100	0.7 ± 0.1	0.8 ± 0.3			
	D200	0.8 ± 0.3	0.8 ± 0.3			
	B500	0.7 ± 0.2	0.7 ± 0.2			

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Comprehensive metabolic panels were analyzed in collected serum samples. As seen in Table 7, analysis revealed significant main effects of time for glucose ($p = 0.024$), sodium ($p < 0.001$), potassium ($p = 0.007$), and chloride ($p = 0.024$) and these changes were within clinically accepted norms. A significant group × time interaction was revealed for creatinine ($p = 0.008$), and follow-up simple main effects testing revealed significant changes over time within the D100 ($p = 0.031$) and D200 ($p = 0.030$) conditions. Within the D100 condition at 120 min, creatinine measures were higher than baseline, and within the D200 condition, baseline measures were higher than 120 min, however, these measures are within clinically accepted norms. Additionally, there were significant creatinine differences between conditions at baseline ($p = 0.039$) and 120 min ($p = 0.009$) measures. At baseline, D200 ($p = 0.030$) and B500 ($p = 0.016$) conditions had higher creatinine measures compared to D100 and these differences were within clinically accepted norms. Lastly, a significant main effect of condition was observed for creatinine measures ($p = 0.040$) where D100 was higher than placebo ($p = 0.035$) but was within clinically accepted norms.

Table 7.

Comprehensive metabolic panel.

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Glucose (mg/dL)	PLA	90.6 ± 5.3	65.6 ± 11.0	0.66	0.02	0.17
	D100	92.0 ± 5.7	68.8 ± 10.0			
	D200	81.2 ± 13.6	73.2 ± 20.1			
	B500	89.2 ± 4.2	73.8 ± 15.4			
Blood Urea Nitrogen (g/dL)	PLA	16.2 ± 2.3	15.6 ± 2.3	0.14	0.11	0.46
	D100	13.8 ± 1.5	13.8 ± 1.3			
	D200	15.4 ± 1.9	14.8 ± 2.2			
	B500	15.8 ± 2.2	15.2 ± 2.5			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Creatinine (mg/dL)	PLA	1.0 ± 0.1	1.0 ± 0.1	0.04	0.55	0.008
	D100	0.9 ± 0.1	1.0 ± 0.1			
	D200	1.0 ± 0.1	0.9 ± 0.1			
	B500	1.0 ± 0.1	0.9 ± 0.1			
Glomerular Filtrate Rate	PLA	107.2 ± 12.0	102.2 ± 12.8	0.03	0.30	0.02
	D100	115.6 ± 8.4	109.4 ± 8.8			
	D200	106.2 ± 13.0	111.0 ± 11.9			
	B500	106.6 ± 13.3	109.2 ±12.8			
Sodium (mM)	PLA	137 ± 1	139 ± 2	0.20	<0.001	0.82
	D100	136 ± 2	139 ± 2			
	D200	135 ± 2	137 ± 2			
	B500	136 ± 2	138 ± 3			
Potassium (mM)	PLA	4.3 ± 0.2	4.0 ± 0.1	0.36	0.007	0.90
	D100	4.2 ± 0.2	3.9 ± 0.2			
	D200	4.3 ± 0.2	4.0 ± 0.2			
	B500	4.2 ± 0.3	3.9 ± 0.2			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Chloride (mM)	PLA	102 ± 2	102 ± 1	0.45	0.02	0.18
	D100	103 ± 2	103 ± 2			
	D200	101 ± 2	103 ± 2			
	B500	101 ± 3	103 ± 2			
Carbon Dioxide (mM)	PLA	25 ± 2	26 ± 3	0.62	0.16	0.78
	D100	25 ± 1	26 ± 1			
	D200	26 ± 1	27 ± 2			
	B500	25 ± 1	26 ± 3			
Calcium (mg/dL)	PLA	9.3 ± 0.4	9.7 ± 0.2	0.26	0.12	0.049
	D100	9.4 ± 0.2	9.5 ± 0.3			
	D200	9.3 ± 0.5	9.4 ± 0.3			
	B500	9.5 ± 0.3	9.5 ± 0.3			
Total Protein (g/dL)	PLA	6.8 ± 0.4	6.9 ± 0.3	0.94	0.74	0.22
	D100	6.8 ± 0.3	6.9 ± 0.3			
	D200	6.8 ± 0.6	6.8 ± 0.4			
	B500	7.0 ± 0.5	6.8 ± 0.5			
Albumin (g/dL)	PLA	4.6 ± 0.2	4.5 ± 0.1	0.42	0.38	0.80
	D100	4.5 ± 0.1	4.4 ± 0.2			
	D200	4.5 ± 0.2	4.4 ± 0.0			
	B500	4.6 ± 0.1	4.5 ± 0.1			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Globulin (g/dL)	PLA	2.2 ± 0.3	2.3 ± 0.3	0.62	0.26	0.17
	D100	2.3 ± 0.3	2.4 ± 0.3			
	D200	2.4 ± 0.4	2.4 ± 0.3			
	B500	2.4 ± 0.4	2.3 ± 0.5			
Alb/Glob Ratio	PLA	2.1 ± 0.2	2.0 ± 0.2	0.37	0.11	0.28
	D100	2.0 ± 0.3	1.8 ± 0.3			
	D200	1.9 ± 0.3	1.9 ± 0.3			
	B500	1.9 ± 0.3	2.0 ± 0.4			
Total Bilirubin (mg/dL)	PLA	0.9 ± 0.3	0.8 ± 0.3	0.52	0.14	0.37
	D100	0.8 ± 0.3	0.8 ± 0.3			
	D200	0.9 ± 0.4	0.8 ± 0.3			
	B500	1.0 ± 0.4	0.9 ± 0.4			
Alkaline Phosphatase (U/L)	PLA	53 ± 10	51 ± 10	0.13	0.16	0.45
	D100	51 ± 11	52 ± 10			
	D200	51 ± 13	50 ± 10			
	B500	54 ± 12	53 ± 13			
AST (U/L)	PLA	22 ± 5	22 ± 5	0.22	0.18	0.55
	D100	24 ± 7	24 ± 7			
	D200	24 ± 6	23 ± 5			
	B500	20 ± 3	20 ± 3			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
ALT (U/L)	PLA	19 ± 6	19 ± 5	0.93	1.00	0.60
	D100	19 ± 7	20 ± 7			
	D200	19 ± 4	18 ± 3			
	B500	18 ± 6	18 ± 5			

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A significant group x time interaction was revealed for globular filtration rate ($p = 0.016$) and follow-up simple main effects testing revealed significant changes over time within the D100 ($p = 0.047$) and D200 ($p = 0.037$) conditions. Within the D100 condition, baseline globular filtration rate measures were higher than 120 min, and within the D200 condition 120 min measures were higher than baseline, however, these measures are within clinically accepted norms. Additionally, follow-up simple main effects testing from the interaction revealed significant differences between conditions for globular filtration rate measures at baseline ($p = 0.050$) and 120 min ($p = 0.005$). At baseline, globular filtration rate measures were higher in D100 compared to D200 ($p = 0.048$) and B500 ($p = 0.038$), which were within clinically accepted norms. At 120 min, globular filtration rate measures were higher in D200 ($p = 0.024$), D100 ($p = 0.024$), and B500 ($p = 0.047$) compared to placebo and were within clinically accepted norms. Lastly, a significant main effect of condition was observed for globular filtration rate measures ($p = 0.033$) where D100 was higher than placebo ($p = 0.028$) but was within clinically accepted norms.

A significant group x time interaction was revealed for calcium ($p = 0.049$) and follow-up simple main effects testing revealed significant changes over time within the placebo condition ($p = 0.009$), the levels of which were higher at 120 min compared to baseline, but within clinically accepted norms. Furthermore, a follow-up simple main effects revealed significant differences between conditions for calcium measures at 120 min ($p = 0.030$) where placebo was higher than D200 ($p = 0.040$) and all measures were within clinically accepted norms. All other variables for the comprehensive metabolic panel did not change over time, between conditions, or reveal any interactions ($p > 0.05$).

4. Discussion

The intent of this project was to evaluate the observed differences in plasma berberine concentrations after oral ingestion of two doses (100 mg [D100] and 200 mg [D200],

respectively) of dihydroberberine and one dose (500 mg) of berberine (B500). Findings from this pilot approach revealed that area under the curve calculations for berberine were significantly greater when either dose of dihydroberberine was ingested in comparison to the values observed for berberine and placebo (PLA). In addition, peak berberine concentrations (C_{Max}) tended to be different between all conditions. Specifically, the 100 mg dose of dihydroberberine was significantly greater than peak values observed in PLA and B500 while peak concentrations for the 200 mg dose of dihydroberberine tended to be different than what was observed in the berberine group.

Of the available human research that has explored efficacy and safety of oral berberine ingestion, the majority of these studies have employed a dosing regimen that consists of two to three daily doses with meals (to reduce incidence of gastrointestinal complications) at a dosage of 500 mg per dose. For example, Perez-Rubio et al. [7] had 24 metabolic syndrome patients randomly ingest, in a double-blind, placebo-controlled fashion, three daily doses of 500 mg/dose (total daily dosage of 1500 mg/day) for three months. Similarly, Yin et al. [6] also randomly assigned type 2 diabetes patients to ingest three daily 500 mg doses for a total of 13 weeks. Finally, Zhang et al. [17] randomly assigned 116 individuals diagnosed with type 2 diabetes to consume two 500 mg daily doses (1000 mg total) over a 3-month period. While results of these studies outline efficacy outcomes in various clinical populations, very limited human data are available that has outlined the absorption pattern and plasma kinetics after oral berberine ingestion. In this respect, it has been previously reported that oral bioavailability of berberine is extremely low (<1%) in both animal [9,10] and human models largely due to poor intestinal absorption and high levels of first-pass removal in the intestines and liver [5]. This has led to the identification of various strategies to increase bioavailability, including administration of dihydroberberine. Currently, no human research has appeared in the peer-reviewed literature that has examined oral ingestion of dihydroberberine. Towards this end, Buchanan et al. [16] compared the pharmacokinetics associated with transdermal administration of berberine and dihydroberberine in Sprague-Dawley rats. Using this model and over an 8-h period, transdermal application of dihydroberberine resulted in greater AUC values than what was observed for both transdermal and oral administration of berberine. Moreover, transdermal delivery of berberine resulted in greater AUC values than what was observed for oral administration, further highlighting the poor bioavailability of oral berberine ingestion.

Other highlights from the present study include the statistically significant higher outcomes at baseline (after three doses, but prior to the 4th dose in the present study) in D100 and D200 when compared to PLA and B500. When viewed in comparison to PLA, this outcome was not surprising and some might deem it expected considering that dihydroberberine was administered, however, the finding of greater levels in comparison to berberine seemingly

speaks to the ability of dihydroberberine to maintain berberine levels in the blood better than oral berberine ingestion. Whether or not these increases in plasma berberine translate beyond one day or measurement or improvements in efficacy outcomes commonly associated with berberine supplementation cannot be determined from this project. In this respect, more research is certainly needed with dihydroberberine to determine how well the greater plasma berberine levels improve dyslipidemia and glucose and insulin homeostasis as commonly reported in the literature.

A key limitation with the present study is the sample size on this project. While the fully randomized, crossover, double-blind, and placebo-controlled approach was a sound approach for this pilot assessment, it is readily acknowledged by the authors that more study participants are needed on future studies to continue to establish the safety and efficacy of berberine and dihydroberberine. Further to the point and beyond just having a low sample size, one needs to closely consider our plasma berberine data as two participants in the D200 at the 60-min time point exhibited high values, which nearly achieved traditional definitions of being a statistical outlier (>±3 of standardized residual estimates). When combined with the low sample size, these data exerted substantial impact on the distribution of these data. As such, traditional approaches to transforming the data were unsuccessful at achieving normality and all data were subsequently analyzed using a parametric approach. Furthermore, all analysis was also completed using an intent-to-treat approach with those values removed and the high values replaced with the previous data point and no meaningful differences were noted in the outcomes of the project. Another limitation might be considered to be the small number of doses ingested prior to assessing the absorption kinetics. This was an a priori consideration as our primary focus for this pilot project was to assess and compare the absorption kinetics of two different doses of oral dihydroberberine in comparison to oral ingestion of berberine and placebo.

5. Conclusions

In conclusion, results from this study represent what we believe are the first data in humans to evaluate the absorption kinetics of berberine and two doses of dihydroberberine. Results from this study demonstrate that dihydroberberine, irrespective of what dose is delivered, achieves greater area under the curve as well as peak berberine concentrations when compared to oral ingestion of berberine or placebo. Since berberine use has traditionally spanned several months to establish the observed efficacy outcomes known for berberine (improved glucose and insulin homeostasis and lipid parameters), several follow-up approaches are recommended. First, can the increased levels of berberine seen from dihydroberberine ingestion usher in faster improvements in outcomes of interest. In a similar vein, are the

outcomes improved as a result of dihydroberberine ingestion in comparison to berberine ingestion.

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Author Contributions

Designed study: C.M.K.; data collection: J.M.M., A.M.H., K.M.R. and R.A.S.; analyzed data: P.W.M.; prepared initial draft: C.M.K., A.M.H., J.M.M. and P.W.M.; approved final version: all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Lindenwood University: IRB-20-173, approval date: 22 June 2020.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data and/or statistical analyses are available upon request on a case-by-case basis for non-commercial scientific inquiry and/or educational use.

Conflicts of Interest

C.M.K. serves as a paid advisor for NNB Nutrition. A conflict-of-interest management plan was filed and approved by the Lindenwood University IRB prior to commencing any data collection. As part of this plan, C.M.K. was not involved in any part of the consenting,

randomization, or data collection process. All statistical analysis performed by C.M.K. was done while data was still blinded and all analysis and interpretation was confirmed by P.W.M. As such, P.W.M. and C.M.K. prepared all statistical analysis and interpretation. All other authors declare that they have no competing interests.

Footnotes

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Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Data Availability Statement

Data and/or statistical analyses are available upon request on a case-by-case basis for non-commercial scientific inquiry and/or educational use.

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Absorption Kinetics of Berberine and Dihydroberberine and Their Impact on Glycemia: A Randomized, Controlled, Crossover Pilot Trial

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Abstract

Berberine is a natural alkaloid used to improve glycemia but displays poor bioavailability and increased rates of gastrointestinal distress at higher doses. Recently, dihydroberberine has been developed to combat these challenges. This study was designed to determine the rate and extent to which berberine appeared in human plasma after oral ingestion of a 500 mg dose of berberine (B500) or 100 mg and 200 mg doses of dihydroberberine (D100 and D200). In a randomized, double-blind, crossover fashion, five males (26 ± 2.6 years; 184.2 ± 11.6 cm; 91.8 ± 10.1 kg; 17.1 ± 3.5% fat) completed a four-dose supplementation protocol of placebo (PLA), B500, D100, and D200. The day prior to their scheduled visit, participants ingested three separate doses with breakfast, lunch, and dinner. Participants fasted overnight (8–10 h) and consumed their fourth dose with a standardized test meal (30 g glucose solution, 3 slices white bread) after arrival. Venous blood samples were collected 0, 20, 40, 60, 90, and 120 minutes (min) after ingestion and analyzed for BBR, glucose, and insulin. Peak concentration (C_{Max}) and area under the curve (AUC) were calculated for all variables. Baseline berberine levels were different between groups ($p = 0.006$), with pairwise comparisons indicating that baseline levels of PLA and B500 were different than D100. Berberine C_{Max} tended to be different ($p = 0.06$) between all conditions. Specifically, the observed C_{Max} for D100 (3.76 ± 1.4 ng/mL) was different than PLA (0.22 ± 0.18 ng/mL, $p = 0.005$) and B500 (0.4 ± 0.17 ng/mL, $p = 0.005$). C_{Max} for D200 (12.0 ± 10.1 ng/mL) tended ($p = 0.06$) to be different than B500. No difference in C_{Max} was found between D100 and D200 ($p = 0.11$). Significant differences in berberine AUC were found between D100 (284.4 ± 115.9 ng/mL × 120 min) and PLA (20.2 ± 16.2 ng/mL × 120 min, $p = 0.007$) and between D100 and B500 (42.3 ± 17.6 ng/mL × 120 min, $p = 0.04$). Significant differences in D100 BBR AUC (284.4 ± 115.9 ng/mL×120 min) were found between PLA (20.2 ± 16.2 ng/mL × 120 min, $p = 0.042$) and B500 (42.3 ± 17.6 ng/mL × 120 min, $p = 0.045$). Berberine AUC values between D100 and D200 tended ($p = 0.073$) to be different. No significant differences in the levels of glucose ($p = 0.97$) and insulin ($p = 0.24$) were observed across the study protocol. These results provide preliminary evidence that four doses of a 100 mg dose of dihydroberberine and 200 mg dose

of dihydroberberine produce significantly greater concentrations of plasma berberine across of two-hour measurement window when compared to a 500 mg dose of berberine or a placebo. The lack of observed changes in glucose and insulin were likely due to the short duration of supplementation and insulin responsive nature of study participants. Follow-up efficacy studies on glucose and insulin changes should be completed to assess the impact of berberine and dihydroberberine supplementation in overweight, glucose intolerant populations.

Keywords: absorption, safety, plants, glucose, insulin

1. Introduction

Berberine is an isoquinoline derivative alkaloid present in various parts (root, stem, fruit, bark) of multiple plants including, in particular, species found in the Coptis, Hydrastis, and Berberis genus [1]. Various animal and culture models have reported on the ability of berberine to exert lipid and glucose-lowering effects in addition to helping with weight loss and improving glucose tolerance [2,3]. For this reason, interest in berberine has suggested it may be beneficial in treating diabetes and obesity. While still being fully characterized, mechanistic actions of berberine consist of it reducing blood lipid levels via action on hepatocyte nuclear factor 1 alpha (HNF-1 alpha) and hepatic LDL receptors, intestinal absorption of cholesterol [4], and glucose levels while increasing insulin sensitivity.

Moreover, berberine is a known activator of AMP Kinase [1,5], which contributes to increases in fatty acid oxidation and a reduction of lipogenic gene expression.

Yin and colleagues [6] published results from two separate studies that both examined the impact of berberine at improving various biomarkers associated with glucose homeostasis. Throughout the first study, berberine (500 mg/dose, 3 doses/day) or metformin was administered over a 3-month period to 36 adults who were recently diagnosed with type 2 diabetes. Levels of hemoglobin A1C, fasting blood glucose, postprandial blood glucose, and triglycerides were all significantly improved in comparison to pre-treatment, and, importantly, the magnitude of changes was similar to what was observed with metformin. An additional investigation was reported where berberine was added to the established treatment plan 48 type 2 diabetes patients. Over a 13-week period, blood glucose (both fasting and postprandial), hemoglobin A1C, fasting, insulin, and HOMA-IR were all improved when berberine was added. These results align with the Perez-Rubio et al. [7] investigation that reported improvements in waist circumference, blood pressure, triglycerides, and area under the curve values for both glucose and insulin (after a 75-g glucose load over a two-hour period) in 24 patients with metabolic syndrome. In this study, berberine was randomly assigned in a double-blind, placebo-controlled fashion at a daily dosage of 1500 mg/day (500

mg/dose, 3 doses/day with meals) for three months. Similar outcomes using berberine have been reported by Rao et al. [8] after completing a randomized, open-label study on 41 diabetic patients where various indicators of glucose and insulin metabolism were improved over the three-month study period.

One challenge associated with oral ingestion of berberine has been its low bioavailability (<1%) reported in both animal [9,10] and human models largely due to poor intestinal absorption and high levels of first-pass removal in the intestines and liver [5]. To overcome this limitation, higher doses of berberine are commonly administered, which has led to a consistent reporting of gastrointestinal adverse events. For example, Yin et al. [6] reported that gastrointestinal adverse events were 34.5% during a 13-week study which provided berberine hydrochloride as a monotherapy or in combination with other treatments. Interestingly all adverse events were reported when berberine was combined with other treatments (metformin, insulin, etc.) and all incidents occurred during the first four weeks of treatment.

For this reason, alternative approaches have been explored to help improve its poor bioavailability. For example, techniques such as encapsulation [11,12], packaging with other nutrients and compounds [13], and spray drying [14] have all been reported on in the literature. In addition, the reduced derivative of berberine, dihydroberberine, has been suggested as a natural alternative to improve bioavailability [15]. Buchanan and investigators [16] examined the ability of transdermal application of dihydroberberine in 40 Sprague-Dawley rats to improve bioavailability, improve glucose homeostasis parameters, and reduce adverse events in comparison to oral berberine gavage and transdermal applications of berberine. Results from both acute and chronic (14 days) pharmacokinetic approaches indicated that transdermal dihydroberberine achieved higher bioavailability than both transdermal and oral berberine administration. Oral administration of dihydroberberine has yet to be reported in human literature, thus initial studies should provide some level of insight into its impact on absorption kinetics, preliminary safety indicators, and adverse events associated with its ingestion. Consequently, the purpose of this study was to conduct a randomized, double-blind, placebo-controlled investigation to examine the dose-dependent impact on absorption kinetics, clinical safety parameters, and adverse events of oral dihydroberberine ingestion in humans.

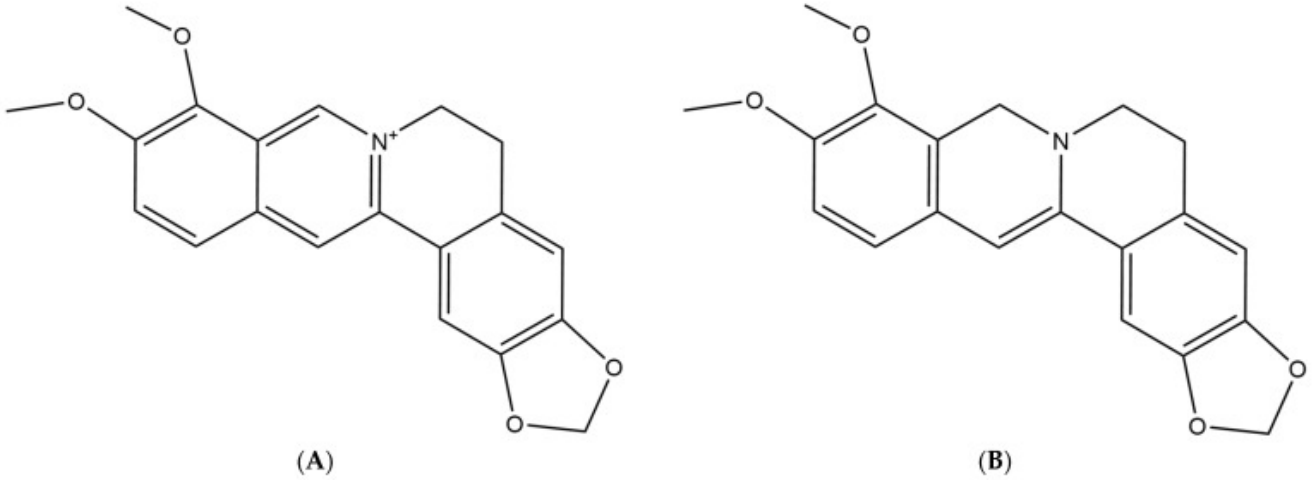
2. Methods

2.1. Overview of Research Design

This study was completed using a randomized, double-blind, crossover study design. Prior to beginning, all participants signed an IRB-approved informed consent document (Lindenwood

University: IRB-20-173, approval date: 22 June 2020) and completed a healthy history questionnaire to determine study eligibility. A power analysis was not computed as this study was considered a proof of concept, pilot investigation. This study protocol and design was retrospectively registered on Clinicaltrials.gov on 25 August 2021 as NCT05021341 (https://clinicaltrials.gov/ct2/show/NCT05021341 accessed on 24 December 2021). All four supplementation conditions were completed by each participant in a crossover fashion with a minimum washout of 72 h. For each supplementation condition, four total doses were consumed with three doses being consumed the day before testing (with breakfast, lunch, and dinner) and the fourth dose being consumed the morning of testing with a standardized test meal after observing an overnight (8 to 10 h) fast (Figure 1). Participants were scheduled to begin each visit at approximately the same times and reported to the laboratory between 600 and 1000 h. Using a randomized, double-blind, placebo-controlled fashion, study participants consumed either a placebo (resistance dextrin) (PLA), 500 mg of berberine (B500), 100 mg dihydroberberine (D100), or 200 mg dihydroberberine (D200). To minimize any order effects from testing, participants were randomized using an online randomization software program (www.random.org accessed on 1 July 2020). Upon arrival for each study visit, participants had their height, body mass, body composition, and hemodynamics (resting heart rate and blood pressure) measured along with an assessment of capillary glucose levels approximately 30 min prior to supplementation ingestion to evaluate fasting status. An indwelling catheter was then implanted into a forearm vein and flushed with saline to remain patent. The fourth and final dose of their assigned supplement was then consumed with 240 milliliters of cold tap water. After supplement ingestion, participants consumed a standardized test meal consisting of a 30-g glucose solution and four slices of white bread. Immediately after ingestion of the test meal, venous blood samples were collected 20, 40, 60, 90, and 120 min (Figure 2). Participants were provided 200 mL of cold water to ingest after each blood collection. Upon processing, all blood samples were stored at −80 °C. Throughout each visit, study participants were asked to report the occurrence of any adverse events (dizziness, headache, nausea, upset stomach, cramping, diarrhea, etc.) that occur throughout completion of the study protocol. All subsequent study visits were completed in an identical fashion and scheduled with at least 72 h between visits.

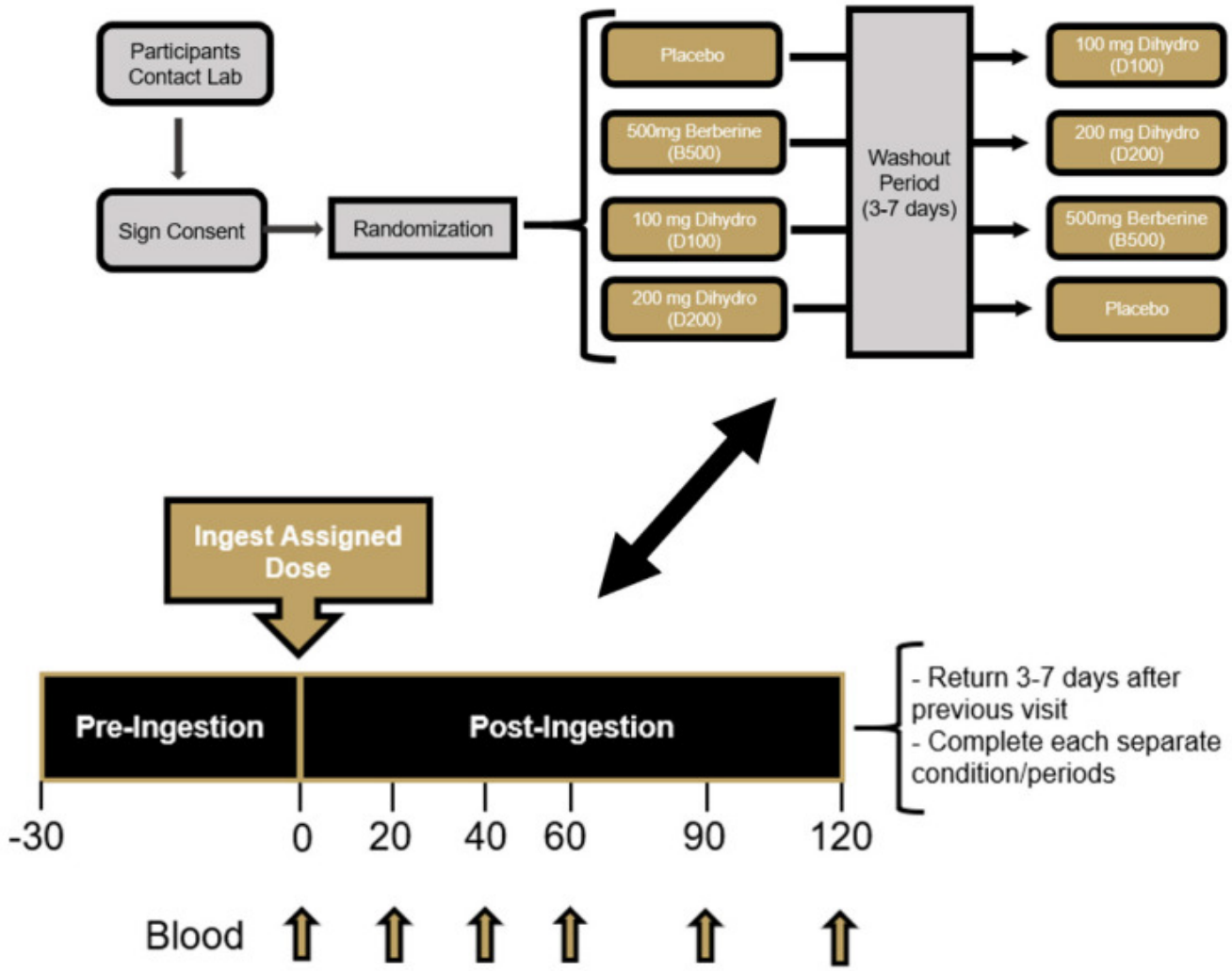
Figure 1.



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(**A**) Chemical structure of berberine. (**B**) Chemical structure of dihydroberberine.

Figure 2.



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Overview of research design.

2.2. Study Participants

Prior to participation, all recruited individuals provided signed informed consent using an IRB-approved consent form (Lindenwood University: IRB-20-173, approval date: 22 June 2020). Five healthy males (26.0 ± 2.6 years, 184.2 ± 11.6 cm, 91.8 ± 10.1 kg, 17.1 ± 3.5% fat) successfully completed all study visits (see Table 1 for full participant characteristics). Inclusion criteria included age (18–45 years), healthy and free of disease (as reported by the health screening questionnaire), and physically active (reported at least 30 min of moderate exercise three days a week). Any individual diagnosed with or being treated for cardiac, respiratory, circulatory, musculoskeletal, metabolic, obesity (defined as body mass index > 30 kg/m^2 and body fat greater than 30%), immune, autoimmune, psychiatric, hematological, neurological, or endocrinological disorder or disease were not allowed to participate in the

current study. Any participant taking any dietary supplement besides a multi-vitamin/mineral or a protein supplement were required to abstain from consuming any further doses for 30 days prior to beginning the study protocol.

Table 1.

Baseline age, gender, height (cm), weight (kg), body mass index, % fat, heart rate, systolic blood pressure, diastolic blood press, energy, carbohydrates, proteins, and fat intake.

	Mean	SD	Minimum	Maximum
Age	26.0	2.6	23	30
Height (cm)	184.2	11.6	171	202
Weight (kg)	91.8	10.1	82.4	108.5
Body mass index (kg/m^2)	27.1	3.9	22.6	33.1
% fat	17.1	3.5	12.5	20.9
Heart rate (beats/minute)	61.0	12.0	41	70
Systolic blood pressure (mm Hg)	122.3	11.4	111	138
Diastolic blood pressure (mm Hg)	72.4	8.8	64	70
Energy intake (kcals/day)	2433	810	1503	3695
Carbohydrate intake (grams/day)	217	62	144	312
Protein intake (grams/day)	121	34	66	153
Fat intake (grams/day)	112	45	75	186

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2.3. Supplementation Protocol

Using a randomized, double-blind, placebo-controlled fashion, study participants consumed either a placebo (resistance dextrin) (PLA), 500 mg of berberine (B500), 100 mg dihydroberberine (D100), or 200 mg dihydroberberine (D200). The dihydroberberine was manufactured by NNB Nutrition (Nanjing, China) and is marketed as Glucovantage®.

Berberine (≥99%) was extracted from *Berberis Aristata* in the hydrochloride form and was used as raw material. Dihydroberberine was obtained by a patented preparation method (CN108997332A). Neither molecule contains any crystalline water. Study participants supplemented consumed four total doses prior to having the absorption kinetics determined. This regimen started the morning before their scheduled visit with participants consuming one dose each with breakfast, lunch, and dinner. In addition to consuming with a meal, all doses were consumed with at least eight fluid ounces of cold tap water. Participants were then required to follow an overnight fast (approximately 8 to 10 h). Upon arrival, capillary glucose levels were determined from a finger-stick blood sample and a handheld glucose monitor (CVS Health Advanced Glucose Monitor, Agamatrix, Inc., Salem, NH, USA). After fasting status was confirmed, the fourth and final dose of their assigned supplement was consumed in front of a study team member with approximately eight fluid ounces of cold tap water. A catheter was then inserted into a forearm vein and approximately 30 min after consuming their final dose a standardized test meal was ingested which consisted of four slices of white bread (approximately 264 kcals, 3.2 g of fat, 50.6 g of carbohydrates and 7.6 g of protein) and 30 g of glucose powder that was mixed with approximately 8–12 fluid ounces of water. The test meal was ingested within five minutes. The ingestion of the test meal was considered to be the 0-min time point and all subsequent measurements occurred as outlined.

All participants were required to complete a supplementation log to document when each dose was consumed. No doses were reported being missed by any participant throughout the study protocol. All study materials were prepared into gelatin capsules of identical size, color, shape, and transparency. All doses were prepared into a single capsule which required each participant to consume a single capsule for each required dose. A resistant dextrin was used as the placebo. All doses were placed into individually labeled bags for each condition for each participant throughout the entire study protocol.

2.4. Procedures

2.4.1. Baseline Demographics and Hemodynamics

During the initial visit, after providing written consent, participants were instructed to rest quietly for approximately 10 min before measuring resting heart rate and blood pressure. Resting heart rate was measured by palpating the radial artery for a period of 60 s. While still resting, blood pressure (Omron BP785, Omron Corporation, Kyoto, Japan) measurements were taken before participants resumed normal activity. Participants then had their body mass determined (Tanita BWB-627A, Tokyo, Japan) and recorded to the nearest ±0.1 kg upon arrival prior to each study visit. All recorded body masses were compared to ensure the participant was weight stable. Any participant whose body mass changed by more than 2% between consecutive study visits was excluded from participation. Following body mass

measurements, height was measured using a standard wall-mounted stadiometer (Tanita, HR-200, Tokyo, Japan) and recorded to the nearest ±0.5 cm (cm). Fat and fat-free mass was determined using a bioelectrical impedance analyzer (InBody 570, Beverly Hills, CA, USA). Participants were required to observe an overnight fast to ensure an accurate determination of body composition. Body composition analysis occurred between 600 and 1000 h by trained research personnel. All assessments were completed according to device specifications.

2.4.2. Dietary Monitoring

Prior to their baseline visit, study participants completed a hand-written four-day food record (three weekdays and one weekend day). The four-day food log was provided to allow participants to replicate their diets. In addition to the four-day food log, participants were instructed on how to complete the ASA24 online dietary assessment tool (https://epi.grants.cancer.gov/asa24/ accessed on 23 December 2021), for determination of baseline caloric and macronutrient intake. From this information, study participants were asked to replicate their dietary intake prior to each subsequent testing visit. Study participants reported 100% compliance to this protocol.

2.4.3. Venous Blood Collection and Processing

To confirm a fasting state prior to catheter insertion and supplement administration, capillary blood was collected from a finger on their non-dominant hand. The collected blood was analyzed using a hand-held glucose analyzer (CVS Health Advanced Glucose Monitor, Agamatrix, Inc., Salem, NH, USA). Fasting was confirmed if glucose was <110 mg/dL. Within each supplementation condition, study participants had their venous blood collected on six different occasions: 0, 20, 40, 60, 90, and 120 min after ingestion of their assigned study agent. Blood was collected via a forearm vein using standard phlebotomy techniques into serum and ethylenediaminetetraacetic acid EDTA-coated Vacutainer™ tubes. To establish safety, whole blood samples were collected into EDTA and serum tubes for assessment of complete blood count and comprehensive metabolic count, respectively. Blood samples collected into EDTA were gently inverted ten times while blood samples collected into serum tubes were gently inverted ten times and allowed to clot at room temperature. For same day analysis, these samples were placed into a chilled container and sent to a commercial diagnostic laboratory (Quest Diagnostics). Plasma samples used for berberine determination were collected into EDTA tubes, gently inverted ten times and centrifuged at 3000 revolutions per minute (rpm) (MegaFuge XFR, Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C for 20 min. After centrifugation, 400 µL aliquots of plasma were removed and frozen at −80 °C within four hours of collection.

2.4.4. Complete Blood Count

To assess adverse events reported in response to the assigned supplementation, whole blood samples were collected in EDTA tubes and analyzed at a commercial diagnostic laboratory (Quest Diagnostics) for changes in red blood cell count, white blood cell count, platelet count, hemoglobin, hematocrit, red blood cell dimension width (RDW), mean corpuscle volume (MCV), mean corpuscle hemoglobin (MCH), mean corpuscle hemoglobin content (MCHC), neutrophil % and cell count, lymphocytes % and cell count, monocytes % and cell count, eosinophils % and cell count, and basophils % and cell count (granulocytes → neutrophils, eosinophils, basophils). Complete blood counts were only assessed at the 0 and 120-min time points.

2.4.5. Comprehensive Metabolic Panel

To assess adverse events in response to the assigned supplementation, serum samples were assayed at a commercial diagnostic laboratory (Quest Diagnostics) for a comprehensive metabolic panel (albumin, albumin/globulin ratio (calculated), alkaline phosphatase, ALT, AST, BUN/creatinine ratio (calculated), calcium, carbon dioxide, chloride, creatinine with estimated GFR, globulin, glucose, potassium, sodium, total bilirubin, total protein, and urea nitrogen). Comprehensive metabolic panels were only assessed at the 0 and 120-min time points.

2.4.6. Glucose and Insulin Determination

Each plasma sample was analyzed in duplicate for glucose and insulin concentration. Plasma glucose was analyzed at a commercial diagnostic laboratory (Quest Diagnostics) using automated clinical analyzers while insulin concentrations were assessed using a standard ELISA technique (DRG International, EIA2935, Springfield, NJ, USA). Briefly, all standards, samples, and controls were analyzed in duplicate across a range of 1.76–100 µIU/mL. Plate to plate controls were employed between all analyzed plates and exhibited a 6.3% coefficient of variation between the plates.

2.4.7. *Plasma Berberine Determination*

Berberine analysis was performed in plasma samples by Heartland Assays (Iowa State University Research Park, Ames, IA, USA) using coupled liquid chromatography-mass spectrometry-mass spectrometry technology (LC-MS-MS) following the procedures of Buchanan et al. [16]. Briefly, BBR and d_6-BBR (Sigma-Aldrich, St. Louis, MO, USA and Toronto Research Chemicals, Toronto, Canada, respectively) standards were prepared using gravimetric determination and were dissolved in 50:50 methanol: water. As previously published, plasma samples and controls were removed from freezer storage and allowed to thaw at room temperature. An amount of 100 µL of plasma was transferred to a 13 × 100

glass test tube followed by the addition of 15.0 µL of d_6-BBR internal standard (ISTD, 0.5 ng/µL). Next, samples were treated with 400 µL of acetonitrile containing 3% acetic acid, vortexed and centrifuged. The supernatant was transferred to 12 × 75 glass test tube and dried in a vacuum dryer. A standard curve (0.3–1000.0 ng/mL) was prepared and dried. Samples, controls, and standard curve were reconstituted in 50 µL of LC/MS/MS organic and 50 µL aqueous mobile phase solutions (Mobile phase B, LCMS-grade methanol and Mobile phase A, LCMS-grade water, both containing 0.2% formic acid and 0.1% 10 M ammonium formate solution) vortexed and transferred into LC/MS/MS vials. Chromatographic separation of BBR and d_6-BBR was conducted on an Agilent Poroshell C18 column (2.1 × 50 mm, 2.7 µm) using an Agilent 6460 triple quadrupole LC/MS/MS system (Santa Clara, CA, USA). The initial gradient conditions were 45% B from 0 to 2 min and advanced to 95% B over 1 min with a flow rate of 0.40 mL/minute. BBR and d_6-BBR data were collected using the Multiple Reaction Monitoring (MRM) mode with Mass Hunter acquisition software (Agilent, Santa Clara, CA, USA). The MRM pairs used for BBR were 337.1 → 322.1 and 337.1 → 293.1 and 343.1 → 325.2 and 343.1 → 307.1 for d_6-BBR. Mass Hunter Quantitation software was used to quantitate the unknown plasma samples based on best fit standard curves. Quality control samples were run at two levels in each assay (4.0 and 100.0 ng/mL). Average recovery was 102% and ranged from 95% to 108%. The average accuracy (bias from nominal) was 1.0%. The average precision (CV) was 2.1% and 1.5% for intra- and inter-assay precision, respectively.

2.5. Statistical Analysis

The primary outcome for this study protocol was berberine AUC while secondary outcomes were considered to the maximum berberine concentration (C_{Max}) as well as the AUC and C_{Max} for glucose and insulin. Area under the curve (AUC) for berberine, glucose, and insulin were calculated using the trapezoidal method (Equation (1)), where C_0 is the unknown concentration at the first time point of interest, C_1 is the unknown concentration at the second time point of interest, and $Time_{1-0}$ is the total time interval between the two time points when the unknown was measured. These individual AUC values for each time interval were then summed to determine the total AUC for berberine, glucose, and insulin (Equation (2)).

(1) (2)

All analyses were completed using Microsoft Excel and the Statistical Package for the Social Sciences (v26; SPSS Inc., Chicago, IL, USA) while provided figures were developed using GraphPad (La Jolla, CA, USA). For all dependent measures, descriptive statistics are presented herein as mean ± standard deviations. Before any statistical tests were completed, normality was assessed using a Shapiro-Wilk test for all dependent variables. All non-normal

data were transformed using natural logarithms, cubed, and then square root transformation approaches and was retested for normality. Data were then analyzed using both parametric and non-parametric approaches. In all such situations, the final statistical decision was identical whether parametric or non-parametric approaches were completed. All reported *p*-values are computed using parametric approaches. Blood markers were analyzed with a mixed factorial ANOVA, and if a significant interaction was observed then follow-up simple effects testing was performed using a repeated measures ANOVA followed by post-hoc testing consisting of paired samples *t*-tests. Body mass, blood pressure, AUC, and C_{Max} variables were analyzed with a repeated measures ANOVA followed by post-hoc testing consisting of paired samples t-tests. For all statistical tests, data were considered statistically significant when the probability of type I error was 0.05 or less. Trends were highlighted and discussed when *p*-values were between 0.05 and 0.10.

3. Results

3.1. Pre-Supplementation Status

Table 2 outlines the pre-supplementation values for body mass and hemodynamics. Body mass was significantly different between groups ($p = 0.045$), and post-hoc testing revealed differences between PLA (90.6 ± 10.1 kg) and D200 (91.4 ± 9.7 kg). Resting heart rate ($p = 0.21$), systolic blood pressure ($p = 0.07$), and diastolic blood pressure ($p = 0.87$) were not different.

Table 2.

Baseline body mass, hemodynamic, glucose, insulin, and berberine.

Raw Data	Group	Pre	*p*-Value
Body Mass (kg)	PLA	90.6 ± 10.1 ‡	0.043
	D100	91.6 ± 10.3	
	D200	91.4 ± 9.7	
	B500	90.8 ± 10.0	

Raw Data	Group	Pre	*p*-Value
Resting Heart Rate (beats/minute)	PLA	64 ± 10	0.21
	D100	62 ± 6	
	D200	60 ± 5	
	B500	57 ± 9	
Systolic Blood Pressure (mm Hg)	PLA	120 ± 8	0.07
	D100	115 ± 10	
	D200	118 ± 11	
	B500	124 ± 10	
Diastolic Blood Pressure (mm Hg)	PLA	72 ± 5	0.87
	D100	70 ± 5	
	D200	70 ± 10	
	B500	72 ± 7	
Glucose (mg/dL)	PLA	91.8 ± 7.6	0.59
	D100	92.0 ± 8.5	
	D200	90.6 ± 3.4	
	B500	89.8 ± 1.8	
Berberine (ng/mL)	PLA	0.14 ± 0.09 †,‡	0.006
	D100	2.60 ± 1.06	
	D200	4.54 ± 2.24	
	B500	0.30 ± 0.12 †,‡	

Raw Data	Group	Pre	*p*-Value
Insulin (μIU/mL)	PLA	15.4 ± 5.4	0.20
	D100	16.7 ± 9.1	
	D200	14.8 ± 4.4	
	B500	18.4 ± 6.3	

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† = Different than D100 ($p < 0.05$); ‡ = Different than D200 ($p < 0.05$).

3.2. Protocol Compliance

Compliance to the supplementation regimen was 100% with all participants reporting that all supplements were ingested as instructed with the final dose being ingested in front of investigators. All study participants were asked to record their food and fluid intake for two days. Compliance was reported at 87.5% as one participant did not record their food and fluid intake on one of the days. Dietary intake is provided in Table 1.

3.3. Adverse Events

A total of 11 adverse events were reported throughout the study protocol by two or 40% of the study participants. The largest number of adverse events ($n = 6$) were reported in D100, $n = 3$ were reported in PLA, $n = 1$ for D200, and $n = 1$ for B500. All but one reported adverse event (10/11 or 90.9%) was deemed by study participants to be mild in severity and one being moderate severity (See Table 3).

Table 3.

Adverse event table.

	PLA	D100	D200	B500
n	5	5	5	5
# of AE's Reported				
Mild	3	5	1	1
Moderate	0	1	0	0

	PLA	D100	D200	B500
Severe	0	0	0	0
	Adverse Events Breakdown			
Gastrointestinal				
Diarrhea	0	0	0	0
Nausea	0	2	0	0
Upset stomach	0	0	1	0
Stomach cramping	2	0	0	0
Pain				
Headache	0	2	0	1
Cardiovascular				
Abnormal heart rhythm	1	0	0	0
Dizziness	0	2	0	0
Constitutional Symptoms				
Nervousness	0	0	0	0
Blurred vision	0	0	0	0
Summary				
Total Adverse Events	3	6	1	1
# of Subjects Reporting AE	2	2	1	1
% of Subjects Reporting AE	40%	40%	20%	20%

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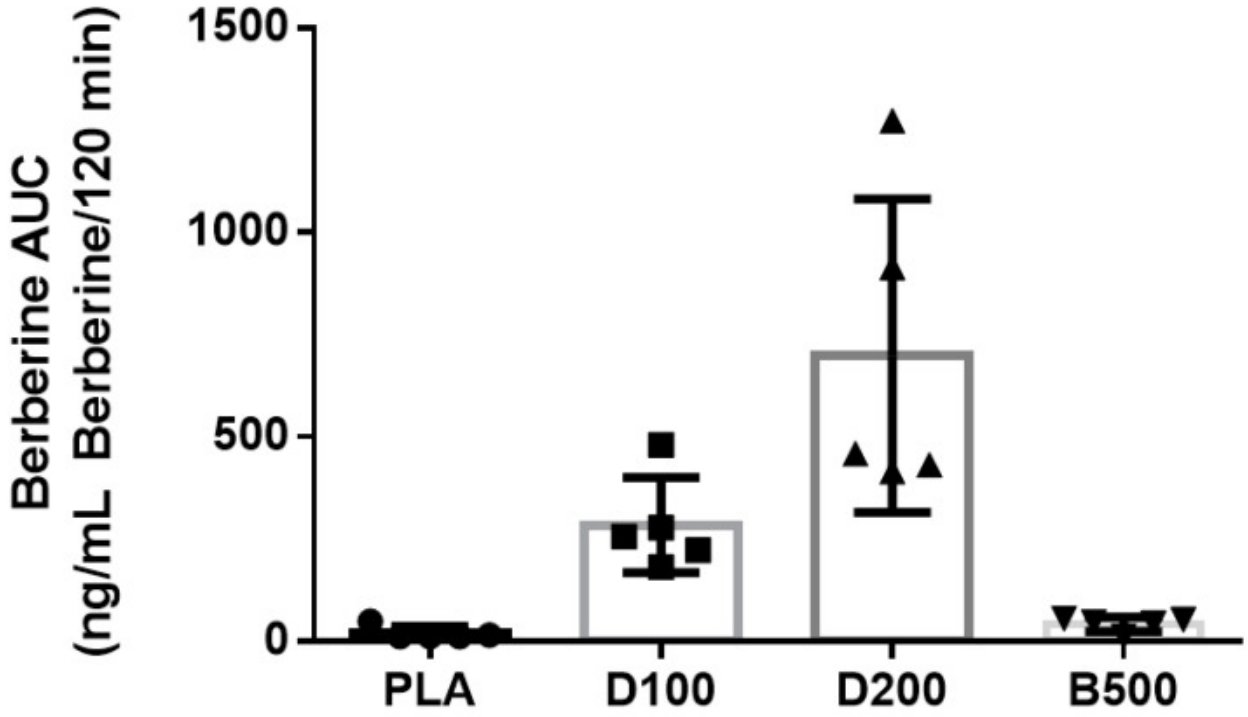
PLA = Placebo; D100 = 100 mg dose of dihydroberberine; D200 = 200 mg dose of dihydroberberine; B500 = 500 mg dose of berberine.

3.4. Berberine

Berberine data were not-normally distributed. The data were transformed using the aforementioned methods and reassessed for normality, which was unable to be achieved due to two high values in the D200 condition at the 60-min timepoint. Subsequently, all berberine data were analyzed using parametric approaches. A significant group x time interaction was revealed for berberine ($p = 0.05$). Simple main effects tests revealed no changes over time within each condition ($p > 0.05$), but significant changes between conditions at the following time points: baseline ($p = 0.006$), 20-min ($p < 0.001$), and 40-min ($p = 0.039$). At baseline, both D100 (2.60 ± 1.06 ng/mL) and D200 (4.54 ± 2.24 ng/mL) conditions had higher berberine values compared to placebo (0.14 ± 0.09 ng/mL; D100, $p = 0.007$; D200, $p = 0.011$) and B500 (0.30 ± 0.12 ng/mL; D100, $p = 0.006$; D200, $p = 0.013$). At the 20-min time point, both D100 (3.00 ± 1.85 ng/mL) and D200 (6.06 ± 2.50 ng/mL) conditions had higher berberine values compared to placebo (0.18 ± 0.18 ng/mL; D100, $p = 0.030$; D200, $p = 0.005$) and B500 (0.32 ± 0.20 ng/mL; D100, $p = 0.008$; D200, $p = 0.029$), but D200 had higher berberine values compared to D100 ($p = 0.050$). Lastly, at 40 min both D100 (2.32 ± 1.27 ng/mL) and D200 (6.90 ± 4.94 ng/mL) conditions had higher berberine values compared to placebo (0.20 ± 0.14 ng/mL; D100, $p = 0.018$; D200, $p = 0.037$) and B500 (0.36 ± 0.15 ng/mL; D100, $p = 0.022$; D200, $p = 0.040$), but D200 had higher berberine values compared to D100 ($p = 0.050$). There was a significant main effect for condition ($p = 0.04$), but no significant main effect for time ($p = 0.18$) was observed.

Berberine AUC values (Figure 3) were significantly difference between conditions ($p = 0.045$). Follow-up analysis indicated that higher berberine AUC values were identified in D200 (929 ± 694 ng/mL berberine/120 min) when compared to PLA (20.2 ± 16.2 ng/mL Berberine/120 min, $p = 0.042$) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min, $p = 0.045$). Significantly higher berberine AUC values were identified for D100 (284.2 ± 115.9 ng/mL Berberine/120 min) when compared to placebo (20.2 ± 16.2 ng/mL Berberine/120 min, $p = 0.007$) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min, $p = 0.007$). Berberine AUC values between D100 (284.2 ± 115.9) and D200 (929 ± 694 ng/mL Berberine/120 min) approached statistical significance ($p = 0.07$). Berberine AUC values between PLA (20.2 ± 16.2 ng/mL Berberine/120 min) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min) tended ($p = 0.096$) to be different. Berberine C_{Max} values tended to be different between conditions ($p = 0.064$). Further, differences in C_{Max} between D100 and PLA were significant $p = 0.005$) while differences between PLA and D200 ($p = 0.06$) as well as PLA and B500 ($p = 0.09$) tended to be different.

Figure 3.



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Individual and aggregated mean values for berberine AUC by condition.

Each individual symbol represents the individual AUC response for one study participant. For all graphs below, *n* = 5 participants completed each condition. PLA = Placebo; D100 = 100 mg dose of dihydroberberine; D200 = 200 mg dose of dihydroberberine; B500 = 500 mg dose of berberine. Data are presented as mean ± SD.

3.5. Glucose

There was no significant group x time interaction ($p = 0.97$) for glucose measures. However, a significant time effect ($p < 0.001$) was observed where glucose concentrations were significantly elevated at the 40-min timepoint (129.25 ± 27.43 mg/dL) compared to the baseline timepoint (91.05 ± 5.61 mg/dL, $p < 0.022$). Furthermore, both the 20-min timepoint (119.5 ± 21.27 mg/dL) and the 40-min timepoint (129.25 ± 27.43 mg/dL) had elevated glucose concentrations compared to the 90-min timepoint (85.40 ± 18.15 mg/dL; 20 min, $p = 0.045$; 40 min, $p = 0.007$), and the 120-min timepoint (69.6 ± 10.19 mg/dL; 20 min, $p = 0.044$; 40 min, $p < 0.001$). The 60-min timepoint (105.45 ± 23.55 mg/dL) had elevated glucose concentrations compared to the 120-min timepoint (69.60 ± 13.59 mg/dL, $p = 0.033$). Both AUC and C_{Max} values were calculated for all four conditions. Oneway ANOVA indicated no significant differences were observed for AUC ($p = 0.92$) and C_{Max} ($p = 0.77$) values (See Table 4 and Table 5).

Table 4.

Berberine (ng/mL), glucose (mg/dL), and insulin (μIU/mL) data.

Berberine (ng/mL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	0.14 ± 0.09	0.18 ± 0.18	0.20 ± 0.14	0.16 ± 0.13	0.16 ± 0.13	0.16 ± 0.13	Group	0.002
B500	0.30 ± 0.12	0.32 ± 0.20	0.36 ± 0.15	0.38 ± 0.16	0.39 ± 0.16	0.32 ± 0.13	Time	0.18
D100	2.6 ± 1.1 †,‡	3.0 ± 1.9 †,‡	2.3 ± 1.3 †,‡	2.5 ± 1.0	2.1 ± 0.8	1.9 ± 0.9	G x T	0.050
D200	4.5 ± 2.2 †,‡	6.1 ± 2.5 †,‡,*	6.9 ± 4.9 †,‡,*	11.4 ± 10.7	7.8 ± 6.5	7.2 ± 6.5		
Glucose (mg/dL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	91.8 ± 7.6	120.4 ± 25.4	129.6 ± 33.0	102.8 ± 27.6	92.0 ± 23.9	63.4 ± 9.3	Group	0.92
B500	89.8 ± 1.8	116.4 ± 30.1	125.6 ± 35.7	101.8 ± 30.9	84.0 ± 25.1	73.0 ± 13.7	Time	<0.001
D100	92.0 ± 8.5	121.8 ± 20.9	126.8 ± 24.5	106.4 ± 14.9	81.2 ± 10.1	69.4 ± 7.9	G x T	0.97
D200	90.6 ± 3.4	119.2 ± 11.8	135.0 ± 23.3	110.8 ± 25.2	84.4 ± 13.4	72.6 ± 21.6		
Insulin (μIU/mL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	15.4 ± 5.4	47.9 ± 31.3	106.9 ± 26.8	85.0 ± 21.0	78.5 ± 23.5	35.4 ± 19.2	Group	0.43
B500	18.4 ± 6.3	38.4 ± 26.9	87.1 ± 24.5	78.1 ± 23.0	58.0 ± 23.5	58.0 ± 23.5	Time	<0.001

Berberine (ng/mL)	0 min	20 min	40 min	60 min	90 min	120 min		*p*
D100	16.7 ± 9.1	47.2 ± 17.6	93.0 ± 35.2	84.5 ± 33.2	60.9 ± 23.1	34.6 ± 14.8	G x T	0.10
D200	14.8 ± 4.4	69.6 ± 28.6	85.0 ± 9.0	83.9 ± 27.4	78.9 ± 12.6	53.3 ± 29.6		

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† = Different than placebo ($p < 0.05$); ‡ = Different than B500 ($p < 0.05$); * = Different than D100 ($p < 0.05$).

Table 5.

Area under the curve (AUC) and concentration max (C_{Max}) for berberine, insulin, and glucose.

Berberine	AUC(ng/mL × 120 min)	C_{Max}(ng/mL)
PLA	20.2 ± 16.2	0.22 ± 0.18
B500	42.3 ± 17.6	0.40 ± 0.17
D100	284.2 ± 115.9	3.8 ± 1.4
D200	929 ± 694	12.0 ± 10.1
p	0.045	0.06
Insulin	**AUC (μIU/mL × 120 min)**	**C_{Max} (μIU/mL)**
PLA	8260 ± 420	110.6 ± 24.3
B500	7429 ± 1519	99.1 ± 6.6
D100	8502 ± 717	107.2 ± 21.4
D200	7242 ± 1263	98.1 ± 3.4
p	0.22	0.36

Berberine	AUC(ng/mL × 120 min)	C_{Max}(ng/mL)
Glucose	**AUC (mg/dL × 120 min)**	**C_{Max} (mg/dL)**
PLA	12,199 ± 1602	146.2 ± 13.9
B500	12,029 ± 194	139.4 ± 32.8
D100	12,381 ± 1128	140.8 ± 8.8
D200	11,898 ± 2058	139.0 ± 16.6
p	0.92	0.77

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3.6. Insulin

There was no significant group x time interaction ($p = 0.095$) for insulin measures. A significant time effect ($p < 0.001$) was observed where insulin concentrations were significantly elevated at 20 min (50.8 ± 20.7 µIU/mL, $p = 0.02$), 40 min (93.0 ± 18.2 µIU/mL, $p = 0.02$, and 60 min (82.9 ± 13.6 µIU/mL, $p = 0.003$) in comparison to baseline (16.3 ± 6.1 µIU/mL). Additionally, insulin concentrations were significantly higher at 40 min (93.0 ± 18.2 µIU/mL) and 60 min (82.9 ± 13.6 µIU/mL) compared to 20 min (50.8 ± 20.7 µIU/mL; 40 min, $p = 0.002$; 60 min, $p = 0.018$) and 120 min (45.1 ± 24.5 µIU/mL; 40 min, $p = 0.002$; 60 min, $p = 0.013$). No significant condition effect ($p > 0.05$) was identified. No significant differences were observed for AUC ($p = 0.22$) and C_{Max} ($p = 0.36$) insulin values (See Table 4 and Table 5).

3.7. Whole Blood and Serum Markers

Complete blood count analysis was completed on collected whole-blood samples to assess safety and presence of any adverse responses to supplementation. As seen in Table 6, absolute lymphocytes analysis revealed a significant main effect of condition ($p = 0.011$) where D100 had elevated concentrations compared to D200 ($p = 0.032$) but were in clinically accepted norms. Absolute monocytes analysis revealed a significant group × time interaction ($p = 0.047$) and follow-up simple main effects testing revealed significant changes over time within the Placebo ($p = 0.042$) and D100 ($p = 0.029$) conditions. Specifically, at 120 min within the Placebo and D100 conditions absolute monocytes were higher than baseline albeit they were within clinically accepted norms. Furthermore, no significant changes between conditions were revealed at any time point for absolute monocytes ($p > 0.05$). All other

variables for complete blood count did not change over time, between conditions, or reveal any interactions ($p > 0.05$).

Table 6.

Complete blood count.

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)*(p)*	G x T(*p*)
White Blood Cell Count (10^3 × cells/μL)	PLA	5.6 ± 1.0	5.7 ± 1.3	0.74	0.45	0.20
	D100	5.7 ± 1.1	5.9 ± 1.3			
	D200	6.6 ± 2.4	6.2 ± 2.1			
	B500	5.8 ± 1.2	5.3 ± 1.2			
Red Blood Cell Count (10^3 × cells/μL)	PLA	4.8 ± 0.2	4.8 ± 0.1	0.10	0.54	0.30
	D100	4.7 ±0.3	4.7 ± 0.3			
	D200	4.7 ± 0.3	4.7 ± 0.2			
	B500	5.0 ± 0.3	4.8 ± 0.4			
Hemoglobin (g/dL)	PLA	14.8 ± 0.5	14.8 ± 0.7	0.15	0.47	0.20
	D100	14.3 ± 0.4	14.5 ± 0.5			
	D200	14.3 ± 0.6	14.2 ± 0.4			
	B500	15.2 ± 1.0	14.8 ± 0.8			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Hematocrit (%)	PLA	43.2 ± 0.8	43.4 ± 1.4	0.20	0.73	0.19
	D100	42.2 ± 0.7	42.6 ± 1.4			
	D200	42.1 ± 1.3	42.0 ± 0.4			
	B500	44.6 ± 2.8	43.7 ± 3.0			
Mean Corpuscular Volume	PLA	89.4 ± 3.5	90.2 ± 3.8	0.87	0.44	0.71
	D100	90.1 ±4.7	90.0 ± 4.3			
	D200	90.4 ± 3.2	90.4 ± 3.8			
	B500	90.2 ± 2.8	90.5 ± 3.7			
Mean Corpuscular Hemoglobin	PLA	30.6 ± 1.6	30.7 ± 1.5	0.86	0.88	0.81
	D100	30.6 ± 1.6	30.5 ± 1.6			
	D200	30.7 ± 1.3	30.6 ± 1.6			
	B500	30.6 ± 1.6	30.7 ± 1.4			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Mean Corpuscular Hemoglobin Content	PLA	34.2 ± 0.6	34.1 ± 0.8	0.91	0.43	0.93
	D100	34.0 ± 0.6	34.0 ± 0.5			
	D200	34.0 ± 0.8	33.8 ± 0.7			
	B500	34.0 ± 0.9	33.9 ± 0.7			
Red Cell Distribution Width (%)	PLA	12.1 ± 0.4	12.0 ± 0.5	0.93	0.08	0.55
	D100	12.1 ± 0.4	12.0 ± 0.5			
	D200	12.1 ±0.4	12.1 ± 0.4			
	B500	12.0 ± 0.4	12.0 ± 0.4			
Platelet Count	PLA	242.2 ± 32.6	239.0 ± 37.3	0.85	0.91	0.55
	D100	243.4 ± 26.7	250.2 ± 26.5			
	D200	242.8 ± 23.9	242.0 ± 19.1			
	B500	247.4 ± 36.1	245.8 ± 30.9			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Abs Neutrophils (cells/uL)	PLA	2790 ± 1318	3046 ± 1295	0.45	0.88	0.38
	D100	2902 ± 719	2962 ± 949			
	D200	3926 ± 2324	3723 ± 2020			
	B500	2743 ± 737	2675 ± 702			
Abs Lymphocytes (cells/uL)	PLA	1893 ± 435	1805 ± 297	0.01	0.31	0.28
	D100	2068 ± 719	2173 ± 253			
	D200	1852 ± 528	1721 ± 149			
	B500	2268 ± 524	1899 ± 423			
Abs Monocytes (cells/µL)	PLA	477 ± 134	549 ± 168	0.92	0.04	0.007
	D100	494 ± 159	597 ± 225			
	D200	549 ± 152	504 ± 96			
	B500	545 ± 178	530 ± 205			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Abs Eosinophils (cells/μL)	PLA	218 ± 162	214 ± 162	0.17	0.09	0.62
	D100	179 ± 116	165 ± 96			
	D200	225 ± 148	188 ± 106			
	B500	183 ± 119	179 ± 161			
Abs Basophils (cells/μL)	PLA	42.8 ± 12.4	45.6 ± 10.9	0.47	0.94	0.67
	D100	38.4 ± 7.6	43.8 ± 12.2			
	D200	49.0 ± 18.5	44.6 ± 9.3			
	B500	41.2 ± 20.7	36.8 ± 11.6			
Neutrophils (%)	PLA	52.1 ± 13.2	52.2 ± 11.7	0.24	0.42	0.29
	D100	50.7 ± 7.3	49.1 ± 6.7			
	D200	56.7 ±14.4	57.3 ± 12.0			
	B500	47.5 ± 7.9	50.4 ± 7.8			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Lymphocytes (%)	PLA	34.7 ± 9.9	33.1 ± 7.8	0.17	0.26	0.32
	D100	36.7 ± 6.2	37.4 ± 5.2			
	D200	30.0 ± 9.5	29.8 ± 7.6			
	B500	39.5 ± 7.1	36.0 ± 4.7			
Monocytes (%)	PLA	8.5 ± 2.1	10.0 ± 3.1	0.12	0.15	0.60
	D100	9.0 ± 1.1	10.0 ± 2.9			
	D200	8.9 ± 2.7	8.8 ± 2.7			
	B500	9.3 ± 1.8	9.8 ± 2.4			
Eosinophils (%)	PLA	4.0 ± 3.0	3.9 ± 3.1	0.30	0.25	0.72
	D100	3.0 ± 1.4	2.7 ± 1.3			
	D200	3.6 ± 2.8	3.3 ± 2.5			
	B500	3.0 ± 1.5	3.1 ± 2.3			
Basophils (%)	PLA	0.8 ± 0.3	0.8 ± 0.3	0.37	0.43	0.95
	D100	0.7 ± 0.1	0.8 ± 0.3			
	D200	0.8 ± 0.3	0.8 ± 0.3			
	B500	0.7 ± 0.2	0.7 ± 0.2			

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Comprehensive metabolic panels were analyzed in collected serum samples. As seen in Table 7, analysis revealed significant main effects of time for glucose ($p = 0.024$), sodium ($p < 0.001$), potassium ($p = 0.007$), and chloride ($p = 0.024$) and these changes were within clinically accepted norms. A significant group × time interaction was revealed for creatinine ($p = 0.008$), and follow-up simple main effects testing revealed significant changes over time within the D100 ($p = 0.031$) and D200 ($p = 0.030$) conditions. Within the D100 condition at 120 min, creatinine measures were higher than baseline, and within the D200 condition, baseline measures were higher than 120 min, however, these measures are within clinically accepted norms. Additionally, there were significant creatinine differences between conditions at baseline ($p = 0.039$) and 120 min ($p = 0.009$) measures. At baseline, D200 ($p = 0.030$) and B500 ($p = 0.016$) conditions had higher creatinine measures compared to D100 and these differences were within clinically accepted norms. Lastly, a significant main effect of condition was observed for creatinine measures ($p = 0.040$) where D100 was higher than placebo ($p = 0.035$) but was within clinically accepted norms.

Table 7.

Comprehensive metabolic panel.

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Glucose (mg/dL)	PLA	90.6 ± 5.3	65.6 ± 11.0	0.66	0.02	0.17
	D100	92.0 ± 5.7	68.8 ± 10.0			
	D200	81.2 ± 13.6	73.2 ± 20.1			
	B500	89.2 ± 4.2	73.8 ± 15.4			
Blood Urea Nitrogen (g/dL)	PLA	16.2 ± 2.3	15.6 ± 2.3	0.14	0.11	0.46
	D100	13.8 ± 1.5	13.8 ± 1.3			
	D200	15.4 ± 1.9	14.8 ± 2.2			
	B500	15.8 ± 2.2	15.2 ± 2.5			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Creatinine (mg/dL)	PLA	1.0 ± 0.1	1.0 ± 0.1	0.04	0.55	0.008
	D100	0.9 ± 0.1	1.0 ± 0.1			
	D200	1.0 ± 0.1	0.9 ± 0.1			
	B500	1.0 ± 0.1	0.9 ± 0.1			
Glomerular Filtrate Rate	PLA	107.2 ± 12.0	102.2 ± 12.8	0.03	0.30	0.02
	D100	115.6 ± 8.4	109.4 ± 8.8			
	D200	106.2 ± 13.0	111.0 ± 11.9			
	B500	106.6 ± 13.3	109.2 ±12.8			
Sodium (mM)	PLA	137 ± 1	139 ± 2	0.20	<0.001	0.82
	D100	136 ± 2	139 ± 2			
	D200	135 ± 2	137 ± 2			
	B500	136 ± 2	138 ± 3			
Potassium (mM)	PLA	4.3 ± 0.2	4.0 ± 0.1	0.36	0.007	0.90
	D100	4.2 ± 0.2	3.9 ± 0.2			
	D200	4.3 ± 0.2	4.0 ± 0.2			
	B500	4.2 ± 0.3	3.9 ± 0.2			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Chloride (mM)	PLA	102 ± 2	102 ± 1	0.45	0.02	0.18
	D100	103 ± 2	103 ± 2			
	D200	101 ± 2	103 ± 2			
	B500	101 ± 3	103 ± 2			
Carbon Dioxide (mM)	PLA	25 ± 2	26 ± 3	0.62	0.16	0.78
	D100	25 ± 1	26 ± 1			
	D200	26 ± 1	27 ± 2			
	B500	25 ± 1	26 ± 3			
Calcium (mg/dL)	PLA	9.3 ± 0.4	9.7 ± 0.2	0.26	0.12	0.049
	D100	9.4 ± 0.2	9.5 ± 0.3			
	D200	9.3 ± 0.5	9.4 ± 0.3			
	B500	9.5 ± 0.3	9.5 ± 0.3			
Total Protein (g/dL)	PLA	6.8 ± 0.4	6.9 ± 0.3	0.94	0.74	0.22
	D100	6.8 ± 0.3	6.9 ± 0.3			
	D200	6.8 ± 0.6	6.8 ± 0.4			
	B500	7.0 ± 0.5	6.8 ± 0.5			
Albumin (g/dL)	PLA	4.6 ± 0.2	4.5 ± 0.1	0.42	0.38	0.80
	D100	4.5 ± 0.1	4.4 ± 0.2			
	D200	4.5 ± 0.2	4.4 ± 0.0			
	B500	4.6 ± 0.1	4.5 ± 0.1			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Globulin (g/dL)	PLA	2.2 ± 0.3	2.3 ± 0.3	0.62	0.26	0.17
	D100	2.3 ± 0.3	2.4 ± 0.3			
	D200	2.4 ± 0.4	2.4 ± 0.3			
	B500	2.4 ± 0.4	2.3 ± 0.5			
Alb/Glob Ratio	PLA	2.1 ± 0.2	2.0 ± 0.2	0.37	0.11	0.28
	D100	2.0 ± 0.3	1.8 ± 0.3			
	D200	1.9 ± 0.3	1.9 ± 0.3			
	B500	1.9 ± 0.3	2.0 ± 0.4			
Total Bilirubin (mg/dL)	PLA	0.9 ± 0.3	0.8 ± 0.3	0.52	0.14	0.37
	D100	0.8 ± 0.3	0.8 ± 0.3			
	D200	0.9 ± 0.4	0.8 ± 0.3			
	B500	1.0 ± 0.4	0.9 ± 0.4			
Alkaline Phosphatase (U/L)	PLA	53 ± 10	51 ± 10	0.13	0.16	0.45
	D100	51 ± 11	52 ± 10			
	D200	51 ± 13	50 ± 10			
	B500	54 ± 12	53 ± 13			
AST (U/L)	PLA	22 ± 5	22 ± 5	0.22	0.18	0.55
	D100	24 ± 7	24 ± 7			
	D200	24 ± 6	23 ± 5			
	B500	20 ± 3	20 ± 3			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
ALT (U/L)	PLA	19 ± 6	19 ± 5	0.93	1.00	0.60
	D100	19 ± 7	20 ± 7			
	D200	19 ± 4	18 ± 3			
	B500	18 ± 6	18 ± 5			

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A significant group x time interaction was revealed for globular filtration rate ($p = 0.016$) and follow-up simple main effects testing revealed significant changes over time within the D100 ($p = 0.047$) and D200 ($p = 0.037$) conditions. Within the D100 condition, baseline globular filtration rate measures were higher than 120 min, and within the D200 condition 120 min measures were higher than baseline, however, these measures are within clinically accepted norms. Additionally, follow-up simple main effects testing from the interaction revealed significant differences between conditions for globular filtration rate measures at baseline ($p = 0.050$) and 120 min ($p = 0.005$). At baseline, globular filtration rate measures were higher in D100 compared to D200 ($p = 0.048$) and B500 ($p = 0.038$), which were within clinically accepted norms. At 120 min, globular filtration rate measures were higher in D200 ($p = 0.024$), D100 ($p = 0.024$), and B500 ($p = 0.047$) compared to placebo and were within clinically accepted norms. Lastly, a significant main effect of condition was observed for globular filtration rate measures ($p = 0.033$) where D100 was higher than placebo ($p = 0.028$) but was within clinically accepted norms.

A significant group x time interaction was revealed for calcium ($p = 0.049$) and follow-up simple main effects testing revealed significant changes over time within the placebo condition ($p = 0.009$), the levels of which were higher at 120 min compared to baseline, but within clinically accepted norms. Furthermore, a follow-up simple main effects revealed significant differences between conditions for calcium measures at 120 min ($p = 0.030$) where placebo was higher than D200 ($p = 0.040$) and all measures were within clinically accepted norms. All other variables for the comprehensive metabolic panel did not change over time, between conditions, or reveal any interactions ($p > 0.05$).

4. Discussion

The intent of this project was to evaluate the observed differences in plasma berberine concentrations after oral ingestion of two doses (100 mg [D100] and 200 mg [D200],

respectively) of dihydroberberine and one dose (500 mg) of berberine (B500). Findings from this pilot approach revealed that area under the curve calculations for berberine were significantly greater when either dose of dihydroberberine was ingested in comparison to the values observed for berberine and placebo (PLA). In addition, peak berberine concentrations (C_{Max}) tended to be different between all conditions. Specifically, the 100 mg dose of dihydroberberine was significantly greater than peak values observed in PLA and B500 while peak concentrations for the 200 mg dose of dihydroberberine tended to be different than what was observed in the berberine group.

Of the available human research that has explored efficacy and safety of oral berberine ingestion, the majority of these studies have employed a dosing regimen that consists of two to three daily doses with meals (to reduce incidence of gastrointestinal complications) at a dosage of 500 mg per dose. For example, Perez-Rubio et al. [7] had 24 metabolic syndrome patients randomly ingest, in a double-blind, placebo-controlled fashion, three daily doses of 500 mg/dose (total daily dosage of 1500 mg/day) for three months. Similarly, Yin et al. [6] also randomly assigned type 2 diabetes patients to ingest three daily 500 mg doses for a total of 13 weeks. Finally, Zhang et al. [17] randomly assigned 116 individuals diagnosed with type 2 diabetes to consume two 500 mg daily doses (1000 mg total) over a 3-month period. While results of these studies outline efficacy outcomes in various clinical populations, very limited human data are available that has outlined the absorption pattern and plasma kinetics after oral berberine ingestion. In this respect, it has been previously reported that oral bioavailability of berberine is extremely low (<1%) in both animal [9,10] and human models largely due to poor intestinal absorption and high levels of first-pass removal in the intestines and liver [5]. This has led to the identification of various strategies to increase bioavailability, including administration of dihydroberberine. Currently, no human research has appeared in the peer-reviewed literature that has examined oral ingestion of dihydroberberine. Towards this end, Buchanan et al. [16] compared the pharmacokinetics associated with transdermal administration of berberine and dihydroberberine in Sprague-Dawley rats. Using this model and over an 8-h period, transdermal application of dihydroberberine resulted in greater AUC values than what was observed for both transdermal and oral administration of berberine. Moreover, transdermal delivery of berberine resulted in greater AUC values than what was observed for oral administration, further highlighting the poor bioavailability of oral berberine ingestion.

Other highlights from the present study include the statistically significant higher outcomes at baseline (after three doses, but prior to the 4th dose in the present study) in D100 and D200 when compared to PLA and B500. When viewed in comparison to PLA, this outcome was not surprising and some might deem it expected considering that dihydroberberine was administered, however, the finding of greater levels in comparison to berberine seemingly

speaks to the ability of dihydroberberine to maintain berberine levels in the blood better than oral berberine ingestion. Whether or not these increases in plasma berberine translate beyond one day or measurement or improvements in efficacy outcomes commonly associated with berberine supplementation cannot be determined from this project. In this respect, more research is certainly needed with dihydroberberine to determine how well the greater plasma berberine levels improve dyslipidemia and glucose and insulin homeostasis as commonly reported in the literature.

A key limitation with the present study is the sample size on this project. While the fully randomized, crossover, double-blind, and placebo-controlled approach was a sound approach for this pilot assessment, it is readily acknowledged by the authors that more study participants are needed on future studies to continue to establish the safety and efficacy of berberine and dihydroberberine. Further to the point and beyond just having a low sample size, one needs to closely consider our plasma berberine data as two participants in the D200 at the 60-min time point exhibited high values, which nearly achieved traditional definitions of being a statistical outlier (>±3 of standardized residual estimates). When combined with the low sample size, these data exerted substantial impact on the distribution of these data. As such, traditional approaches to transforming the data were unsuccessful at achieving normality and all data were subsequently analyzed using a parametric approach. Furthermore, all analysis was also completed using an intent-to-treat approach with those values removed and the high values replaced with the previous data point and no meaningful differences were noted in the outcomes of the project. Another limitation might be considered to be the small number of doses ingested prior to assessing the absorption kinetics. This was an a priori consideration as our primary focus for this pilot project was to assess and compare the absorption kinetics of two different doses of oral dihydroberberine in comparison to oral ingestion of berberine and placebo.

5. Conclusions

In conclusion, results from this study represent what we believe are the first data in humans to evaluate the absorption kinetics of berberine and two doses of dihydroberberine. Results from this study demonstrate that dihydroberberine, irrespective of what dose is delivered, achieves greater area under the curve as well as peak berberine concentrations when compared to oral ingestion of berberine or placebo. Since berberine use has traditionally spanned several months to establish the observed efficacy outcomes known for berberine (improved glucose and insulin homeostasis and lipid parameters), several follow-up approaches are recommended. First, can the increased levels of berberine seen from dihydroberberine ingestion usher in faster improvements in outcomes of interest. In a similar vein, are the

outcomes improved as a result of dihydroberberine ingestion in comparison to berberine ingestion.

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Author Contributions

Designed study: C.M.K.; data collection: J.M.M., A.M.H., K.M.R. and R.A.S.; analyzed data: P.W.M.; prepared initial draft: C.M.K., A.M.H., J.M.M. and P.W.M.; approved final version: all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Lindenwood University: IRB-20-173, approval date: 22 June 2020.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data and/or statistical analyses are available upon request on a case-by-case basis for non-commercial scientific inquiry and/or educational use.

Conflicts of Interest

C.M.K. serves as a paid advisor for NNB Nutrition. A conflict-of-interest management plan was filed and approved by the Lindenwood University IRB prior to commencing any data collection. As part of this plan, C.M.K. was not involved in any part of the consenting,

randomization, or data collection process. All statistical analysis performed by C.M.K. was done while data was still blinded and all analysis and interpretation was confirmed by P.W.M. As such, P.W.M. and C.M.K. prepared all statistical analysis and interpretation. All other authors declare that they have no competing interests.

Footnotes

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Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Data Availability Statement

Data and/or statistical analyses are available upon request on a case-by-case basis for non-commercial scientific inquiry and/or educational use.

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Absorption Kinetics of Berberine and Dihydroberberine and Their Impact on Glycemia: A Randomized, Controlled, Crossover Pilot Trial

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Abstract

Berberine is a natural alkaloid used to improve glycemia but displays poor bioavailability and increased rates of gastrointestinal distress at higher doses. Recently, dihydroberberine has been developed to combat these challenges. This study was designed to determine the rate and extent to which berberine appeared in human plasma after oral ingestion of a 500 mg dose of berberine (B500) or 100 mg and 200 mg doses of dihydroberberine (D100 and D200). In a randomized, double-blind, crossover fashion, five males (26 ± 2.6 years; 184.2 ± 11.6 cm; 91.8 ± 10.1 kg; 17.1 ± 3.5% fat) completed a four-dose supplementation protocol of placebo (PLA), B500, D100, and D200. The day prior to their scheduled visit, participants ingested three separate doses with breakfast, lunch, and dinner. Participants fasted overnight (8–10 h) and consumed their fourth dose with a standardized test meal (30 g glucose solution, 3 slices white bread) after arrival. Venous blood samples were collected 0, 20, 40, 60, 90, and 120 minutes (min) after ingestion and analyzed for BBR, glucose, and insulin. Peak concentration (C_{Max}) and area under the curve (AUC) were calculated for all variables. Baseline berberine levels were different between groups ($p = 0.006$), with pairwise comparisons indicating that baseline levels of PLA and B500 were different than D100. Berberine C_{Max} tended to be different ($p = 0.06$) between all conditions. Specifically, the observed C_{Max} for D100 (3.76 ± 1.4 ng/mL) was different than PLA (0.22 ± 0.18 ng/mL, $p = 0.005$) and B500 (0.4 ± 0.17 ng/mL, $p = 0.005$). C_{Max} for D200 (12.0 ± 10.1 ng/mL) tended ($p = 0.06$) to be different than B500. No difference in C_{Max} was found between D100 and D200 ($p = 0.11$). Significant differences in berberine AUC were found between D100 (284.4 ± 115.9 ng/mL × 120 min) and PLA (20.2 ± 16.2 ng/mL × 120 min, $p = 0.007$) and between D100 and B500 (42.3 ± 17.6 ng/mL × 120 min, $p = 0.04$). Significant differences in D100 BBR AUC (284.4 ± 115.9 ng/mL×120 min) were found between PLA (20.2 ± 16.2 ng/mL × 120 min, $p = 0.042$) and B500 (42.3 ± 17.6 ng/mL × 120 min, $p = 0.045$). Berberine AUC values between D100 and D200 tended ($p = 0.073$) to be different. No significant differences in the levels of glucose ($p = 0.97$) and insulin ($p = 0.24$) were observed across the study protocol. These results provide preliminary evidence that four doses of a 100 mg dose of dihydroberberine and 200 mg dose

of dihydroberberine produce significantly greater concentrations of plasma berberine across of two-hour measurement window when compared to a 500 mg dose of berberine or a placebo. The lack of observed changes in glucose and insulin were likely due to the short duration of supplementation and insulin responsive nature of study participants. Follow-up efficacy studies on glucose and insulin changes should be completed to assess the impact of berberine and dihydroberberine supplementation in overweight, glucose intolerant populations.

Keywords: absorption, safety, plants, glucose, insulin

1. Introduction

Berberine is an isoquinoline derivative alkaloid present in various parts (root, stem, fruit, bark) of multiple plants including, in particular, species found in the Coptis, Hydrastis, and Berberis genus [1]. Various animal and culture models have reported on the ability of berberine to exert lipid and glucose-lowering effects in addition to helping with weight loss and improving glucose tolerance [2,3]. For this reason, interest in berberine has suggested it may be beneficial in treating diabetes and obesity. While still being fully characterized, mechanistic actions of berberine consist of it reducing blood lipid levels via action on hepatocyte nuclear factor 1 alpha (HNF-1 alpha) and hepatic LDL receptors, intestinal absorption of cholesterol [4], and glucose levels while increasing insulin sensitivity.

Moreover, berberine is a known activator of AMP Kinase [1,5], which contributes to increases in fatty acid oxidation and a reduction of lipogenic gene expression.

Yin and colleagues [6] published results from two separate studies that both examined the impact of berberine at improving various biomarkers associated with glucose homeostasis. Throughout the first study, berberine (500 mg/dose, 3 doses/day) or metformin was administered over a 3-month period to 36 adults who were recently diagnosed with type 2 diabetes. Levels of hemoglobin A1C, fasting blood glucose, postprandial blood glucose, and triglycerides were all significantly improved in comparison to pre-treatment, and, importantly, the magnitude of changes was similar to what was observed with metformin. An additional investigation was reported where berberine was added to the established treatment plan 48 type 2 diabetes patients. Over a 13-week period, blood glucose (both fasting and postprandial), hemoglobin A1C, fasting, insulin, and HOMA-IR were all improved when berberine was added. These results align with the Perez-Rubio et al. [7] investigation that reported improvements in waist circumference, blood pressure, triglycerides, and area under the curve values for both glucose and insulin (after a 75-g glucose load over a two-hour period) in 24 patients with metabolic syndrome. In this study, berberine was randomly assigned in a double-blind, placebo-controlled fashion at a daily dosage of 1500 mg/day (500

mg/dose, 3 doses/day with meals) for three months. Similar outcomes using berberine have been reported by Rao et al. [8] after completing a randomized, open-label study on 41 diabetic patients where various indicators of glucose and insulin metabolism were improved over the three-month study period.

One challenge associated with oral ingestion of berberine has been its low bioavailability (<1%) reported in both animal [9,10] and human models largely due to poor intestinal absorption and high levels of first-pass removal in the intestines and liver [5]. To overcome this limitation, higher doses of berberine are commonly administered, which has led to a consistent reporting of gastrointestinal adverse events. For example, Yin et al. [6] reported that gastrointestinal adverse events were 34.5% during a 13-week study which provided berberine hydrochloride as a monotherapy or in combination with other treatments. Interestingly all adverse events were reported when berberine was combined with other treatments (metformin, insulin, etc.) and all incidents occurred during the first four weeks of treatment.

For this reason, alternative approaches have been explored to help improve its poor bioavailability. For example, techniques such as encapsulation [11,12], packaging with other nutrients and compounds [13], and spray drying [14] have all been reported on in the literature. In addition, the reduced derivative of berberine, dihydroberberine, has been suggested as a natural alternative to improve bioavailability [15]. Buchanan and investigators [16] examined the ability of transdermal application of dihydroberberine in 40 Sprague-Dawley rats to improve bioavailability, improve glucose homeostasis parameters, and reduce adverse events in comparison to oral berberine gavage and transdermal applications of berberine. Results from both acute and chronic (14 days) pharmacokinetic approaches indicated that transdermal dihydroberberine achieved higher bioavailability than both transdermal and oral berberine administration. Oral administration of dihydroberberine has yet to be reported in human literature, thus initial studies should provide some level of insight into its impact on absorption kinetics, preliminary safety indicators, and adverse events associated with its ingestion. Consequently, the purpose of this study was to conduct a randomized, double-blind, placebo-controlled investigation to examine the dose-dependent impact on absorption kinetics, clinical safety parameters, and adverse events of oral dihydroberberine ingestion in humans.

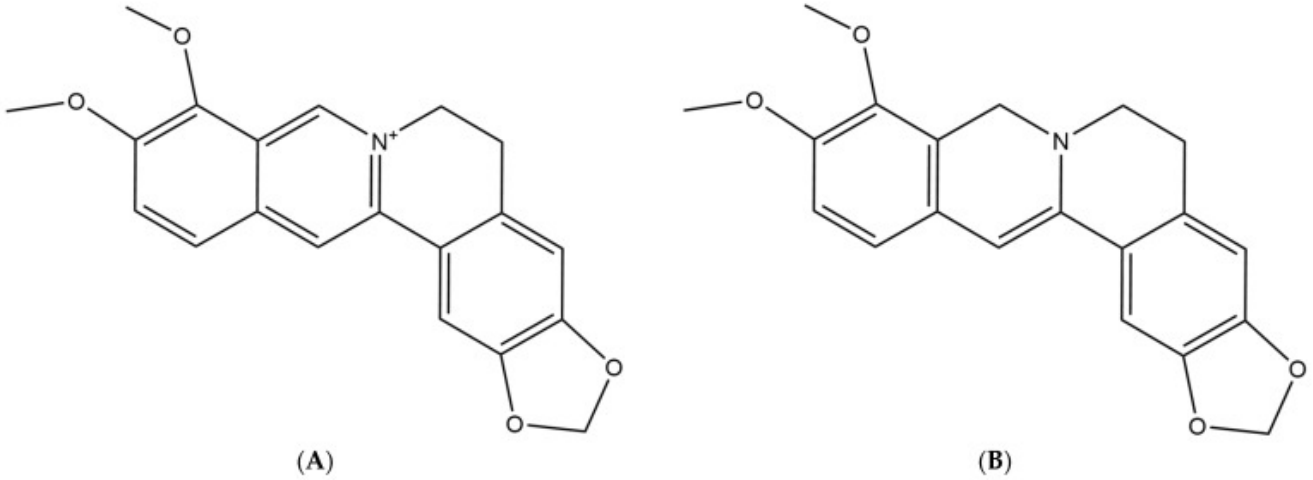
2. Methods

2.1. Overview of Research Design

This study was completed using a randomized, double-blind, crossover study design. Prior to beginning, all participants signed an IRB-approved informed consent document (Lindenwood

University: IRB-20-173, approval date: 22 June 2020) and completed a healthy history questionnaire to determine study eligibility. A power analysis was not computed as this study was considered a proof of concept, pilot investigation. This study protocol and design was retrospectively registered on Clinicaltrials.gov on 25 August 2021 as NCT05021341 (https://clinicaltrials.gov/ct2/show/NCT05021341 accessed on 24 December 2021). All four supplementation conditions were completed by each participant in a crossover fashion with a minimum washout of 72 h. For each supplementation condition, four total doses were consumed with three doses being consumed the day before testing (with breakfast, lunch, and dinner) and the fourth dose being consumed the morning of testing with a standardized test meal after observing an overnight (8 to 10 h) fast (Figure 1). Participants were scheduled to begin each visit at approximately the same times and reported to the laboratory between 600 and 1000 h. Using a randomized, double-blind, placebo-controlled fashion, study participants consumed either a placebo (resistance dextrin) (PLA), 500 mg of berberine (B500), 100 mg dihydroberberine (D100), or 200 mg dihydroberberine (D200). To minimize any order effects from testing, participants were randomized using an online randomization software program (www.random.org accessed on 1 July 2020). Upon arrival for each study visit, participants had their height, body mass, body composition, and hemodynamics (resting heart rate and blood pressure) measured along with an assessment of capillary glucose levels approximately 30 min prior to supplementation ingestion to evaluate fasting status. An indwelling catheter was then implanted into a forearm vein and flushed with saline to remain patent. The fourth and final dose of their assigned supplement was then consumed with 240 milliliters of cold tap water. After supplement ingestion, participants consumed a standardized test meal consisting of a 30-g glucose solution and four slices of white bread. Immediately after ingestion of the test meal, venous blood samples were collected 20, 40, 60, 90, and 120 min (Figure 2). Participants were provided 200 mL of cold water to ingest after each blood collection. Upon processing, all blood samples were stored at −80 °C. Throughout each visit, study participants were asked to report the occurrence of any adverse events (dizziness, headache, nausea, upset stomach, cramping, diarrhea, etc.) that occur throughout completion of the study protocol. All subsequent study visits were completed in an identical fashion and scheduled with at least 72 h between visits.

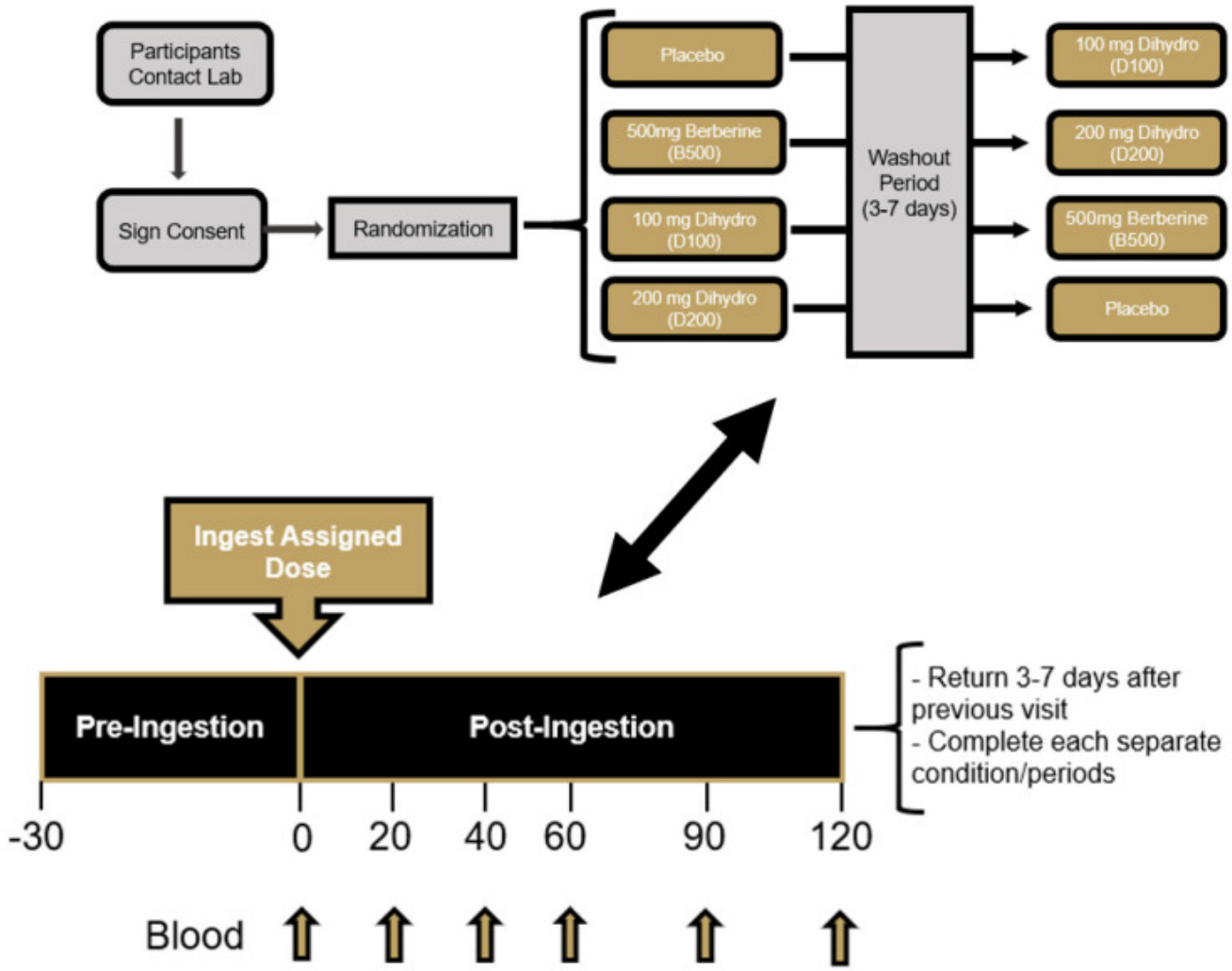
Figure 1.



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(**A**) Chemical structure of berberine. (**B**) Chemical structure of dihydroberberine.

Figure 2.



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Overview of research design.

2.2. Study Participants

Prior to participation, all recruited individuals provided signed informed consent using an IRB-approved consent form (Lindenwood University: IRB-20-173, approval date: 22 June 2020). Five healthy males (26.0 ± 2.6 years, 184.2 ± 11.6 cm, 91.8 ± 10.1 kg, 17.1 ± 3.5% fat) successfully completed all study visits (see Table 1 for full participant characteristics). Inclusion criteria included age (18–45 years), healthy and free of disease (as reported by the health screening questionnaire), and physically active (reported at least 30 min of moderate exercise three days a week). Any individual diagnosed with or being treated for cardiac, respiratory, circulatory, musculoskeletal, metabolic, obesity (defined as body mass index > 30 kg/m^2 and body fat greater than 30%), immune, autoimmune, psychiatric, hematological, neurological, or endocrinological disorder or disease were not allowed to participate in the

current study. Any participant taking any dietary supplement besides a multi-vitamin/mineral or a protein supplement were required to abstain from consuming any further doses for 30 days prior to beginning the study protocol.

Table 1.

Baseline age, gender, height (cm), weight (kg), body mass index, % fat, heart rate, systolic blood pressure, diastolic blood press, energy, carbohydrates, proteins, and fat intake.

	Mean	SD	Minimum	Maximum
Age	26.0	2.6	23	30
Height (cm)	184.2	11.6	171	202
Weight (kg)	91.8	10.1	82.4	108.5
Body mass index (kg/m^2)	27.1	3.9	22.6	33.1
% fat	17.1	3.5	12.5	20.9
Heart rate (beats/minute)	61.0	12.0	41	70
Systolic blood pressure (mm Hg)	122.3	11.4	111	138
Diastolic blood pressure (mm Hg)	72.4	8.8	64	70
Energy intake (kcals/day)	2433	810	1503	3695
Carbohydrate intake (grams/day)	217	62	144	312
Protein intake (grams/day)	121	34	66	153
Fat intake (grams/day)	112	45	75	186

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2.3. Supplementation Protocol

Using a randomized, double-blind, placebo-controlled fashion, study participants consumed either a placebo (resistance dextrin) (PLA), 500 mg of berberine (B500), 100 mg dihydroberberine (D100), or 200 mg dihydroberberine (D200). The dihydroberberine was manufactured by NNB Nutrition (Nanjing, China) and is marketed as Glucovantage®.

Berberine (≥99%) was extracted from *Berberis Aristata* in the hydrochloride form and was used as raw material. Dihydroberberine was obtained by a patented preparation method (CN108997332A). Neither molecule contains any crystalline water. Study participants supplemented consumed four total doses prior to having the absorption kinetics determined. This regimen started the morning before their scheduled visit with participants consuming one dose each with breakfast, lunch, and dinner. In addition to consuming with a meal, all doses were consumed with at least eight fluid ounces of cold tap water. Participants were then required to follow an overnight fast (approximately 8 to 10 h). Upon arrival, capillary glucose levels were determined from a finger-stick blood sample and a handheld glucose monitor (CVS Health Advanced Glucose Monitor, Agamatrix, Inc., Salem, NH, USA). After fasting status was confirmed, the fourth and final dose of their assigned supplement was consumed in front of a study team member with approximately eight fluid ounces of cold tap water. A catheter was then inserted into a forearm vein and approximately 30 min after consuming their final dose a standardized test meal was ingested which consisted of four slices of white bread (approximately 264 kcals, 3.2 g of fat, 50.6 g of carbohydrates and 7.6 g of protein) and 30 g of glucose powder that was mixed with approximately 8–12 fluid ounces of water. The test meal was ingested within five minutes. The ingestion of the test meal was considered to be the 0-min time point and all subsequent measurements occurred as outlined.

All participants were required to complete a supplementation log to document when each dose was consumed. No doses were reported being missed by any participant throughout the study protocol. All study materials were prepared into gelatin capsules of identical size, color, shape, and transparency. All doses were prepared into a single capsule which required each participant to consume a single capsule for each required dose. A resistant dextrin was used as the placebo. All doses were placed into individually labeled bags for each condition for each participant throughout the entire study protocol.

2.4. Procedures

2.4.1. Baseline Demographics and Hemodynamics

During the initial visit, after providing written consent, participants were instructed to rest quietly for approximately 10 min before measuring resting heart rate and blood pressure. Resting heart rate was measured by palpating the radial artery for a period of 60 s. While still resting, blood pressure (Omron BP785, Omron Corporation, Kyoto, Japan) measurements were taken before participants resumed normal activity. Participants then had their body mass determined (Tanita BWB-627A, Tokyo, Japan) and recorded to the nearest ±0.1 kg upon arrival prior to each study visit. All recorded body masses were compared to ensure the participant was weight stable. Any participant whose body mass changed by more than 2% between consecutive study visits was excluded from participation. Following body mass

measurements, height was measured using a standard wall-mounted stadiometer (Tanita, HR-200, Tokyo, Japan) and recorded to the nearest ±0.5 cm (cm). Fat and fat-free mass was determined using a bioelectrical impedance analyzer (InBody 570, Beverly Hills, CA, USA). Participants were required to observe an overnight fast to ensure an accurate determination of body composition. Body composition analysis occurred between 600 and 1000 h by trained research personnel. All assessments were completed according to device specifications.

2.4.2. Dietary Monitoring

Prior to their baseline visit, study participants completed a hand-written four-day food record (three weekdays and one weekend day). The four-day food log was provided to allow participants to replicate their diets. In addition to the four-day food log, participants were instructed on how to complete the ASA24 online dietary assessment tool (https://epi.grants.cancer.gov/asa24/ accessed on 23 December 2021), for determination of baseline caloric and macronutrient intake. From this information, study participants were asked to replicate their dietary intake prior to each subsequent testing visit. Study participants reported 100% compliance to this protocol.

2.4.3. Venous Blood Collection and Processing

To confirm a fasting state prior to catheter insertion and supplement administration, capillary blood was collected from a finger on their non-dominant hand. The collected blood was analyzed using a hand-held glucose analyzer (CVS Health Advanced Glucose Monitor, Agamatrix, Inc., Salem, NH, USA). Fasting was confirmed if glucose was <110 mg/dL. Within each supplementation condition, study participants had their venous blood collected on six different occasions: 0, 20, 40, 60, 90, and 120 min after ingestion of their assigned study agent. Blood was collected via a forearm vein using standard phlebotomy techniques into serum and ethylenediaminetetraacetic acid EDTA-coated Vacutainer™ tubes. To establish safety, whole blood samples were collected into EDTA and serum tubes for assessment of complete blood count and comprehensive metabolic count, respectively. Blood samples collected into EDTA were gently inverted ten times while blood samples collected into serum tubes were gently inverted ten times and allowed to clot at room temperature. For same day analysis, these samples were placed into a chilled container and sent to a commercial diagnostic laboratory (Quest Diagnostics). Plasma samples used for berberine determination were collected into EDTA tubes, gently inverted ten times and centrifuged at 3000 revolutions per minute (rpm) (MegaFuge XFR, Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C for 20 min. After centrifugation, 400 µL aliquots of plasma were removed and frozen at −80 °C within four hours of collection.

2.4.4. Complete Blood Count

To assess adverse events reported in response to the assigned supplementation, whole blood samples were collected in EDTA tubes and analyzed at a commercial diagnostic laboratory (Quest Diagnostics) for changes in red blood cell count, white blood cell count, platelet count, hemoglobin, hematocrit, red blood cell dimension width (RDW), mean corpuscle volume (MCV), mean corpuscle hemoglobin (MCH), mean corpuscle hemoglobin content (MCHC), neutrophil % and cell count, lymphocytes % and cell count, monocytes % and cell count, eosinophils % and cell count, and basophils % and cell count (granulocytes → neutrophils, eosinophils, basophils). Complete blood counts were only assessed at the 0 and 120-min time points.

2.4.5. Comprehensive Metabolic Panel

To assess adverse events in response to the assigned supplementation, serum samples were assayed at a commercial diagnostic laboratory (Quest Diagnostics) for a comprehensive metabolic panel (albumin, albumin/globulin ratio (calculated), alkaline phosphatase, ALT, AST, BUN/creatinine ratio (calculated), calcium, carbon dioxide, chloride, creatinine with estimated GFR, globulin, glucose, potassium, sodium, total bilirubin, total protein, and urea nitrogen). Comprehensive metabolic panels were only assessed at the 0 and 120-min time points.

2.4.6. Glucose and Insulin Determination

Each plasma sample was analyzed in duplicate for glucose and insulin concentration. Plasma glucose was analyzed at a commercial diagnostic laboratory (Quest Diagnostics) using automated clinical analyzers while insulin concentrations were assessed using a standard ELISA technique (DRG International, EIA2935, Springfield, NJ, USA). Briefly, all standards, samples, and controls were analyzed in duplicate across a range of 1.76–100 µIU/mL. Plate to plate controls were employed between all analyzed plates and exhibited a 6.3% coefficient of variation between the plates.

2.4.7. *Plasma Berberine Determination*

Berberine analysis was performed in plasma samples by Heartland Assays (Iowa State University Research Park, Ames, IA, USA) using coupled liquid chromatography-mass spectrometry-mass spectrometry technology (LC-MS-MS) following the procedures of Buchanan et al. [16]. Briefly, BBR and d_6-BBR (Sigma-Aldrich, St. Louis, MO, USA and Toronto Research Chemicals, Toronto, Canada, respectively) standards were prepared using gravimetric determination and were dissolved in 50:50 methanol: water. As previously published, plasma samples and controls were removed from freezer storage and allowed to thaw at room temperature. An amount of 100 µL of plasma was transferred to a 13 × 100

glass test tube followed by the addition of 15.0 µL of d_6-BBR internal standard (ISTD, 0.5 ng/µL). Next, samples were treated with 400 µL of acetonitrile containing 3% acetic acid, vortexed and centrifuged. The supernatant was transferred to 12 × 75 glass test tube and dried in a vacuum dryer. A standard curve (0.3–1000.0 ng/mL) was prepared and dried. Samples, controls, and standard curve were reconstituted in 50 µL of LC/MS/MS organic and 50 µL aqueous mobile phase solutions (Mobile phase B, LCMS-grade methanol and Mobile phase A, LCMS-grade water, both containing 0.2% formic acid and 0.1% 10 M ammonium formate solution) vortexed and transferred into LC/MS/MS vials. Chromatographic separation of BBR and d_6-BBR was conducted on an Agilent Poroshell C18 column (2.1 × 50 mm, 2.7 µm) using an Agilent 6460 triple quadrupole LC/MS/MS system (Santa Clara, CA, USA). The initial gradient conditions were 45% B from 0 to 2 min and advanced to 95% B over 1 min with a flow rate of 0.40 mL/minute. BBR and d_6-BBR data were collected using the Multiple Reaction Monitoring (MRM) mode with Mass Hunter acquisition software (Agilent, Santa Clara, CA, USA). The MRM pairs used for BBR were 337.1 → 322.1 and 337.1 → 293.1 and 343.1 → 325.2 and 343.1 → 307.1 for d_6-BBR. Mass Hunter Quantitation software was used to quantitate the unknown plasma samples based on best fit standard curves. Quality control samples were run at two levels in each assay (4.0 and 100.0 ng/mL). Average recovery was 102% and ranged from 95% to 108%. The average accuracy (bias from nominal) was 1.0%. The average precision (CV) was 2.1% and 1.5% for intra- and inter-assay precision, respectively.

2.5. Statistical Analysis

The primary outcome for this study protocol was berberine AUC while secondary outcomes were considered to the maximum berberine concentration (C_{Max}) as well as the AUC and C_{Max} for glucose and insulin. Area under the curve (AUC) for berberine, glucose, and insulin were calculated using the trapezoidal method (Equation (1)), where C_0 is the unknown concentration at the first time point of interest, C_1 is the unknown concentration at the second time point of interest, and $Time_{1-0}$ is the total time interval between the two time points when the unknown was measured. These individual AUC values for each time interval were then summed to determine the total AUC for berberine, glucose, and insulin (Equation (2)).

(1) (2)

All analyses were completed using Microsoft Excel and the Statistical Package for the Social Sciences (v26; SPSS Inc., Chicago, IL, USA) while provided figures were developed using GraphPad (La Jolla, CA, USA). For all dependent measures, descriptive statistics are presented herein as mean ± standard deviations. Before any statistical tests were completed, normality was assessed using a Shapiro-Wilk test for all dependent variables. All non-normal

data were transformed using natural logarithms, cubed, and then square root transformation approaches and was retested for normality. Data were then analyzed using both parametric and non-parametric approaches. In all such situations, the final statistical decision was identical whether parametric or non-parametric approaches were completed. All reported *p*-values are computed using parametric approaches. Blood markers were analyzed with a mixed factorial ANOVA, and if a significant interaction was observed then follow-up simple effects testing was performed using a repeated measures ANOVA followed by post-hoc testing consisting of paired samples *t*-tests. Body mass, blood pressure, AUC, and C_{Max} variables were analyzed with a repeated measures ANOVA followed by post-hoc testing consisting of paired samples t-tests. For all statistical tests, data were considered statistically significant when the probability of type I error was 0.05 or less. Trends were highlighted and discussed when *p*-values were between 0.05 and 0.10.

3. Results

3.1. Pre-Supplementation Status

Table 2 outlines the pre-supplementation values for body mass and hemodynamics. Body mass was significantly different between groups ($p = 0.045$), and post-hoc testing revealed differences between PLA (90.6 ± 10.1 kg) and D200 (91.4 ± 9.7 kg). Resting heart rate ($p = 0.21$), systolic blood pressure ($p = 0.07$), and diastolic blood pressure ($p = 0.87$) were not different.

Table 2.

Baseline body mass, hemodynamic, glucose, insulin, and berberine.

Raw Data	Group	Pre	*p*-Value
Body Mass (kg)	PLA	90.6 ± 10.1 ‡	0.043
	D100	91.6 ± 10.3	
	D200	91.4 ± 9.7	
	B500	90.8 ± 10.0	

Raw Data	Group	Pre	*p*-Value
Resting Heart Rate (beats/minute)	PLA	64 ± 10	0.21
	D100	62 ± 6	
	D200	60 ± 5	
	B500	57 ± 9	
Systolic Blood Pressure (mm Hg)	PLA	120 ± 8	0.07
	D100	115 ± 10	
	D200	118 ± 11	
	B500	124 ± 10	
Diastolic Blood Pressure (mm Hg)	PLA	72 ± 5	0.87
	D100	70 ± 5	
	D200	70 ± 10	
	B500	72 ± 7	
Glucose (mg/dL)	PLA	91.8 ± 7.6	0.59
	D100	92.0 ± 8.5	
	D200	90.6 ± 3.4	
	B500	89.8 ± 1.8	
Berberine (ng/mL)	PLA	0.14 ± 0.09 †,‡	0.006
	D100	2.60 ± 1.06	
	D200	4.54 ± 2.24	
	B500	0.30 ± 0.12 †,‡	

Raw Data	Group	Pre	*p*-Value
Insulin (μIU/mL)	PLA	15.4 ± 5.4	0.20
	D100	16.7 ± 9.1	
	D200	14.8 ± 4.4	
	B500	18.4 ± 6.3	

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† = Different than D100 ($p < 0.05$); ‡ = Different than D200 ($p < 0.05$).

3.2. Protocol Compliance

Compliance to the supplementation regimen was 100% with all participants reporting that all supplements were ingested as instructed with the final dose being ingested in front of investigators. All study participants were asked to record their food and fluid intake for two days. Compliance was reported at 87.5% as one participant did not record their food and fluid intake on one of the days. Dietary intake is provided in Table 1.

3.3. Adverse Events

A total of 11 adverse events were reported throughout the study protocol by two or 40% of the study participants. The largest number of adverse events ($n = 6$) were reported in D100, $n = 3$ were reported in PLA, $n = 1$ for D200, and $n = 1$ for B500. All but one reported adverse event (10/11 or 90.9%) was deemed by study participants to be mild in severity and one being moderate severity (See Table 3).

Table 3.

Adverse event table.

	PLA	D100	D200	B500
n	5	5	5	5
# of AE's Reported				
Mild	3	5	1	1
Moderate	0	1	0	0

	PLA	D100	D200	B500
Severe	0	0	0	0
	Adverse Events Breakdown			
Gastrointestinal				
Diarrhea	0	0	0	0
Nausea	0	2	0	0
Upset stomach	0	0	1	0
Stomach cramping	2	0	0	0
Pain				
Headache	0	2	0	1
Cardiovascular				
Abnormal heart rhythm	1	0	0	0
Dizziness	0	2	0	0
Constitutional Symptoms				
Nervousness	0	0	0	0
Blurred vision	0	0	0	0
Summary				
Total Adverse Events	3	6	1	1
# of Subjects Reporting AE	2	2	1	1
% of Subjects Reporting AE	40%	40%	20%	20%

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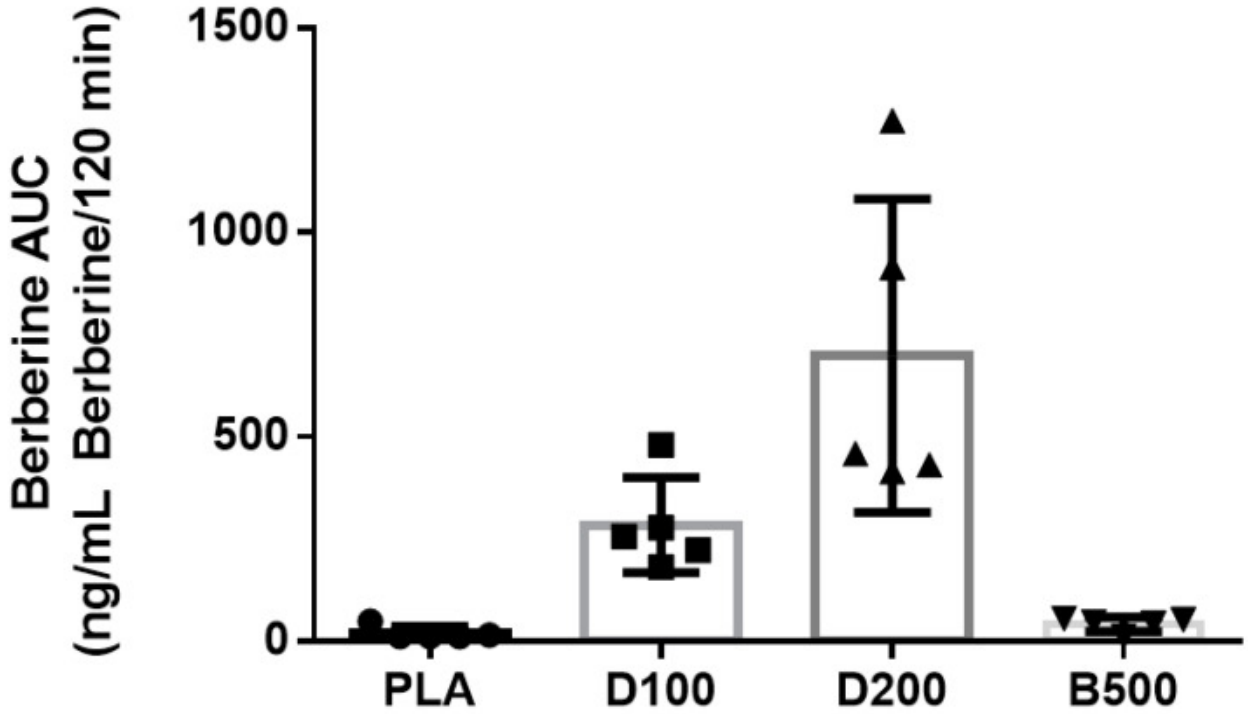
PLA = Placebo; D100 = 100 mg dose of dihydroberberine; D200 = 200 mg dose of dihydroberberine; B500 = 500 mg dose of berberine.

3.4. Berberine

Berberine data were not-normally distributed. The data were transformed using the aforementioned methods and reassessed for normality, which was unable to be achieved due to two high values in the D200 condition at the 60-min timepoint. Subsequently, all berberine data were analyzed using parametric approaches. A significant group x time interaction was revealed for berberine ($p = 0.05$). Simple main effects tests revealed no changes over time within each condition ($p > 0.05$), but significant changes between conditions at the following time points: baseline ($p = 0.006$), 20-min ($p < 0.001$), and 40-min ($p = 0.039$). At baseline, both D100 (2.60 ± 1.06 ng/mL) and D200 (4.54 ± 2.24 ng/mL) conditions had higher berberine values compared to placebo (0.14 ± 0.09 ng/mL; D100, $p = 0.007$; D200, $p = 0.011$) and B500 (0.30 ± 0.12 ng/mL; D100, $p = 0.006$; D200, $p = 0.013$). At the 20-min time point, both D100 (3.00 ± 1.85 ng/mL) and D200 (6.06 ± 2.50 ng/mL) conditions had higher berberine values compared to placebo (0.18 ± 0.18 ng/mL; D100, $p = 0.030$; D200, $p = 0.005$) and B500 (0.32 ± 0.20 ng/mL; D100, $p = 0.008$; D200, $p = 0.029$), but D200 had higher berberine values compared to D100 ($p = 0.050$). Lastly, at 40 min both D100 (2.32 ± 1.27 ng/mL) and D200 (6.90 ± 4.94 ng/mL) conditions had higher berberine values compared to placebo (0.20 ± 0.14 ng/mL; D100, $p = 0.018$; D200, $p = 0.037$) and B500 (0.36 ± 0.15 ng/mL; D100, $p = 0.022$; D200, $p = 0.040$), but D200 had higher berberine values compared to D100 ($p = 0.050$). There was a significant main effect for condition ($p = 0.04$), but no significant main effect for time ($p = 0.18$) was observed.

Berberine AUC values (Figure 3) were significantly difference between conditions ($p = 0.045$). Follow-up analysis indicated that higher berberine AUC values were identified in D200 (929 ± 694 ng/mL berberine/120 min) when compared to PLA (20.2 ± 16.2 ng/mL Berberine/120 min, $p = 0.042$) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min, $p = 0.045$). Significantly higher berberine AUC values were identified for D100 (284.2 ± 115.9 ng/mL Berberine/120 min) when compared to placebo (20.2 ± 16.2 ng/mL Berberine/120 min, $p = 0.007$) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min, $p = 0.007$). Berberine AUC values between D100 (284.2 ± 115.9) and D200 (929 ± 694 ng/mL Berberine/120 min) approached statistical significance ($p = 0.07$). Berberine AUC values between PLA (20.2 ± 16.2 ng/mL Berberine/120 min) and B500 (42.3 ± 17.5 ng/mL Berberine/120 min) tended ($p = 0.096$) to be different. Berberine C_{Max} values tended to be different between conditions ($p = 0.064$). Further, differences in C_{Max} between D100 and PLA were significant $p = 0.005$) while differences between PLA and D200 ($p = 0.06$) as well as PLA and B500 ($p = 0.09$) tended to be different.

Figure 3.



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Individual and aggregated mean values for berberine AUC by condition.

Each individual symbol represents the individual AUC response for one study participant. For all graphs below, *n* = 5 participants completed each condition. PLA = Placebo; D100 = 100 mg dose of dihydroberberine; D200 = 200 mg dose of dihydroberberine; B500 = 500 mg dose of berberine. Data are presented as mean ± SD.

3.5. Glucose

There was no significant group x time interaction ($p = 0.97$) for glucose measures. However, a significant time effect ($p < 0.001$) was observed where glucose concentrations were significantly elevated at the 40-min timepoint (129.25 ± 27.43 mg/dL) compared to the baseline timepoint (91.05 ± 5.61 mg/dL, $p < 0.022$). Furthermore, both the 20-min timepoint (119.5 ± 21.27 mg/dL) and the 40-min timepoint (129.25 ± 27.43 mg/dL) had elevated glucose concentrations compared to the 90-min timepoint (85.40 ± 18.15 mg/dL; 20 min, $p = 0.045$; 40 min, $p = 0.007$), and the 120-min timepoint (69.6 ± 10.19 mg/dL; 20 min, $p = 0.044$; 40 min, $p < 0.001$). The 60-min timepoint (105.45 ± 23.55 mg/dL) had elevated glucose concentrations compared to the 120-min timepoint (69.60 ± 13.59 mg/dL, $p = 0.033$). Both AUC and C_{Max} values were calculated for all four conditions. Oneway ANOVA indicated no significant differences were observed for AUC ($p = 0.92$) and C_{Max} ($p = 0.77$) values (See Table 4 and Table 5).

Table 4.

Berberine (ng/mL), glucose (mg/dL), and insulin (μIU/mL) data.

Berberine (ng/mL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	0.14 ± 0.09	0.18 ± 0.18	0.20 ± 0.14	0.16 ± 0.13	0.16 ± 0.13	0.16 ± 0.13	Group	0.002
B500	0.30 ± 0.12	0.32 ± 0.20	0.36 ± 0.15	0.38 ± 0.16	0.39 ± 0.16	0.32 ± 0.13	Time	0.18
D100	2.6 ± 1.1 †,‡	3.0 ± 1.9 †,‡	2.3 ± 1.3 †,‡	2.5 ± 1.0	2.1 ± 0.8	1.9 ± 0.9	G x T	0.050
D200	4.5 ± 2.2 †,‡	6.1 ± 2.5 †,‡,*	6.9 ± 4.9 †,‡,*	11.4 ± 10.7	7.8 ± 6.5	7.2 ± 6.5		
Glucose (mg/dL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	91.8 ± 7.6	120.4 ± 25.4	129.6 ± 33.0	102.8 ± 27.6	92.0 ± 23.9	63.4 ± 9.3	Group	0.92
B500	89.8 ± 1.8	116.4 ± 30.1	125.6 ± 35.7	101.8 ± 30.9	84.0 ± 25.1	73.0 ± 13.7	Time	<0.001
D100	92.0 ± 8.5	121.8 ± 20.9	126.8 ± 24.5	106.4 ± 14.9	81.2 ± 10.1	69.4 ± 7.9	G x T	0.97
D200	90.6 ± 3.4	119.2 ± 11.8	135.0 ± 23.3	110.8 ± 25.2	84.4 ± 13.4	72.6 ± 21.6		
Insulin (μIU/mL)	**0 min**	**20 min**	**40 min**	**60 min**	**90 min**	**120 min**		***p***
PLA	15.4 ± 5.4	47.9 ± 31.3	106.9 ± 26.8	85.0 ± 21.0	78.5 ± 23.5	35.4 ± 19.2	Group	0.43
B500	18.4 ± 6.3	38.4 ± 26.9	87.1 ± 24.5	78.1 ± 23.0	58.0 ± 23.5	58.0 ± 23.5	Time	<0.001

Berberine (ng/mL)	0 min	20 min	40 min	60 min	90 min	120 min		*p*
D100	16.7 ± 9.1	47.2 ± 17.6	93.0 ± 35.2	84.5 ± 33.2	60.9 ± 23.1	34.6 ± 14.8	G x T	0.10
D200	14.8 ± 4.4	69.6 ± 28.6	85.0 ± 9.0	83.9 ± 27.4	78.9 ± 12.6	53.3 ± 29.6		

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† = Different than placebo ($p < 0.05$); ‡ = Different than B500 ($p < 0.05$); * = Different than D100 ($p < 0.05$).

Table 5.

Area under the curve (AUC) and concentration max (C_{Max}) for berberine, insulin, and glucose.

Berberine	AUC(ng/mL × 120 min)	C_{Max}(ng/mL)
PLA	20.2 ± 16.2	0.22 ± 0.18
B500	42.3 ± 17.6	0.40 ± 0.17
D100	284.2 ± 115.9	3.8 ± 1.4
D200	929 ± 694	12.0 ± 10.1
p	0.045	0.06
Insulin	**AUC (μIU/mL × 120 min)**	**C_{Max} (μIU/mL)**
PLA	8260 ± 420	110.6 ± 24.3
B500	7429 ± 1519	99.1 ± 6.6
D100	8502 ± 717	107.2 ± 21.4
D200	7242 ± 1263	98.1 ± 3.4
p	0.22	0.36

Berberine	AUC(ng/mL × 120 min)	C_{Max}(ng/mL)
Glucose	**AUC (mg/dL × 120 min)**	**C_{Max} (mg/dL)**
PLA	12,199 ± 1602	146.2 ± 13.9
B500	12,029 ± 194	139.4 ± 32.8
D100	12,381 ± 1128	140.8 ± 8.8
D200	11,898 ± 2058	139.0 ± 16.6
p	0.92	0.77

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3.6. Insulin

There was no significant group x time interaction ($p = 0.095$) for insulin measures. A significant time effect ($p < 0.001$) was observed where insulin concentrations were significantly elevated at 20 min (50.8 ± 20.7 µIU/mL, $p = 0.02$), 40 min (93.0 ± 18.2 µIU/mL, $p = 0.02$, and 60 min (82.9 ± 13.6 µIU/mL, $p = 0.003$) in comparison to baseline (16.3 ± 6.1 µIU/mL). Additionally, insulin concentrations were significantly higher at 40 min (93.0 ± 18.2 µIU/mL) and 60 min (82.9 ± 13.6 µIU/mL) compared to 20 min (50.8 ± 20.7 µIU/mL; 40 min, $p = 0.002$; 60 min, $p = 0.018$) and 120 min (45.1 ± 24.5 µIU/mL; 40 min, $p = 0.002$; 60 min, $p = 0.013$). No significant condition effect ($p > 0.05$) was identified. No significant differences were observed for AUC ($p = 0.22$) and C_{Max} ($p = 0.36$) insulin values (See Table 4 and Table 5).

3.7. Whole Blood and Serum Markers

Complete blood count analysis was completed on collected whole-blood samples to assess safety and presence of any adverse responses to supplementation. As seen in Table 6, absolute lymphocytes analysis revealed a significant main effect of condition ($p = 0.011$) where D100 had elevated concentrations compared to D200 ($p = 0.032$) but were in clinically accepted norms. Absolute monocytes analysis revealed a significant group × time interaction ($p = 0.047$) and follow-up simple main effects testing revealed significant changes over time within the Placebo ($p = 0.042$) and D100 ($p = 0.029$) conditions. Specifically, at 120 min within the Placebo and D100 conditions absolute monocytes were higher than baseline albeit they were within clinically accepted norms. Furthermore, no significant changes between conditions were revealed at any time point for absolute monocytes ($p > 0.05$). All other

variables for complete blood count did not change over time, between conditions, or reveal any interactions ($p > 0.05$).

Table 6.

Complete blood count.

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)*(p)*	G x T(*p*)
White Blood Cell Count (10^3 × cells/μL)	PLA	5.6 ± 1.0	5.7 ± 1.3	0.74	0.45	0.20
	D100	5.7 ± 1.1	5.9 ± 1.3			
	D200	6.6 ± 2.4	6.2 ± 2.1			
	B500	5.8 ± 1.2	5.3 ± 1.2			
Red Blood Cell Count (10^3 × cells/μL)	PLA	4.8 ± 0.2	4.8 ± 0.1	0.10	0.54	0.30
	D100	4.7 ±0.3	4.7 ± 0.3			
	D200	4.7 ± 0.3	4.7 ± 0.2			
	B500	5.0 ± 0.3	4.8 ± 0.4			
Hemoglobin (g/dL)	PLA	14.8 ± 0.5	14.8 ± 0.7	0.15	0.47	0.20
	D100	14.3 ± 0.4	14.5 ± 0.5			
	D200	14.3 ± 0.6	14.2 ± 0.4			
	B500	15.2 ± 1.0	14.8 ± 0.8			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)*(p)*	G x T(*p*)
Hematocrit (%)	PLA	43.2 ± 0.8	43.4 ± 1.4	0.20	0.73	0.19
	D100	42.2 ± 0.7	42.6 ± 1.4			
	D200	42.1 ± 1.3	42.0 ± 0.4			
	B500	44.6 ± 2.8	43.7 ± 3.0			
Mean Corpuscular Volume	PLA	89.4 ± 3.5	90.2 ± 3.8	0.87	0.44	0.71
	D100	90.1 ±4.7	90.0 ± 4.3			
	D200	90.4 ± 3.2	90.4 ± 3.8			
	B500	90.2 ± 2.8	90.5 ± 3.7			
Mean Corpuscular Hemoglobin	PLA	30.6 ± 1.6	30.7 ± 1.5	0.86	0.88	0.81
	D100	30.6 ± 1.6	30.5 ± 1.6			
	D200	30.7 ± 1.3	30.6 ± 1.6			
	B500	30.6 ± 1.6	30.7 ± 1.4			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Mean Corpuscular Hemoglobin Content	PLA	34.2 ± 0.6	34.1 ± 0.8	0.91	0.43	0.93
	D100	34.0 ± 0.6	34.0 ± 0.5			
	D200	34.0 ± 0.8	33.8 ± 0.7			
	B500	34.0 ± 0.9	33.9 ± 0.7			
Red Cell Distribution Width (%)	PLA	12.1 ± 0.4	12.0 ± 0.5	0.93	0.08	0.55
	D100	12.1 ± 0.4	12.0 ± 0.5			
	D200	12.1 ±0.4	12.1 ± 0.4			
	B500	12.0 ± 0.4	12.0 ± 0.4			
Platelet Count	PLA	242.2 ± 32.6	239.0 ± 37.3	0.85	0.91	0.55
	D100	243.4 ± 26.7	250.2 ± 26.5			
	D200	242.8 ± 23.9	242.0 ± 19.1			
	B500	247.4 ± 36.1	245.8 ± 30.9			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Abs Neutrophils (cells/uL)	PLA	2790 ± 1318	3046 ± 1295	0.45	0.88	0.38
	D100	2902 ± 719	2962 ± 949			
	D200	3926 ± 2324	3723 ± 2020			
	B500	2743 ± 737	2675 ± 702			
Abs Lymphocytes (cells/uL)	PLA	1893 ± 435	1805 ± 297	0.01	0.31	0.28
	D100	2068 ± 719	2173 ± 253			
	D200	1852 ± 528	1721 ± 149			
	B500	2268 ± 524	1899 ± 423			
Abs Monocytes (cells/µL)	PLA	477 ± 134	549 ± 168	0.92	0.04	0.007
	D100	494 ± 159	597 ± 225			
	D200	549 ± 152	504 ± 96			
	B500	545 ± 178	530 ± 205			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Abs Eosinophils (cells/μL)	PLA	218 ± 162	214 ± 162	0.17	0.09	0.62
	D100	179 ± 116	165 ± 96			
	D200	225 ± 148	188 ± 106			
	B500	183 ± 119	179 ± 161			
Abs Basophils (cells/μL)	PLA	42.8 ± 12.4	45.6 ± 10.9	0.47	0.94	0.67
	D100	38.4 ± 7.6	43.8 ± 12.2			
	D200	49.0 ± 18.5	44.6 ± 9.3			
	B500	41.2 ± 20.7	36.8 ± 11.6			
Neutrophils (%)	PLA	52.1 ± 13.2	52.2 ± 11.7	0.24	0.42	0.29
	D100	50.7 ± 7.3	49.1 ± 6.7			
	D200	56.7 ±14.4	57.3 ± 12.0			
	B500	47.5 ± 7.9	50.4 ± 7.8			

Variable Name	Group	Pre	Post	Group (G)(*p*)	Time (T)(*p*)	G x T(*p*)
Lymphocytes (%)	PLA	34.7 ± 9.9	33.1 ± 7.8	0.17	0.26	0.32
	D100	36.7 ± 6.2	37.4 ± 5.2			
	D200	30.0 ± 9.5	29.8 ± 7.6			
	B500	39.5 ± 7.1	36.0 ± 4.7			
Monocytes (%)	PLA	8.5 ± 2.1	10.0 ± 3.1	0.12	0.15	0.60
	D100	9.0 ± 1.1	10.0 ± 2.9			
	D200	8.9 ± 2.7	8.8 ± 2.7			
	B500	9.3 ± 1.8	9.8 ± 2.4			
Eosinophils (%)	PLA	4.0 ± 3.0	3.9 ± 3.1	0.30	0.25	0.72
	D100	3.0 ± 1.4	2.7 ± 1.3			
	D200	3.6 ± 2.8	3.3 ± 2.5			
	B500	3.0 ± 1.5	3.1 ± 2.3			
Basophils (%)	PLA	0.8 ± 0.3	0.8 ± 0.3	0.37	0.43	0.95
	D100	0.7 ± 0.1	0.8 ± 0.3			
	D200	0.8 ± 0.3	0.8 ± 0.3			
	B500	0.7 ± 0.2	0.7 ± 0.2			

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Comprehensive metabolic panels were analyzed in collected serum samples. As seen in Table 7, analysis revealed significant main effects of time for glucose ($p = 0.024$), sodium ($p < 0.001$), potassium ($p = 0.007$), and chloride ($p = 0.024$) and these changes were within clinically accepted norms. A significant group × time interaction was revealed for creatinine ($p = 0.008$), and follow-up simple main effects testing revealed significant changes over time within the D100 ($p = 0.031$) and D200 ($p = 0.030$) conditions. Within the D100 condition at 120 min, creatinine measures were higher than baseline, and within the D200 condition, baseline measures were higher than 120 min, however, these measures are within clinically accepted norms. Additionally, there were significant creatinine differences between conditions at baseline ($p = 0.039$) and 120 min ($p = 0.009$) measures. At baseline, D200 ($p = 0.030$) and B500 ($p = 0.016$) conditions had higher creatinine measures compared to D100 and these differences were within clinically accepted norms. Lastly, a significant main effect of condition was observed for creatinine measures ($p = 0.040$) where D100 was higher than placebo ($p = 0.035$) but was within clinically accepted norms.

Table 7.

Comprehensive metabolic panel.

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Glucose (mg/dL)	PLA	90.6 ± 5.3	65.6 ± 11.0	0.66	0.02	0.17
	D100	92.0 ± 5.7	68.8 ± 10.0			
	D200	81.2 ± 13.6	73.2 ± 20.1			
	B500	89.2 ± 4.2	73.8 ± 15.4			
Blood Urea Nitrogen (g/dL)	PLA	16.2 ± 2.3	15.6 ± 2.3	0.14	0.11	0.46
	D100	13.8 ± 1.5	13.8 ± 1.3			
	D200	15.4 ± 1.9	14.8 ± 2.2			
	B500	15.8 ± 2.2	15.2 ± 2.5			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Creatinine (mg/dL)	PLA	1.0 ± 0.1	1.0 ± 0.1	0.04	0.55	0.008
	D100	0.9 ± 0.1	1.0 ± 0.1			
	D200	1.0 ± 0.1	0.9 ± 0.1			
	B500	1.0 ± 0.1	0.9 ± 0.1			
Glomerular Filtrate Rate	PLA	107.2 ± 12.0	102.2 ± 12.8	0.03	0.30	0.02
	D100	115.6 ± 8.4	109.4 ± 8.8			
	D200	106.2 ± 13.0	111.0 ± 11.9			
	B500	106.6 ± 13.3	109.2 ±12.8			
Sodium (mM)	PLA	137 ± 1	139 ± 2	0.20	<0.001	0.82
	D100	136 ± 2	139 ± 2			
	D200	135 ± 2	137 ± 2			
	B500	136 ± 2	138 ± 3			
Potassium (mM)	PLA	4.3 ± 0.2	4.0 ± 0.1	0.36	0.007	0.90
	D100	4.2 ± 0.2	3.9 ± 0.2			
	D200	4.3 ± 0.2	4.0 ± 0.2			
	B500	4.2 ± 0.3	3.9 ± 0.2			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Chloride (mM)	PLA	102 ± 2	102 ± 1	0.45	0.02	0.18
	D100	103 ± 2	103 ± 2			
	D200	101 ± 2	103 ± 2			
	B500	101 ± 3	103 ± 2			
Carbon Dioxide (mM)	PLA	25 ± 2	26 ± 3	0.62	0.16	0.78
	D100	25 ± 1	26 ± 1			
	D200	26 ± 1	27 ± 2			
	B500	25 ± 1	26 ± 3			
Calcium (mg/dL)	PLA	9.3 ± 0.4	9.7 ± 0.2	0.26	0.12	0.049
	D100	9.4 ± 0.2	9.5 ± 0.3			
	D200	9.3 ± 0.5	9.4 ± 0.3			
	B500	9.5 ± 0.3	9.5 ± 0.3			
Total Protein (g/dL)	PLA	6.8 ± 0.4	6.9 ± 0.3	0.94	0.74	0.22
	D100	6.8 ± 0.3	6.9 ± 0.3			
	D200	6.8 ± 0.6	6.8 ± 0.4			
	B500	7.0 ± 0.5	6.8 ± 0.5			
Albumin (g/dL)	PLA	4.6 ± 0.2	4.5 ± 0.1	0.42	0.38	0.80
	D100	4.5 ± 0.1	4.4 ± 0.2			
	D200	4.5 ± 0.2	4.4 ± 0.0			
	B500	4.6 ± 0.1	4.5 ± 0.1			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
Globulin (g/dL)	PLA	2.2 ± 0.3	2.3 ± 0.3	0.62	0.26	0.17
	D100	2.3 ± 0.3	2.4 ± 0.3			
	D200	2.4 ± 0.4	2.4 ± 0.3			
	B500	2.4 ± 0.4	2.3 ± 0.5			
Alb/Glob Ratio	PLA	2.1 ± 0.2	2.0 ± 0.2	0.37	0.11	0.28
	D100	2.0 ± 0.3	1.8 ± 0.3			
	D200	1.9 ± 0.3	1.9 ± 0.3			
	B500	1.9 ± 0.3	2.0 ± 0.4			
Total Bilirubin (mg/dL)	PLA	0.9 ± 0.3	0.8 ± 0.3	0.52	0.14	0.37
	D100	0.8 ± 0.3	0.8 ± 0.3			
	D200	0.9 ± 0.4	0.8 ± 0.3			
	B500	1.0 ± 0.4	0.9 ± 0.4			
Alkaline Phosphatase (U/L)	PLA	53 ± 10	51 ± 10	0.13	0.16	0.45
	D100	51 ± 11	52 ± 10			
	D200	51 ± 13	50 ± 10			
	B500	54 ± 12	53 ± 13			
AST (U/L)	PLA	22 ± 5	22 ± 5	0.22	0.18	0.55
	D100	24 ± 7	24 ± 7			
	D200	24 ± 6	23 ± 5			
	B500	20 ± 3	20 ± 3			

Variable Name	Group	Pre	Post	Group (G) (*p*)	Time (T) *(p)*	G x T(*p*)
ALT (U/L)	PLA	19 ± 6	19 ± 5	0.93	1.00	0.60
	D100	19 ± 7	20 ± 7			
	D200	19 ± 4	18 ± 3			
	B500	18 ± 6	18 ± 5			

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A significant group x time interaction was revealed for globular filtration rate ($p = 0.016$) and follow-up simple main effects testing revealed significant changes over time within the D100 ($p = 0.047$) and D200 ($p = 0.037$) conditions. Within the D100 condition, baseline globular filtration rate measures were higher than 120 min, and within the D200 condition 120 min measures were higher than baseline, however, these measures are within clinically accepted norms. Additionally, follow-up simple main effects testing from the interaction revealed significant differences between conditions for globular filtration rate measures at baseline ($p = 0.050$) and 120 min ($p = 0.005$). At baseline, globular filtration rate measures were higher in D100 compared to D200 ($p = 0.048$) and B500 ($p = 0.038$), which were within clinically accepted norms. At 120 min, globular filtration rate measures were higher in D200 ($p = 0.024$), D100 ($p = 0.024$), and B500 ($p = 0.047$) compared to placebo and were within clinically accepted norms. Lastly, a significant main effect of condition was observed for globular filtration rate measures ($p = 0.033$) where D100 was higher than placebo ($p = 0.028$) but was within clinically accepted norms.

A significant group x time interaction was revealed for calcium ($p = 0.049$) and follow-up simple main effects testing revealed significant changes over time within the placebo condition ($p = 0.009$), the levels of which were higher at 120 min compared to baseline, but within clinically accepted norms. Furthermore, a follow-up simple main effects revealed significant differences between conditions for calcium measures at 120 min ($p = 0.030$) where placebo was higher than D200 ($p = 0.040$) and all measures were within clinically accepted norms. All other variables for the comprehensive metabolic panel did not change over time, between conditions, or reveal any interactions ($p > 0.05$).

4. Discussion

The intent of this project was to evaluate the observed differences in plasma berberine concentrations after oral ingestion of two doses (100 mg [D100] and 200 mg [D200],

respectively) of dihydroberberine and one dose (500 mg) of berberine (B500). Findings from this pilot approach revealed that area under the curve calculations for berberine were significantly greater when either dose of dihydroberberine was ingested in comparison to the values observed for berberine and placebo (PLA). In addition, peak berberine concentrations (C_{Max}) tended to be different between all conditions. Specifically, the 100 mg dose of dihydroberberine was significantly greater than peak values observed in PLA and B500 while peak concentrations for the 200 mg dose of dihydroberberine tended to be different than what was observed in the berberine group.

Of the available human research that has explored efficacy and safety of oral berberine ingestion, the majority of these studies have employed a dosing regimen that consists of two to three daily doses with meals (to reduce incidence of gastrointestinal complications) at a dosage of 500 mg per dose. For example, Perez-Rubio et al. [7] had 24 metabolic syndrome patients randomly ingest, in a double-blind, placebo-controlled fashion, three daily doses of 500 mg/dose (total daily dosage of 1500 mg/day) for three months. Similarly, Yin et al. [6] also randomly assigned type 2 diabetes patients to ingest three daily 500 mg doses for a total of 13 weeks. Finally, Zhang et al. [17] randomly assigned 116 individuals diagnosed with type 2 diabetes to consume two 500 mg daily doses (1000 mg total) over a 3-month period. While results of these studies outline efficacy outcomes in various clinical populations, very limited human data are available that has outlined the absorption pattern and plasma kinetics after oral berberine ingestion. In this respect, it has been previously reported that oral bioavailability of berberine is extremely low (<1%) in both animal [9,10] and human models largely due to poor intestinal absorption and high levels of first-pass removal in the intestines and liver [5]. This has led to the identification of various strategies to increase bioavailability, including administration of dihydroberberine. Currently, no human research has appeared in the peer-reviewed literature that has examined oral ingestion of dihydroberberine. Towards this end, Buchanan et al. [16] compared the pharmacokinetics associated with transdermal administration of berberine and dihydroberberine in Sprague-Dawley rats. Using this model and over an 8-h period, transdermal application of dihydroberberine resulted in greater AUC values than what was observed for both transdermal and oral administration of berberine. Moreover, transdermal delivery of berberine resulted in greater AUC values than what was observed for oral administration, further highlighting the poor bioavailability of oral berberine ingestion.

Other highlights from the present study include the statistically significant higher outcomes at baseline (after three doses, but prior to the 4th dose in the present study) in D100 and D200 when compared to PLA and B500. When viewed in comparison to PLA, this outcome was not surprising and some might deem it expected considering that dihydroberberine was administered, however, the finding of greater levels in comparison to berberine seemingly

speaks to the ability of dihydroberberine to maintain berberine levels in the blood better than oral berberine ingestion. Whether or not these increases in plasma berberine translate beyond one day or measurement or improvements in efficacy outcomes commonly associated with berberine supplementation cannot be determined from this project. In this respect, more research is certainly needed with dihydroberberine to determine how well the greater plasma berberine levels improve dyslipidemia and glucose and insulin homeostasis as commonly reported in the literature.

A key limitation with the present study is the sample size on this project. While the fully randomized, crossover, double-blind, and placebo-controlled approach was a sound approach for this pilot assessment, it is readily acknowledged by the authors that more study participants are needed on future studies to continue to establish the safety and efficacy of berberine and dihydroberberine. Further to the point and beyond just having a low sample size, one needs to closely consider our plasma berberine data as two participants in the D200 at the 60-min time point exhibited high values, which nearly achieved traditional definitions of being a statistical outlier (>±3 of standardized residual estimates). When combined with the low sample size, these data exerted substantial impact on the distribution of these data. As such, traditional approaches to transforming the data were unsuccessful at achieving normality and all data were subsequently analyzed using a parametric approach. Furthermore, all analysis was also completed using an intent-to-treat approach with those values removed and the high values replaced with the previous data point and no meaningful differences were noted in the outcomes of the project. Another limitation might be considered to be the small number of doses ingested prior to assessing the absorption kinetics. This was an a priori consideration as our primary focus for this pilot project was to assess and compare the absorption kinetics of two different doses of oral dihydroberberine in comparison to oral ingestion of berberine and placebo.

5. Conclusions

In conclusion, results from this study represent what we believe are the first data in humans to evaluate the absorption kinetics of berberine and two doses of dihydroberberine. Results from this study demonstrate that dihydroberberine, irrespective of what dose is delivered, achieves greater area under the curve as well as peak berberine concentrations when compared to oral ingestion of berberine or placebo. Since berberine use has traditionally spanned several months to establish the observed efficacy outcomes known for berberine (improved glucose and insulin homeostasis and lipid parameters), several follow-up approaches are recommended. First, can the increased levels of berberine seen from dihydroberberine ingestion usher in faster improvements in outcomes of interest. In a similar vein, are the

outcomes improved as a result of dihydroberberine ingestion in comparison to berberine ingestion.

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Author Contributions

Designed study: C.M.K.; data collection: J.M.M., A.M.H., K.M.R. and R.A.S.; analyzed data: P.W.M.; prepared initial draft: C.M.K., A.M.H., J.M.M. and P.W.M.; approved final version: all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Lindenwood University: IRB-20-173, approval date: 22 June 2020.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data and/or statistical analyses are available upon request on a case-by-case basis for non-commercial scientific inquiry and/or educational use.

Conflicts of Interest

C.M.K. serves as a paid advisor for NNB Nutrition. A conflict-of-interest management plan was filed and approved by the Lindenwood University IRB prior to commencing any data collection. As part of this plan, C.M.K. was not involved in any part of the consenting,

randomization, or data collection process. All statistical analysis performed by C.M.K. was done while data was still blinded and all analysis and interpretation was confirmed by P.W.M. As such, P.W.M. and C.M.K. prepared all statistical analysis and interpretation. All other authors declare that they have no competing interests.

Footnotes

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Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Data Availability Statement

Data and/or statistical analyses are available upon request on a case-by-case basis for non-commercial scientific inquiry and/or educational use.

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Interaction of Herbal Compounds with Biological Targets: A Case Study with Berberine

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Abstract

Berberine is one of the main alkaloids found in the Chinese herb Huang lian (*Rhizoma Coptidis*), which has been reported to have multiple pharmacological activities. This study aimed to analyze the molecular targets of berberine based on literature data followed by a pathway analysis using the PANTHER program. PANTHER analysis of berberine targets showed that the most classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor-linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved. In addition, berberine was found to interact with a mixture of biological pathways, such as Alzheimer's disease-presenilin and -secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Hungtington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways. We also explored the possible mechanism of action for the anti-diabetic effect of berberine. Further studies are warranted to elucidate the mechanisms of action of berberine using systems biology approach.

1. Introduction

The majority of clinical drugs achieve their effect by binding to a cavity and regulating the cavity, of its protein targets [1]. In general, drugs act on four main types of regulatory proteins that mediate the actions of hormones, neurotransmitters, and autacoids. These four types of regulatory proteins are carriers, proteins, ion channels, and receptors [2]. Certain characteristics are expected for therapeutic targets [3]. A potential target needs only not to be druggable but also linked to disease, most preferably playing critical and inimitable roles in disease state. Binding sites are to have certain structural and physiochemical properties to accommodate high-affinity site-specific binding and subsequent regulation of protein activity by drugs. They are not significantly involved in other important biological processes to avoid potential side effects. Useful information about these targets may be investigated by analysing their sequence properties, protein families, structural folds, biochemical classes, similarity proteins, gene location in the human genome, and associated pathways [4]. This information can be potentially useful in derivation of rule and developing predictive tools in the search for druggable and potential targets [4].

The number of molecular targets acted on by current drug therapy is still in dispute. In 1996, Drews and Ryser identified a total of 483 drug targets addressed by drug therapy [5, 6]. Approximately 45% are cell membrane receptors, 28% are enzymes, and the remaining classes comprise hormones (11%), ion channels (5%), nuclear receptors (2%), and DNA (2%). About 7% of the targets are not known biochemically. Later, Hopkins and Groom challenged this figure and suggested that “rule-of-five” compliant drugs acted primarily through only 120 underlying molecular targets [3, 7]. However, the statistical analysis of disease genes and related proteins suggested that the total number of the estimated potential targets in the human genome ranges from 600 to 1,500 [3]. In the meantime, another report showed the estimated total number of distinct targets is in the range of 1,700–3,000 [8]. Chen et al. reported targets collected in the Therapeutic Target Database [9] is 997 distinct proteins, 1,494 distinct protein subtypes, and 41 nucleic acids, which are only targeted by at least one marketed drug and 1,267 research targets, which are only targeted by investigational agents that are not approved for clinical use at present [4]. Targets for

neoplasm diseases, circulatory system diseases, infectious diseases, and nervous system and sense organs disorders constitute the largest number of targets [1]. An increase in target numbers is made possible by advances in genomics, proteomics, better molecular understanding of diseases, and increased effort in the exploration of new therapeutic targets as well as increased knowledge of unknown or unreported targets of previous existing drugs. An improvement in technology for target identification and validation also contributes greatly.

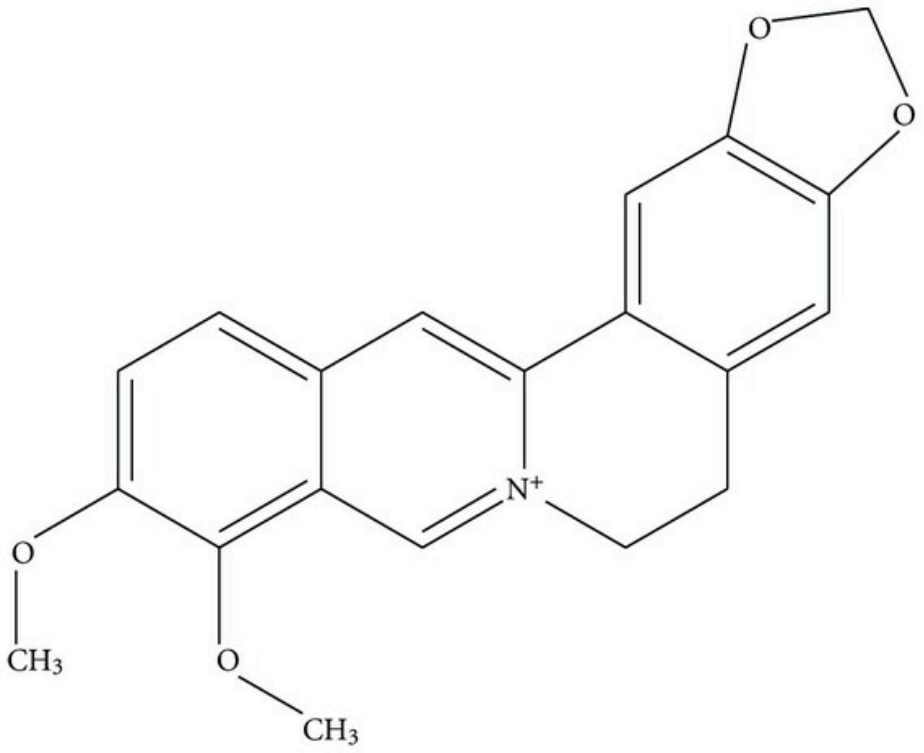
Chinese herbal medicine (CHM) has always been an integral part of traditional Chinese medicine (TCM), which has been practiced in the east for thousands of years. Chinese herbs are usually in the forms of dried whole plants or parts of the plants (roots, leaves, body, etc.); sometimes shells and even minerals are used. Chinese herbs are often used in a compound formula, consisting of several different herbs hosting different roles according to the principle of Jun-Chen-Zuo-Shi described by the ancient Chinese. Each of Jun, Chen, Zuo, and Shi function together to harmonise the body, with Shi (courier) herbs are included in many formulae to ensure that all components in the prescription are well absorbed and to help to deliver or guide them to the target organs [10]. On some level, the guiding function of Shi herbs relates to modern drug delivery techniques, guiding the drug compound to target tissues. In the modern world, complementary medicine has gained vast popularity in the West. There has been increased use of herbal medicine to manage chronic diseases and promote wellbeing, in countries such as Australia, New Zealand, USA, and Europe [11]. Reports show that 18.9% of the American population used natural products in the precedent year [12]. This increase in popularity is closely related to its proven effectiveness in clinical practice over the past centuries. To date, more than 11,000 species of plants are used medicinally and about 300 are commonly used [13].

Despite its widespread use, CHM is associated with high levels of uncertainty. This is mainly due to lack of evidence, base of efficacy, targets, and safety data. During the process of therapeutic drug development, owing to the preselection of targets, researchers have a basic if not full understanding of which molecular structures the drug will react with or which biological pathway in the body it might alter. Knowledge on molecular interactions and modulations of the drug is anticipated and researched on. However, this is not the case for CHM. There is no preselection of molecular targets in the body but CHM has been used for thousands of years and is proven to be effective. The exact mechanism of the herbs actions is yet to be elucidated.

The proven clinical efficacy of some herbal medicines is considered to be due to the interaction of pharmacologically active components from the herbs with molecular targets in the body. Similar to synthetic drugs, active compounds of herbal medicine may bind to and undergo interactions with molecular structures or herbal targets to produce therapeutic or adverse effects. However, there is a lack of understanding of how CHMs exert their biological and clinical effects at a molecular level, which impedes development of CHMs and the incorporation of CHMs into mainstream medicine in the West.

Berberine (Figure 1, molecular formula $C_{20}H_{19}NO_5$ and a molecular weight of 353.36) is an isoquinoline alkaloid found in many medicinal plants [14]. It is a major constituent of many medicinal plants of families Papaveraceae, Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, and Annonaceae [15]. It is present in *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). The berberine alkaloid can be found in the roots, rhizomes, and stem bark of the plants. Berberine is one of the main alkaloids found in the Chinese herb Huang Lian (*Rhizoma coptidis*) [16]. Huang Lian has traditionally been used to treat diarrhoea and diabetes. In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhoea [14, 17]. In recent years, there has been a growing interest in the pharmacological activities of berberine and many studies have been carried out to elucidate the mechanisms of action of berberine. This study aims to review molecular targets of berberine based on *in vitro* studies. Berberine has shown to have good hypoglycaemic effects, so we also reviewed the effects of berberine in animal and human studies, with a focus on diabetes mellitus.

Figure 1.



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Chemical structure of berberine.

2. Methods

2.1. Data Retrieval from the Literature

In vitro studies related to berberine and its targets were searched using Pubmed (from inception to April 2012). Search terms used were a combination of “berberine,” “*in vitro,*” “human cell,” and “mechanism.” Only studies using human cell lines were used to extract current berberine targets. Studies using animal cell lines or berberine derivatives or in a language other than English were excluded. Information extracted from these studies includes molecular targets of berberine (name and gene symbols), cell type, effects of berberine, and possible clinical applications.

2.2. PANTHER Analysis

Using the PANTHER Classification System, *in vitro* berberine targets were analysed using three approaches: molecular function, biological process, and pathway involvement Table 2. PANTHER is a publicly available database that relates protein sequence evolution to evolution of protein functions and biological roles (http://www.pantherdb.org/).

Table 2.

Berberine's target classification based on PANTHER.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Multidrug resistance protein 1 (Pgp, Pgp-170)	*ABCB1*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity	Immune system process, extracellular transport, carbohydrate metabolic process, response to toxin	ATP-binding cassette (ABC) transporter
ATP-binding cassette sub-family G member 2	*ABCG2*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity, anion channel activity	Immune system process, anion transport, lipid transport, oxygen and reactive oxygen species, metabolic process, lipid metabolic process, response to stress	N/A
Acetyl-coenzyme A carboxylase-*α*	*ACACA*	Other ligase	Gluconeogenesis, monosaccharide metabolism, fatty acid biosynthesis, coenzyme metabolism	N/A
α-Fetoprotein	*AFP*	Other transfer/carrier protein	Transport, mesoderm development, oncogenesis	N/A
Transcription factor AP-1	*AP-1*	DNA binding, transcription factor activity	Cell cycle, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle, signal transduction	Toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway, oxidative stress response, angiogenesis, TGF-beta signaling pathway, T-cell activation, B-cell activation, Ras Pathway, FAS signaling pathway, PDGF signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Amyloid-β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Other signaling molecules	Other signal transduction, cell communication, other intracellular protein traffic	Alzheimer disease-amyloid secretase pathway, Alzheimer disease-presenilin pathway, blood coagulation, Alzheimer disease-presenilin pathway, Alzheimer disease-amyloid secretase pathway
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	DNA binding, transcription factor activity	Transcription factor activity, immune system process, neurological system process, induction of apoptosis, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Apoptosis signaling pathway
Bcl2 antagonist of cell death	*BAD*	N/A	N/A	PDGF signaling pathway, apoptosis signaling pathway, angiogenesis, PI3 kinase pathway, VEGF signaling pathway, interleukin signaling pathway
BCL2-associated X protein	*BAX*	Other signaling molecule	Induction of apoptosis, gametogenesis, hematopoiesis, cell cycle control, cell proliferation and differentiation, tumor suppressor	p53 pathway, apoptosis signaling pathway, Huntington disease
B-cell CLL/lymphoma 2	*BCL2*	Other signaling molecule	Inhibition of apoptosis, oncogenesis	Oxidative stress response, apoptosis signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Apoptosis regulator Bcl-X	*BCL2L1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
B-cell lymphoma 3-encoded protein	*Bcl-3*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Inflammation mediated by chemokine and cytokine signaling pathway
BH3-interacting domain death agonist p11	*BID*	N/A	N/A	Apoptosis signaling pathway, FAS signaling pathway
Baculoviral IAP repeat-containing protein 2 (anti-apoptosis factor c-IPA-1)	*BIRC2*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 3	*BIRC3*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	N/A	N/A	Angiogenesis
Caspase 3, apoptosis-related cysteine peptidase	*CASP3*	Cysteine protease	Proteolysis, apoptosis	Huntington disease, FAS signaling pathway, apoptosis signaling pathway
Caspase 8, apoptosis-related cysteine peptidase	*CASP8*	Cysteine protease	Proteolysis, apoptosis	Apoptosis signaling pathway, FAS signaling pathway, Huntington disease

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Caspase 9, apoptosis-related cysteine peptidase	*CASP9*	Cysteine protease	Proteolysis, apoptosis	Angiogenesis, apoptosis signaling pathway, FAS signaling pathway, VEGF signaling pathway, PI3 kinase pathway
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Nonreceptor serine/threonine, protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
G2/mitotic-specific cyclin-B1	*CCNB1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	Cell cycle, p53 pathway
G1/S-specific cyclin-D1	*CCND1*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle, Wnt signaling pathway
G1/S-specific cyclin-D2	*CCND2*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle
Cyclin E1	*CCNE1*	Kinase activator	Cell cycle control, mitosis, cell proliferation and differentiation	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2
G1/S-specific cyclin-E1	*CCNE1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Interleukin-2 receptor alpha chain	*IL2RA/CD25*	Cytokine receptor activity	Immune system process, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Interleukin signaling pathway
Platelet glycoprotein 4	*CD36/FAT*	Receptor activity	Macrophage activation, lipid transport, apoptosis, signal transduction, cell adhesion, lipid metabolic process, signal transduction, cell adhesion, cellular component, morphogenesis	N/A
Early activation antigen CD69	*CD69*	Receptor activity, receptor binding	B-cell-mediated immunity, natural killer cell activation, cellular defense response	Membrane-bound signaling molecule
M-phase inducer phosphatase 1	*CDC25A*	Hydrolase activity, acting on ester bonds, phosphatase activity	Phosphatase activity cell cycle, phosphate metabolic process, protein metabolic process, cell cycle	p53 pathway
Cdc42 effector protein 1	*CDC42EP1*	N/A	N/A	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Cyclin dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	p53 pathway
Cyclin-dependent kinase 2	*CDK2*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	p53 pathway, p53 pathway feedback loops 2
Cyclin-dependent kinase 4	*CDK4*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
Cell division protein kinase 6	*CDK6*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	N/A
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway, p53 pathway feedback loops 2, p53 pathway
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway
CASP8-and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Peptidase activity, protein binding, peptidase inhibitor activity	Apoptosis, protein metabolic process	Apoptosis signaling pathway, FAS signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Inhibitor of NF-κB kinase subunit alpha (IκB kinase)	*CHUK(IKK)*	Kinase activity	Immune response, intracellular signaling cascade, protein metabolic process, signal transduction, response to stimulus	Interleukin signaling pathway, apoptosis signaling pathway, T-cell activation, toll receptor signaling pathway, B-cell activation
SDF-1-α (3–67) (SDF-1)	*CXCL12*	N/A	N/A	Axon guidance-mediated by Slit/Robo
Cytochrome c-1	*CYC1*	Reductase	Oxidative phosphorylation	FAS signaling pathway, ATP synthesis, Huntington disease
Cytochrome P450, family 2, subfamily C, polypeptide 9	*CYP2C9*	Oxygenase	Fatty acid metabolism, steroid metabolism, electron transport	N/A
Cytochrome P450, family 2, subfamily D, polypeptide 6	*CYP2D6*	Oxygenase	Other lipid, fatty acid and steroid metabolism, steroid metabolism, electron transport	Vitamin D metabolism and pathway
Cytochrome P450, family 3, subfamily A, polypeptide 4	*CYP3A4*	Oxygenase	Steroid hormone metabolism, electron transport	N/A
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Serine protease	Proteolysis, cell surface receptor mediated signal transduction, T-cell-mediated immunity	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Epidermal growth factor receptor	*EGFR*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Apoptosis signaling pathway, Alzheimer disease-amyloid secretase pathway, B-cell activation, Ras pathway, interleukin signaling pathway, angiogenesis, T-cell activation, toll receptor signaling pathway, insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade, FGF signaling pathway, Parkinson disease, PDGF signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, VEGF signaling pathway, interferon-gamma signaling pathway, endothelin signaling pathway, angiogenesis, TGF-beta signaling pathway, integrin signalling pathway, EGF receptor signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, interleukin signaling pathway, angiogenesis, VEGF signaling pathway, integrin signalling pathway
Ezrin	*EZR*	Structural constituent of cytoskeleton	Cellular component, morphogenesis	N/A
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	N/A	N/A	Oxidative stress response
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	Receptor binding	Signal transduction, cell-cell signalling, carbohydrate metabolic process, lipid metabolic process, signal transduction, cell-cell signalling, cellular glucose homeostasis	Peptide hormone

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Receptor binding	Female gamete generation, cell surface receptor linked signal transduction, signal transduction, ectoderm development, mesoderm development, skeletal system development, heart development, muscle organ development	TGF-beta signaling pathway
Hypoxia-inducible factor 1*α*	*HIF1A*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, ectoderm development, nervous system development	Hypoxia response via HIF activation, VEGF signaling pathway, angiogenesis
Interferon-*β*	*IFNB1*	Receptor binding	Response to interferon-gamma, induction of apoptosis, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Toll receptor signaling pathway
Interleukin-1*β*	*IL1B*	Receptor binding	Immune response, macrophage activation, cell surface receptor linked signal transduction, cell-cell signalling, signal transduction, cell-cell signalling, response to stimulus	Inflammation mediated by chemokine and cytokine signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Interleukin-6	*IL6*	Receptor binding	Immune system process, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling signal transduction, cell-cell signaling	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Interleukin 8	*IL8*	Chemokine	Cytokine- and chemokine-mediated signaling pathways, calcium-mediated signalling, NF-kappaB cascade, ligand-mediated signalling, T-cell-mediated immunity, macrophage-mediated immunity, granulocyte-mediated immunity, angiogenesis, cell proliferation and differentiation, cell motility	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Receptor activity, cation transmembrane transporter activity, voltage-gated potassium channel activity, cation channel activity, cyclic nucleotide-gated ion channel activity	Cation transport, signal transduction	Ligand-gated ion channel
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	Other receptor	Oogenesis	Alzheimer disease-presenilin pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, Ras pathway, EGF receptor signaling pathway, Parkinson disease, angiogenesis, FGF signaling pathway, FAS signaling pathway, toll receptor signaling pathway, TGF-beta signaling pathway, PDGF signaling pathway, Huntington disease, integrin signalling pathway, T-cell activation, B-cell activation, interferon-gamma signaling pathway, oxidative stress response, apoptosis signaling pathway, integrin signalling pathway
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Peptidase activity	Protein metabolic process	Plasminogen activating cascade, Alzheimer disease-presenilin pathway, plasminogen activating cascade
Matrix metalloproteinase-16	*MMP16*	Peptidase activity	Protein metabolic process	Alzheimer disease-presenilin pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
72 kDa type IV collagenase	*MMP2*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway, plasminogen activating cascade
Myc proto-oncogene protein	*MYC*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle	Oxidative stress response, p53 pathway feedback loops 2, Wnt signaling pathway, interleukin signaling pathway, PDGF signaling pathway
Arylamine *N*-acetyltransferase 1	*NAT*	Acyltransferase activity	Metabolic process	Acetyltransferase
Nuclear factor NF-*κ*B p50 subunit (NF-*κ*B)	*NFKB1*	DNA binding, transcription factor activity	B-cell-mediated immunity, negative regulation of apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, cellular defense response	T-cell activation, B-cell activation, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
NF-κB inhibitor-α	*NFKBIA*	Protein binding	Immune system process, intracellular protein transport apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, response to stress	Apoptosis signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, T-cell activation, B-cell activation
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	N/A
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	Nuclear hormone receptor, transcription factor, nucleic acid binding	N/A	N/A
Poly(ADP-ribose) polymerase family, member 1	*PARP*	Glycosyltransferase	DNA repair, protein ADP-ribosylation, stress response	FAS signaling pathway
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	Serine protease	Proteolysis	N/A
Urokinase-plasminogen activator	*PLAU*	Peptidase activity	Immune system process, signal transduction, protein metabolic process, cell motion, signal transduction, blood coagulation	Blood coagulation, plasminogen activating cascade

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Peroxisome proliferator-activated receptor-γ	*PPARG*	Nuclear hormone receptor, transcription factor, nucleic acid binding	Monosaccharide metabolism, regulation of lipid, fatty acid, and steroid metabolism, mRNA transcription regulation, ligand-mediated signalling, stress response, developmental processes, cell proliferation and differentiation	N/A
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Oxidoreductase activity	Immune system process	Endothelin signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Ras-related C3 botulinum toxin substrate 1	*RAC1*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, integrin signalling pathway, inflammation mediated by chemokine and cytokine signaling pathway, Huntington disease, axon guidance mediated by Slit/Robo, FGF signaling pathway, T-cell activation, axon guidance mediated by netrin, EGF receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase, aAxon guidance mediated by semaphorins, cytoskeletal regulation by Rho GTPase, B-cell activation, Ras pathway
Rho-associated protein kinase 1	*ROCK1/RHO*	Kinase activity	Mitosis, intracellular signaling cascade, cell adhesion, protein metabolic process, cell motion, mitosis, signal transduction, cell adhesion, embryonic development	Inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Transforming protein RhoA	*RHOA*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, angiogenesis, heterotrimeric G-protein signaling pathway-Gq alpha; and Go alpha mediated pathway, axon guidance mediated by semaphorins, inflammation mediated by chemokine and cytokine signaling pathway, integrin signalling pathway, Ras pathway, cytoskeletal regulation by Rho GTPase, PDGF signaling pathway
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, visual perception, sensory perception, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cycle, signal transduction, ectoderm development, mesoderm development, embryonic development, nervous system development	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Runt-related transcription factor 2	*RUNX2*	DNA binding, transcription factor activity	Mesoderm development, skeletal system development, hemopoiesis	N/A
Sucrase-isomaltase (Alpha-glucosidase)	*SI*	Hydrolase activity, hydrolyzing O-glycosyl compounds	Carbohydrate metabolic process, protein metabolic process	N/A
Processed sterol regulatory element-binding protein 2	*SREBP2*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, lipid metabolic process	Basic helix-loop-helix transcription factor
Tumor necrosis factor/tumor necrosis factor-*α*	*TNFA*	Tumor necrosis factor family member	Cytokine- and chemokine-mediated signaling pathways, ligand-mediated signalling, immunity and defense, induction of apoptosis	Wnt signaling pathway, apoptosis signaling pathway
Topoisomerase (DNA) I	*Top1*	DNA topoisomerase	DNA replication, general mRNA transcription activities	DNA replication
Cellular tumor antigen p53	*TP53*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, cell cycle	Apoptosis signaling pathway, Huntington disease, P53 pathway feedback loops 1, p53 pathway, p53 pathway by glucose deprivation, p53 pathway feedback loops 2, Wnt signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Vascular endothelial growth factor A	*VEGFA*	Receptor binding	Immune system process, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell cyclesignal transduction, mesoderm development, angiogenesis, response to stress	Angiogenesis, VEGF signaling pathway
Wee1-like protein kinase	*Wee1*	Kinase activity	Mitosis, protein metabolic process	Protein kinase

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3. Results

3.1. Targets of Berberine

A total of 90 berberine targets were identified in our literature search, as shown in Table 1.

Table 1.

Potential targets of berberine.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
72 kDa type IV collagenase	*MMP2*	HUVECs, tongue cancer SCC-4 cells, gastric carcinoma SNU-5 cells, lung cancer A549 cells, and U-87 glioma cells	Downregulation of MMP2 mRNA and protein expression, reduced MMP-2 levels	Antimetastatic	[18, 69–72]
Acetyl-Coenzyme A carboxylase-*α*	*ACACA*	HepG2 hepatoma cells	Phosphorylation	Antihyperlipidemic	[73]
α-Fetoprotein	*AFP*	HepG2 hepatoma cells	Reduced secretion of alpha fetoprotein	Apoptosis	[74]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Amyloid-β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Neuroglioma H4 cells	Reduces amyloid-β peptide (Aβ) levels via modulation of APP	Alzheimer disease	[75]
Bcl-X	*BCL2L1*	Colonic carcinoma cells, HepG2 cells/A549 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells	JNK/p38 pathway and induction of ROS production. Decreased expression	Cell apoptosis, anticancer, and anti-inflammatory	[19–21, 30, 76, 77]
Arylamine *N*-acetyltransferase 1	*NAT*	Leukemia HL-60 cells, colon tumour cells, brain tumour cells (G95/VGH and GBM 8401)	Decrease in *N*-acetyltransferase (NAT) protein and expression of mRNA	Anticancer (leukemia, colon cancer, brain tumour, etc.)	[78–80]
ATP-binding cassette subfamily G member 2	*ABCG2*	MCF-7 breast cancer cells	Decrease in ABCG2 expression	Breast cancer	[81]
Baculoviral IAP repeat-containing protein 2 (antiapoptosis factor c-IPA-1)	*BIRC2*	Jurkat cells, colonic carcinoma cells (SW620)	Suppresses expression of antiapoptosis factor IAP1	Anticancer	[19, 77]
Baculoviral IAP repeat-containing protein 3	*BIRC3*	Jurkat cells	Suppresses expression of antiapoptosis factor IAP2	Anticancer	[77]
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	Jurkat cells	Suppresses expression of survivin	Anticancer and anti-inflammatory agent	[77]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
B-cell CLL/lymphoma 2	*BCL2*	HepG2 cells, oral squamous cell carcinoma, tongue cancer SCC-4 cells, colonic carcinoma cells, lung cancer cells, breast cancer MCF-7 (estrogen receptor+) cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Bcl-2 Downregulation	Cell apoptosis, cancer, and ER antagonist adjuvant therapy	[19–23, 30, 34, 82, 83]
B-cell lymphoma 3-encoded protein	*Bcl-3*	Gastric carcinoma SNU-5 cells	Downregulation of Bcl-3	Gastric cancer	[24]
Bcl2 antagonist of cell death	*BAD*	Human oral squamous cell carcinoma	Increased expression of proapoptotic BAD protein	Antitumour	[23]
BCL2-associated X protein	*BAX*	Gastric carcinoma SNU-5 cells, prostate carcinoma cells (DU145, PC-3, LNCaP and PWR-1E), leukemia HL-60, tongue cancer SCC-4 cells, lung cancer cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Upregulation of Bax, increased expression. G2/M phase arrest	Cell apoptosis, gastric cancer	[18, 20, 21, 24, 29, 30, 83, 84]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
BH3-interacting domain death agonist p11	*BID*	Colonic carcinoma cells/HepG2 cells	JNK/p38 pathway and induction of ROS production	Induction of apoptosis	[19, 76]
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	Cervical cancerCa Ski cells	Induced expression of GADD153	Cervical cancer	[85]
CASP8 and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Jurkat cells	Suppresses expression of cFLIP	Anticancer and anti-inflammatory	[77]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Caspase 3	*CASP3*	Tongue cancer SCC-4 cells, neuroblastoma (SK-N-SH), glioblastoma T98G cells, gastric carcinoma SNU-5 cells, HL-60 cells, prostate carcinoma cells (DU145, PWR-1E, PC-3 and LNCaP), colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma, promonocytic U937 cells, lung cancer A549, H1301 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase-3, G2/M phase arrest	Anticancer	[18–21, 23–25, 30, 33, 76, 83, 86–89]
Caspase 8	*CASP8*	Tongue cancer SCC-4 cells, colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma	Activated caspase 8	Anticancer	[18, 19, 23, 76]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Caspase 9	*CASP9*	Tongue cancer SCC-4 cells, glioblastoma T98G, oral squamous carcinoma, promonocytic U937 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase 9	Cell apoptosis, anticancer	[18, 21, 23, 30, 33, 83, 86, 87, 89, 90]
Cdc42 effector protein 1	*CDC42EP1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Cdc42)	Cancer metastasis inhibition	[91]
Cell division protein kinase 6	*CDK6*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk6	Cell apoptosis, cancer	[21, 30, 83]
Cellular tumor antigen p53	*TP53*	Gastric carcinoma SNU-5 cells, osteosarcoma	Increased expression of p53 protein, cell cycle arrest at G1G2/M phase arrest	Anticancer (gastric cancer, osteosarcoma)	[24, 92]
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Retinal pigment epithelial cell line	Inhibits CCL2 (MCP-1) expression	Anti-inflammatory	[93]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	Colorectal cancer cells	Induces ATF3 expression	Colorectal cancer	[94]
Cyclin-dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	HL-60 cell, gastric carcinoma SNU-5 cells	Inactivation of Cdc2 (CDK1) or decreased protein expression	Antiproliferative and proapoptotic	[24, 95]
Cyclin E1	*CCNE1*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Anticancer	[25, 83, 90]
Cyclin-dependent kinase 2	*CDK2*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk2	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]
Cyclin-dependent kinase 4	*CDK4*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk4	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Brest cancer MCF-7 (estrogen receptor+) cells, epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of p21	Breast cancer, ER antagonist adjuvant therapy	[21, 30, 82, 83]
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of Cdki proteins	Cell apoptosis, cancer	[21, 30, 83]
Cytochrome c-1	*CYC1*	Tongue cancer SCC-4 cells, colonic carcinoma cells, promyelocytic leukemia HL-60 cells	Release of cytochrome c-1	Anticancer	[18, 19, 84, 86]
CYP2C9	*CYP2C9*	Recombinant CYP	Inhibition of CYP2C9	Drug interactions	[96]
CYP2D6	*CYP2D6*	Recombinant CYP	Inhibition of CYP2D6	Drug interactions	[96]
CYP3A4	*CYP3A4*	Caco-2 cells, patients	CYP3A4 Downregulation and inhibition	Drug interactions	[97, 98]
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Recombinant DPP4	Inhibition of DPP4	—	[99]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Early activation antigen CD69	*CD69*	Human peripheral lymphocytes	Reduced expression of CD69	Immunosuppressive agent	[100]
Epidermal growth factor receptor	*EGFR*	Brest cancer MCF-7 (estrogen receptor+) cells	EGFRdownregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Ezrin	*EZR*	Nasopharyngeal carcinoma 5–8F cells	Ezrin inhibition	Anticancer	[26]
G1/S-specific cyclin-D1	*CCND1*	Giant cell carcinoma cell line, HL-60 cell, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells, neuroblastoma (SK-N-SK), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Inhibits expression of cyclin D1	Antiproliferative and proapoptotic, anticancer, anti-inflammatory	[21, 25, 30, 32, 77, 83, 95]
G1/S-specific cyclin-D2	*CCND2*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin D2	Cell apoptosis, cancer	[21, 30, 83]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
G1/S-specific cyclin-E1	*CCNE1*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Cell apoptosis, cancer	[21, 30, 83]
G2/mitotic-specific cyclin-B1	*CCNB1*	Gastric carcinoma SNU-5 cells	Decreased cyclin B, G2/M phase arrest	Cell apoptosis, anticancer	[24]
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	NCI-H716 cells	Enhanced glucagon-like peptide (GLP)-1	Antidiabetic agent	[42]
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Colorectal cancer cells	Induces NAG-1 expression	Colorectal cancer	[94]
Hypoxia-inducible factor 1*α*	*HIF1A*	HUVECs, HepG2 cells	Prevention and reduction of HIF-1 alpha expression	Tumour angiogenesis	[101, 102]
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Oral cancer cells	Inhibition of Mcl-1 expression	Induced apoptosis	[103]
Inhibitor of NF-*κ*B kinase subunit alpha (I*κ*B kinase)	*CHUK(IKK)*	Jurkat cells	Inhibition of I*κ*B kinase (IKK)	Anticancer and anti-inflammatory agent	[77]
Interferon-*γ*	*IFNB1*	Brest cancer MCF-7 (estrogen receptor+) cells	IFN-beta upregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Interleukin 8	*IL8*	Retinal pigment epithelial cell line	Inhibits IL-8 expression	Anti-inflammatory	[93]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Interleukin-1β	*IL1B*	Fibroblasts (HFL1)	Induces IL-1B productions	Pulmonary inflammation	[104]
Interleukin-2 receptor α-chain	*IL2RA/CD25*	Human peripheral lymphocytes	Reduced expression of CD25	Immunosuppressive agent	[100]
Interleukin-6	*IL6*	Keratinocytes	Reduces and IL-6 expression	Antiskin ageing agent	[105]
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	HepG2 cells	Increased mRNA and protein expression	Hyperlipidemia	[106–108]
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Dermal fibroblasts, U-87 glioma cells	MMP-1 expression decreased	Antiskin ageing agent, anticancer	[70, 109]
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Tongue cancer SCC-4 cells, keratinocytes, gastric carcinoma SNU-5	Inhibition	Anticancer	[34, 70, 105]
Matrix metalloproteinase-16	*MMP16*	Jurkat cells	Suppresses expression of MMP-16	Anticancer and anti-inflammatory agent	[77]
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Peripheral blood monocytes (PBMC)	ERK1 protein expression inhibition	Antiatherosclerotic effects	[110]
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Peripheral blood monocytes (PBMC)	ERK2 protein expression inhibition	Antiatherosclerotic effects	[110]
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Peripheral blood monocytes (PBMC)	Jun N-terminal kinase (JNK) protein expression inhibited at high levels of BBR	Antiatherosclerotic effects	[19, 110]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
M-phase inducer phosphatase 1	*CDC25A*	HL-60 cell	Phosphorylation and degradation of Cdc25A	Antiproliferative and proapoptotic	[95]
Multidrug resistance protein 1 (P-gp, P-gp-170)	*ABCB1*	Tumour cell lines	Significant inhibited P-gp multidrug resistance (MDR) activity	MDR activity reversal	[111]
		HepatomaHepG2 cells	Upregulated multidrug resistance transporter (P-gp-170) expression	Reduced retention of chemotherapeutic agents	[112]
Myc proto-oncogene protein	*MYC*	U-87 glioma cells	Myc level decreased	Malignant glioma and cancer development	[71]
NF-κB inhibitor-α	*NFKBIA*	Lung epithelial cells (A-549)	Inhibition of κB-α phosphorylation and degradation	Pulmonary inflammation	[104]
Nuclear factor NF-κB p50 subunit (NF-κB)	*NFKB1*	Jurkat cells, osteoblastic cells, HepG2 cells	Inhibit NF-κB production and suppress NF-κB	Anticancer and anti-inflammatory agent, alcohol liver disease, osteoclast formation	[77, 113–115]
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	HepG2 cells	Reduced GR levels	Cell growth arrest	[74]
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	Leukemia HL-60 cells	Downregulation of nucleophosmin/B23 and telomerase activity	Cancer	[116]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Peroxisome proliferator-activated receptor-γ	*PPARG*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes, 3T3-L1 preadipocytes	Decreased expression	Antidiabetic	[37, 117]
Platelet glycoprotein 4	*CD36/FAT*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes	Decreased expression	Antidiabetic	[37]
Poly (ADP-ribose) polymerase family, member 1	*PARP*	HepG2 cells/hepatoma cells, colonic carcinoma cells, prostate cancer cells (PC-3), prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Cleavage of poly (ADP-ribose) polymerase. Activation of PARP	Cell apoptosis, Anticancer	[19, 21, 22, 30, 76, 83, 87]
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Leukemic stem cells (LSCs)	Inhibits HERG1 K (+) channels of leukemic cells	Inhibits AML cell migration	[35]
Processed sterol regulatory element-binding protein 2	*SREBP2*	HepG2 cells	Reduction of SREBP2	Hyperlipidemia	[101]
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	HepG2 cells	Suppression of PCSK9 mRNA and protein levels	Hyperlipidemia	[101, 107]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Peripheral blood monocytes (PBMC), oral cancer cell lines OC2 and KB cells, breast cancer MCF-7 (estrogen receptor+) cells, Jurkat cells, colon cancer cells	Decrease of Cox-2 mRNA and protein expression	Antiatherosclerotic effects, anti-inflammatory, anticancer, breast cancer ER antagonist adjuvant therapy, Anticancer	[27, 77, 82, 103, 110, 118]
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	HUVECs	Inhibition of ROS generation	Protects LDL oxidation and prevents ox-LDL-induced cellular dysfunction	[19, 119]
Ras-related C3 botulinum toxin substrate 1	*RAC1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Rac1)	Cancer metastasis inhibition	[91]
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Brest cancer MCF-7 (estrogen receptor+) cells	HER2 downregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Rho-associated protein kinase 1	*ROCK1/RHO*	Nasopharyngeal carcinoma 5–8F cells	Suppression of Rho kinase activity	Anticancer	[91]
Runt-related transcription factor 2	*RUNX2*	Osteoblast cells	Promotes transcriptional activity of Runx2	Osteoblast differentiation and bone formation in osteoporosis	[120]
SDF-1-*α* (3–67) (SDF-1)	*CXCL12*	Acute myeloid leukemia (AML)	Reduces SDF-1 chemokine	Inhibits AML cell migration	[35]
Sucrase-isomaltase (*α*-glucosidase)	*SI*	Caco-2 cells	Inhibit alpha-glucosidase	Antihyperglycaemic	[39]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Topoisomerase (DNA) I	*Top1*	Recombinant human topoisomerase I	Top1 inhibition	Anticancer	[121]
Transcription factor AP-1	*AP-1*	Hepatoma cells, MDA-MB-231 breast cancer cells, giant cell carcinoma cell line, colon cancer cells, U-87 glioma cells, HeLa cells	Inhibition of AP-1 activity, AP-1 DNA suppression	Antitumor activity, Anticancer	[27, 32, 71, 115, 118, 122–124]
Transforming protein RhoA	*RHOA*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (RhoA)	Cancer metastasis inhibition	[91]
Tumor necrosis factor-*α*	*TNFA*	Macrophages, fibroblasts (HFL1)	Inhibition of TNF-*α*	Anti-inflammatory	[104, 125]
Urokinase-plasminogen activator	*PLAU*	Lung cancer A549 cells, tongue cancer SCC-4 cells	Reduced urokinase-plasminogen activator (u-PA)	Antimetastatic, Anticancer	[34, 72]
Vascular endothelial growth factor A	*VEGFA*	HUVECs	Prevention of VEGF expression	Tumour angiogenesis	[102]
Wee1-like protein kinase	*Wee1*	Gastric carcinoma SNU-5 cells	Increased expression of Wee1protein, G2/M phase arrest	Gastric cancer	[24]

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Extensive research has been carried out to study the effects of berberine on cancer cells *in vitro*. This may be related to recent discovery of anti-cancer drugs with natural compound origin, for example, paclitaxel and topotecan.

Various human cancer cell lines were used to demonstrate the anti-cancer effects of berberine *in vitro*. These include cancer cell lines of the tongue, stomach, lung, colon, liver, breast, prostate, nasopharyngeal, neurones, epidermal, and blood [18–28]. Berberine has shown to induce cancer cell death via several mechanisms such as regulation of apoptosis proteins and cell cycle arrest.

Berberine treatment increased the expression of apoptotic cell death proteins, promotes cell cycle arrest, and induces cell death in human cancer cell lines. For instance, in human prostate epithelial cells (PWR-1E), berberine-increased expression of BCL2-associated X protein (Bax) was observed after berberine treatment, inducing cell death and demonstrating pro-apoptotic properties [29]. Similar effects of berberine were observed in prostate carcinoma cells (DU145, PC-3, and LNCaP) [21, 30]. Berberine also increased levels of Bax in promyelocytic leukemia cells [31], gastric carcinoma cells [24], and lung cancer cells [20].

Berberine can also promote cell death by the regulation of antiapoptotic proteins. Decreased expression of antiapoptotic Bcl-2 protein was observed in human oral squamous cell carcinoma after berberine treatment [23]. Studies done in other cancer cell lines such as lung cancer, gastric cancer, and prostate cancer also showed reduced levels of Bcl-2 after berberine treatment [20, 21, 24, 30]. Cell cycle arrest at different phases has also been observed in human cancer cell lines after treatment with berberine. In giant cell carcinoma and prostate carcinoma cells, berberine also decreased G_0/G_1 phase-associated cyclins (D_1, D_2, E, Cdk2, Cdk4, and Cdk6), inducing G_0/G_1 arrest and suppressing cell proliferation [21, 25, 30, 32]. Further, in HepG2 cells, berberine acted on B-cell CLL/lymphoma 2 (BCL2), procaspase-3 and -9, and poly (ADP-ribose) polymerase (PARP), induced cell cycle arrest at G_2/M phase and inhibited cell proliferation [22].

Further, berberine can promote cell death via the regulation of pro- and antiapoptotic proteins. In addition to this, berberine can also promote apoptosis via mitochondrial/caspase pathway. In cancer cell lines (tongue cancer, oral squamous cell carcinoma and prostate epithelial) [18, 23, 29, 33], activation of caspases-3 & -9 promotes G_1 cell cycle arrest in different human cancer cell lines (lung, stomach, and prostate) [20, 21, 24, 30, 33].

Berberine also showed anti-metastatic properties in several cancer cell lines, acting on 72 kDa type IV collagenase (MMP2), Cdc42 effector protein 1 (CDC42EP1), and ras-related C3 botulinum toxin substrate 1 (RAC1), transforming protein RhoA (RHOA) and urokinase-plasminogen activator A (PLAU) [34, 35]. Further, berberine showed antitopoisomerase I properties [36]; this observation can be useful as topoisomerase I is responsible for DNA replication and antitopoisomerase I compounds can be effective in cancer treatments.

In addition to its effects on cancer cells, berberine also acts on molecular targets related to insulin resistance. In free-fatty-acid-induced insulin resistance muscle cells, berberine improves insulin resistance and improves glucose uptake by decreasing PPARγ and FAT/CD36 protein expression [37]. Another study reported increased insulin receptor (InsR) mRNA and protein expression increases insulin sensitivity in liver cells after berberine treatment [38]. In Caco-2 cells, berberine inhibited alpha-glucosidase and disaccharidases activities, leading to reduced glucose levels [39]. In Hep G_2 cells, berberine also improved insulin signal transduction through various mechanisms such as decreased phosphorylation of PERK and eLF2-α, increased phosphorylation of IRS-1 tyrosine and AKT serine [40]. In intestinal NCI-H716 cells, berberine enhanced glucagon-like peptide 1 (GLP-1) release and promotes proglucagon mRNA expression [41]. These results demonstrate that berberine has great potential for insulin resistance treatment and should be explored further in animal and human studies.

3.2. PANTHER Analysis of Berberine Targets

Distribution of berberine therapeutic targets *in vitro* varied in each of these functional classifications. Tables 3, 4, and 5 show various distributions of the most frequent occurring berberine targets *in vitro* based on molecular functions, biological processes, and pathways, respectively.

Table 3.

Distribution of berberine's targets *in vitro* according to molecular functions.

PANTHER molecular function	Numberof targets
Acyltransferase activity	1
Anion channel activity	1
ATPase activity, coupled to transmembrane movement of substances	2
Cation channel activity	1
Cation transmembrane transporter activity	1
Chemokine	1
Cyclic nucleotide-gated ion channel activity	1
Cysteine protease	3
Cytokine receptor activity	1
DNA binding	9
DNA topoisomerase	1
Glycosyltransferase	1
GTPase activity	2
Hydrolase activity, acting on ester bonds	1
Hydrolase activity, hydrolyzing O-glycosyl compounds	1
Kinase activator	1
Kinase activator activity	4
Kinase activity	11
Kinase inhibitor activity	2
Kinase regulator activity	6
Metalloprotease	2
Not classified	10
Non-receptor serine/threonine protein kinase	3
Nuclear hormone receptor	2
Nucleic acid binding	2
Other extracellular matrix	2
Other ligase	1

PANTHER molecular function	Numberof targets
Other receptor	1
Other signaling molecule	3
Other transfer/carrier protein	1
Oxidoreductase activity	1
Oxygenase	3
Peptidase activity	4
Peptidase inhibitor activity	1
Phosphatase activity	1
Protein binding	10
Receptor activity	3
Receptor binding	12
Reductase	1
Serine protease	2
Structural constituent of cytoskeleton	1
Transmembrane transporter activity	2
Transcription factor	2
Transcription factor activity	9
Transmembrane receptor protein kinase activity	3
Transmembrane receptor protein tyrosine kinase activity	3
Tumor necrosis factor family member	1
Voltage-gated potassium channel activity	1

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Table 4.

Distribution of berberine's targets *in vitro* according to biological functions.

PANTHER biological functions	Number of targets
Angiogenesis	2
Anion transport	1

PANTHER biological functions	Number of targets
Apoptosis	6
B-cell-mediated immunity	2
Blood coagulation	1
Calcium-mediated signaling	1
Carbohydrate metabolic process	7
Cation transport	1
Cell adhesion	3
Cell communication	1
Cell cycle	11
Cell cycle control	5
Cell cycle intracellular signaling cascade	1
Cell cycle signal transduction	1
Cell motility	1
Cell motion	10
Cell proliferation and differentiation	3
Cell proliferation and differentiation	1
Cell surface receptor linked signal transduction	14
Cell surface receptor-mediated signal transduction	1
Cell-cell adhesion	3
Cell-cell signaling	9
Cellular component morphogenesis	2
Cellular defense response	4
Cellular glucose homeostasis	1
Coenzyme metabolism	1
Cytokine- and chemokine-mediated signaling pathways	2
Developmental processes	1
DNA repair	1

PANTHER biological functions	Number of targets
DNA replication	2
Dorsal/ventral axis specification	1
Ectoderm development	1
Ectoderm development	8
Electron transport	3
Embryonic development	7
Endocytosis	2
Extracellular transport	2
Fatty acid biosynthesis	1
Fatty acid metabolism	1
Female gamete generation	4
Gamete generation	2
Gametogenesis	1
General mRNA transcription activities	1
Gluconeogenesis	1
Granulocyte-mediated immunity	1
Heart development	1
Hematopoiesis	1
Hemopoiesis	3
Immune response	2
Immune system process	16
Immune system processMitosis	1
Immunity and defense	1
Induction of apoptosis	9
Intracellular protein transport	3
Intracellular signaling cascade	18
Ligand-mediated signaling	3

PANTHER biological functions	Number of targets
Lipid metabolic process	4
Lipid transport	2
Macrophage activation	2
Macrophage-mediated immunity	1
Mesoderm development	12
Metabolic process	1
Mitosis	4
Monosaccharide metabolism	2
mRNA transcription regulation	1
Muscle organ development	1
Not classified	9
Natural killer cell activation	1
Negative regulation of apoptosis	8
Nervous system development	7
Neurological system process	1
NF-κB cascade	1
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	10
Oncogenesis	3
Other intracellular protein traffic	1
Other lipid, fatty acid and steroid metabolism	1
Other signal transduction	1
Oxidative phosphorylation	1
Oxygen and reactive oxygen species metabolic process	1
Phosphatase activity cell cycle	1
Phosphate metabolic process	1
Protein ADP-ribosylation	1
Protein metabolic process	17

PANTHER biological functions	Number of targets
Protein phosphorylation	3
Proteolysis	7
Regulation of lipid, fatty acid and steroid metabolism	1
Response to interferon-*γ*	1
Response to stimulus	2
Response to stress	8
Response to toxin	2
Segment specification	3
Sensory perception	1
Signal transduction	25
Skeletal system development	2
Spermatogenesis	2
Steroid hormone metabolism	1
Steroid metabolism	2
Stress response	2
T-cell-mediated immunity	2
Transcription factor activity immune system process	1
Transport	1
Tumor suppressor	1
Visual perception	1

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Table 5.

Distribution of berberine's targets *in vitro* according to pathway categories.

PANTHER pathway categories	Number of targets
Acetyltransferase	1
Alzheimer disease-amyloid secretase pathway	11
Alzheimer disease-presenilin pathway	14

PANTHER pathway categories	Number of targets
Angiogenesis	11
Apoptosis signaling pathway	21
ATP synthesis	1
ATP-binding cassette (ABC) transporter	2
Axon guidance mediated by netrin	1
Axon guidance mediated by semaphorins	1
Axon guidance mediated by Slit/Robo	4
B-cell activation	7
Basic helix-loop-helix transcription factor	1
Blood coagulation	3
Cadherin signaling pathway	2
Cell cycle	4
Cytoskeletal regulation by Rho GTPase	3
DNA replication	2
EGF receptor signaling pathway	4
Endothelin signaling pathway	2
FAS signaling pathway	13
FGF signaling pathway	4
Heterotrimeric G-protein signaling pathway—Gq alpha- and Go alpha-mediated pathway	1
Huntington disease	9
Hypoxia response via HIF activation	1
Inflammation mediated by chemokine and cytokine signaling pathways	13
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	1
Integrin signalling pathway	6
Interferon-gamma signaling pathway	2
Interleukin signaling pathway	10
Ligand-gated ion channel	1

PANTHER pathway categories	Number of targets
Membrane-bound signaling molecule	1
Pathway unclassified	19
Oxidative stress response	5
p53 pathway	12
p53 pathway by glucose deprivation	1
p53 pathway feedback loops	1
P53 pathway feedback loops 1	1
p53 pathway feedback loops 2	4
Parkinson disease	3
PDGF signaling pathway	6
Peptide hormone	1
PI3 kinase pathway	4
Plasminogen activating cascade	8
Protein kinase	1
Ras Pathway	5
T-cell activation	7
TGF-*β* signaling pathway	4
Toll receptor signaling pathway	9
VEGF signaling pathway	7
Vitamin D metabolism and pathway	1
Wnt signaling pathway	4

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As shown in Table 3, berberine acts on a diverse range of molecular targets *in vitro*. The most common classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Known berberine targets *in vitro* from the receptor binding class include epidermal growth factor receptor (EGFR), vascular endothelial growth factor A (VEGFA), interleukin-1*β* (IL1B) and interleukin-6 (IL6), growth/differentiation factor 15 (NAG-1), and glucagon-like peptide (GLP1).

Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved (Table 4). *In vitro* berberine

targets involved signal transduction include cyclin-dependant kinases (CDK1 and CDK6), inhibitor of nuclear factor kappa-B kinase subunit alpha (CHUK), epidermal growth factor receptor (EFGR), receptor tyrosine-protein kinase (ERBB2), glucagon-like peptide (GCG), growth/differentiation factor 15 (GDF15), interferon beta (IFNB1), interleukins (IL1B, IL2RA, and IL6), potassium voltage-gated channel subfamily H member 2 (KCNH1), mitogen-activated protein kinases (ERK1, ERK2, and MAPK8), nuclear factor-kappa-B p50 subunit (NFKB1), NF-kappa-B inhibitor alpha (NFKB1A), urokinase-plasminogen activator (PLAU), Ras-related C3 botulinum toxin substrate 1 (RAC1), Rho-associated protein kinase 4 (RHO), transforming protein RhoA (RHOA), proto-oncogene tyrosine-protein kinase ROS (ROS1), vascular endothelial growth factor A (VEGFA).

According to the PANTHER Classification System, *in vitro* berberine targets correlate with a mixture of biological pathways, such as Alzheimer disease-presenilin and secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Huntington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways (Table 5).

The targets of berberine distributed across a large number of PANTHER classifications of molecular functions, biological processes, and pathways. This can be an advantage in terms of drug discovery using berberine. Seen that berberine targets are involved in a wide range of molecular activities, in turn, can alter many pathological states of the body. Thus, berberine can be explored for the treatment of different diseases. On the other hand, the nature of multitargeting of berberine lacks in target specificity which can become difficult for drug design. Further, because berberine can have interactions with so many molecular structures and involve in different pathways, much attention must be paid to avoid interactions with other therapeutic drugs.

3.3. Data from *In Vivo* Studies with a Focus on Diabetes Mellitus

In China, Huang Lian (*Rhizoma coptidis*) has been used to treat diabetes for more than 1,400 years [16]. Berberine is one of the main active alkaloids present in *Rhizoma Coptidis* and has shown to have good hypoglycaemic effects *in vitro* [37–39, 42]. Further, the chemical structure of berberine is different from the commonly used other hypoglycaemic agents such as sulphonylureas, biguanides, thiazolidinediones, or acarbose [14]. Thus, it is meaningful to investigate the efficacy and safety of berberine treatments for diabetes mellitus to confirm the possibility of berberine serving as a new class of antidiabetic medications. Extensive research has been done to investigate the hypoglycaemic effects of berberine in animal models. This section will highlight the effects of berberine in diabetic animal studies, focusing on different mechanisms of actions of berberine.

Hyperglycemia is a hallmark metabolic abnormality associated with metabolic diseases such as type 2 diabetes. Berberine has shown to significantly decrease fasting blood glucose levels in diabetic rats (diet or drug induced), this has been observed in a number of studies [43–46]. Berberine can reduce fasting blood glucose level via different mechanisms. For example, Liu et al. [43] reported that berberine reduced fasting blood glucose (FBG) levels by inhibiting intestinal disaccharidases in a concentration-dependent manner. Xia et al. [46] reported berberine reduced fasting glucose level via the inhibition of gluconeogenesis, via decreased *PEPCK* and *G6Pase* genes in the liver, reduced hepatic steatosis, and inhibition of FAS expression.

Current diabetes therapies do not address the key driver of this condition, *β*-cell dysfunction [47, 48], and do not alter the progressive nature of insulin secretory deficit [49]. Berberine increased pancreatic *β*-cell numbers and *β*-cell mass in streptozotocin-induced diabetic rats [41, 50]. It also reversed pathological changes of pancreatic *β*-cells in diabetic rats induced by streptozotocin and diet [51]. Further, in berberine treated diabetic rats, the pancreatic and plasma insulin levels increased after glucose load, reducing blood glucose levels [41, 50]. These observations are significant as berberine may be explored further as an additional therapy to existing antidiabetic drugs to effectively preserve *β*-cell functions, reverse *β*-cell damage, and promote insulin secretion in diabetes patients.

Further to *β*-cell dysfunction and insulin secretory deficit in diabetes, defects in insulin receptor (InsR) expression or function can cause insulin resistance and diabetes mellitus [52]. Thus, regulation of InsR expression may improve insulin resistance in diabetes mellitus. Berberine increases InsR mRNA and protein expression in human liver cells and in animal model in a dose- and time-dependent manner [38]. Berberine upregulates InsR and leads to enhanced insulin signalling pathway, confirming berberine as an insulin sensitiser.

Glucagon-like peptide 1 (GLP-1) is an intestinal peptide hormone released in response to food ingestion [53]. GLP-1 enhances meal-related insulin secretion and promotes glucose tolerance. In streptozotocin-induced rats, berberine enhanced GLP-1 release and promotes proglucagon mRNA expression, increased beta cell mass and pancreas insulin levels after glucose load [41]. This observation was in line with the groups, previous experiments *in vitro*. Lu et al. [50] also reported that berberine increased proglucagon mRNA expression and plasma insulin levels in streptozotocin-induced diabetic rats. The glucagon gene encodes GLP-1 and the increased expression of proglucagon mRNA assists in controlling the blood glucose homeostasis.

Berberine also reduced body weight and caused a significant improvement in glucose tolerance without altering food intake in *db/db* mice [54]. Oral glucose tolerance improvement in diabetic rats after berberine treatment has also been observed in other studies [55, 56].

Long-term hyperglycaemia can lead to increased risk of cardiovascular complications. In hyperglycemia and hypercholesterolemia rats with injured cardiac functions, berberine (15, 30 mg/kg/day, i.g for 6 weeks) increased cardiac output, left ventricular systolic pressure, and $+dp/dt_{max}$ by 64, 16, and 79%, but decreased left ventricular end diastolic pressure and $-dp/dt_{max}$ by 121 and 61% in the rats receiving HSFD/streptozotocin, respectively, when compared with the untreated rats of hyperglycemia and hypercholesterolemia [57]. Berberine caused significant increase in cardiac fatty acid transport protein-1 (159%), fatty acid transport proteins (56%), fatty acid beta-oxidase (52%), and glucose transporter-4. These results demonstrate the cardioprotective functions of berberine in hyperglycemia/hypercholesterolemia through alleviating cardiac lipid accumulation and promoting glucose transport 4 [57]. Another study also showed improved vasorelaxation in impaired aorta in diabetic rats after berberine treatment (100 mg/kg/day, 8 weeks) [45]. Thus, in addition to its hypoglycaemic effects, berberine can also be investigated for cardiomyopathy in diabetes.

Berberine also regulates lipid metabolism which is closely related to diabetes. In rats with induced diabetic hyperlipidemia, berberine (75, 150, 300 mg/kg/day for 16 weeks) effectively reduced liver weight and liver/body weight ratio, levels of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol [58]. In rats with a high fat diet, berberine significantly reduced body weight, alleviated liver steatosis, and improved insulin resistance [59]. This observation indicates that berberine can be an effective treatment for diabetes with obesity.

Clinically, preeminent factors for monitoring glycaemia and evaluating the risks of complications of diabetes include FBG, haemoglobin A_{1c} (HbA_{1c}) [60]. Triglyceride synthesis is closely associated with glucose metabolism so serum triglyceride levels are determined. Clinical studies often measure FBG, HbA_{1c}, and triglyceride levels, along with other factors to study the hypoglycaemic effects of berberine. The efficacies of berberine in type 2 diabetes patients have been reported. Through literature search, key clinical studies on berberine effects on type 2 diabetes patients are summarised.

Zhang et al. [61] conducted a randomized, double-blind, placebo-controlled multicenter trial (n = 116). The authors found that when berberine (1.0 g daily) was administered for 3 months in type 2 diabetes patients with dyslipidemia, the fasting and postload plasma glucose levels decreased from 7.0 ± 0.8 to 5.6 ± 0.9 and from 12.0 ± 2.7 to 8.9 ± 2.8 mM/L, HbA_{1c} from 7.5 ± 1.0% to 6.6 ± 0.7%. Further, in the treatment group, triglyceride levels were reduced from 2.51 ± 2.04 to 1.61 ± 1.0 mM/L, total cholesterol from 5.31 ± 0.98 to 4.35 ± 0.96 mM/L, and LDL-cholesterol from 3.23 ± 0.81 to 2.55 ± 0.77 mM/L. Results from the treatment group was significant compared to the control group. In the

treatment group, patient's body weight was also significantly reduced. Mild-to-moderate constipation was reported in 5 patients from the treatment group and 1 patient from the control group; however, this finding was not statistically significant. No other adverse events were reported. At 3 months, berberine was found to be effective in lowering blood glucose, lipids, body weight, and blood pressure with a good safety profile.

Yin et al. reported a 3-month study comparing berberine to antidiabetic drug metformin (0.5 g t.i.d) [14]. In this study, berberine exhibited identical effect as metformin in the regulation of glucose metabolism, significant decreases in HbA_{1c} (by 2%, $P < 0.01$), FBG (by 3.8 mmol/L; $P < 0.01$), and postprandial blood glucose (PBG) (by 8.8 mmol/L; $P < 0.01$). Further, the regulation of lipid metabolism was better in the berberine group than the metformin group. Triglycerides and total cholesterol levels were significantly lower than in the metformin group ($P < 0.05$). At the same time, the same group of researchers used berberine as a combination therapy to evaluate its additive or synergistic effects on the commonly used hypoglycemic agents, such as sulphonylureas, biguanides, thiazolidinediones, and acarbose. Patients were given 500 mg berberine three times daily for 3 months in addition to their previous treatment. At week 5, berberine significantly ($P < 0.01$) reduced HbA_{1c} (from 8.1% to 7.3%), FBG, PBG, and fasting insulin levels. Blood lipids including triglyceride, total cholesterol, and LDL-C decreased significantly lowered compared to baseline. In both studies, incidences of gastrointestinal adverse events were observed, including diarrhea, constipation, flatulence, and abdominal pain. Interestingly, patients did not suffer from severe gastrointestinal adverse events when berberine was used alone and in combination therapy; adverse effects disappeared after berberine dosage was reduced. No pronounced elevation in liver enzymes or creatinine was observed, suggesting that berberine did not cause damage to the liver or kidneys.

Another clinical study [62] randomly divided 97 type 2 diabetes mellitus patients into berberine treatment (1 g/day) for 2 months, using metformin therapy (1.5 g/day) and rosiglitazone group (4 mg/b.i.d) as reference groups. Blood samples were taking before and after treatments to measure FBG, HbA_{1c}, triglyceride, and serum insulin levels. Compared to values prior to treatment, berberine significantly lowered FBG by 25.9% ($P < 0.001$), HbA_{1c} by 18.1% ($P < 0.00$), and triglycerides by 17.6% ($P < 0.01$). The hypoglycaemic effects of berberine were comparable to metformin and rosiglitazone. Serum insulin level was declined significantly ($P < 0.01$) by 28.2%; this indicates increased insulin sensitivity in peripheral tissues by berberine treatment. Peripheral blood lymphocytes from berberine treated patients were isolated to examine the InsR expression. The surface expression of InsR significantly elevated by 3.6-fold after berberine treatment.

Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. So the effect of berberine was tested in hyperglycaemic patients with hepatitis. Hepatitis B and C patients with hyperglycaemia received berberine at 1 g/day for 2 months. In both diabetic hepatitis B and C patients, berberine significantly reduced FBG and triglyceride levels. Berberine treatment also reduced the elevated alanine transaminase and aspartate aminotransferase levels in these patients. Overall, berberine is safe and effective in hyperglycaemic patients with liver function damage.

Table 6 compares clinical studies of berberine in diabetes patients. Across the studies, berberine has shown to significantly reduce FBG, PBG, and HbA_{1c} levels. Berberine also demonstrated ability to reduce triglyceride and cholesterol levels. Minimal gastrointestinal side effects were shown but no liver or kidney damage was observed. These observations in diabetes patients demonstrate that berberine is a safe and effective antidiabetic agent.

Table 6.

Comparison of clinical studies of berberine in diabetes patients.

Study type	Study subjects	Berberine dosage	Control treatment	Major findings	Side effects	Reference
Randomised, double-blind, placebo-controlled, multiple-center	Type 2 diabetes and dyslipidemia (*n* = 116)	0.5 g, b.i.d for 3 months	Placebo	Significantly reduced fasting and postload plasma glucose, HbA_{1c} Significantly reduced triglyceride, total cholesterol, and LDL-cholesterol	Mild to moderate constipation in 5 patients	[61]
Randomised, blinded, placebo-controlled	Type 2 diabetes (*n* = 36)	0.5 g, t.i.d for 3 months	Metformin (0.5 g t.i.d)	Significantly reduced FBG, PBG, and HbA_{1c} Significantly reduced plasma triglycerides	Transient gastrointestinal adverse effects. No liver or kidney damage	[14]
	Type 2 diabetes poorly controlled (*n* = 48)	0.5 g, t.i.d for 3 months	Existing anti-diabetic treatment	Lowered FBG and PBG Significantly decreased HbA_{1c} Significantly reduced fasting plasma insulin and HOMA-IR		
Randomised	Type 2 diabetes (*n* = 97)	1 g/day for 2 months	Metformin (1.5 g/day); rosiglitazone (4 mg/day)	Significantly reduced FBG, HbA_{1c}, and triglycerides Serum insulin level was declined significantly ($P < 0.01$), increased insulin sensitivity in peripheral tissues Significantly elevated surface expression of InsR by 3.6-fold	No adverse events	[62]
	Type 2 diabetes with chronic hepatitis C virus infection (*n* = 35)	1 g/day for 2 months	N/A	Significantly reduced FBG and triglyceride levels Reduced the elevated ALT and aspartate aminotransferase levels		

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b.i.d: twice daily; t.i.d: three times daily; FBG: fasting blood glucose; HOMA-IR: homeostasis model of assessment—insulin resistance; PBG: postprandial blood glucose.

4. Discussion

The “rule-of-five” analysis by Lipinski et al. [7] shows that poor absorption or permeation of a compound is more likely when there are more than five hydrogen-bond donors; the molecular mass is more than 500 Da; the lipophilicity is high (expressed as *c*Log *P* > 5); the sum of nitrogen and oxygen is more than 10. Specific structural and physiochemical properties, such as “rule-of-five,” are required for clinical drugs to have sufficient levels of efficacy, bioavailability, and safety, which define target sites to which drug-like molecules can bind [4].

Plant compounds exhibit enormous structural diversity and only a small portion of the diversity has been explored for its pharmacological potential [64]. In recent years, herbal compounds have been source of new drugs [64]. Approximately 28% of new molecular entities (NMEs) between 1981 and 2002 were natural products or natural product derived; further to this, 20% of these NMEs were natural product mimics [65]. There are a number of successful plant-derived drugs, especially in anti-cancer treatment. Medicinal herbal compounds have become an important source for the discovery of new drugs. Further, drugs derived from medicinal plants can also be used as drug leads suitable for optimization by medicinal and synthetic chemists [65].

As Chinese herbal medicine becomes increasingly popular in the west, researchers are spending more time looking into mechanisms of actions of crude extracts and herbal compounds such as berberine. In recent years, extensive research has been done to explore the effects of berberine on various cell lines *in vitro*. In cell-based studies, berberine has shown effects on multiple molecular targets and alters various biological pathways. Berberine associates with a range of conditions, particularly diabetes, hyperlipidemia, and cancer. Many *in vitro* studies showed potent anticancer properties of berberine against various cancer cells. This observation is valuable in the search for new anti-cancer therapeutics with potent anti-cancer effects but reduced side effect. So berberine may potentially be developed into an anticancer agent, like other natural compounds (taxol, camptothecin) that have been developed and used as anticancer agents.

Diabetes mellitus is a major health problem around the world and its prevalence is on the rise. Diabetes mellitus drug therapy is limited by availability of effective medications, as existing oral hypoglycaemic agents often have side effects and fails in long-term administration [14]. Berberine has shown significant results in fasting blood glucose levels reduction, increase in insulin sensitivity, and improvement in insulin resistance *in vitro*, in diabetic animal models and in diabetic patients. Further, berberine shows mechanism that current antidiabetic drugs do not have. For instance, berberine has shown effects on pancreatic *β*-cell number and mass improvement [41, 50, 51]. In addition, berberine has a good safety profile and does not show side effects such as hypoglycaemia, weight gain, or liver and kidney damage. Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. Berberine has shown to be effective in the reduction of blood glucose level and is safe in diabetic patients with viral hepatitis [62]. Berberine can therefore be investigated as an effective diabetes therapy with patients with liver function damage. In addition to its hypoglycemic effects in diabetic patients, berberine also reduced triglyceride and cholesterol levels. Abnormalities in lipid metabolism often deteriorate diabetes and cause complications. The regulation of lipid metabolism in diabetes patients by berberine may have clinical significance in managing diabetic patients with hyperlipidemia. Although there are only a small number of clinical studies and evidence is limited, current reports still show a promising future for berberine being developed into a new antidiabetic agent.

In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhea [14, 17]. However, *in vitro* and *in vivo* studies have shown that berberine has potent anti-cancer, antidiabetic, antilipidemic, and anti-inflammatory effects. Therefore, further clinical studies are warranted to investigate the potential of berberine in the application of cancer and diabetes treatments in the future.

Pharmacological activity of CHMs begins with the binding of the active components to their molecular targets. CHMs are considered as typical multitherapeutics that can interact simultaneously with multiple targets. The origins and the

progression of diseases are multifactorial. Complex disorders such as cancer, cardiovascular disease, and depression tend to result from multiple molecular abnormalities, not from a single defect [66]. Biochemical and genetic studies revealed the molecular mechanism that underlie common illnesses [66]. Reports show that targets for neoplasm diseases, circulatory system diseases, infectious diseases, and nervous system and sense organs disorders constitute the largest number of targets [1]. Because drug targets are presented at the molecular level, increased knowledge of herbal targets can facilitate deeper understanding of complex diseases at its fundamental level. In turn, it is likely to determine the optimal molecular targets for therapeutic intervention [6].

Further to assisting the molecular dissection of the mechanism of action of CHMs, knowledge on herbal targets makes it possible to use disease specific targets and design more desirable herbal drugs/formulas with increased specificity and efficacy. Target-oriented synthesis in drug discovery involves in preselected protein targets [67]. Binding of drugs to preselected protein target/s is dependent on which biological pathway the drug is aimed to modulate the target or the diseased pathway(s) [67]. Target and disease specific drug design results in improved efficacy and reduced side effects, especially in high impact diseases that require more effective and more treatment options. However, due to the fact that diseases often involve in multiple molecular abnormalities, diversity-oriented syntheses are used in efforts to identify simultaneously therapeutic protein targets and their small-molecule regulators [67]. Target-oriented drug design allows more focused drug design, which in turn costs less time and money for pharmaceutical companies.

Protein structure of well-validated old and new targets should be able to guide the chemical effort directed at new drugs [68]. Study of various aspects of known targets including molecular mechanism of their binding agents and related adverse effects is useful for finding clues to new target identification [9]. Based on the knowledge of molecular targets and molecular understanding of disease state and using this knowledge will allow some direction in identifying potential targets. Potential herbal targets may come from the same class as confirmed therapeutic targets and have similar physiological functions, or maybe a structure along a biological pathway. Additionally, with increased number of potential targets from ~500 to >5,000, the nature of pharmaceutical research has changed. This increase in numbers has given researchers more opportunities to discover and design new and improved drugs.

Target selection may be one of the most important determinants of attrition and the overall R&D productivity. There are few ways to overcome this challenge and improve the target selection process, in turn, improving R&D productivity. First of all, researchers can discover new target classes. Targets of herbal medicine are becoming a popular resource to find new target classes. In addition, increased understanding of genetic variations/polymorphisms of drug targets or drug metabolising enzymes can assist in target selection and drug metabolism. Further, the use of new technology can help to speed up the early exploratory discovery phase of drug discovery.

In summary, updated knowledge of herbal targets is valuable contribution to complex disease understanding and clinical responses. Further, drug discovery and development from herbal medicines can be supported by new target discovery and target-focused drug design. This will speed up the exploratory phase of drug R&D and benefit the pharmaceutical industry in terms of cost and time.

Authors' Contribution

Xiao-Wu Chen and Yuan Ming Di contributed equally to this work.

Abbreviations

Bax:
BCL2-associated X protein

FBG:
Fasting blood glucose

GLP-1:
Glucagon like peptide 1

HbA_{1c}:
Hemoglobin A_{1c}

InsR:
Insulin receptor

PBG:
Postprandial blood glucose.

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Interaction of Herbal Compounds with Biological Targets: A Case Study with Berberine

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Abstract

Berberine is one of the main alkaloids found in the Chinese herb Huang lian (*Rhizoma Coptidis*), which has been reported to have multiple pharmacological activities. This study aimed to analyze the molecular targets of berberine based on literature data followed by a pathway analysis using the PANTHER program. PANTHER analysis of berberine targets showed that the most classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor-linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved. In addition, berberine was found to interact with a mixture of biological pathways, such as Alzheimer's disease-presenilin and -secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Hungtington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways. We also explored the possible mechanism of action for the anti-diabetic effect of berberine. Further studies are warranted to elucidate the mechanisms of action of berberine using systems biology approach.

1. Introduction

The majority of clinical drugs achieve their effect by binding to a cavity and regulating the cavity, of its protein targets [1]. In general, drugs act on four main types of regulatory proteins that mediate the actions of hormones, neurotransmitters, and autacoids. These four types of regulatory proteins are carriers, proteins, ion channels, and receptors [2]. Certain characteristics are expected for therapeutic targets [3]. A potential target needs only not to be druggable but also linked to disease, most preferably playing critical and inimitable roles in disease state. Binding sites are to have certain structural and physiochemical properties to accommodate high-affinity site-specific binding and subsequent regulation of protein activity by drugs. They are not significantly involved in other important biological processes to avoid potential side effects. Useful information about these targets may be investigated by analysing their sequence properties, protein families, structural folds, biochemical classes, similarity proteins, gene location in the human genome, and associated pathways [4]. This information can be potentially useful in derivation of rule and developing predictive tools in the search for druggable and potential targets [4].

The number of molecular targets acted on by current drug therapy is still in dispute. In 1996, Drews and Ryser identified a total of 483 drug targets addressed by drug therapy [5, 6]. Approximately 45% are cell membrane receptors, 28% are enzymes, and the remaining classes comprise hormones (11%), ion channels (5%), nuclear receptors (2%), and DNA (2%). About 7% of the targets are not known biochemically. Later, Hopkins and Groom challenged this figure and suggested that “rule-of-five” compliant drugs acted primarily through only 120 underlying molecular targets [3, 7]. However, the statistical analysis of disease genes and related proteins suggested that the total number of the estimated potential targets in the human genome ranges from 600 to 1,500 [3]. In the meantime, another report showed the estimated total number of distinct targets is in the range of 1,700–3,000 [8]. Chen et al. reported targets collected in the Therapeutic Target Database [9] is 997 distinct proteins, 1,494 distinct protein subtypes, and 41 nucleic acids, which are only targeted by at least one marketed drug and 1,267 research targets, which are only targeted by investigational agents that are not approved for clinical use at present [4]. Targets for

neoplasm diseases, circulatory system diseases, infectious diseases, and nervous system and sense organs disorders constitute the largest number of targets [1]. An increase in target numbers is made possible by advances in genomics, proteomics, better molecular understanding of diseases, and increased effort in the exploration of new therapeutic targets as well as increased knowledge of unknown or unreported targets of previous existing drugs. An improvement in technology for target identification and validation also contributes greatly.

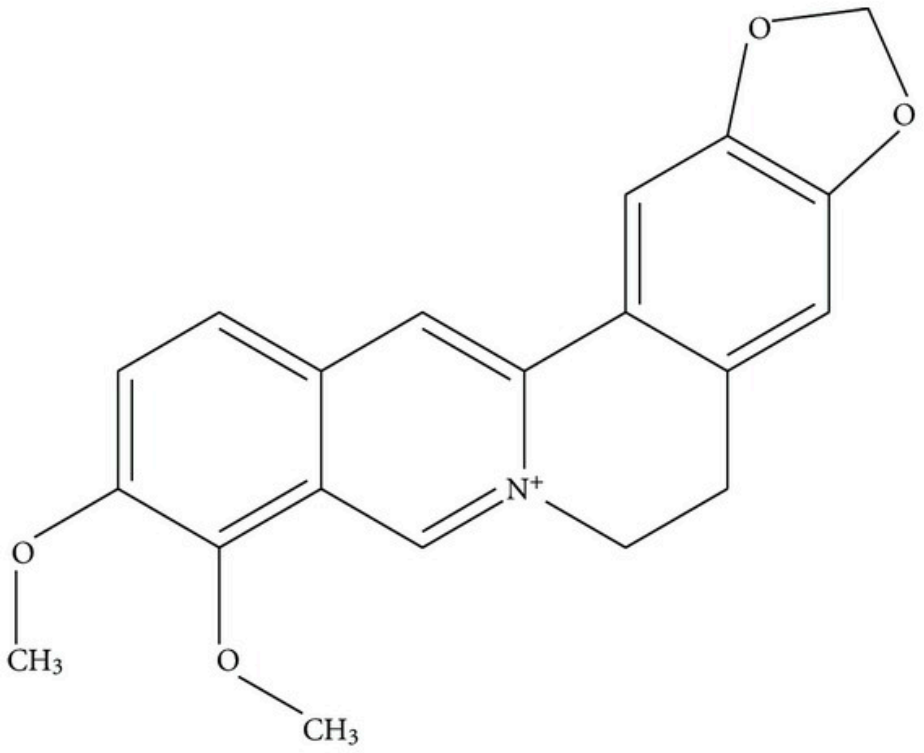
Chinese herbal medicine (CHM) has always been an integral part of traditional Chinese medicine (TCM), which has been practiced in the east for thousands of years. Chinese herbs are usually in the forms of dried whole plants or parts of the plants (roots, leaves, body, etc.); sometimes shells and even minerals are used. Chinese herbs are often used in a compound formula, consisting of several different herbs hosting different roles according to the principle of Jun-Chen-Zuo-Shi described by the ancient Chinese. Each of Jun, Chen, Zuo, and Shi function together to harmonise the body, with Shi (courier) herbs are included in many formulae to ensure that all components in the prescription are well absorbed and to help to deliver or guide them to the target organs [10]. On some level, the guiding function of Shi herbs relates to modern drug delivery techniques, guiding the drug compound to target tissues. In the modern world, complementary medicine has gained vast popularity in the West. There has been increased use of herbal medicine to manage chronic diseases and promote wellbeing, in countries such as Australia, New Zealand, USA, and Europe [11]. Reports show that 18.9% of the American population used natural products in the precedent year [12]. This increase in popularity is closely related to its proven effectiveness in clinical practice over the past centuries. To date, more than 11,000 species of plants are used medicinally and about 300 are commonly used [13].

Despite its widespread use, CHM is associated with high levels of uncertainty. This is mainly due to lack of evidence, base of efficacy, targets, and safety data. During the process of therapeutic drug development, owing to the preselection of targets, researchers have a basic if not full understanding of which molecular structures the drug will react with or which biological pathway in the body it might alter. Knowledge on molecular interactions and modulations of the drug is anticipated and researched on. However, this is not the case for CHM. There is no preselection of molecular targets in the body but CHM has been used for thousands of years and is proven to be effective. The exact mechanism of the herbs actions is yet to be elucidated.

The proven clinical efficacy of some herbal medicines is considered to be due to the interaction of pharmacologically active components from the herbs with molecular targets in the body. Similar to synthetic drugs, active compounds of herbal medicine may bind to and undergo interactions with molecular structures or herbal targets to produce therapeutic or adverse effects. However, there is a lack of understanding of how CHMs exert their biological and clinical effects at a molecular level, which impedes development of CHMs and the incorporation of CHMs into mainstream medicine in the West.

Berberine (Figure 1, molecular formula $C_{20}H_{19}NO_5$ and a molecular weight of 353.36) is an isoquinoline alkaloid found in many medicinal plants [14]. It is a major constituent of many medicinal plants of families Papaveraceae, Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, and Annonaceae [15]. It is present in *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). The berberine alkaloid can be found in the roots, rhizomes, and stem bark of the plants. Berberine is one of the main alkaloids found in the Chinese herb Huang Lian (*Rhizoma coptidis*) [16]. Huang Lian has traditionally been used to treat diarrhoea and diabetes. In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhoea [14, 17]. In recent years, there has been a growing interest in the pharmacological activities of berberine and many studies have been carried out to elucidate the mechanisms of action of berberine. This study aims to review molecular targets of berberine based on *in vitro* studies. Berberine has shown to have good hypoglycaemic effects, so we also reviewed the effects of berberine in animal and human studies, with a focus on diabetes mellitus.

Figure 1.



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Chemical structure of berberine.

2. Methods

2.1. Data Retrieval from the Literature

In vitro studies related to berberine and its targets were searched using Pubmed (from inception to April 2012). Search terms used were a combination of “berberine,” “*in vitro,*” “human cell,” and “mechanism.” Only studies using human cell lines were used to extract current berberine targets. Studies using animal cell lines or berberine derivatives or in a language other than English were excluded. Information extracted from these studies includes molecular targets of berberine (name and gene symbols), cell type, effects of berberine, and possible clinical applications.

2.2. PANTHER Analysis

Using the PANTHER Classification System, *in vitro* berberine targets were analysed using three approaches: molecular function, biological process, and pathway involvement Table 2. PANTHER is a publicly available database that relates protein sequence evolution to evolution of protein functions and biological roles (http://www.pantherdb.org/).

Table 2.

Berberine's target classification based on PANTHER.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Multidrug resistance protein 1 (Pgp, Pgp-170)	*ABCB1*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity	Immune system process, extracellular transport, carbohydrate metabolic process, response to toxin	ATP-binding cassette (ABC) transporter
ATP-binding cassette sub-family G member 2	*ABCG2*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity, anion channel activity	Immune system process, anion transport, lipid transport, oxygen and reactive oxygen species, metabolic process, lipid metabolic process, response to stress	N/A
Acetyl-coenzyme A carboxylase-*α*	*ACACA*	Other ligase	Gluconeogenesis, monosaccharide metabolism, fatty acid biosynthesis, coenzyme metabolism	N/A
α-Fetoprotein	*AFP*	Other transfer/carrier protein	Transport, mesoderm development, oncogenesis	N/A
Transcription factor AP-1	*AP-1*	DNA binding, transcription factor activity	Cell cycle, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle, signal transduction	Toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway, oxidative stress response, angiogenesis, TGF-beta signaling pathway, T-cell activation, B-cell activation, Ras Pathway, FAS signaling pathway, PDGF signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Amyloid-*β* (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Other signaling molecules	Other signal transduction, cell communication, other intracellular protein traffic	Alzheimer disease-amyloid secretase pathway, Alzheimer disease-presenilin pathway, blood coagulation, Alzheimer disease-presenilin pathway, Alzheimer disease-amyloid secretase pathway
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	DNA binding, transcription factor activity	Transcription factor activity, immune system process, neurological system process, induction of apoptosis, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Apoptosis signaling pathway
Bcl2 antagonist of cell death	*BAD*	N/A	N/A	PDGF signaling pathway, apoptosis signaling pathway, angiogenesis, PI3 kinase pathway, VEGF signaling pathway, interleukin signaling pathway
BCL2-associated X protein	*BAX*	Other signaling molecule	Induction of apoptosis, gametogenesis, hematopoiesis, cell cycle control, cell proliferation and differentiation, tumor suppressor	p53 pathway, apoptosis signaling pathway, Huntington disease
B-cell CLL/lymphoma 2	*BCL2*	Other signaling molecule	Inhibition of apoptosis, oncogenesis	Oxidative stress response, apoptosis signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Apoptosis regulator Bcl-X	*BCL2L1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
B-cell lymphoma 3-encoded protein	*Bcl-3*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Inflammation mediated by chemokine and cytokine signaling pathway
BH3-interacting domain death agonist p11	*BID*	N/A	N/A	Apoptosis signaling pathway, FAS signaling pathway
Baculoviral IAP repeat-containing protein 2 (anti-apoptosis factor c-IPA-1)	*BIRC2*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 3	*BIRC3*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	N/A	N/A	Angiogenesis
Caspase 3, apoptosis-related cysteine peptidase	*CASP3*	Cysteine protease	Proteolysis, apoptosis	Huntington disease, FAS signaling pathway, apoptosis signaling pathway
Caspase 8, apoptosis-related cysteine peptidase	*CASP8*	Cysteine protease	Proteolysis, apoptosis	Apoptosis signaling pathway, FAS signaling pathway, Huntington disease

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Caspase 9, apoptosis-related cysteine peptidase	*CASP9*	Cysteine protease	Proteolysis, apoptosis	Angiogenesis, apoptosis signaling pathway, FAS signaling pathway, VEGF signaling pathway, PI3 kinase pathway
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Nonreceptor serine/threonine, protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
G2/mitotic-specific cyclin-B1	*CCNB1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	Cell cycle, p53 pathway
G1/S-specific cyclin-D1	*CCND1*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle, Wnt signaling pathway
G1/S-specific cyclin-D2	*CCND2*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle
Cyclin E1	*CCNE1*	Kinase activator	Cell cycle control, mitosis, cell proliferation and differentiation	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2
G1/S-specific cyclin-E1	*CCNE1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Interleukin-2 receptor alpha chain	*IL2RA/CD25*	Cytokine receptor activity	Immune system process, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Interleukin signaling pathway
Platelet glycoprotein 4	*CD36/FAT*	Receptor activity	Macrophage activation, lipid transport, apoptosis, signal transduction, cell adhesion, lipid metabolic process, signal transduction, cell adhesion, cellular component, morphogenesis	N/A
Early activation antigen CD69	*CD69*	Receptor activity, receptor binding	B-cell-mediated immunity, natural killer cell activation, cellular defense response	Membrane-bound signaling molecule
M-phase inducer phosphatase 1	*CDC25A*	Hydrolase activity, acting on ester bonds, phosphatase activity	Phosphatase activity cell cycle, phosphate metabolic process, protein metabolic process, cell cycle	p53 pathway
Cdc42 effector protein 1	*CDC42EP1*	N/A	N/A	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Cyclin dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	p53 pathway
Cyclin-dependent kinase 2	*CDK2*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	p53 pathway, p53 pathway feedback loops 2
Cyclin-dependent kinase 4	*CDK4*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
Cell division protein kinase 6	*CDK6*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	N/A
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway, p53 pathway feedback loops 2, p53 pathway
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway
CASP8-and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Peptidase activity, protein binding, peptidase inhibitor activity	Apoptosis, protein metabolic process	Apoptosis signaling pathway, FAS signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Inhibitor of NF-κB kinase subunit alpha (IκB kinase)	*CHUK(IKK)*	Kinase activity	Immune response, intracellular signaling cascade, protein metabolic process, signal transduction, response to stimulus	Interleukin signaling pathway, apoptosis signaling pathway, T-cell activation, toll receptor signaling pathway, B-cell activation
SDF-1-α (3–67) (SDF-1)	*CXCL12*	N/A	N/A	Axon guidance-mediated by Slit/Robo
Cytochrome c-1	*CYC1*	Reductase	Oxidative phosphorylation	FAS signaling pathway, ATP synthesis, Huntington disease
Cytochrome P450, family 2, subfamily C, polypeptide 9	*CYP2C9*	Oxygenase	Fatty acid metabolism, steroid metabolism, electron transport	N/A
Cytochrome P450, family 2, subfamily D, polypeptide 6	*CYP2D6*	Oxygenase	Other lipid, fatty acid and steroid metabolism, steroid metabolism, electron transport	Vitamin D metabolism and pathway
Cytochrome P450, family 3, subfamily A, polypeptide 4	*CYP3A4*	Oxygenase	Steroid hormone metabolism, electron transport	N/A
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Serine protease	Proteolysis, cell surface receptor mediated signal transduction, T-cell-mediated immunity	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Epidermal growth factor receptor	*EGFR*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Apoptosis signaling pathway, Alzheimer disease-amyloid secretase pathway, B-cell activation, Ras pathway, interleukin signaling pathway, angiogenesis, T-cell activation, toll receptor signaling pathway, insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade, FGF signaling pathway, Parkinson disease, PDGF signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, VEGF signaling pathway, interferon-gamma signaling pathway, endothelin signaling pathway, angiogenesis, TGF-beta signaling pathway, integrin signalling pathway, EGF receptor signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, interleukin signaling pathway, angiogenesis, VEGF signaling pathway, integrin signalling pathway
Ezrin	*EZR*	Structural constituent of cytoskeleton	Cellular component, morphogenesis	N/A
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	N/A	N/A	Oxidative stress response
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	Receptor binding	Signal transduction, cell-cell signalling, carbohydrate metabolic process, lipid metabolic process, signal transduction, cell-cell signalling, cellular glucose homeostasis	Peptide hormone

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Receptor binding	Female gamete generation, cell surface receptor linked signal transduction, signal transduction, ectoderm development, mesoderm development, skeletal system development, heart development, muscle organ development	TGF-beta signaling pathway
Hypoxia-inducible factor 1*α*	*HIF1A*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, ectoderm development, nervous system development	Hypoxia response via HIF activation, VEGF signaling pathway, angiogenesis
Interferon-*β*	*IFNB1*	Receptor binding	Response to interferon-gamma, induction of apoptosis, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Toll receptor signaling pathway
Interleukin-1*β*	*IL1B*	Receptor binding	Immune response, macrophage activation, cell surface receptor linked signal transduction, cell-cell signalling, signal transduction, cell-cell signalling, response to stimulus	Inflammation mediated by chemokine and cytokine signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Interleukin-6	*IL6*	Receptor binding	Immune system process, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling signal transduction, cell-cell signaling	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Interleukin 8	*IL8*	Chemokine	Cytokine- and chemokine-mediated signaling pathways, calcium-mediated signalling, NF-kappaB cascade, ligand-mediated signalling, T-cell-mediated immunity, macrophage-mediated immunity, granulocyte-mediated immunity, angiogenesis, cell proliferation and differentiation, cell motility	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Receptor activity, cation transmembrane transporter activity, voltage-gated potassium channel activity, cation channel activity, cyclic nucleotide-gated ion channel activity	Cation transport, signal transduction	Ligand-gated ion channel
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	Other receptor	Oogenesis	Alzheimer disease-presenilin pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, Ras pathway, EGF receptor signaling pathway, Parkinson disease, angiogenesis, FGF signaling pathway, FAS signaling pathway, toll receptor signaling pathway, TGF-beta signaling pathway, PDGF signaling pathway, Huntington disease, integrin signalling pathway, T-cell activation, B-cell activation, interferon-gamma signaling pathway, oxidative stress response, apoptosis signaling pathway, integrin signalling pathway
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Peptidase activity	Protein metabolic process	Plasminogen activating cascade, Alzheimer disease-presenilin pathway, plasminogen activating cascade
Matrix metalloproteinase-16	*MMP16*	Peptidase activity	Protein metabolic process	Alzheimer disease-presenilin pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
72 kDa type IV collagenase	*MMP2*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway, plasminogen activating cascade
Myc proto-oncogene protein	*MYC*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle	Oxidative stress response, p53 pathway feedback loops 2, Wnt signaling pathway, interleukin signaling pathway, PDGF signaling pathway
Arylamine *N*-acetyltransferase 1	*NAT*	Acyltransferase activity	Metabolic process	Acetyltransferase
Nuclear factor NF-*κ*B p50 subunit (NF-*κ*B)	*NFKB1*	DNA binding, transcription factor activity	B-cell-mediated immunity, negative regulation of apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, cellular defense response	T-cell activation, B-cell activation, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
NF-κB inhibitor-α	*NFKBIA*	Protein binding	Immune system process, intracellular protein transport apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, response to stress	Apoptosis signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, T-cell activation, B-cell activation
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	N/A
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	Nuclear hormone receptor, transcription factor, nucleic acid binding	N/A	N/A
Poly(ADP-ribose) polymerase family, member 1	*PARP*	Glycosyltransferase	DNA repair, protein ADP-ribosylation, stress response	FAS signaling pathway
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	Serine protease	Proteolysis	N/A
Urokinase-plasminogen activator	*PLAU*	Peptidase activity	Immune system process, signal transduction, protein metabolic process, cell motion, signal transduction, blood coagulation	Blood coagulation, plasminogen activating cascade

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Peroxisome proliferator-activated receptor-γ	*PPARG*	Nuclear hormone receptor, transcription factor, nucleic acid binding	Monosaccharide metabolism, regulation of lipid, fatty acid, and steroid metabolism, mRNA transcription regulation, ligand-mediated signalling, stress response, developmental processes, cell proliferation and differentiation	N/A
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Oxidoreductase activity	Immune system process	Endothelin signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Ras-related C3 botulinum toxin substrate 1	*RAC1*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, integrin signalling pathway, inflammation mediated by chemokine and cytokine signaling pathway, Huntington disease, axon guidance mediated by Slit/Robo, FGF signaling pathway, T-cell activation, axon guidance mediated by netrin, EGF receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase, aAxon guidance mediated by semaphorins, cytoskeletal regulation by Rho GTPase, B-cell activation, Ras pathway
Rho-associated protein kinase 1	*ROCK1/RHO*	Kinase activity	Mitosis, intracellular signaling cascade, cell adhesion, protein metabolic process, cell motion, mitosis, signal transduction, cell adhesion, embryonic development	Inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Transforming protein RhoA	*RHOA*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, angiogenesis, heterotrimeric G-protein signaling pathway-Gq alpha; and Go alpha mediated pathway, axon guidance mediated by semaphorins, inflammation mediated by chemokine and cytokine signaling pathway, integrin signalling pathway, Ras pathway, cytoskeletal regulation by Rho GTPase, PDGF signaling pathway
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, visual perception, sensory perception, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cycle, signal transduction, ectoderm development, mesoderm development, embryonic development, nervous system development	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Runt-related transcription factor 2	*RUNX2*	DNA binding, transcription factor activity	Mesoderm development, skeletal system development, hemopoiesis	N/A
Sucrase-isomaltase (Alpha-glucosidase)	*SI*	Hydrolase activity, hydrolyzing O-glycosyl compounds	Carbohydrate metabolic process, protein metabolic process	N/A
Processed sterol regulatory element-binding protein 2	*SREBP2*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, lipid metabolic process	Basic helix-loop-helix transcription factor
Tumor necrosis factor/tumor necrosis factor-*α*	*TNFA*	Tumor necrosis factor family member	Cytokine- and chemokine-mediated signaling pathways, ligand-mediated signalling, immunity and defense, induction of apoptosis	Wnt signaling pathway, apoptosis signaling pathway
Topoisomerase (DNA) I	*Top1*	DNA topoisomerase	DNA replication, general mRNA transcription activities	DNA replication
Cellular tumor antigen p53	*TP53*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, cell cycle	Apoptosis signaling pathway, Huntington disease, P53 pathway feedback loops 1, p53 pathway, p53 pathway by glucose deprivation, p53 pathway feedback loops 2, Wnt signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Vascular endothelial growth factor A	*VEGFA*	Receptor binding	Immune system process, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell cyclesignal transduction, mesoderm development, angiogenesis, response to stress	Angiogenesis, VEGF signaling pathway
Wee1-like protein kinase	*Wee1*	Kinase activity	Mitosis, protein metabolic process	Protein kinase

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3. Results

3.1. Targets of Berberine

A total of 90 berberine targets were identified in our literature search, as shown in Table 1.

Table 1.

Potential targets of berberine.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
72 kDa type IV collagenase	*MMP2*	HUVECs, tongue cancer SCC-4 cells, gastric carcinoma SNU-5 cells, lung cancer A549 cells, and U-87 glioma cells	Downregulation of MMP2 mRNA and protein expression, reduced MMP-2 levels	Antimetastatic	[18, 69–72]
Acetyl-Coenzyme A carboxylase-*α*	*ACACA*	HepG2 hepatoma cells	Phosphorylation	Antihyperlipidemic	[73]
α-Fetoprotein	*AFP*	HepG2 hepatoma cells	Reduced secretion of alpha fetoprotein	Apoptosis	[74]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Amyloid-β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Neuroglioma H4 cells	Reduces amyloid-β peptide (Aβ) levels via modulation of APP	Alzheimer disease	[75]
Bcl-X	*BCL2L1*	Colonic carcinoma cells, HepG2 cells/A549 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells	JNK/p38 pathway and induction of ROS production. Decreased expression	Cell apoptosis, anticancer, and anti-inflammatory	[19–21, 30, 76, 77]
Arylamine *N*-acetyltransferase 1	*NAT*	Leukemia HL-60 cells, colon tumour cells, brain tumour cells (G95/VGH and GBM 8401)	Decrease in *N*-acetyltransferase (NAT) protein and expression of mRNA	Anticancer (leukemia, colon cancer, brain tumour, etc.)	[78–80]
ATP-binding cassette subfamily G member 2	*ABCG2*	MCF-7 breast cancer cells	Decrease in ABCG2 expression	Breast cancer	[81]
Baculoviral IAP repeat-containing protein 2 (antiapoptosis factor c-IPA-1)	*BIRC2*	Jurkat cells, colonic carcinoma cells (SW620)	Suppresses expression of antiapoptosis factor IAP1	Anticancer	[19, 77]
Baculoviral IAP repeat-containing protein 3	*BIRC3*	Jurkat cells	Suppresses expression of antiapoptosis factor IAP2	Anticancer	[77]
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	Jurkat cells	Suppresses expression of survivin	Anticancer and anti-inflammatory agent	[77]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
B-cell CLL/lymphoma 2	*BCL2*	HepG2 cells, oral squamous cell carcinoma, tongue cancer SCC-4 cells, colonic carcinoma cells, lung cancer cells, breast cancer MCF-7 (estrogen receptor+) cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Bcl-2 Downregulation	Cell apoptosis, cancer, and ER antagonist adjuvant therapy	[19–23, 30, 34, 82, 83]
B-cell lymphoma 3-encoded protein	*Bcl-3*	Gastric carcinoma SNU-5 cells	Downregulation of Bcl-3	Gastric cancer	[24]
Bcl2 antagonist of cell death	*BAD*	Human oral squamous cell carcinoma	Increased expression of proapoptotic BAD protein	Antitumour	[23]
BCL2-associated X protein	*BAX*	Gastric carcinoma SNU-5 cells, prostate carcinoma cells (DU145, PC-3, LNCaP and PWR-1E), leukemia HL-60, tongue cancer SCC-4 cells, lung cancer cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Upregulation of Bax, increased expression. G2/M phase arrest	Cell apoptosis, gastric cancer	[18, 20, 21, 24, 29, 30, 83, 84]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
BH3-interacting domain death agonist p11	*BID*	Colonic carcinoma cells/HepG2 cells	JNK/p38 pathway and induction of ROS production	Induction of apoptosis	[19, 76]
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	Cervical cancerCa Ski cells	Induced expression of GADD153	Cervical cancer	[85]
CASP8 and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Jurkat cells	Suppresses expression of cFLIP	Anticancer and anti-inflammatory	[77]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Caspase 3	*CASP3*	Tongue cancer SCC-4 cells, neuroblastoma (SK-N-SH), glioblastoma T98G cells, gastric carcinoma SNU-5 cells, HL-60 cells, prostate carcinoma cells (DU145, PWR-1E, PC-3 and LNCaP), colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma, promonocytic U937 cells, lung cancer A549, H1301 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase-3, G2/M phase arrest	Anticancer	[18–21, 23–25, 30, 33, 76, 83, 86–89]
Caspase 8	*CASP8*	Tongue cancer SCC-4 cells, colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma	Activated caspase 8	Anticancer	[18, 19, 23, 76]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Caspase 9	*CASP9*	Tongue cancer SCC-4 cells, glioblastoma T98G, oral squamous carcinoma, promonocytic U937 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase 9	Cell apoptosis, anticancer	[18, 21, 23, 30, 33, 83, 86, 87, 89, 90]
Cdc42 effector protein 1	*CDC42EP1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Cdc42)	Cancer metastasis inhibition	[91]
Cell division protein kinase 6	*CDK6*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk6	Cell apoptosis, cancer	[21, 30, 83]
Cellular tumor antigen p53	*TP53*	Gastric carcinoma SNU-5 cells, osteosarcoma	Increased expression of p53 protein, cell cycle arrest at G1G2/M phase arrest	Anticancer (gastric cancer, osteosarcoma)	[24, 92]
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Retinal pigment epithelial cell line	Inhibits CCL2 (MCP-1) expression	Anti-inflammatory	[93]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	Colorectal cancer cells	Induces ATF3 expression	Colorectal cancer	[94]
Cyclin-dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	HL-60 cell, gastric carcinoma SNU-5 cells	Inactivation of Cdc2 (CDK1) or decreased protein expression	Antiproliferative and proapoptotic	[24, 95]
Cyclin E1	*CCNE1*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Anticancer	[25, 83, 90]
Cyclin-dependent kinase 2	*CDK2*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk2	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]
Cyclin-dependent kinase 4	*CDK4*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk4	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Brest cancer MCF-7 (estrogen receptor+) cells, epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of p21	Breast cancer, ER antagonist adjuvant therapy	[21, 30, 82, 83]
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of Cdki proteins	Cell apoptosis, cancer	[21, 30, 83]
Cytochrome c-1	*CYC1*	Tongue cancer SCC-4 cells, colonic carcinoma cells, promyelocytic leukemia HL-60 cells	Release of cytochrome c-1	Anticancer	[18, 19, 84, 86]
CYP2C9	*CYP2C9*	Recombinant CYP	Inhibition of CYP2C9	Drug interactions	[96]
CYP2D6	*CYP2D6*	Recombinant CYP	Inhibition of CYP2D6	Drug interactions	[96]
CYP3A4	*CYP3A4*	Caco-2 cells, patients	CYP3A4 Downregulation and inhibition	Drug interactions	[97, 98]
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Recombinant DPP4	Inhibition of DPP4	—	[99]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Early activation antigen CD69	*CD69*	Human peripheral lymphocytes	Reduced expression of CD69	Immunosuppressive agent	[100]
Epidermal growth factor receptor	*EGFR*	Brest cancer MCF-7 (estrogen receptor+) cells	EGFRdownregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Ezrin	*EZR*	Nasopharyngeal carcinoma 5–8F cells	Ezrin inhibition	Anticancer	[26]
G1/S-specific cyclin-D1	*CCND1*	Giant cell carcinoma cell line, HL-60 cell, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells, neuroblastoma (SK-N-SK), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Inhibits expression of cyclin D1	Antiproliferative and proapoptotic, anticancer, anti-inflammatory	[21, 25, 30, 32, 77, 83, 95]
G1/S-specific cyclin-D2	*CCND2*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin D2	Cell apoptosis, cancer	[21, 30, 83]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
G1/S-specific cyclin-E1	*CCNE1*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Cell apoptosis, cancer	[21, 30, 83]
G2/mitotic-specific cyclin-B1	*CCNB1*	Gastric carcinoma SNU-5 cells	Decreased cyclin B, G2/M phase arrest	Cell apoptosis, anticancer	[24]
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	NCI-H716 cells	Enhanced glucagon-like peptide (GLP)-1	Antidiabetic agent	[42]
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Colorectal cancer cells	Induces NAG-1 expression	Colorectal cancer	[94]
Hypoxia-inducible factor 1*α*	*HIF1A*	HUVECs, HepG2 cells	Prevention and reduction of HIF-1 alpha expression	Tumour angiogenesis	[101, 102]
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Oral cancer cells	Inhibition of Mcl-1 expression	Induced apoptosis	[103]
Inhibitor of NF-*κ*B kinase subunit alpha (I*κ*B kinase)	*CHUK(IKK)*	Jurkat cells	Inhibition of I*κ*B kinase (IKK)	Anticancer and anti-inflammatory agent	[77]
Interferon-*γ*	*IFNB1*	Brest cancer MCF-7 (estrogen receptor+) cells	IFN-beta upregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Interleukin 8	*IL8*	Retinal pigment epithelial cell line	Inhibits IL-8 expression	Anti-inflammatory	[93]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Interleukin-1β	*IL1B*	Fibroblasts (HFL1)	Induces IL-1B productions	Pulmonary inflammation	[104]
Interleukin-2 receptor α-chain	*IL2RA/CD25*	Human peripheral lymphocytes	Reduced expression of CD25	Immunosuppressive agent	[100]
Interleukin-6	*IL6*	Keratinocytes	Reduces and IL-6 expression	Antiskin ageing agent	[105]
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	HepG2 cells	Increased mRNA and protein expression	Hyperlipidemia	[106–108]
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Dermal fibroblasts, U-87 glioma cells	MMP-1 expression decreased	Antiskin ageing agent, anticancer	[70, 109]
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Tongue cancer SCC-4 cells, keratinocytes, gastric carcinoma SNU-5	Inhibition	Anticancer	[34, 70, 105]
Matrix metalloproteinase-16	*MMP16*	Jurkat cells	Suppresses expression of MMP-16	Anticancer and anti-inflammatory agent	[77]
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Peripheral blood monocytes (PBMC)	ERK1 protein expression inhibition	Antiatherosclerotic effects	[110]
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Peripheral blood monocytes (PBMC)	ERK2 protein expression inhibition	Antiatherosclerotic effects	[110]
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Peripheral blood monocytes (PBMC)	Jun N-terminal kinase (JNK) protein expression inhibited at high levels of BBR	Antiatherosclerotic effects	[19, 110]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
M-phase inducer phosphatase 1	*CDC25A*	HL-60 cell	Phosphorylation and degradation of Cdc25A	Antiproliferative and proapoptotic	[95]
Multidrug resistance protein 1 (P-gp, P-gp-170)	*ABCB1*	Tumour cell lines	Significant inhibited P-gp multidrug resistance (MDR) activity	MDR activity reversal	[111]
		HepatomaHepG2 cells	Upregulated multidrug resistance transporter (P-gp-170) expression	Reduced retention of chemotherapeutic agents	[112]
Myc proto-oncogene protein	*MYC*	U-87 glioma cells	Myc level decreased	Malignant glioma and cancer development	[71]
NF-κB inhibitor-α	*NFKBIA*	Lung epithelial cells (A-549)	Inhibition of κB-α phosphorylation and degradation	Pulmonary inflammation	[104]
Nuclear factor NF-κB p50 subunit (NF-κB)	*NFKB1*	Jurkat cells, osteoblastic cells, HepG2 cells	Inhibit NF-κB production and suppress NF-κB	Anticancer and anti-inflammatory agent, alcohol liver disease, osteoclast formation	[77, 113–115]
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	HepG2 cells	Reduced GR levels	Cell growth arrest	[74]
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	Leukemia HL-60 cells	Downregulation of nucleophosmin/B23 and telomerase activity	Cancer	[116]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Peroxisome proliferator-activated receptor-γ	*PPARG*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes, 3T3-L1 preadipocytes	Decreased expression	Antidiabetic	[37, 117]
Platelet glycoprotein 4	*CD36/FAT*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes	Decreased expression	Antidiabetic	[37]
Poly (ADP-ribose) polymerase family, member 1	*PARP*	HepG2 cells/hepatoma cells, colonic carcinoma cells, prostate cancer cells (PC-3), prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Cleavage of poly (ADP-ribose) polymerase. Activation of PARP	Cell apoptosis, Anticancer	[19, 21, 22, 30, 76, 83, 87]
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Leukemic stem cells (LSCs)	Inhibits HERG1 K (+) channels of leukemic cells	Inhibits AML cell migration	[35]
Processed sterol regulatory element-binding protein 2	*SREBP2*	HepG2 cells	Reduction of SREBP2	Hyperlipidemia	[101]
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	HepG2 cells	Suppression of PCSK9 mRNA and protein levels	Hyperlipidemia	[101, 107]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Peripheral blood monocytes (PBMC), oral cancer cell lines OC2 and KB cells, breast cancer MCF-7 (estrogen receptor+) cells, Jurkat cells, colon cancer cells	Decrease of Cox-2 mRNA and protein expression	Antiatherosclerotic effects, anti-inflammatory, anticancer, breast cancer ER antagonist adjuvant therapy, Anticancer	[27, 77, 82, 103, 110, 118]
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	HUVECs	Inhibition of ROS generation	Protects LDL oxidation and prevents ox-LDL-induced cellular dysfunction	[19, 119]
Ras-related C3 botulinum toxin substrate 1	*RAC1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Rac1)	Cancer metastasis inhibition	[91]
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Brest cancer MCF-7 (estrogen receptor+) cells	HER2 downregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Rho-associated protein kinase 1	*ROCK1/RHO*	Nasopharyngeal carcinoma 5–8F cells	Suppression of Rho kinase activity	Anticancer	[91]
Runt-related transcription factor 2	*RUNX2*	Osteoblast cells	Promotes transcriptional activity of Runx2	Osteoblast differentiation and bone formation in osteoporosis	[120]
SDF-1-*α* (3–67) (SDF-1)	*CXCL12*	Acute myeloid leukemia (AML)	Reduces SDF-1 chemokine	Inhibits AML cell migration	[35]
Sucrase-isomaltase (*α*-glucosidase)	*SI*	Caco-2 cells	Inhibit alpha-glucosidase	Antihyperglycaemic	[39]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Topoisomerase (DNA) I	*Top1*	Recombinant human topoisomerase I	Top1 inhibition	Anticancer	[121]
Transcription factor AP-1	*AP-1*	Hepatoma cells, MDA-MB-231 breast cancer cells, giant cell carcinoma cell line, colon cancer cells, U-87 glioma cells, HeLa cells	Inhibition of AP-1 activity, AP-1 DNA suppression	Antitumor activity, Anticancer	[27, 32, 71, 115, 118, 122–124]
Transforming protein RhoA	*RHOA*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (RhoA)	Cancer metastasis inhibition	[91]
Tumor necrosis factor-*α*	*TNFA*	Macrophages, fibroblasts (HFL1)	Inhibition of TNF-*α*	Anti-inflammatory	[104, 125]
Urokinase-plasminogen activator	*PLAU*	Lung cancer A549 cells, tongue cancer SCC-4 cells	Reduced urokinase-plasminogen activator (u-PA)	Antimetastatic, Anticancer	[34, 72]
Vascular endothelial growth factor A	*VEGFA*	HUVECs	Prevention of VEGF expression	Tumour angiogenesis	[102]
Wee1-like protein kinase	*Wee1*	Gastric carcinoma SNU-5 cells	Increased expression of Wee1protein, G2/M phase arrest	Gastric cancer	[24]

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Extensive research has been carried out to study the effects of berberine on cancer cells *in vitro*. This may be related to recent discovery of anti-cancer drugs with natural compound origin, for example, paclitaxel and topotecan.

Various human cancer cell lines were used to demonstrate the anti-cancer effects of berberine *in vitro*. These include cancer cell lines of the tongue, stomach, lung, colon, liver, breast, prostate, nasopharyngeal, neurones, epidermal, and blood [18–28]. Berberine has shown to induce cancer cell death via several mechanisms such as regulation of apoptosis proteins and cell cycle arrest.

Berberine treatment increased the expression of apoptotic cell death proteins, promotes cell cycle arrest, and induces cell death in human cancer cell lines. For instance, in human prostate epithelial cells (PWR-1E), berberine-increased expression of BCL2-associated X protein (Bax) was observed after berberine treatment, inducing cell death and demonstrating pro-apoptotic properties [29]. Similar effects of berberine were observed in prostate carcinoma cells (DU145, PC-3, and LNCaP) [21, 30]. Berberine also increased levels of Bax in promyelocytic leukemia cells [31], gastric carcinoma cells [24], and lung cancer cells [20].

Berberine can also promote cell death by the regulation of antiapoptotic proteins. Decreased expression of antiapoptotic Bcl-2 protein was observed in human oral squamous cell carcinoma after berberine treatment [23]. Studies done in other cancer cell lines such as lung cancer, gastric cancer, and prostate cancer also showed reduced levels of Bcl-2 after berberine treatment [20, 21, 24, 30]. Cell cycle arrest at different phases has also been observed in human cancer cell lines after treatment with berberine. In giant cell carcinoma and prostate carcinoma cells, berberine also decreased G_0/G_1 phase-associated cyclins (D_1, D_2, E, Cdk2, Cdk4, and Cdk6), inducing G_0/G_1 arrest and suppressing cell proliferation [21, 25, 30, 32]. Further, in HepG2 cells, berberine acted on B-cell CLL/lymphoma 2 (BCL2), procaspase-3 and -9, and poly (ADP-ribose) polymerase (PARP), induced cell cycle arrest at G_2/M phase and inhibited cell proliferation [22].

Further, berberine can promote cell death via the regulation of pro- and antiapoptotic proteins. In addition to this, berberine can also promote apoptosis via mitochondrial/caspase pathway. In cancer cell lines (tongue cancer, oral squamous cell carcinoma and prostate epithelial) [18, 23, 29, 33], activation of caspases-3 & -9 promotes G_1 cell cycle arrest in different human cancer cell lines (lung, stomach, and prostate) [20, 21, 24, 30, 33].

Berberine also showed anti-metastatic properties in several cancer cell lines, acting on 72 kDa type IV collagenase (MMP2), Cdc42 effector protein 1 (CDC42EP1), and ras-related C3 botulinum toxin substrate 1 (RAC1), transforming protein RhoA (RHOA) and urokinase-plasminogen activator A (PLAU) [34, 35]. Further, berberine showed antitopoisomerase I properties [36]; this observation can be useful as topoisomerase I is responsible for DNA replication and antitopoisomerase I compounds can be effective in cancer treatments.

In addition to its effects on cancer cells, berberine also acts on molecular targets related to insulin resistance. In free-fatty-acid-induced insulin resistance muscle cells, berberine improves insulin resistance and improves glucose uptake by decreasing PPARγ and FAT/CD36 protein expression [37]. Another study reported increased insulin receptor (InsR) mRNA and protein expression increases insulin sensitivity in liver cells after berberine treatment [38]. In Caco-2 cells, berberine inhibited alpha-glucosidase and disaccharidases activities, leading to reduced glucose levels [39]. In Hep G_2 cells, berberine also improved insulin signal transduction through various mechanisms such as decreased phosphorylation of PERK and eLF2-α, increased phosphorylation of IRS-1 tyrosine and AKT serine [40]. In intestinal NCI-H716 cells, berberine enhanced glucagon-like peptide 1 (GLP-1) release and promotes proglucagon mRNA expression [41]. These results demonstrate that berberine has great potential for insulin resistance treatment and should be explored further in animal and human studies.

3.2. PANTHER Analysis of Berberine Targets

Distribution of berberine therapeutic targets *in vitro* varied in each of these functional classifications. Tables 3, 4, and 5 show various distributions of the most frequent occurring berberine targets *in vitro* based on molecular functions, biological processes, and pathways, respectively.

Table 3.

Distribution of berberine's targets *in vitro* according to molecular functions.

PANTHER molecular function	Numberof targets
Acyltransferase activity	1
Anion channel activity	1
ATPase activity, coupled to transmembrane movement of substances	2
Cation channel activity	1
Cation transmembrane transporter activity	1
Chemokine	1
Cyclic nucleotide-gated ion channel activity	1
Cysteine protease	3
Cytokine receptor activity	1
DNA binding	9
DNA topoisomerase	1
Glycosyltransferase	1
GTPase activity	2
Hydrolase activity, acting on ester bonds	1
Hydrolase activity, hydrolyzing O-glycosyl compounds	1
Kinase activator	1
Kinase activator activity	4
Kinase activity	11
Kinase inhibitor activity	2
Kinase regulator activity	6
Metalloprotease	2
Not classified	10
Non-receptor serine/threonine protein kinase	3
Nuclear hormone receptor	2
Nucleic acid binding	2
Other extracellular matrix	2
Other ligase	1

PANTHER molecular function	Numberof targets
Other receptor	1
Other signaling molecule	3
Other transfer/carrier protein	1
Oxidoreductase activity	1
Oxygenase	3
Peptidase activity	4
Peptidase inhibitor activity	1
Phosphatase activity	1
Protein binding	10
Receptor activity	3
Receptor binding	12
Reductase	1
Serine protease	2
Structural constituent of cytoskeleton	1
Transmembrane transporter activity	2
Transcription factor	2
Transcription factor activity	9
Transmembrane receptor protein kinase activity	3
Transmembrane receptor protein tyrosine kinase activity	3
Tumor necrosis factor family member	1
Voltage-gated potassium channel activity	1

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Table 4.

Distribution of berberine's targets *in vitro* according to biological functions.

PANTHER biological functions	Number of targets
Angiogenesis	2
Anion transport	1

PANTHER biological functions	Number of targets
Apoptosis	6
B-cell-mediated immunity	2
Blood coagulation	1
Calcium-mediated signaling	1
Carbohydrate metabolic process	7
Cation transport	1
Cell adhesion	3
Cell communication	1
Cell cycle	11
Cell cycle control	5
Cell cycle intracellular signaling cascade	1
Cell cycle signal transduction	1
Cell motility	1
Cell motion	10
Cell proliferation and differentiation	3
Cell proliferation and differentiation	1
Cell surface receptor linked signal transduction	14
Cell surface receptor-mediated signal transduction	1
Cell-cell adhesion	3
Cell-cell signaling	9
Cellular component morphogenesis	2
Cellular defense response	4
Cellular glucose homeostasis	1
Coenzyme metabolism	1
Cytokine- and chemokine-mediated signaling pathways	2
Developmental processes	1
DNA repair	1

PANTHER biological functions	Number of targets
DNA replication	2
Dorsal/ventral axis specification	1
Ectoderm development	1
Ectoderm development	8
Electron transport	3
Embryonic development	7
Endocytosis	2
Extracellular transport	2
Fatty acid biosynthesis	1
Fatty acid metabolism	1
Female gamete generation	4
Gamete generation	2
Gametogenesis	1
General mRNA transcription activities	1
Gluconeogenesis	1
Granulocyte-mediated immunity	1
Heart development	1
Hematopoiesis	1
Hemopoiesis	3
Immune response	2
Immune system process	16
Immune system processMitosis	1
Immunity and defense	1
Induction of apoptosis	9
Intracellular protein transport	3
Intracellular signaling cascade	18
Ligand-mediated signaling	3

PANTHER biological functions	Number of targets
Lipid metabolic process	4
Lipid transport	2
Macrophage activation	2
Macrophage-mediated immunity	1
Mesoderm development	12
Metabolic process	1
Mitosis	4
Monosaccharide metabolism	2
mRNA transcription regulation	1
Muscle organ development	1
Not classified	9
Natural killer cell activation	1
Negative regulation of apoptosis	8
Nervous system development	7
Neurological system process	1
NF-κB cascade	1
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	10
Oncogenesis	3
Other intracellular protein traffic	1
Other lipid, fatty acid and steroid metabolism	1
Other signal transduction	1
Oxidative phosphorylation	1
Oxygen and reactive oxygen species metabolic process	1
Phosphatase activity cell cycle	1
Phosphate metabolic process	1
Protein ADP-ribosylation	1
Protein metabolic process	17

PANTHER biological functions	Number of targets
Protein phosphorylation	3
Proteolysis	7
Regulation of lipid, fatty acid and steroid metabolism	1
Response to interferon-*γ*	1
Response to stimulus	2
Response to stress	8
Response to toxin	2
Segment specification	3
Sensory perception	1
Signal transduction	25
Skeletal system development	2
Spermatogenesis	2
Steroid hormone metabolism	1
Steroid metabolism	2
Stress response	2
T-cell-mediated immunity	2
Transcription factor activity immune system process	1
Transport	1
Tumor suppressor	1
Visual perception	1

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Table 5.

Distribution of berberine's targets *in vitro* according to pathway categories.

PANTHER pathway categories	Number of targets
Acetyltransferase	1
Alzheimer disease-amyloid secretase pathway	11
Alzheimer disease-presenilin pathway	14

PANTHER pathway categories	Number of targets
Angiogenesis	11
Apoptosis signaling pathway	21
ATP synthesis	1
ATP-binding cassette (ABC) transporter	2
Axon guidance mediated by netrin	1
Axon guidance mediated by semaphorins	1
Axon guidance mediated by Slit/Robo	4
B-cell activation	7
Basic helix-loop-helix transcription factor	1
Blood coagulation	3
Cadherin signaling pathway	2
Cell cycle	4
Cytoskeletal regulation by Rho GTPase	3
DNA replication	2
EGF receptor signaling pathway	4
Endothelin signaling pathway	2
FAS signaling pathway	13
FGF signaling pathway	4
Heterotrimeric G-protein signaling pathway—Gq alpha- and Go alpha-mediated pathway	1
Huntington disease	9
Hypoxia response via HIF activation	1
Inflammation mediated by chemokine and cytokine signaling pathways	13
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	1
Integrin signalling pathway	6
Interferon-gamma signaling pathway	2
Interleukin signaling pathway	10
Ligand-gated ion channel	1

PANTHER pathway categories	Number of targets
Membrane-bound signaling molecule	1
Pathway unclassified	19
Oxidative stress response	5
p53 pathway	12
p53 pathway by glucose deprivation	1
p53 pathway feedback loops	1
P53 pathway feedback loops 1	1
p53 pathway feedback loops 2	4
Parkinson disease	3
PDGF signaling pathway	6
Peptide hormone	1
PI3 kinase pathway	4
Plasminogen activating cascade	8
Protein kinase	1
Ras Pathway	5
T-cell activation	7
TGF-*β* signaling pathway	4
Toll receptor signaling pathway	9
VEGF signaling pathway	7
Vitamin D metabolism and pathway	1
Wnt signaling pathway	4

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As shown in Table 3, berberine acts on a diverse range of molecular targets *in vitro*. The most common classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Known berberine targets *in vitro* from the receptor binding class include epidermal growth factor receptor (EGFR), vascular endothelial growth factor A (VEGFA), interleukin-1*β* (IL1B) and interleukin-6 (IL6), growth/differentiation factor 15 (NAG-1), and glucagon-like peptide (GLP1).

Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved (Table 4). *In vitro* berberine

targets involved signal transduction include cyclin-dependant kinases (CDK1 and CDK6), inhibitor of nuclear factor kappa-B kinase subunit alpha (CHUK), epidermal growth factor receptor (EFGR), receptor tyrosine-protein kinase (ERBB2), glucagon-like peptide (GCG), growth/differentiation factor 15 (GDF15), interferon beta (IFNB1), interleukins (IL1B, IL2RA, and IL6), potassium voltage-gated channel subfamily H member 2 (KCNH1), mitogen-activated protein kinases (ERK1, ERK2, and MAPK8), nuclear factor-kappa-B p50 subunit (NFKB1), NF-kappa-B inhibitor alpha (NFKB1A), urokinase-plasminogen activator (PLAU), Ras-related C3 botulinum toxin substrate 1 (RAC1), Rho-associated protein kinase 4 (RHO), transforming protein RhoA (RHOA), proto-oncogene tyrosine-protein kinase ROS (ROS1), vascular endothelial growth factor A (VEGFA).

According to the PANTHER Classification System, *in vitro* berberine targets correlate with a mixture of biological pathways, such as Alzheimer disease-presenilin and secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Huntington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways (Table 5).

The targets of berberine distributed across a large number of PANTHER classifications of molecular functions, biological processes, and pathways. This can be an advantage in terms of drug discovery using berberine. Seen that berberine targets are involved in a wide range of molecular activities, in turn, can alter many pathological states of the body. Thus, berberine can be explored for the treatment of different diseases. On the other hand, the nature of multitargeting of berberine lacks in target specificity which can become difficult for drug design. Further, because berberine can have interactions with so many molecular structures and involve in different pathways, much attention must be paid to avoid interactions with other therapeutic drugs.

3.3. Data from *In Vivo* Studies with a Focus on Diabetes Mellitus

In China, Huang Lian (*Rhizoma coptidis*) has been used to treat diabetes for more than 1,400 years [16]. Berberine is one of the main active alkaloids present in *Rhizoma Coptidis* and has shown to have good hypoglycaemic effects *in vitro* [37–39, 42]. Further, the chemical structure of berberine is different from the commonly used other hypoglycaemic agents such as sulphonylureas, biguanides, thiazolidinediones, or acarbose [14]. Thus, it is meaningful to investigate the efficacy and safety of berberine treatments for diabetes mellitus to confirm the possibility of berberine serving as a new class of antidiabetic medications. Extensive research has been done to investigate the hypoglycaemic effects of berberine in animal models. This section will highlight the effects of berberine in diabetic animal studies, focusing on different mechanisms of actions of berberine.

Hyperglycemia is a hallmark metabolic abnormality associated with metabolic diseases such as type 2 diabetes. Berberine has shown to significantly decrease fasting blood glucose levels in diabetic rats (diet or drug induced), this has been observed in a number of studies [43–46]. Berberine can reduce fasting blood glucose level via different mechanisms. For example, Liu et al. [43] reported that berberine reduced fasting blood glucose (FBG) levels by inhibiting intestinal disaccharidases in a concentration-dependent manner. Xia et al. [46] reported berberine reduced fasting glucose level via the inhibition of gluconeogenesis, via decreased *PEPCK* and *G6Pase* genes in the liver, reduced hepatic steatosis, and inhibition of FAS expression.

Current diabetes therapies do not address the key driver of this condition, *β*-cell dysfunction [47, 48], and do not alter the progressive nature of insulin secretory deficit [49]. Berberine increased pancreatic *β*-cell numbers and *β*-cell mass in streptozotocin-induced diabetic rats [41, 50]. It also reversed pathological changes of pancreatic *β*-cells in diabetic rats induced by streptozotocin and diet [51]. Further, in berberine treated diabetic rats, the pancreatic and plasma insulin levels increased after glucose load, reducing blood glucose levels [41, 50]. These observations are significant as berberine may be explored further as an additional therapy to existing antidiabetic drugs to effectively preserve *β*-cell functions, reverse *β*-cell damage, and promote insulin secretion in diabetes patients.

Further to *β*-cell dysfunction and insulin secretory deficit in diabetes, defects in insulin receptor (InsR) expression or function can cause insulin resistance and diabetes mellitus [52]. Thus, regulation of InsR expression may improve insulin resistance in diabetes mellitus. Berberine increases InsR mRNA and protein expression in human liver cells and in animal model in a dose- and time-dependent manner [38]. Berberine upregulates InsR and leads to enhanced insulin signalling pathway, confirming berberine as an insulin sensitiser.

Glucagon-like peptide 1 (GLP-1) is an intestinal peptide hormone released in response to food ingestion [53]. GLP-1 enhances meal-related insulin secretion and promotes glucose tolerance. In streptozotocin-induced rats, berberine enhanced GLP-1 release and promotes proglucagon mRNA expression, increased beta cell mass and pancreas insulin levels after glucose load [41]. This observation was in line with the groups, previous experiments *in vitro*. Lu et al. [50] also reported that berberine increased proglucagon mRNA expression and plasma insulin levels in streptozotocin-induced diabetic rats. The glucagon gene encodes GLP-1 and the increased expression of proglucagon mRNA assists in controlling the blood glucose homeostasis.

Berberine also reduced body weight and caused a significant improvement in glucose tolerance without altering food intake in *db/db* mice [54]. Oral glucose tolerance improvement in diabetic rats after berberine treatment has also been observed in other studies [55, 56].

Long-term hyperglycaemia can lead to increased risk of cardiovascular complications. In hyperglycemia and hypercholesterolemia rats with injured cardiac functions, berberine (15, 30 mg/kg/day, i.g for 6 weeks) increased cardiac output, left ventricular systolic pressure, and $+dp/dt_{max}$ by 64, 16, and 79%, but decreased left ventricular end diastolic pressure and $-dp/dt_{max}$ by 121 and 61% in the rats receiving HSFD/streptozotocin, respectively, when compared with the untreated rats of hyperglycemia and hypercholesterolemia [57]. Berberine caused significant increase in cardiac fatty acid transport protein-1 (159%), fatty acid transport proteins (56%), fatty acid beta-oxidase (52%), and glucose transporter-4. These results demonstrate the cardioprotective functions of berberine in hyperglycemia/hypercholesterolemia through alleviating cardiac lipid accumulation and promoting glucose transport 4 [57]. Another study also showed improved vasorelaxation in impaired aorta in diabetic rats after berberine treatment (100 mg/kg/day, 8 weeks) [45]. Thus, in addition to its hypoglycaemic effects, berberine can also be investigated for cardiomyopathy in diabetes.

Berberine also regulates lipid metabolism which is closely related to diabetes. In rats with induced diabetic hyperlipidemia, berberine (75, 150, 300 mg/kg/day for 16 weeks) effectively reduced liver weight and liver/body weight ratio, levels of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol [58]. In rats with a high fat diet, berberine significantly reduced body weight, alleviated liver steatosis, and improved insulin resistance [59]. This observation indicates that berberine can be an effective treatment for diabetes with obesity.

Clinically, preeminent factors for monitoring glycaemia and evaluating the risks of complications of diabetes include FBG, haemoglobin A_{1c} (HbA_{1c}) [60]. Triglyceride synthesis is closely associated with glucose metabolism so serum triglyceride levels are determined. Clinical studies often measure FBG, HbA_{1c}, and triglyceride levels, along with other factors to study the hypoglycaemic effects of berberine. The efficacies of berberine in type 2 diabetes patients have been reported. Through literature search, key clinical studies on berberine effects on type 2 diabetes patients are summarised.

Zhang et al. [61] conducted a randomized, double-blind, placebo-controlled multicenter trial (n = 116). The authors found that when berberine (1.0 g daily) was administered for 3 months in type 2 diabetes patients with dyslipidemia, the fasting and postload plasma glucose levels decreased from 7.0 ± 0.8 to 5.6 ± 0.9 and from 12.0 ± 2.7 to 8.9 ± 2.8 mM/L, HbA_{1c} from 7.5 ± 1.0% to 6.6 ± 0.7%. Further, in the treatment group, triglyceride levels were reduced from 2.51 ± 2.04 to 1.61 ± 1.0 mM/L, total cholesterol from 5.31 ± 0.98 to 4.35 ± 0.96 mM/L, and LDL-cholesterol from 3.23 ± 0.81 to 2.55 ± 0.77 mM/L. Results from the treatment group was significant compared to the control group. In the

treatment group, patient's body weight was also significantly reduced. Mild-to-moderate constipation was reported in 5 patients from the treatment group and 1 patient from the control group; however, this finding was not statistically significant. No other adverse events were reported. At 3 months, berberine was found to be effective in lowering blood glucose, lipids, body weight, and blood pressure with a good safety profile.

Yin et al. reported a 3-month study comparing berberine to antidiabetic drug metformin (0.5 g t.i.d) [14]. In this study, berberine exhibited identical effect as metformin in the regulation of glucose metabolism, significant decreases in HbA_{1c} (by 2%, $P < 0.01$), FBG (by 3.8 mmol/L; $P < 0.01$), and postprandial blood glucose (PBG) (by 8.8 mmol/L; $P < 0.01$). Further, the regulation of lipid metabolism was better in the berberine group than the metformin group. Triglycerides and total cholesterol levels were significantly lower than in the metformin group ($P < 0.05$). At the same time, the same group of researchers used berberine as a combination therapy to evaluate its additive or synergistic effects on the commonly used hypoglycemic agents, such as sulphonylureas, biguanides, thiazolidinediones, and acarbose. Patients were given 500 mg berberine three times daily for 3 months in addition to their previous treatment. At week 5, berberine significantly ($P < 0.01$) reduced HbA_{1c} (from 8.1% to 7.3%), FBG, PBG, and fasting insulin levels. Blood lipids including triglyceride, total cholesterol, and LDL-C decreased significantly lowered compared to baseline. In both studies, incidences of gastrointestinal adverse events were observed, including diarrhea, constipation, flatulence, and abdominal pain. Interestingly, patients did not suffer from severe gastrointestinal adverse events when berberine was used alone and in combination therapy; adverse effects disappeared after berberine dosage was reduced. No pronounced elevation in liver enzymes or creatinine was observed, suggesting that berberine did not cause damage to the liver or kidneys.

Another clinical study [62] randomly divided 97 type 2 diabetes mellitus patients into berberine treatment (1 g/day) for 2 months, using metformin therapy (1.5 g/day) and rosiglitazone group (4 mg/b.i.d) as reference groups. Blood samples were taking before and after treatments to measure FBG, HbA_{1c}, triglyceride, and serum insulin levels. Compared to values prior to treatment, berberine significantly lowered FBG by 25.9% ($P < 0.001$), HbA_{1c} by 18.1% ($P < 0.00$), and triglycerides by 17.6% ($P < 0.01$). The hypoglycaemic effects of berberine were comparable to metformin and rosiglitazone. Serum insulin level was declined significantly ($P < 0.01$) by 28.2%; this indicates increased insulin sensitivity in peripheral tissues by berberine treatment. Peripheral blood lymphocytes from berberine treated patients were isolated to examine the InsR expression. The surface expression of InsR significantly elevated by 3.6-fold after berberine treatment.

Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. So the effect of berberine was tested in hyperglycaemic patients with hepatitis. Hepatitis B and C patients with hyperglycaemia received berberine at 1 g/day for 2 months. In both diabetic hepatitis B and C patients, berberine significantly reduced FBG and triglyceride levels. Berberine treatment also reduced the elevated alanine transaminase and aspartate aminotransferase levels in these patients. Overall, berberine is safe and effective in hyperglycaemic patients with liver function damage.

Table 6 compares clinical studies of berberine in diabetes patients. Across the studies, berberine has shown to significantly reduce FBG, PBG, and HbA_{1c} levels. Berberine also demonstrated ability to reduce triglyceride and cholesterol levels. Minimal gastrointestinal side effects were shown but no liver or kidney damage was observed. These observations in diabetes patients demonstrate that berberine is a safe and effective antidiabetic agent.

Table 6.

Comparison of clinical studies of berberine in diabetes patients.

Study type	Study subjects	Berberine dosage	Control treatment	Major findings	Side effects	Reference
Randomised, double-blind, placebo-controlled, multiple-center	Type 2 diabetes and dyslipidemia (*n* = 116)	0.5 g, b.i.d for 3 months	Placebo	Significantly reduced fasting and postload plasma glucose, HbA_{1c} Significantly reduced triglyceride, total cholesterol, and LDL-cholesterol	Mild to moderate constipation in 5 patients	[61]
Randomised, blinded, placebo-controlled	Type 2 diabetes (*n* = 36)	0.5 g, t.i.d for 3 months	Metformin (0.5 g t.i.d)	Significantly reduced FBG, PBG, and HbA_{1c} Significantly reduced plasma triglycerides	Transient gastrointestinal adverse effects. No liver or kidney damage	[14]
	Type 2 diabetes poorly controlled (*n* = 48)	0.5 g, t.i.d for 3 months	Existing anti-diabetic treatment	Lowered FBG and PBG Significantly decreased HbA_{1c} Significantly reduced fasting plasma insulin and HOMA-IR		
Randomised	Type 2 diabetes (*n* = 97)	1 g/day for 2 months	Metformin (1.5 g/day); rosiglitazone (4 mg/day)	Significantly reduced FBG, HbA_{1c}, and triglycerides Serum insulin level was declined significantly ($P < 0.01$), increased insulin sensitivity in peripheral tissues Significantly elevated surface expression of InsR by 3.6-fold	No adverse events	[62]
	Type 2 diabetes with chronic hepatitis C virus infection (*n* = 35)	1 g/day for 2 months	N/A	Significantly reduced FBG and triglyceride levels Reduced the elevated ALT and aspartate aminotransferase levels		

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b.i.d: twice daily; t.i.d: three times daily; FBG: fasting blood glucose; HOMA-IR: homeostasis model of assessment—insulin resistance; PBG: postprandial blood glucose.

4. Discussion

The “rule-of-five” analysis by Lipinski et al. [7] shows that poor absorption or permeation of a compound is more likely when there are more than five hydrogen-bond donors; the molecular mass is more than 500 Da; the lipophilicity is high (expressed as *c*Log *P* > 5); the sum of nitrogen and oxygen is more than 10. Specific structural and physiochemical properties, such as “rule-of-five,” are required for clinical drugs to have sufficient levels of efficacy, bioavailability, and safety, which define target sites to which drug-like molecules can bind [4].

Plant compounds exhibit enormous structural diversity and only a small portion of the diversity has been explored for its pharmacological potential [64]. In recent years, herbal compounds have been source of new drugs [64]. Approximately 28% of new molecular entities (NMEs) between 1981 and 2002 were natural products or natural product derived; further to this, 20% of these NMEs were natural product mimics [65]. There are a number of successful plant-derived drugs, especially in anti-cancer treatment. Medicinal herbal compounds have become an important source for the discovery of new drugs. Further, drugs derived from medicinal plants can also be used as drug leads suitable for optimization by medicinal and synthetic chemists [65].

As Chinese herbal medicine becomes increasingly popular in the west, researchers are spending more time looking into mechanisms of actions of crude extracts and herbal compounds such as berberine. In recent years, extensive research has been done to explore the effects of berberine on various cell lines *in vitro*. In cell-based studies, berberine has shown effects on multiple molecular targets and alters various biological pathways. Berberine associates with a range of conditions, particularly diabetes, hyperlipidemia, and cancer. Many *in vitro* studies showed potent anticancer properties of berberine against various cancer cells. This observation is valuable in the search for new anti-cancer therapeutics with potent anti-cancer effects but reduced side effect. So berberine may potentially be developed into an anticancer agent, like other natural compounds (taxol, camptothecin) that have been developed and used as anticancer agents.

Diabetes mellitus is a major health problem around the world and its prevalence is on the rise. Diabetes mellitus drug therapy is limited by availability of effective medications, as existing oral hypoglycaemic agents often have side effects and fails in long-term administration [14]. Berberine has shown significant results in fasting blood glucose levels reduction, increase in insulin sensitivity, and improvement in insulin resistance *in vitro*, in diabetic animal models and in diabetic patients. Further, berberine shows mechanism that current antidiabetic drugs do not have. For instance, berberine has shown effects on pancreatic *β*-cell number and mass improvement [41, 50, 51]. In addition, berberine has a good safety profile and does not show side effects such as hypoglycaemia, weight gain, or liver and kidney damage. Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. Berberine has shown to be effective in the reduction of blood glucose level and is safe in diabetic patients with viral hepatitis [62]. Berberine can therefore be investigated as an effective diabetes therapy with patients with liver function damage. In addition to its hypoglycemic effects in diabetic patients, berberine also reduced triglyceride and cholesterol levels. Abnormalities in lipid metabolism often deteriorate diabetes and cause complications. The regulation of lipid metabolism in diabetes patients by berberine may have clinical significance in managing diabetic patients with hyperlipidemia. Although there are only a small number of clinical studies and evidence is limited, current reports still show a promising future for berberine being developed into a new antidiabetic agent.

In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhea [14, 17]. However, *in vitro* and *in vivo* studies have shown that berberine has potent anti-cancer, antidiabetic, antilipidemic, and anti-inflammatory effects. Therefore, further clinical studies are warranted to investigate the potential of berberine in the application of cancer and diabetes treatments in the future.

Pharmacological activity of CHMs begins with the binding of the active components to their molecular targets. CHMs are considered as typical multitherapeutics that can interact simultaneously with multiple targets. The origins and the

progression of diseases are multifactorial. Complex disorders such as cancer, cardiovascular disease, and depression tend to result from multiple molecular abnormalities, not from a single defect [66]. Biochemical and genetic studies revealed the molecular mechanism that underlie common illnesses [66]. Reports show that targets for neoplasm diseases, circulatory system diseases, infectious diseases, and nervous system and sense organs disorders constitute the largest number of targets [1]. Because drug targets are presented at the molecular level, increased knowledge of herbal targets can facilitate deeper understanding of complex diseases at its fundamental level. In turn, it is likely to determine the optimal molecular targets for therapeutic intervention [6].

Further to assisting the molecular dissection of the mechanism of action of CHMs, knowledge on herbal targets makes it possible to use disease specific targets and design more desirable herbal drugs/formulas with increased specificity and efficacy. Target-oriented synthesis in drug discovery involves in preselected protein targets [67]. Binding of drugs to preselected protein target/s is dependent on which biological pathway the drug is aimed to modulate the target or the diseased pathway(s) [67]. Target and disease specific drug design results in improved efficacy and reduced side effects, especially in high impact diseases that require more effective and more treatment options. However, due to the fact that diseases often involve in multiple molecular abnormalities, diversity-oriented syntheses are used in efforts to identify simultaneously therapeutic protein targets and their small-molecule regulators [67]. Target-oriented drug design allows more focused drug design, which in turn costs less time and money for pharmaceutical companies.

Protein structure of well-validated old and new targets should be able to guide the chemical effort directed at new drugs [68]. Study of various aspects of known targets including molecular mechanism of their binding agents and related adverse effects is useful for finding clues to new target identification [9]. Based on the knowledge of molecular targets and molecular understanding of disease state and using this knowledge will allow some direction in identifying potential targets. Potential herbal targets may come from the same class as confirmed therapeutic targets and have similar physiological functions, or maybe a structure along a biological pathway. Additionally, with increased number of potential targets from ~500 to >5,000, the nature of pharmaceutical research has changed. This increase in numbers has given researchers more opportunities to discover and design new and improved drugs.

Target selection may be one of the most important determinants of attrition and the overall R&D productivity. There are few ways to overcome this challenge and improve the target selection process, in turn, improving R&D productivity. First of all, researchers can discover new target classes. Targets of herbal medicine are becoming a popular resource to find new target classes. In addition, increased understanding of genetic variations/polymorphisms of drug targets or drug metabolising enzymes can assist in target selection and drug metabolism. Further, the use of new technology can help to speed up the early exploratory discovery phase of drug discovery.

In summary, updated knowledge of herbal targets is valuable contribution to complex disease understanding and clinical responses. Further, drug discovery and development from herbal medicines can be supported by new target discovery and target-focused drug design. This will speed up the exploratory phase of drug R&D and benefit the pharmaceutical industry in terms of cost and time.

Authors' Contribution

Xiao-Wu Chen and Yuan Ming Di contributed equally to this work.

Abbreviations

Bax:
BCL2-associated X protein

FBG:
Fasting blood glucose

GLP-1:
Glucagon like peptide 1

HbA_{1c}:
Hemoglobin A_{1c}

InsR:
Insulin receptor

PBG:
Postprandial blood glucose.

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Interaction of Herbal Compounds with Biological Targets: A Case Study with Berberine

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Abstract

Berberine is one of the main alkaloids found in the Chinese herb Huang lian (*Rhizoma Coptidis*), which has been reported to have multiple pharmacological activities. This study aimed to analyze the molecular targets of berberine based on literature data followed by a pathway analysis using the PANTHER program. PANTHER analysis of berberine targets showed that the most classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor-linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved. In addition, berberine was found to interact with a mixture of biological pathways, such as Alzheimer's disease-presenilin and -secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Hungtington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways. We also explored the possible mechanism of action for the anti-diabetic effect of berberine. Further studies are warranted to elucidate the mechanisms of action of berberine using systems biology approach.

1. Introduction

The majority of clinical drugs achieve their effect by binding to a cavity and regulating the cavity, of its protein targets [1]. In general, drugs act on four main types of regulatory proteins that mediate the actions of hormones, neurotransmitters, and autacoids. These four types of regulatory proteins are carriers, proteins, ion channels, and receptors [2]. Certain characteristics are expected for therapeutic targets [3]. A potential target needs only not to be druggable but also linked to disease, most preferably playing critical and inimitable roles in disease state. Binding sites are to have certain structural and physiochemical properties to accommodate high-affinity site-specific binding and subsequent regulation of protein activity by drugs. They are not significantly involved in other important biological processes to avoid potential side effects. Useful information about these targets may be investigated by analysing their sequence properties, protein families, structural folds, biochemical classes, similarity proteins, gene location in the human genome, and associated pathways [4]. This information can be potentially useful in derivation of rule and developing predictive tools in the search for druggable and potential targets [4].

The number of molecular targets acted on by current drug therapy is still in dispute. In 1996, Drews and Ryser identified a total of 483 drug targets addressed by drug therapy [5, 6]. Approximately 45% are cell membrane receptors, 28% are enzymes, and the remaining classes comprise hormones (11%), ion channels (5%), nuclear receptors (2%), and DNA (2%). About 7% of the targets are not known biochemically. Later, Hopkins and Groom challenged this figure and suggested that “rule-of-five” compliant drugs acted primarily through only 120 underlying molecular targets [3, 7]. However, the statistical analysis of disease genes and related proteins suggested that the total number of the estimated potential targets in the human genome ranges from 600 to 1,500 [3]. In the meantime, another report showed the estimated total number of distinct targets is in the range of 1,700–3,000 [8]. Chen et al. reported targets collected in the Therapeutic Target Database [9] is 997 distinct proteins, 1,494 distinct protein subtypes, and 41 nucleic acids, which are only targeted by at least one marketed drug and 1,267 research targets, which are only targeted by investigational agents that are not approved for clinical use at present [4]. Targets for

neoplasm diseases, circulatory system diseases, infectious diseases, and nervous system and sense organs disorders constitute the largest number of targets [1]. An increase in target numbers is made possible by advances in genomics, proteomics, better molecular understanding of diseases, and increased effort in the exploration of new therapeutic targets as well as increased knowledge of unknown or unreported targets of previous existing drugs. An improvement in technology for target identification and validation also contributes greatly.

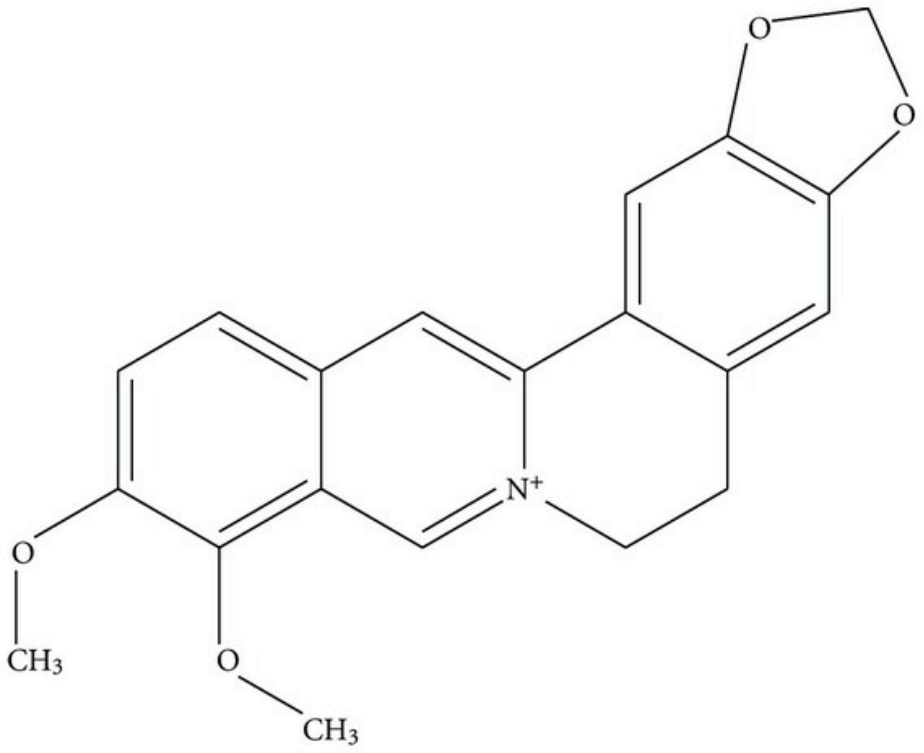
Chinese herbal medicine (CHM) has always been an integral part of traditional Chinese medicine (TCM), which has been practiced in the east for thousands of years. Chinese herbs are usually in the forms of dried whole plants or parts of the plants (roots, leaves, body, etc.); sometimes shells and even minerals are used. Chinese herbs are often used in a compound formula, consisting of several different herbs hosting different roles according to the principle of Jun-Chen-Zuo-Shi described by the ancient Chinese. Each of Jun, Chen, Zuo, and Shi function together to harmonise the body, with Shi (courier) herbs are included in many formulae to ensure that all components in the prescription are well absorbed and to help to deliver or guide them to the target organs [10]. On some level, the guiding function of Shi herbs relates to modern drug delivery techniques, guiding the drug compound to target tissues. In the modern world, complementary medicine has gained vast popularity in the West. There has been increased use of herbal medicine to manage chronic diseases and promote wellbeing, in countries such as Australia, New Zealand, USA, and Europe [11]. Reports show that 18.9% of the American population used natural products in the precedent year [12]. This increase in popularity is closely related to its proven effectiveness in clinical practice over the past centuries. To date, more than 11,000 species of plants are used medicinally and about 300 are commonly used [13].

Despite its widespread use, CHM is associated with high levels of uncertainty. This is mainly due to lack of evidence, base of efficacy, targets, and safety data. During the process of therapeutic drug development, owing to the preselection of targets, researchers have a basic if not full understanding of which molecular structures the drug will react with or which biological pathway in the body it might alter. Knowledge on molecular interactions and modulations of the drug is anticipated and researched on. However, this is not the case for CHM. There is no preselection of molecular targets in the body but CHM has been used for thousands of years and is proven to be effective. The exact mechanism of the herbs actions is yet to be elucidated.

The proven clinical efficacy of some herbal medicines is considered to be due to the interaction of pharmacologically active components from the herbs with molecular targets in the body. Similar to synthetic drugs, active compounds of herbal medicine may bind to and undergo interactions with molecular structures or herbal targets to produce therapeutic or adverse effects. However, there is a lack of understanding of how CHMs exert their biological and clinical effects at a molecular level, which impedes development of CHMs and the incorporation of CHMs into mainstream medicine in the West.

Berberine (Figure 1, molecular formula $C_{20}H_{19}NO_5$ and a molecular weight of 353.36) is an isoquinoline alkaloid found in many medicinal plants [14]. It is a major constituent of many medicinal plants of families Papaveraceae, Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, and Annonaceae [15]. It is present in *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). The berberine alkaloid can be found in the roots, rhizomes, and stem bark of the plants. Berberine is one of the main alkaloids found in the Chinese herb Huang Lian (*Rhizoma coptidis*) [16]. Huang Lian has traditionally been used to treat diarrhoea and diabetes. In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhoea [14, 17]. In recent years, there has been a growing interest in the pharmacological activities of berberine and many studies have been carried out to elucidate the mechanisms of action of berberine. This study aims to review molecular targets of berberine based on *in vitro* studies. Berberine has shown to have good hypoglycaemic effects, so we also reviewed the effects of berberine in animal and human studies, with a focus on diabetes mellitus.

Figure 1.



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Chemical structure of berberine.

2. Methods

2.1. Data Retrieval from the Literature

In vitro studies related to berberine and its targets were searched using Pubmed (from inception to April 2012). Search terms used were a combination of “berberine,” “*in vitro,*” “human cell,” and “mechanism.” Only studies using human cell lines were used to extract current berberine targets. Studies using animal cell lines or berberine derivatives or in a language other than English were excluded. Information extracted from these studies includes molecular targets of berberine (name and gene symbols), cell type, effects of berberine, and possible clinical applications.

2.2. PANTHER Analysis

Using the PANTHER Classification System, *in vitro* berberine targets were analysed using three approaches: molecular function, biological process, and pathway involvement Table 2. PANTHER is a publicly available database that relates protein sequence evolution to evolution of protein functions and biological roles (http://www.pantherdb.org/).

Table 2.

Berberine's target classification based on PANTHER.

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Multidrug resistance protein 1 (Pgp, Pgp-170)	*ABCB1*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity	Immune system process, extracellular transport, carbohydrate metabolic process, response to toxin	ATP-binding cassette (ABC) transporter
ATP-binding cassette sub-family G member 2	*ABCG2*	ATPase activity, coupled to transmembrane movement of substances, transmembrane transporter activity, anion channel activity	Immune system process, anion transport, lipid transport, oxygen and reactive oxygen species, metabolic process, lipid metabolic process, response to stress	N/A
Acetyl-coenzyme A carboxylase-*α*	*ACACA*	Other ligase	Gluconeogenesis, monosaccharide metabolism, fatty acid biosynthesis, coenzyme metabolism	N/A
α-Fetoprotein	*AFP*	Other transfer/carrier protein	Transport, mesoderm development, oncogenesis	N/A
Transcription factor AP-1	*AP-1*	DNA binding, transcription factor activity	Cell cycle, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle, signal transduction	Toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway, oxidative stress response, angiogenesis, TGF-beta signaling pathway, T-cell activation, B-cell activation, Ras Pathway, FAS signaling pathway, PDGF signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Amyloid-β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Other signaling molecules	Other signal transduction, cell communication, other intracellular protein traffic	Alzheimer disease-amyloid secretase pathway, Alzheimer disease-presenilin pathway, blood coagulation, Alzheimer disease-presenilin pathway, Alzheimer disease-amyloid secretase pathway
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	DNA binding, transcription factor activity	Transcription factor activity, immune system process, neurological system process, induction of apoptosis, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Apoptosis signaling pathway
Bcl2 antagonist of cell death	*BAD*	N/A	N/A	PDGF signaling pathway, apoptosis signaling pathway, angiogenesis, PI3 kinase pathway, VEGF signaling pathway, interleukin signaling pathway
BCL2-associated X protein	*BAX*	Other signaling molecule	Induction of apoptosis, gametogenesis, hematopoiesis, cell cycle control, cell proliferation and differentiation, tumor suppressor	p53 pathway, apoptosis signaling pathway, Huntington disease
B-cell CLL/lymphoma 2	*BCL2*	Other signaling molecule	Inhibition of apoptosis, oncogenesis	Oxidative stress response, apoptosis signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Apoptosis regulator Bcl-X	*BCL2L1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
B-cell lymphoma 3-encoded protein	*Bcl-3*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	Inflammation mediated by chemokine and cytokine signaling pathway
BH3-interacting domain death agonist p11	*BID*	N/A	N/A	Apoptosis signaling pathway, FAS signaling pathway
Baculoviral IAP repeat-containing protein 2 (anti-apoptosis factor c-IPA-1)	*BIRC2*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 3	*BIRC3*	N/A	N/A	Apoptosis signaling pathway
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	N/A	N/A	Angiogenesis
Caspase 3, apoptosis-related cysteine peptidase	*CASP3*	Cysteine protease	Proteolysis, apoptosis	Huntington disease, FAS signaling pathway, apoptosis signaling pathway
Caspase 8, apoptosis-related cysteine peptidase	*CASP8*	Cysteine protease	Proteolysis, apoptosis	Apoptosis signaling pathway, FAS signaling pathway, Huntington disease

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Caspase 9, apoptosis-related cysteine peptidase	*CASP9*	Cysteine protease	Proteolysis, apoptosis	Angiogenesis, apoptosis signaling pathway, FAS signaling pathway, VEGF signaling pathway, PI3 kinase pathway
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Nonreceptor serine/threonine, protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
G2/mitotic-specific cyclin-B1	*CCNB1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	Cell cycle, p53 pathway
G1/S-specific cyclin-D1	*CCND1*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle, Wnt signaling pathway
G1/S-specific cyclin-D2	*CCND2*	Protein binding, kinase activator activity, kinase regulator activity	Spermatogenesis, mitosis	PI3 kinase pathway, cell cycle
Cyclin E1	*CCNE1*	Kinase activator	Cell cycle control, mitosis, cell proliferation and differentiation	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2
G1/S-specific cyclin-E1	*CCNE1*	Protein binding, kinase activator activity, kinase regulator activity	Mitosis	p53 pathway, cell cycle, Parkinson disease, p53 pathway feedback loops 2

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Interleukin-2 receptor alpha chain	*IL2RA/CD25*	Cytokine receptor activity	Immune system process, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Interleukin signaling pathway
Platelet glycoprotein 4	*CD36/FAT*	Receptor activity	Macrophage activation, lipid transport, apoptosis, signal transduction, cell adhesion, lipid metabolic process, signal transduction, cell adhesion, cellular component, morphogenesis	N/A
Early activation antigen CD69	*CD69*	Receptor activity, receptor binding	B-cell-mediated immunity, natural killer cell activation, cellular defense response	Membrane-bound signaling molecule
M-phase inducer phosphatase 1	*CDC25A*	Hydrolase activity, acting on ester bonds, phosphatase activity	Phosphatase activity cell cycle, phosphate metabolic process, protein metabolic process, cell cycle	p53 pathway
Cdc42 effector protein 1	*CDC42EP1*	N/A	N/A	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Cyclin dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	p53 pathway
Cyclin-dependent kinase 2	*CDK2*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	p53 pathway, p53 pathway feedback loops 2
Cyclin-dependent kinase 4	*CDK4*	Nonreceptor serine/threonine protein kinase	Protein phosphorylation, cell cycle control, mitosis	N/A
Cell division protein kinase 6	*CDK6*	Kinase activity	Immune system process, mitosis, intracellular signaling cascade, protein metabolic process, cell motion, mitosis, signal transduction, response to stress	N/A
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway, p53 pathway feedback loops 2, p53 pathway
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Protein binding, kinase inhibitor activity, kinase regulator activity	Cell cycle	Interleukin signaling pathway
CASP8-and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Peptidase activity, protein binding, peptidase inhibitor activity	Apoptosis, protein metabolic process	Apoptosis signaling pathway, FAS signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Inhibitor of NF-κB kinase subunit alpha (IκB kinase)	*CHUK(IKK)*	Kinase activity	Immune response, intracellular signaling cascade, protein metabolic process, signal transduction, response to stimulus	Interleukin signaling pathway, apoptosis signaling pathway, T-cell activation, toll receptor signaling pathway, B-cell activation
SDF-1-α (3–67) (SDF-1)	*CXCL12*	N/A	N/A	Axon guidance-mediated by Slit/Robo
Cytochrome c-1	*CYC1*	Reductase	Oxidative phosphorylation	FAS signaling pathway, ATP synthesis, Huntington disease
Cytochrome P450, family 2, subfamily C, polypeptide 9	*CYP2C9*	Oxygenase	Fatty acid metabolism, steroid metabolism, electron transport	N/A
Cytochrome P450, family 2, subfamily D, polypeptide 6	*CYP2D6*	Oxygenase	Other lipid, fatty acid and steroid metabolism, steroid metabolism, electron transport	Vitamin D metabolism and pathway
Cytochrome P450, family 3, subfamily A, polypeptide 4	*CYP3A4*	Oxygenase	Steroid hormone metabolism, electron transport	N/A
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Serine protease	Proteolysis, cell surface receptor mediated signal transduction, T-cell-mediated immunity	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Epidermal growth factor receptor	*EGFR*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor-linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cyclesignal transduction, cell-cell signalling, dorsal/ventral axis specification, ectoderm development, mesoderm development, embryonic development, nervous system development	EGF receptor signaling pathway, cadherin signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Apoptosis signaling pathway, Alzheimer disease-amyloid secretase pathway, B-cell activation, Ras pathway, interleukin signaling pathway, angiogenesis, T-cell activation, toll receptor signaling pathway, insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade, FGF signaling pathway, Parkinson disease, PDGF signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, VEGF signaling pathway, interferon-gamma signaling pathway, endothelin signaling pathway, angiogenesis, TGF-beta signaling pathway, integrin signalling pathway, EGF receptor signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, interleukin signaling pathway, angiogenesis, VEGF signaling pathway, integrin signalling pathway
Ezrin	*EZR*	Structural constituent of cytoskeleton	Cellular component, morphogenesis	N/A
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	N/A	N/A	Oxidative stress response
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	Receptor binding	Signal transduction, cell-cell signalling, carbohydrate metabolic process, lipid metabolic process, signal transduction, cell-cell signalling, cellular glucose homeostasis	Peptide hormone

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Receptor binding	Female gamete generation, cell surface receptor linked signal transduction, signal transduction, ectoderm development, mesoderm development, skeletal system development, heart development, muscle organ development	TGF-beta signaling pathway
Hypoxia-inducible factor 1*α*	*HIF1A*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, ectoderm development, nervous system development	Hypoxia response via HIF activation, VEGF signaling pathway, angiogenesis
Interferon-*β*	*IFNB1*	Receptor binding	Response to interferon-gamma, induction of apoptosis, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, signal transduction, cell-cell signalling, cellular defense response	Toll receptor signaling pathway
Interleukin-1*β*	*IL1B*	Receptor binding	Immune response, macrophage activation, cell surface receptor linked signal transduction, cell-cell signalling, signal transduction, cell-cell signalling, response to stimulus	Inflammation mediated by chemokine and cytokine signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Interleukin-6	*IL6*	Receptor binding	Immune system process, negative regulation of apoptosis, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling signal transduction, cell-cell signaling	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Interleukin 8	*IL8*	Chemokine	Cytokine- and chemokine-mediated signaling pathways, calcium-mediated signalling, NF-kappaB cascade, ligand-mediated signalling, T-cell-mediated immunity, macrophage-mediated immunity, granulocyte-mediated immunity, angiogenesis, cell proliferation and differentiation, cell motility	Inflammation mediated by chemokine and cytokine signaling pathway, interleukin signaling pathway
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Receptor activity, cation transmembrane transporter activity, voltage-gated potassium channel activity, cation channel activity, cyclic nucleotide-gated ion channel activity	Cation transport, signal transduction	Ligand-gated ion channel
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	Other receptor	Oogenesis	Alzheimer disease-presenilin pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Kinase activity	Immune system process, mitosis, cell surface receptor linked signal transduction, intracellular signaling cascade, carbohydrate metabolic process, protein metabolic process, cell motion, mitosis, signal transduction, segment specification, ectoderm development, mesoderm development, embryonic development, nervous system development, response to stress	Alzheimer disease-amyloid secretase pathway, Ras pathway, EGF receptor signaling pathway, Parkinson disease, angiogenesis, FGF signaling pathway, FAS signaling pathway, toll receptor signaling pathway, TGF-beta signaling pathway, PDGF signaling pathway, Huntington disease, integrin signalling pathway, T-cell activation, B-cell activation, interferon-gamma signaling pathway, oxidative stress response, apoptosis signaling pathway, integrin signalling pathway
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Receptor binding	Gamete generation, induction of apoptosis, negative regulation of apoptosis, cell cycle, mesoderm development, hemopoiesis	Apoptosis signaling pathway
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Peptidase activity	Protein metabolic process	Plasminogen activating cascade, Alzheimer disease-presenilin pathway, plasminogen activating cascade
Matrix metalloproteinase-16	*MMP16*	Peptidase activity	Protein metabolic process	Alzheimer disease-presenilin pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
72 kDa type IV collagenase	*MMP2*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Metalloprotease, other extracellular matrix	Proteolysis	Alzheimer disease-presenilin pathway, plasminogen activating cascade
Myc proto-oncogene protein	*MYC*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid, metabolic process, cell cycle	Oxidative stress response, p53 pathway feedback loops 2, Wnt signaling pathway, interleukin signaling pathway, PDGF signaling pathway
Arylamine *N*-acetyltransferase 1	*NAT*	Acyltransferase activity	Metabolic process	Acetyltransferase
Nuclear factor NF-*κ*B p50 subunit (NF-*κ*B)	*NFKB1*	DNA binding, transcription factor activity	B-cell-mediated immunity, negative regulation of apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, cellular defense response	T-cell activation, B-cell activation, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, apoptosis signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
NF-κB inhibitor-α	*NFKBIA*	Protein binding	Immune system process, intracellular protein transport apoptosis, intracellular signaling cascade, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, signal transduction, response to stress	Apoptosis signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, T-cell activation, B-cell activation
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	N/A	Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	N/A
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	Nuclear hormone receptor, transcription factor, nucleic acid binding	N/A	N/A
Poly(ADP-ribose) polymerase family, member 1	*PARP*	Glycosyltransferase	DNA repair, protein ADP-ribosylation, stress response	FAS signaling pathway
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	Serine protease	Proteolysis	N/A
Urokinase-plasminogen activator	*PLAU*	Peptidase activity	Immune system process, signal transduction, protein metabolic process, cell motion, signal transduction, blood coagulation	Blood coagulation, plasminogen activating cascade

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Peroxisome proliferator-activated receptor-γ	*PPARG*	Nuclear hormone receptor, transcription factor, nucleic acid binding	Monosaccharide metabolism, regulation of lipid, fatty acid, and steroid metabolism, mRNA transcription regulation, ligand-mediated signalling, stress response, developmental processes, cell proliferation and differentiation	N/A
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Oxidoreductase activity	Immune system process	Endothelin signaling pathway, toll receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Ras-related C3 botulinum toxin substrate 1	*RAC1*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, integrin signalling pathway, inflammation mediated by chemokine and cytokine signaling pathway, Huntington disease, axon guidance mediated by Slit/Robo, FGF signaling pathway, T-cell activation, axon guidance mediated by netrin, EGF receptor signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase, aAxon guidance mediated by semaphorins, cytoskeletal regulation by Rho GTPase, B-cell activation, Ras pathway
Rho-associated protein kinase 1	*ROCK1/RHO*	Kinase activity	Mitosis, intracellular signaling cascade, cell adhesion, protein metabolic process, cell motion, mitosis, signal transduction, cell adhesion, embryonic development	Inflammation mediated by chemokine and cytokine signaling pathway, cytoskeletal regulation by Rho GTPase

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Transforming protein RhoA	*RHOA*	GTPase activity, protein binding	Intracellular protein transport, endocytosis, cell surface receptor linked signal transduction, intracellular signaling cascade, signal transduction	Axon guidance mediated by Slit/Robo, angiogenesis, heterotrimeric G-protein signaling pathway-Gq alpha; and Go alpha mediated pathway, axon guidance mediated by semaphorins, inflammation mediated by chemokine and cytokine signaling pathway, integrin signalling pathway, Ras pathway, cytoskeletal regulation by Rho GTPase, PDGF signaling pathway
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	Kinase activity, transmembrane receptor protein tyrosine kinase activity, transmembrane receptor protein kinase activity, receptor binding	Female gamete generation, immune system process, visual perception, sensory perception, negative regulation of apoptosis, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell-cell adhesion, protein metabolic process, cell motion, cell cycle, signal transduction, ectoderm development, mesoderm development, embryonic development, nervous system development	N/A

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Runt-related transcription factor 2	*RUNX2*	DNA binding, transcription factor activity	Mesoderm development, skeletal system development, hemopoiesis	N/A
Sucrase-isomaltase (Alpha-glucosidase)	*SI*	Hydrolase activity, hydrolyzing O-glycosyl compounds	Carbohydrate metabolic process, protein metabolic process	N/A
Processed sterol regulatory element-binding protein 2	*SREBP2*	DNA binding, transcription factor activity	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, lipid metabolic process	Basic helix-loop-helix transcription factor
Tumor necrosis factor/tumor necrosis factor-*α*	*TNFA*	Tumor necrosis factor family member	Cytokine- and chemokine-mediated signaling pathways, ligand-mediated signalling, immunity and defense, induction of apoptosis	Wnt signaling pathway, apoptosis signaling pathway
Topoisomerase (DNA) I	*Top1*	DNA topoisomerase	DNA replication, general mRNA transcription activities	DNA replication
Cellular tumor antigen p53	*TP53*	DNA binding, transcription factor activity	Induction of apoptosis, cell cycle, nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, cell cycle	Apoptosis signaling pathway, Huntington disease, P53 pathway feedback loops 1, p53 pathway, p53 pathway by glucose deprivation, p53 pathway feedback loops 2, Wnt signaling pathway

Target names	Target gene symbol	PANTHER molecular function	Biological process	Pathway categories
Vascular endothelial growth factor A	*VEGFA*	Receptor binding	Immune system process, cell cycle, cell surface receptor linked signal transduction, intracellular signaling cascade, cell-cell signalling, cell cyclesignal transduction, mesoderm development, angiogenesis, response to stress	Angiogenesis, VEGF signaling pathway
Wee1-like protein kinase	*Wee1*	Kinase activity	Mitosis, protein metabolic process	Protein kinase

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3. Results

3.1. Targets of Berberine

A total of 90 berberine targets were identified in our literature search, as shown in Table 1.

Table 1.

Potential targets of berberine.

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
72 kDa type IV collagenase	*MMP2*	HUVECs, tongue cancer SCC-4 cells, gastric carcinoma SNU-5 cells, lung cancer A549 cells, and U-87 glioma cells	Downregulation of MMP2 mRNA and protein expression, reduced MMP-2 levels	Antimetastatic	[18, 69–72]
Acetyl-Coenzyme A carboxylase-*α*	*ACACA*	HepG2 hepatoma cells	Phosphorylation	Antihyperlipidemic	[73]
α-Fetoprotein	*AFP*	HepG2 hepatoma cells	Reduced secretion of alpha fetoprotein	Apoptosis	[74]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Amyloid-β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	*APP*	Neuroglioma H4 cells	Reduces amyloid-β peptide (Aβ) levels via modulation of APP	Alzheimer disease	[75]
Bcl-X	*BCL2L1*	Colonic carcinoma cells, HepG2 cells/A549 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells	JNK/p38 pathway and induction of ROS production. Decreased expression	Cell apoptosis, anticancer, and anti-inflammatory	[19–21, 30, 76, 77]
Arylamine *N*-acetyltransferase 1	*NAT*	Leukemia HL-60 cells, colon tumour cells, brain tumour cells (G95/VGH and GBM 8401)	Decrease in *N*-acetyltransferase (NAT) protein and expression of mRNA	Anticancer (leukemia, colon cancer, brain tumour, etc.)	[78–80]
ATP-binding cassette subfamily G member 2	*ABCG2*	MCF-7 breast cancer cells	Decrease in ABCG2 expression	Breast cancer	[81]
Baculoviral IAP repeat-containing protein 2 (antiapoptosis factor c-IPA-1)	*BIRC2*	Jurkat cells, colonic carcinoma cells (SW620)	Suppresses expression of antiapoptosis factor IAP1	Anticancer	[19, 77]
Baculoviral IAP repeat-containing protein 3	*BIRC3*	Jurkat cells	Suppresses expression of antiapoptosis factor IAP2	Anticancer	[77]
Baculoviral IAP repeat-containing protein 5 (Survivin)	*BIRC5*	Jurkat cells	Suppresses expression of survivin	Anticancer and anti-inflammatory agent	[77]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
B-cell CLL/lymphoma 2	*BCL2*	HepG2 cells, oral squamous cell carcinoma, tongue cancer SCC-4 cells, colonic carcinoma cells, lung cancer cells, breast cancer MCF-7 (estrogen receptor+) cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Bcl-2 Downregulation	Cell apoptosis, cancer, and ER antagonist adjuvant therapy	[19–23, 30, 34, 82, 83]
B-cell lymphoma 3-encoded protein	*Bcl-3*	Gastric carcinoma SNU-5 cells	Downregulation of Bcl-3	Gastric cancer	[24]
Bcl2 antagonist of cell death	*BAD*	Human oral squamous cell carcinoma	Increased expression of proapoptotic BAD protein	Antitumour	[23]
BCL2-associated X protein	*BAX*	Gastric carcinoma SNU-5 cells, prostate carcinoma cells (DU145, PC-3, LNCaP and PWR-1E), leukemia HL-60, tongue cancer SCC-4 cells, lung cancer cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Upregulation of Bax, increased expression. G2/M phase arrest	Cell apoptosis, gastric cancer	[18, 20, 21, 24, 29, 30, 83, 84]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
BH3-interacting domain death agonist p11	*BID*	Colonic carcinoma cells/HepG2 cells	JNK/p38 pathway and induction of ROS production	Induction of apoptosis	[19, 76]
C/EBP homologous protein (CHOP) or growth arrest- and DNA damage-inducible gene 153 (GADD153) or DNA damage-inducible transcript 3	*GADD153/DDIT3*	Cervical cancerCa Ski cells	Induced expression of GADD153	Cervical cancer	[85]
CASP8 and FADD-like apoptosis regulator subunit p12	*CFLAR/cFLIP*	Jurkat cells	Suppresses expression of cFLIP	Anticancer and anti-inflammatory	[77]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Caspase 3	*CASP3*	Tongue cancer SCC-4 cells, neuroblastoma (SK-N-SH), glioblastoma T98G cells, gastric carcinoma SNU-5 cells, HL-60 cells, prostate carcinoma cells (DU145, PWR-1E, PC-3 and LNCaP), colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma, promonocytic U937 cells, lung cancer A549, H1301 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase-3, G2/M phase arrest	Anticancer	[18–21, 23–25, 30, 33, 76, 83, 86–89]
Caspase 8	*CASP8*	Tongue cancer SCC-4 cells, colonic carcinoma cells, hepatoma cells, oral squamous cell carcinoma	Activated caspase 8	Anticancer	[18, 19, 23, 76]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Caspase 9	*CASP9*	Tongue cancer SCC-4 cells, glioblastoma T98G, oral squamous carcinoma, promonocytic U937 cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs), BIU-87 and T24 bladder cancer cells	Activation of caspase 9	Cell apoptosis, anticancer	[18, 21, 23, 30, 33, 83, 86, 87, 89, 90]
Cdc42 effector protein 1	*CDC42EP1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Cdc42)	Cancer metastasis inhibition	[91]
Cell division protein kinase 6	*CDK6*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk6	Cell apoptosis, cancer	[21, 30, 83]
Cellular tumor antigen p53	*TP53*	Gastric carcinoma SNU-5 cells, osteosarcoma	Increased expression of p53 protein, cell cycle arrest at G1G2/M phase arrest	Anticancer (gastric cancer, osteosarcoma)	[24, 92]
Chemokine (C-C motif) ligand 2 (monocyte chemotactic protein-1)	*CCL2*	Retinal pigment epithelial cell line	Inhibits CCL2 (MCP-1) expression	Anti-inflammatory	[93]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Cyclic AMP-dependent transcription factor ATF-3	*ATF3*	Colorectal cancer cells	Induces ATF3 expression	Colorectal cancer	[94]
Cyclin-dependant kinase 1/cell division control protein 2 homolog	*CDK1/CDC2*	HL-60 cell, gastric carcinoma SNU-5 cells	Inactivation of Cdc2 (CDK1) or decreased protein expression	Antiproliferative and proapoptotic	[24, 95]
Cyclin E1	*CCNE1*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Anticancer	[25, 83, 90]
Cyclin-dependent kinase 2	*CDK2*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk2	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]
Cyclin-dependent kinase 4	*CDK4*	Neuroblastoma (SK-N-SK), glioblastoma T98G cells, prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in Cdk4	Cell apoptosis, anticancer	[21, 25, 30, 83, 90]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Cyclin-dependent kinase inhibitor 1 (p21)	*CDKN1A*	Brest cancer MCF-7 (estrogen receptor+) cells, epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of p21	Breast cancer, ER antagonist adjuvant therapy	[21, 30, 82, 83]
Cyclin-dependent kinase inhibitor 1B (P27/KIP1)	*CDKN1B*	Epidermoid carcinoma A431 cells, activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Increased expression of Cdki proteins	Cell apoptosis, cancer	[21, 30, 83]
Cytochrome c-1	*CYC1*	Tongue cancer SCC-4 cells, colonic carcinoma cells, promyelocytic leukemia HL-60 cells	Release of cytochrome c-1	Anticancer	[18, 19, 84, 86]
CYP2C9	*CYP2C9*	Recombinant CYP	Inhibition of CYP2C9	Drug interactions	[96]
CYP2D6	*CYP2D6*	Recombinant CYP	Inhibition of CYP2D6	Drug interactions	[96]
CYP3A4	*CYP3A4*	Caco-2 cells, patients	CYP3A4 Downregulation and inhibition	Drug interactions	[97, 98]
Dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	*DPP4*	Recombinant DPP4	Inhibition of DPP4	—	[99]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Early activation antigen CD69	*CD69*	Human peripheral lymphocytes	Reduced expression of CD69	Immunosuppressive agent	[100]
Epidermal growth factor receptor	*EGFR*	Brest cancer MCF-7 (estrogen receptor+) cells	EGFRdownregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Ezrin	*EZR*	Nasopharyngeal carcinoma 5–8F cells	Ezrin inhibition	Anticancer	[26]
G1/S-specific cyclin-D1	*CCND1*	Giant cell carcinoma cell line, HL-60 cell, prostate carcinoma cells (DU145 and PC-3, LNCaP), Jurkat cells, neuroblastoma (SK-N-SK), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Inhibits expression of cyclin D1	Antiproliferative and proapoptotic, anticancer, anti-inflammatory	[21, 25, 30, 32, 77, 83, 95]
G1/S-specific cyclin-D2	*CCND2*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin D2	Cell apoptosis, cancer	[21, 30, 83]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
G1/S-specific cyclin-E1	*CCNE1*	Prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Decrease in cyclin E	Cell apoptosis, cancer	[21, 30, 83]
G2/mitotic-specific cyclin-B1	*CCNB1*	Gastric carcinoma SNU-5 cells	Decreased cyclin B, G2/M phase arrest	Cell apoptosis, anticancer	[24]
Glucagon-like peptide (GCG/GLP-1/GLP-2)	*GCG*	NCI-H716 cells	Enhanced glucagon-like peptide (GLP)-1	Antidiabetic agent	[42]
Growth/differentiation factor 15 (NAG-1)	*GDF15*	Colorectal cancer cells	Induces NAG-1 expression	Colorectal cancer	[94]
Hypoxia-inducible factor 1*α*	*HIF1A*	HUVECs, HepG2 cells	Prevention and reduction of HIF-1 alpha expression	Tumour angiogenesis	[101, 102]
Induced myeloid leukemia cell differentiation protein Mcl-1	*MCL1*	Oral cancer cells	Inhibition of Mcl-1 expression	Induced apoptosis	[103]
Inhibitor of NF-*κ*B kinase subunit alpha (I*κ*B kinase)	*CHUK(IKK)*	Jurkat cells	Inhibition of I*κ*B kinase (IKK)	Anticancer and anti-inflammatory agent	[77]
Interferon-*γ*	*IFNB1*	Brest cancer MCF-7 (estrogen receptor+) cells	IFN-beta upregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Interleukin 8	*IL8*	Retinal pigment epithelial cell line	Inhibits IL-8 expression	Anti-inflammatory	[93]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Interleukin-1β	*IL1B*	Fibroblasts (HFL1)	Induces IL-1B productions	Pulmonary inflammation	[104]
Interleukin-2 receptor α-chain	*IL2RA/CD25*	Human peripheral lymphocytes	Reduced expression of CD25	Immunosuppressive agent	[100]
Interleukin-6	*IL6*	Keratinocytes	Reduces and IL-6 expression	Antiskin ageing agent	[105]
Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	HepG2 cells	Increased mRNA and protein expression	Hyperlipidemia	[106–108]
Matrix metallopeptidase 1 (27 kDa interstitial collagenase)	*MMP1*	Dermal fibroblasts, U-87 glioma cells	MMP-1 expression decreased	Antiskin ageing agent, anticancer	[70, 109]
Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	*MMP9*	Tongue cancer SCC-4 cells, keratinocytes, gastric carcinoma SNU-5	Inhibition	Anticancer	[34, 70, 105]
Matrix metalloproteinase-16	*MMP16*	Jurkat cells	Suppresses expression of MMP-16	Anticancer and anti-inflammatory agent	[77]
Mitogen-activated protein kinase 3	*ERK1/MAPK3*	Peripheral blood monocytes (PBMC)	ERK1 protein expression inhibition	Antiatherosclerotic effects	[110]
Mitogen-activated protein kinase 4	*ERK2/MAPK4*	Peripheral blood monocytes (PBMC)	ERK2 protein expression inhibition	Antiatherosclerotic effects	[110]
Mitogen-activated protein kinase 8 (JNK)	*MAPK8*	Peripheral blood monocytes (PBMC)	Jun N-terminal kinase (JNK) protein expression inhibited at high levels of BBR	Antiatherosclerotic effects	[19, 110]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
M-phase inducer phosphatase 1	*CDC25A*	HL-60 cell	Phosphorylation and degradation of Cdc25A	Antiproliferative and proapoptotic	[95]
Multidrug resistance protein 1 (P-gp, P-gp-170)	*ABCB1*	Tumour cell lines	Significant inhibited P-gp multidrug resistance (MDR) activity	MDR activity reversal	[111]
		HepatomaHepG2 cells	Upregulated multidrug resistance transporter (P-gp-170) expression	Reduced retention of chemotherapeutic agents	[112]
Myc proto-oncogene protein	*MYC*	U-87 glioma cells	Myc level decreased	Malignant glioma and cancer development	[71]
NF-κB inhibitor-α	*NFKBIA*	Lung epithelial cells (A-549)	Inhibition of κB-α phosphorylation and degradation	Pulmonary inflammation	[104]
Nuclear factor NF-κB p50 subunit (NF-κB)	*NFKB1*	Jurkat cells, osteoblastic cells, HepG2 cells	Inhibit NF-κB production and suppress NF-κB	Anticancer and anti-inflammatory agent, alcohol liver disease, osteoclast formation	[77, 113–115]
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*NR3C1*	HepG2 cells	Reduced GR levels	Cell growth arrest	[74]
Nucleophosmin (nucleolar phosphoprotein B23) and telomerase	*NPM1*	Leukemia HL-60 cells	Downregulation of nucleophosmin/B23 and telomerase activity	Cancer	[116]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Peroxisome proliferator-activated receptor-γ	*PPARG*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes, 3T3-L1 preadipocytes	Decreased expression	Antidiabetic	[37, 117]
Platelet glycoprotein 4	*CD36/FAT*	Free-fatty-acid-induced insulin resistance muscle cells-L6 myotubes	Decreased expression	Antidiabetic	[37]
Poly (ADP-ribose) polymerase family, member 1	*PARP*	HepG2 cells/hepatoma cells, colonic carcinoma cells, prostate cancer cells (PC-3), prostate carcinoma cells (DU145 and PC-3, LNCaP), activated rheumatoid arthritis fibroblast-like synoviocytes (RAFLSs)	Cleavage of poly (ADP-ribose) polymerase. Activation of PARP	Cell apoptosis, Anticancer	[19, 21, 22, 30, 76, 83, 87]
Potassium voltage-gated channel subfamily H member 2	*KCNH2/HERG1*	Leukemic stem cells (LSCs)	Inhibits HERG1 K (+) channels of leukemic cells	Inhibits AML cell migration	[35]
Processed sterol regulatory element-binding protein 2	*SREBP2*	HepG2 cells	Reduction of SREBP2	Hyperlipidemia	[101]
Proprotein convertase subtilisin/kexin type 9	*PCSK9*	HepG2 cells	Suppression of PCSK9 mRNA and protein levels	Hyperlipidemia	[101, 107]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Prostaglandin G/H synthase 2	*PTGS2/COX2*	Peripheral blood monocytes (PBMC), oral cancer cell lines OC2 and KB cells, breast cancer MCF-7 (estrogen receptor+) cells, Jurkat cells, colon cancer cells	Decrease of Cox-2 mRNA and protein expression	Antiatherosclerotic effects, anti-inflammatory, anticancer, breast cancer ER antagonist adjuvant therapy, Anticancer	[27, 77, 82, 103, 110, 118]
Proto-oncogene tyrosine-protein kinase ROS	*ROS1*	HUVECs	Inhibition of ROS generation	Protects LDL oxidation and prevents ox-LDL-induced cellular dysfunction	[19, 119]
Ras-related C3 botulinum toxin substrate 1	*RAC1*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (Rac1)	Cancer metastasis inhibition	[91]
Receptor tyrosine-protein kinase erbB-2	*ERBB2/HER2*	Brest cancer MCF-7 (estrogen receptor+) cells	HER2 downregulated	Breast cancer, ER antagonist adjuvant therapy	[82]
Rho-associated protein kinase 1	*ROCK1/RHO*	Nasopharyngeal carcinoma 5–8F cells	Suppression of Rho kinase activity	Anticancer	[91]
Runt-related transcription factor 2	*RUNX2*	Osteoblast cells	Promotes transcriptional activity of Runx2	Osteoblast differentiation and bone formation in osteoporosis	[120]
SDF-1-*α* (3–67) (SDF-1)	*CXCL12*	Acute myeloid leukemia (AML)	Reduces SDF-1 chemokine	Inhibits AML cell migration	[35]
Sucrase-isomaltase (*α*-glucosidase)	*SI*	Caco-2 cells	Inhibit alpha-glucosidase	Antihyperglycaemic	[39]

Target names	Target gene symbol	Cells	Effects	Possible clinical applications	References
Topoisomerase (DNA) I	*Top1*	Recombinant human topoisomerase I	Top1 inhibition	Anticancer	[121]
Transcription factor AP-1	*AP-1*	Hepatoma cells, MDA-MB-231 breast cancer cells, giant cell carcinoma cell line, colon cancer cells, U-87 glioma cells, HeLa cells	Inhibition of AP-1 activity, AP-1 DNA suppression	Antitumor activity, Anticancer	[27, 32, 71, 115, 118, 122–124]
Transforming protein RhoA	*RHOA*	Nasopharyngeal carcinoma (HONE1) cells	Suppression of Rho GTPases activation (RhoA)	Cancer metastasis inhibition	[91]
Tumor necrosis factor-*α*	*TNFA*	Macrophages, fibroblasts (HFL1)	Inhibition of TNF-*α*	Anti-inflammatory	[104, 125]
Urokinase-plasminogen activator	*PLAU*	Lung cancer A549 cells, tongue cancer SCC-4 cells	Reduced urokinase-plasminogen activator (u-PA)	Antimetastatic, Anticancer	[34, 72]
Vascular endothelial growth factor A	*VEGFA*	HUVECs	Prevention of VEGF expression	Tumour angiogenesis	[102]
Wee1-like protein kinase	*Wee1*	Gastric carcinoma SNU-5 cells	Increased expression of Wee1protein, G2/M phase arrest	Gastric cancer	[24]

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Extensive research has been carried out to study the effects of berberine on cancer cells *in vitro*. This may be related to recent discovery of anti-cancer drugs with natural compound origin, for example, paclitaxel and topotecan.

Various human cancer cell lines were used to demonstrate the anti-cancer effects of berberine *in vitro*. These include cancer cell lines of the tongue, stomach, lung, colon, liver, breast, prostate, nasopharyngeal, neurones, epidermal, and blood [18–28]. Berberine has shown to induce cancer cell death via several mechanisms such as regulation of apoptosis proteins and cell cycle arrest.

Berberine treatment increased the expression of apoptotic cell death proteins, promotes cell cycle arrest, and induces cell death in human cancer cell lines. For instance, in human prostate epithelial cells (PWR-1E), berberine-increased expression of BCL2-associated X protein (Bax) was observed after berberine treatment, inducing cell death and demonstrating pro-apoptotic properties [29]. Similar effects of berberine were observed in prostate carcinoma cells (DU145, PC-3, and LNCaP) [21, 30]. Berberine also increased levels of Bax in promyelocytic leukemia cells [31], gastric carcinoma cells [24], and lung cancer cells [20].

Berberine can also promote cell death by the regulation of antiapoptotic proteins. Decreased expression of antiapoptotic Bcl-2 protein was observed in human oral squamous cell carcinoma after berberine treatment [23]. Studies done in other cancer cell lines such as lung cancer, gastric cancer, and prostate cancer also showed reduced levels of Bcl-2 after berberine treatment [20, 21, 24, 30]. Cell cycle arrest at different phases has also been observed in human cancer cell lines after treatment with berberine. In giant cell carcinoma and prostate carcinoma cells, berberine also decreased G_0/G_1 phase-associated cyclins (D_1, D_2, E, Cdk2, Cdk4, and Cdk6), inducing G_0/G_1 arrest and suppressing cell proliferation [21, 25, 30, 32]. Further, in HepG2 cells, berberine acted on B-cell CLL/lymphoma 2 (BCL2), procaspase-3 and -9, and poly (ADP-ribose) polymerase (PARP), induced cell cycle arrest at G_2/M phase and inhibited cell proliferation [22].

Further, berberine can promote cell death via the regulation of pro- and antiapoptotic proteins. In addition to this, berberine can also promote apoptosis via mitochondrial/caspase pathway. In cancer cell lines (tongue cancer, oral squamous cell carcinoma and prostate epithelial) [18, 23, 29, 33], activation of caspases-3 & -9 promotes G_1 cell cycle arrest in different human cancer cell lines (lung, stomach, and prostate) [20, 21, 24, 30, 33].

Berberine also showed anti-metastatic properties in several cancer cell lines, acting on 72 kDa type IV collagenase (MMP2), Cdc42 effector protein 1 (CDC42EP1), and ras-related C3 botulinum toxin substrate 1 (RAC1), transforming protein RhoA (RHOA) and urokinase-plasminogen activator A (PLAU) [34, 35]. Further, berberine showed antitopoisomerase I properties [36]; this observation can be useful as topoisomerase I is responsible for DNA replication and antitopoisomerase I compounds can be effective in cancer treatments.

In addition to its effects on cancer cells, berberine also acts on molecular targets related to insulin resistance. In free-fatty-acid-induced insulin resistance muscle cells, berberine improves insulin resistance and improves glucose uptake by decreasing PPARγ and FAT/CD36 protein expression [37]. Another study reported increased insulin receptor (InsR) mRNA and protein expression increases insulin sensitivity in liver cells after berberine treatment [38]. In Caco-2 cells, berberine inhibited alpha-glucosidase and disaccharidases activities, leading to reduced glucose levels [39]. In Hep G_2 cells, berberine also improved insulin signal transduction through various mechanisms such as decreased phosphorylation of PERK and eLF2-α, increased phosphorylation of IRS-1 tyrosine and AKT serine [40]. In intestinal NCI-H716 cells, berberine enhanced glucagon-like peptide 1 (GLP-1) release and promotes proglucagon mRNA expression [41]. These results demonstrate that berberine has great potential for insulin resistance treatment and should be explored further in animal and human studies.

3.2. PANTHER Analysis of Berberine Targets

Distribution of berberine therapeutic targets *in vitro* varied in each of these functional classifications. Tables 3, 4, and 5 show various distributions of the most frequent occurring berberine targets *in vitro* based on molecular functions, biological processes, and pathways, respectively.

Table 3.

Distribution of berberine's targets *in vitro* according to molecular functions.

PANTHER molecular function	Numberof targets
Acyltransferase activity	1
Anion channel activity	1
ATPase activity, coupled to transmembrane movement of substances	2
Cation channel activity	1
Cation transmembrane transporter activity	1
Chemokine	1
Cyclic nucleotide-gated ion channel activity	1
Cysteine protease	3
Cytokine receptor activity	1
DNA binding	9
DNA topoisomerase	1
Glycosyltransferase	1
GTPase activity	2
Hydrolase activity, acting on ester bonds	1
Hydrolase activity, hydrolyzing O-glycosyl compounds	1
Kinase activator	1
Kinase activator activity	4
Kinase activity	11
Kinase inhibitor activity	2
Kinase regulator activity	6
Metalloprotease	2
Not classified	10
Non-receptor serine/threonine protein kinase	3
Nuclear hormone receptor	2
Nucleic acid binding	2
Other extracellular matrix	2
Other ligase	1

PANTHER molecular function	Numberof targets
Other receptor	1
Other signaling molecule	3
Other transfer/carrier protein	1
Oxidoreductase activity	1
Oxygenase	3
Peptidase activity	4
Peptidase inhibitor activity	1
Phosphatase activity	1
Protein binding	10
Receptor activity	3
Receptor binding	12
Reductase	1
Serine protease	2
Structural constituent of cytoskeleton	1
Transmembrane transporter activity	2
Transcription factor	2
Transcription factor activity	9
Transmembrane receptor protein kinase activity	3
Transmembrane receptor protein tyrosine kinase activity	3
Tumor necrosis factor family member	1
Voltage-gated potassium channel activity	1

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Table 4.

Distribution of berberine's targets *in vitro* according to biological functions.

PANTHER biological functions	Number of targets
Angiogenesis	2
Anion transport	1

PANTHER biological functions	Number of targets
Apoptosis	6
B-cell-mediated immunity	2
Blood coagulation	1
Calcium-mediated signaling	1
Carbohydrate metabolic process	7
Cation transport	1
Cell adhesion	3
Cell communication	1
Cell cycle	11
Cell cycle control	5
Cell cycle intracellular signaling cascade	1
Cell cycle signal transduction	1
Cell motility	1
Cell motion	10
Cell proliferation and differentiation	3
Cell proliferation and differentiation	1
Cell surface receptor linked signal transduction	14
Cell surface receptor-mediated signal transduction	1
Cell-cell adhesion	3
Cell-cell signaling	9
Cellular component morphogenesis	2
Cellular defense response	4
Cellular glucose homeostasis	1
Coenzyme metabolism	1
Cytokine- and chemokine-mediated signaling pathways	2
Developmental processes	1
DNA repair	1

PANTHER biological functions	Number of targets
DNA replication	2
Dorsal/ventral axis specification	1
Ectoderm development	1
Ectoderm development	8
Electron transport	3
Embryonic development	7
Endocytosis	2
Extracellular transport	2
Fatty acid biosynthesis	1
Fatty acid metabolism	1
Female gamete generation	4
Gamete generation	2
Gametogenesis	1
General mRNA transcription activities	1
Gluconeogenesis	1
Granulocyte-mediated immunity	1
Heart development	1
Hematopoiesis	1
Hemopoiesis	3
Immune response	2
Immune system process	16
Immune system processMitosis	1
Immunity and defense	1
Induction of apoptosis	9
Intracellular protein transport	3
Intracellular signaling cascade	18
Ligand-mediated signaling	3

PANTHER biological functions	Number of targets
Lipid metabolic process	4
Lipid transport	2
Macrophage activation	2
Macrophage-mediated immunity	1
Mesoderm development	12
Metabolic process	1
Mitosis	4
Monosaccharide metabolism	2
mRNA transcription regulation	1
Muscle organ development	1
Not classified	9
Natural killer cell activation	1
Negative regulation of apoptosis	8
Nervous system development	7
Neurological system process	1
NF-κB cascade	1
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	10
Oncogenesis	3
Other intracellular protein traffic	1
Other lipid, fatty acid and steroid metabolism	1
Other signal transduction	1
Oxidative phosphorylation	1
Oxygen and reactive oxygen species metabolic process	1
Phosphatase activity cell cycle	1
Phosphate metabolic process	1
Protein ADP-ribosylation	1
Protein metabolic process	17

PANTHER biological functions	Number of targets
Protein phosphorylation	3
Proteolysis	7
Regulation of lipid, fatty acid and steroid metabolism	1
Response to interferon-*γ*	1
Response to stimulus	2
Response to stress	8
Response to toxin	2
Segment specification	3
Sensory perception	1
Signal transduction	25
Skeletal system development	2
Spermatogenesis	2
Steroid hormone metabolism	1
Steroid metabolism	2
Stress response	2
T-cell-mediated immunity	2
Transcription factor activity immune system process	1
Transport	1
Tumor suppressor	1
Visual perception	1

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Table 5.

Distribution of berberine's targets *in vitro* according to pathway categories.

PANTHER pathway categories	Number of targets
Acetyltransferase	1
Alzheimer disease-amyloid secretase pathway	11
Alzheimer disease-presenilin pathway	14

PANTHER pathway categories	Number of targets
Angiogenesis	11
Apoptosis signaling pathway	21
ATP synthesis	1
ATP-binding cassette (ABC) transporter	2
Axon guidance mediated by netrin	1
Axon guidance mediated by semaphorins	1
Axon guidance mediated by Slit/Robo	4
B-cell activation	7
Basic helix-loop-helix transcription factor	1
Blood coagulation	3
Cadherin signaling pathway	2
Cell cycle	4
Cytoskeletal regulation by Rho GTPase	3
DNA replication	2
EGF receptor signaling pathway	4
Endothelin signaling pathway	2
FAS signaling pathway	13
FGF signaling pathway	4
Heterotrimeric G-protein signaling pathway—Gq alpha- and Go alpha-mediated pathway	1
Huntington disease	9
Hypoxia response via HIF activation	1
Inflammation mediated by chemokine and cytokine signaling pathways	13
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	1
Integrin signalling pathway	6
Interferon-gamma signaling pathway	2
Interleukin signaling pathway	10
Ligand-gated ion channel	1

PANTHER pathway categories	Number of targets
Membrane-bound signaling molecule	1
Pathway unclassified	19
Oxidative stress response	5
p53 pathway	12
p53 pathway by glucose deprivation	1
p53 pathway feedback loops	1
P53 pathway feedback loops 1	1
p53 pathway feedback loops 2	4
Parkinson disease	3
PDGF signaling pathway	6
Peptide hormone	1
PI3 kinase pathway	4
Plasminogen activating cascade	8
Protein kinase	1
Ras Pathway	5
T-cell activation	7
TGF-*β* signaling pathway	4
Toll receptor signaling pathway	9
VEGF signaling pathway	7
Vitamin D metabolism and pathway	1
Wnt signaling pathway	4

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As shown in Table 3, berberine acts on a diverse range of molecular targets *in vitro*. The most common classes of molecular functions include receptor binding, kinase activity, protein binding, transcription activity, DNA binding, and kinase regulator activity. Known berberine targets *in vitro* from the receptor binding class include epidermal growth factor receptor (EGFR), vascular endothelial growth factor A (VEGFA), interleukin-1*β* (IL1B) and interleukin-6 (IL6), growth/differentiation factor 15 (NAG-1), and glucagon-like peptide (GLP1).

Based on the biological process classification of *in vitro* berberine targets, those targets related to signal transduction, intracellular signalling cascade, cell surface receptor linked signal transduction, cell motion, cell cycle control, immunity system process, and protein metabolic process are most frequently involved (Table 4). *In vitro* berberine

targets involved signal transduction include cyclin-dependant kinases (CDK1 and CDK6), inhibitor of nuclear factor kappa-B kinase subunit alpha (CHUK), epidermal growth factor receptor (EFGR), receptor tyrosine-protein kinase (ERBB2), glucagon-like peptide (GCG), growth/differentiation factor 15 (GDF15), interferon beta (IFNB1), interleukins (IL1B, IL2RA, and IL6), potassium voltage-gated channel subfamily H member 2 (KCNH1), mitogen-activated protein kinases (ERK1, ERK2, and MAPK8), nuclear factor-kappa-B p50 subunit (NFKB1), NF-kappa-B inhibitor alpha (NFKB1A), urokinase-plasminogen activator (PLAU), Ras-related C3 botulinum toxin substrate 1 (RAC1), Rho-associated protein kinase 4 (RHO), transforming protein RhoA (RHOA), proto-oncogene tyrosine-protein kinase ROS (ROS1), vascular endothelial growth factor A (VEGFA).

According to the PANTHER Classification System, *in vitro* berberine targets correlate with a mixture of biological pathways, such as Alzheimer disease-presenilin and secretase pathways, angiogenesis, apoptosis signalling pathway, FAS signalling pathway, Huntington disease, inflammation mediated by chemokine and cytokine signalling pathways, interleukin signalling pathway, and p53 pathways (Table 5).

The targets of berberine distributed across a large number of PANTHER classifications of molecular functions, biological processes, and pathways. This can be an advantage in terms of drug discovery using berberine. Seen that berberine targets are involved in a wide range of molecular activities, in turn, can alter many pathological states of the body. Thus, berberine can be explored for the treatment of different diseases. On the other hand, the nature of multitargeting of berberine lacks in target specificity which can become difficult for drug design. Further, because berberine can have interactions with so many molecular structures and involve in different pathways, much attention must be paid to avoid interactions with other therapeutic drugs.

3.3. Data from *In Vivo* Studies with a Focus on Diabetes Mellitus

In China, Huang Lian (*Rhizoma coptidis*) has been used to treat diabetes for more than 1,400 years [16]. Berberine is one of the main active alkaloids present in *Rhizoma Coptidis* and has shown to have good hypoglycaemic effects *in vitro* [37–39, 42]. Further, the chemical structure of berberine is different from the commonly used other hypoglycaemic agents such as sulphonylureas, biguanides, thiazolidinediones, or acarbose [14]. Thus, it is meaningful to investigate the efficacy and safety of berberine treatments for diabetes mellitus to confirm the possibility of berberine serving as a new class of antidiabetic medications. Extensive research has been done to investigate the hypoglycaemic effects of berberine in animal models. This section will highlight the effects of berberine in diabetic animal studies, focusing on different mechanisms of actions of berberine.

Hyperglycemia is a hallmark metabolic abnormality associated with metabolic diseases such as type 2 diabetes. Berberine has shown to significantly decrease fasting blood glucose levels in diabetic rats (diet or drug induced), this has been observed in a number of studies [43–46]. Berberine can reduce fasting blood glucose level via different mechanisms. For example, Liu et al. [43] reported that berberine reduced fasting blood glucose (FBG) levels by inhibiting intestinal disaccharidases in a concentration-dependent manner. Xia et al. [46] reported berberine reduced fasting glucose level via the inhibition of gluconeogenesis, via decreased *PEPCK* and *G6Pase* genes in the liver, reduced hepatic steatosis, and inhibition of FAS expression.

Current diabetes therapies do not address the key driver of this condition, *β*-cell dysfunction [47, 48], and do not alter the progressive nature of insulin secretory deficit [49]. Berberine increased pancreatic *β*-cell numbers and *β*-cell mass in streptozotocin-induced diabetic rats [41, 50]. It also reversed pathological changes of pancreatic *β*-cells in diabetic rats induced by streptozotocin and diet [51]. Further, in berberine treated diabetic rats, the pancreatic and plasma insulin levels increased after glucose load, reducing blood glucose levels [41, 50]. These observations are significant as berberine may be explored further as an additional therapy to existing antidiabetic drugs to effectively preserve *β*-cell functions, reverse *β*-cell damage, and promote insulin secretion in diabetes patients.

Further to *β*-cell dysfunction and insulin secretory deficit in diabetes, defects in insulin receptor (InsR) expression or function can cause insulin resistance and diabetes mellitus [52]. Thus, regulation of InsR expression may improve insulin resistance in diabetes mellitus. Berberine increases InsR mRNA and protein expression in human liver cells and in animal model in a dose- and time-dependent manner [38]. Berberine upregulates InsR and leads to enhanced insulin signalling pathway, confirming berberine as an insulin sensitiser.

Glucagon-like peptide 1 (GLP-1) is an intestinal peptide hormone released in response to food ingestion [53]. GLP-1 enhances meal-related insulin secretion and promotes glucose tolerance. In streptozotocin-induced rats, berberine enhanced GLP-1 release and promotes proglucagon mRNA expression, increased beta cell mass and pancreas insulin levels after glucose load [41]. This observation was in line with the groups, previous experiments *in vitro*. Lu et al. [50] also reported that berberine increased proglucagon mRNA expression and plasma insulin levels in streptozotocin-induced diabetic rats. The glucagon gene encodes GLP-1 and the increased expression of proglucagon mRNA assists in controlling the blood glucose homeostasis.

Berberine also reduced body weight and caused a significant improvement in glucose tolerance without altering food intake in *db/db* mice [54]. Oral glucose tolerance improvement in diabetic rats after berberine treatment has also been observed in other studies [55, 56].

Long-term hyperglycaemia can lead to increased risk of cardiovascular complications. In hyperglycemia and hypercholesterolemia rats with injured cardiac functions, berberine (15, 30 mg/kg/day, i.g for 6 weeks) increased cardiac output, left ventricular systolic pressure, and $+dp/dt_{max}$ by 64, 16, and 79%, but decreased left ventricular end diastolic pressure and $-dp/dt_{max}$ by 121 and 61% in the rats receiving HSFD/streptozotocin, respectively, when compared with the untreated rats of hyperglycemia and hypercholesterolemia [57]. Berberine caused significant increase in cardiac fatty acid transport protein-1 (159%), fatty acid transport proteins (56%), fatty acid beta-oxidase (52%), and glucose transporter-4. These results demonstrate the cardioprotective functions of berberine in hyperglycemia/hypercholesterolemia through alleviating cardiac lipid accumulation and promoting glucose transport 4 [57]. Another study also showed improved vasorelaxation in impaired aorta in diabetic rats after berberine treatment (100 mg/kg/day, 8 weeks) [45]. Thus, in addition to its hypoglycaemic effects, berberine can also be investigated for cardiomyopathy in diabetes.

Berberine also regulates lipid metabolism which is closely related to diabetes. In rats with induced diabetic hyperlipidemia, berberine (75, 150, 300 mg/kg/day for 16 weeks) effectively reduced liver weight and liver/body weight ratio, levels of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol [58]. In rats with a high fat diet, berberine significantly reduced body weight, alleviated liver steatosis, and improved insulin resistance [59]. This observation indicates that berberine can be an effective treatment for diabetes with obesity.

Clinically, preeminent factors for monitoring glycaemia and evaluating the risks of complications of diabetes include FBG, haemoglobin A_{1c} (HbA_{1c}) [60]. Triglyceride synthesis is closely associated with glucose metabolism so serum triglyceride levels are determined. Clinical studies often measure FBG, HbA_{1c}, and triglyceride levels, along with other factors to study the hypoglycaemic effects of berberine. The efficacies of berberine in type 2 diabetes patients have been reported. Through literature search, key clinical studies on berberine effects on type 2 diabetes patients are summarised.

Zhang et al. [61] conducted a randomized, double-blind, placebo-controlled multicenter trial (n = 116). The authors found that when berberine (1.0 g daily) was administered for 3 months in type 2 diabetes patients with dyslipidemia, the fasting and postload plasma glucose levels decreased from 7.0 ± 0.8 to 5.6 ± 0.9 and from 12.0 ± 2.7 to 8.9 ± 2.8 mM/L, HbA_{1c} from 7.5 ± 1.0% to 6.6 ± 0.7%. Further, in the treatment group, triglyceride levels were reduced from 2.51 ± 2.04 to 1.61 ± 1.0 mM/L, total cholesterol from 5.31 ± 0.98 to 4.35 ± 0.96 mM/L, and LDL-cholesterol from 3.23 ± 0.81 to 2.55 ± 0.77 mM/L. Results from the treatment group was significant compared to the control group. In the

treatment group, patient's body weight was also significantly reduced. Mild-to-moderate constipation was reported in 5 patients from the treatment group and 1 patient from the control group; however, this finding was not statistically significant. No other adverse events were reported. At 3 months, berberine was found to be effective in lowering blood glucose, lipids, body weight, and blood pressure with a good safety profile.

Yin et al. reported a 3-month study comparing berberine to antidiabetic drug metformin (0.5 g t.i.d) [14]. In this study, berberine exhibited identical effect as metformin in the regulation of glucose metabolism, significant decreases in HbA_{1c} (by 2%, $P < 0.01$), FBG (by 3.8 mmol/L; $P < 0.01$), and postprandial blood glucose (PBG) (by 8.8 mmol/L; $P < 0.01$). Further, the regulation of lipid metabolism was better in the berberine group than the metformin group. Triglycerides and total cholesterol levels were significantly lower than in the metformin group ($P < 0.05$). At the same time, the same group of researchers used berberine as a combination therapy to evaluate its additive or synergistic effects on the commonly used hypoglycemic agents, such as sulphonylureas, biguanides, thiazolidinediones, and acarbose. Patients were given 500 mg berberine three times daily for 3 months in addition to their previous treatment. At week 5, berberine significantly ($P < 0.01$) reduced HbA_{1c} (from 8.1% to 7.3%), FBG, PBG, and fasting insulin levels. Blood lipids including triglyceride, total cholesterol, and LDL-C decreased significantly lowered compared to baseline. In both studies, incidences of gastrointestinal adverse events were observed, including diarrhea, constipation, flatulence, and abdominal pain. Interestingly, patients did not suffer from severe gastrointestinal adverse events when berberine was used alone and in combination therapy; adverse effects disappeared after berberine dosage was reduced. No pronounced elevation in liver enzymes or creatinine was observed, suggesting that berberine did not cause damage to the liver or kidneys.

Another clinical study [62] randomly divided 97 type 2 diabetes mellitus patients into berberine treatment (1 g/day) for 2 months, using metformin therapy (1.5 g/day) and rosiglitazone group (4 mg/b.i.d) as reference groups. Blood samples were taking before and after treatments to measure FBG, HbA_{1c}, triglyceride, and serum insulin levels. Compared to values prior to treatment, berberine significantly lowered FBG by 25.9% ($P < 0.001$), HbA_{1c} by 18.1% ($P < 0.00$), and triglycerides by 17.6% ($P < 0.01$). The hypoglycaemic effects of berberine were comparable to metformin and rosiglitazone. Serum insulin level was declined significantly ($P < 0.01$) by 28.2%; this indicates increased insulin sensitivity in peripheral tissues by berberine treatment. Peripheral blood lymphocytes from berberine treated patients were isolated to examine the InsR expression. The surface expression of InsR significantly elevated by 3.6-fold after berberine treatment.

Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. So the effect of berberine was tested in hyperglycaemic patients with hepatitis. Hepatitis B and C patients with hyperglycaemia received berberine at 1 g/day for 2 months. In both diabetic hepatitis B and C patients, berberine significantly reduced FBG and triglyceride levels. Berberine treatment also reduced the elevated alanine transaminase and aspartate aminotransferase levels in these patients. Overall, berberine is safe and effective in hyperglycaemic patients with liver function damage.

Table 6 compares clinical studies of berberine in diabetes patients. Across the studies, berberine has shown to significantly reduce FBG, PBG, and HbA_{1c} levels. Berberine also demonstrated ability to reduce triglyceride and cholesterol levels. Minimal gastrointestinal side effects were shown but no liver or kidney damage was observed. These observations in diabetes patients demonstrate that berberine is a safe and effective antidiabetic agent.

Table 6.

Comparison of clinical studies of berberine in diabetes patients.

Study type	Study subjects	Berberine dosage	Control treatment	Major findings	Side effects	Reference
Randomised, double-blind, placebo-controlled, multiple-center	Type 2 diabetes and dyslipidemia (*n* = 116)	0.5 g, b.i.d for 3 months	Placebo	Significantly reduced fasting and postload plasma glucose, HbA_{1c} Significantly reduced triglyceride, total cholesterol, and LDL-cholesterol	Mild to moderate constipation in 5 patients	[61]
Randomised, blinded, placebo-controlled	Type 2 diabetes (*n* = 36)	0.5 g, t.i.d for 3 months	Metformin (0.5 g t.i.d)	Significantly reduced FBG, PBG, and HbA_{1c} Significantly reduced plasma triglycerides	Transient gastrointestinal adverse effects. No liver or kidney damage	[14]
	Type 2 diabetes poorly controlled (*n* = 48)	0.5 g, t.i.d for 3 months	Existing anti-diabetic treatment	Lowered FBG and PBG Significantly decreased HbA_{1c} Significantly reduced fasting plasma insulin and HOMA-IR		
Randomised	Type 2 diabetes (*n* = 97)	1 g/day for 2 months	Metformin (1.5 g/day); rosiglitazone (4 mg/day)	Significantly reduced FBG, HbA_{1c}, and triglycerides Serum insulin level was declined significantly ($P < 0.01$), increased insulin sensitivity in peripheral tissues Significantly elevated surface expression of InsR by 3.6-fold	No adverse events	[62]
	Type 2 diabetes with chronic hepatitis C virus infection (*n* = 35)	1 g/day for 2 months	N/A	Significantly reduced FBG and triglyceride levels Reduced the elevated ALT and aspartate aminotransferase levels		

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b.i.d: twice daily; t.i.d: three times daily; FBG: fasting blood glucose; HOMA-IR: homeostasis model of assessment—insulin resistance; PBG: postprandial blood glucose.

4. Discussion

The “rule-of-five” analysis by Lipinski et al. [7] shows that poor absorption or permeation of a compound is more likely when there are more than five hydrogen-bond donors; the molecular mass is more than 500 Da; the lipophilicity is high (expressed as *c*Log *P* > 5); the sum of nitrogen and oxygen is more than 10. Specific structural and physiochemical properties, such as “rule-of-five,” are required for clinical drugs to have sufficient levels of efficacy, bioavailability, and safety, which define target sites to which drug-like molecules can bind [4].

Plant compounds exhibit enormous structural diversity and only a small portion of the diversity has been explored for its pharmacological potential [64]. In recent years, herbal compounds have been source of new drugs [64]. Approximately 28% of new molecular entities (NMEs) between 1981 and 2002 were natural products or natural product derived; further to this, 20% of these NMEs were natural product mimics [65]. There are a number of successful plant-derived drugs, especially in anti-cancer treatment. Medicinal herbal compounds have become an important source for the discovery of new drugs. Further, drugs derived from medicinal plants can also be used as drug leads suitable for optimization by medicinal and synthetic chemists [65].

As Chinese herbal medicine becomes increasingly popular in the west, researchers are spending more time looking into mechanisms of actions of crude extracts and herbal compounds such as berberine. In recent years, extensive research has been done to explore the effects of berberine on various cell lines *in vitro*. In cell-based studies, berberine has shown effects on multiple molecular targets and alters various biological pathways. Berberine associates with a range of conditions, particularly diabetes, hyperlipidemia, and cancer. Many *in vitro* studies showed potent anticancer properties of berberine against various cancer cells. This observation is valuable in the search for new anti-cancer therapeutics with potent anti-cancer effects but reduced side effect. So berberine may potentially be developed into an anticancer agent, like other natural compounds (taxol, camptothecin) that have been developed and used as anticancer agents.

Diabetes mellitus is a major health problem around the world and its prevalence is on the rise. Diabetes mellitus drug therapy is limited by availability of effective medications, as existing oral hypoglycaemic agents often have side effects and fails in long-term administration [14]. Berberine has shown significant results in fasting blood glucose levels reduction, increase in insulin sensitivity, and improvement in insulin resistance *in vitro*, in diabetic animal models and in diabetic patients. Further, berberine shows mechanism that current antidiabetic drugs do not have. For instance, berberine has shown effects on pancreatic *β*-cell number and mass improvement [41, 50, 51]. In addition, berberine has a good safety profile and does not show side effects such as hypoglycaemia, weight gain, or liver and kidney damage. Metformin and rosiglitazone are not recommended for use in diabetic patients with liver function damage [54, 63]. Berberine has shown to be effective in the reduction of blood glucose level and is safe in diabetic patients with viral hepatitis [62]. Berberine can therefore be investigated as an effective diabetes therapy with patients with liver function damage. In addition to its hypoglycemic effects in diabetic patients, berberine also reduced triglyceride and cholesterol levels. Abnormalities in lipid metabolism often deteriorate diabetes and cause complications. The regulation of lipid metabolism in diabetes patients by berberine may have clinical significance in managing diabetic patients with hyperlipidemia. Although there are only a small number of clinical studies and evidence is limited, current reports still show a promising future for berberine being developed into a new antidiabetic agent.

In China, berberine has been manufactured into the over-the-counter drug Huang Lian Su Pian, also known as Coptis Extract Tablets for the treatment of traveler's diarrhea [14, 17]. However, *in vitro* and *in vivo* studies have shown that berberine has potent anti-cancer, antidiabetic, antilipidemic, and anti-inflammatory effects. Therefore, further clinical studies are warranted to investigate the potential of berberine in the application of cancer and diabetes treatments in the future.

Pharmacological activity of CHMs begins with the binding of the active components to their molecular targets. CHMs are considered as typical multitherapeutics that can interact simultaneously with multiple targets. The origins and the

progression of diseases are multifactorial. Complex disorders such as cancer, cardiovascular disease, and depression tend to result from multiple molecular abnormalities, not from a single defect [66]. Biochemical and genetic studies revealed the molecular mechanism that underlie common illnesses [66]. Reports show that targets for neoplasm diseases, circulatory system diseases, infectious diseases, and nervous system and sense organs disorders constitute the largest number of targets [1]. Because drug targets are presented at the molecular level, increased knowledge of herbal targets can facilitate deeper understanding of complex diseases at its fundamental level. In turn, it is likely to determine the optimal molecular targets for therapeutic intervention [6].

Further to assisting the molecular dissection of the mechanism of action of CHMs, knowledge on herbal targets makes it possible to use disease specific targets and design more desirable herbal drugs/formulas with increased specificity and efficacy. Target-oriented synthesis in drug discovery involves in preselected protein targets [67]. Binding of drugs to preselected protein target/s is dependent on which biological pathway the drug is aimed to modulate the target or the diseased pathway(s) [67]. Target and disease specific drug design results in improved efficacy and reduced side effects, especially in high impact diseases that require more effective and more treatment options. However, due to the fact that diseases often involve in multiple molecular abnormalities, diversity-oriented syntheses are used in efforts to identify simultaneously therapeutic protein targets and their small-molecule regulators [67]. Target-oriented drug design allows more focused drug design, which in turn costs less time and money for pharmaceutical companies.

Protein structure of well-validated old and new targets should be able to guide the chemical effort directed at new drugs [68]. Study of various aspects of known targets including molecular mechanism of their binding agents and related adverse effects is useful for finding clues to new target identification [9]. Based on the knowledge of molecular targets and molecular understanding of disease state and using this knowledge will allow some direction in identifying potential targets. Potential herbal targets may come from the same class as confirmed therapeutic targets and have similar physiological functions, or maybe a structure along a biological pathway. Additionally, with increased number of potential targets from ~500 to >5,000, the nature of pharmaceutical research has changed. This increase in numbers has given researchers more opportunities to discover and design new and improved drugs.

Target selection may be one of the most important determinants of attrition and the overall R&D productivity. There are few ways to overcome this challenge and improve the target selection process, in turn, improving R&D productivity. First of all, researchers can discover new target classes. Targets of herbal medicine are becoming a popular resource to find new target classes. In addition, increased understanding of genetic variations/polymorphisms of drug targets or drug metabolising enzymes can assist in target selection and drug metabolism. Further, the use of new technology can help to speed up the early exploratory discovery phase of drug discovery.

In summary, updated knowledge of herbal targets is valuable contribution to complex disease understanding and clinical responses. Further, drug discovery and development from herbal medicines can be supported by new target discovery and target-focused drug design. This will speed up the exploratory phase of drug R&D and benefit the pharmaceutical industry in terms of cost and time.

Authors' Contribution

Xiao-Wu Chen and Yuan Ming Di contributed equally to this work.

Abbreviations

Bax:
BCL2-associated X protein

FBG:
Fasting blood glucose

GLP-1:
Glucagon like peptide 1

HbA_{1c}:
Hemoglobin A_{1c}

InsR:
Insulin receptor

PBG:
Postprandial blood glucose.

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Transforming berberine into its intestine-absorbable form by the gut microbiota

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Abstract

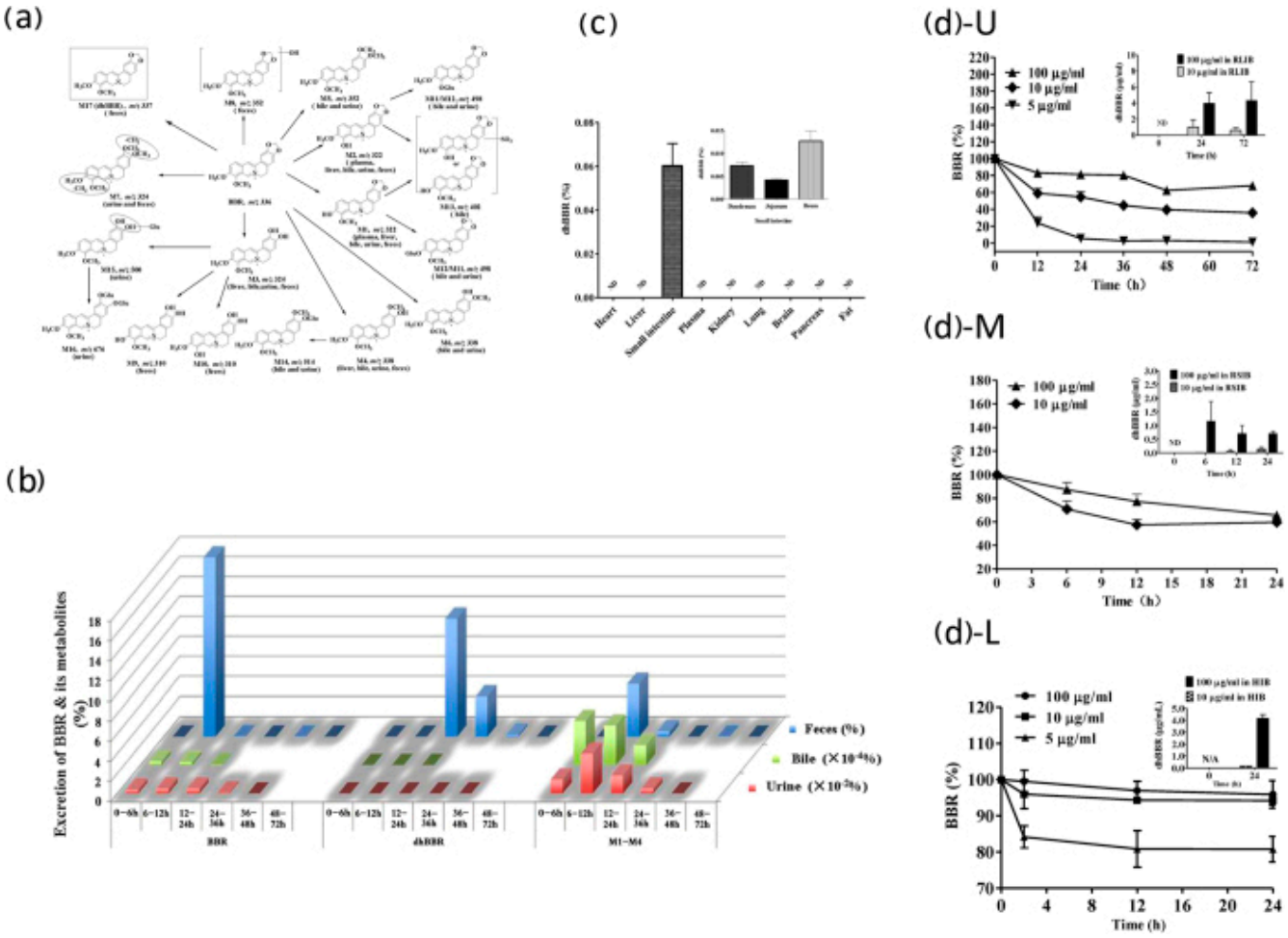
The gut microbiota is important in the pathogenesis of energy-metabolism related diseases. We focused on the interaction between intestinal bacteria and orally administered chemical drugs. Oral administration of berberine (BBR) effectively treats patients with metabolic disorders. However, because BBR exhibits poor solubility, its absorption mechanism remains unknown. Here, we show that the gut microbiota converts BBR into its absorbable form of dihydroberberine (dhBBR), which has an intestinal absorption rate 5-fold that of BBR in animals. The reduction of BBR to dhBBR was performed by nitroreductases of the gut microbiota. DhBBR was unstable in solution and reverted to BBR in intestine tissues via oxidization. Heat inactivation of intestinal homogenate did not inhibit dhBBR oxidization, suggesting the process a non-enzymatic reaction. The diminution of intestinal bacteria via orally treating KK-Ay mice with antibiotics decreased the BBR-to-dhBBR conversion and blood BBR; accordingly, the lipid- and glucose-lowering efficacy of BBR was reduced. Conclusively, the gut microbiota reduces BBR into its absorbable form of dhBBR, which then oxidizes back to BBR after absorption in intestine tissues and enters the blood. Thus, interaction(s) between the gut microbiota and orally administrated drugs may modify the structure and function of chemicals and be important in drug investigation.

The mammalian intestine hosts extremely diverse and vast microorganisms, collectively referred to as the gut microbiota, which mostly comprises four bacterial phyla: the Gram-negative Bacteroidetes and Proteobacteria and the Gram-positive Actinobacteria and Firmicutes[1]. In humans, the gut microbiota consists of more than 10^{14} bacteria and archaea, which cover approximately 1,100 prevalent species[1]. The gut microbiota is considered a “hidden organ” of the body and may be associated with the pathogenesis of diseases such as cardiovascular diseases, obesity, and diabetes[2,3,4]. The microbiota is also an interesting field of research in drug metabolism, particularly with respect to orally administered drugs because

the drug metabolism by gut microbiota may generate metabolites different from those generated by the host organs[5,6]. It is particularly important if the metabolites generated by the gut microbiota possess novel features and bioactivities. Thus, the drug metabolism by intestinal microorganisms is considered attractive in pharmaceutical research. Here, we present berberine (BBR) as an interesting drug example to show the significance of the gut microbiota in drug investigations.

BBR (Fig. 1a) is a medicinal alkaloid isolated from *Coptis chinensis* and has been used orally for decades in China as an over-the-counter (OTC) drug to treat diarrhea with good safety[7]. We and others have previously identified BBR as a new medicine for hyperlipidemia and type 2 diabetes because it reduces blood lipids and glucose in patients[7,8] through a multiple-target mechanism involving low-density-lipoprotein receptor (LDLR), insulin receptor (InsR), AMP-activated protein kinase (AMPK), and proprotein convertase subtilisin kexin 9 (PCSK9), among others[7,8,9,10,11]. The therapeutic effect of BBR on lipid- and glucose-related metabolic disorders was recently confirmed in a large number of patients by independent clinical groups both in and outside China[12], demonstrating the botanic compound to be a promising drug. By contrast, BBR exhibits poor water solubility and is presumably very difficult for intestinal epithelial cells to absorb[13,14]. However, after its oral administration, BBR was detected in nearly all major organs as well as in urine, with the liver exhibiting the highest concentration[15], and these findings raise the question of how BBR is absorbed in the intestine. Our recent study on the metabolism of BBR in the intestine has identified the gut microbiota as the most likely answer, at least in part, to this question, and we consider the finding of general interest in drug discovery and investigation.

Figure 1. BBR is metabolized into dhBBR in the intestine ecosystem.



Open in a new tab

(**a**) *In vivo* BBR metabolites in SD rats; M17 (*m/z* 337) was identified to be dihydroberberine (dhBBR), a BBR metabolite detected in the feces only; the parentheses indicate the sample in which the compound was detected. (**b**) Excretion of BBR, dhBBR and other BBR metabolites in rat feces, urine and bile after 72 h. (**c**) Distribution of dhBBR in organs of SD rats orally treated with BBR (200 mg/kg); ND: not detectable. (**d**) Conversion of BBR into dhBBR by the gut microbiota *in vitro*; U & insert: in rat large intestinal bacteria (RLIB); M & insert: in rat small intestinal bacteria (RSIB); L & insert: in human intestinal bacteria (HIB) *in vitro*. The Y axis shows the percentage of the administered amount of BBR (n = 6).

Results

DhBBR is a BBR metabolite generated in the intestinal ecosystem

To advance our understanding of the *in vivo* fate of BBR after oral administration, we recently investigated the BBR metabolites in the urine, bile and feces in rat. The compound detection was performed using both liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and gas chromatography coupled with mass spectrometry (GC-MS). Among the

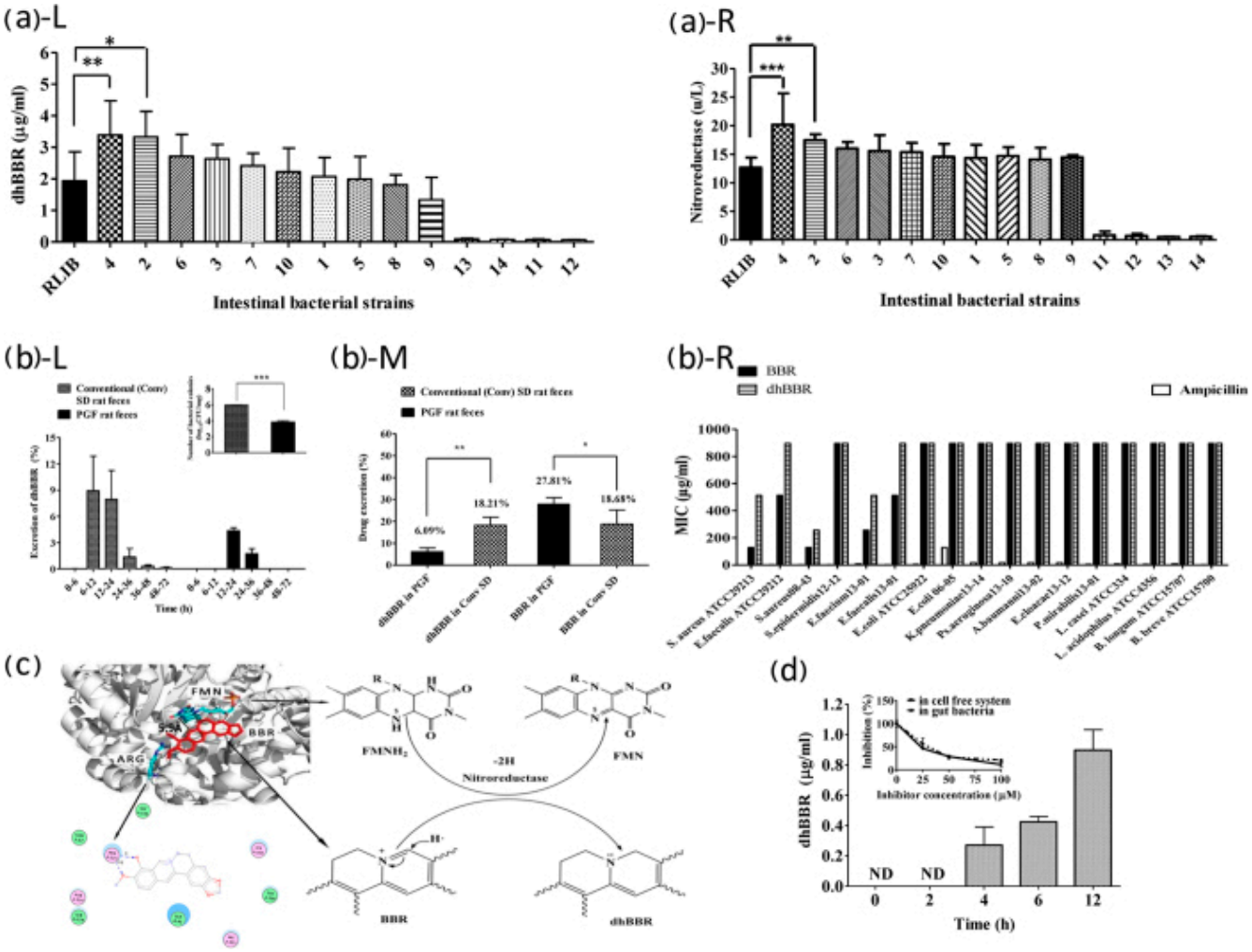
17 BBR metabolites detected in body excretion, a new metabolite, denoted dihydroberberine (dhBBR), caught our attention (Fig. 1a, in frame). First, dhBBR was detectable in feces but not in the bile and urine samples. Second, among these metabolites, dhBBR was the only one that exhibited a change in the backbone structure (Fig. 1a). We assumed that it might be a metabolite generated by the gut microbiota of rats. We then detected BBR, dhBBR and other BBR metabolites in rat excretions over a 72-h period after the oral administration of BBR. As shown in Fig. 1b, although other major BBR metabolites (M1-M4) were detectable in urine, bile and feces, dhBBR was detected only in feces. The amount of dhBBR reached its peak within 12–24 h and then decreased over time. To further confirm that dhBBR is generated within the intestine ecosystem, rat organs were examined for the presence of dhBBR. As shown in Fig. 1c, 24 h after the oral administration of BBR (200 mg/kg) in rats, dhBBR was detected in intestinal tissue only (including duodenum, jejunum and ileum) but not in other organs. Thus, we considered that rat intestinal bacteria might be the “organ” that converts BBR into dhBBR. We then incubated BBR with a large amount of rat intestinal bacteria *in vitro* and found that the amount of BBR decreased over time (Fig. 1d, U) and that, accordingly, dhBBR became detectable within 24 h of incubation (Fig. 1d, U insert). Next, we investigated samples of the rat small intestine by incubating BBR with bacteria isolated from the small intestine of untreated rats. As shown in Fig. 1d (M), the BBR content decreased over time and dhBBR became detectable within 6 h of incubation, suggesting that the bacteria from the small intestine were also active in the conversion of BBR into dhBBR. We also performed the experiment using human gut microbiota, and the results were similar to those obtained with rat gut bacteria (Fig. 1d, L).

Intestinal dhBBR was generated by gut microbiota

To determine whether the gut microbiota is responsible for the BBR-to-dhBBR conversion, we incubated BBR (50 μg/ml) *in vitro* with 14 intestinal bacterial strains (*Staphylococcus aureus, Enterococcus faecium, Enterococcus faecalis, Enterobacter cloacae, Escherichia coli, Staphylococcus, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Acinetobacter baumannii, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium longum, and Bifidobacterium breve*) and then measured the amount of dhBBR in the culture. The first 10 strains were clinical isolates and the last four strains are resident intestinal bacteria. As shown in Fig. 2a–L, all 10 of the clinical isolates produced dhBBR after incubation with BBR for 72 h. *Enterobacter cloacae* and *Enterococcus faecium* produced more dhBBR than did the rat feces, which were used as a positive reference, and *Proteus mirabilis* produced less (Fig. 2a). The four resident intestinal bacteria produced dhBBR at a level markedly lower than that obtained with the clinical isolates. The results suggest that the intestinal microbiota appeared to work as a “tissue” to transform BBR into dhBBR. If this is true, oral treatment of rats with antibiotics should reduce dhBBR production by the intestinal

bacteria. Thus, SD rats were treated orally with cefadroxil (100 mg/kg), terramycin (300 mg/kg) and erythromycin (300 mg/kg), bid, for 3 days. Two days later, the colon contents were collected for bacterial examination. As shown in Fig. 2b (L insert), oral treatment with the antibiotics for 3 days reduced the bacterial colony in the rat feces by 2 logs, indicating a pseudo germ-free (PGF) intestinal environment in the rats after treatment with antibiotics. BBR (200 mg/kg) was then orally administered to the PGF rats using untreated rats as a control; this step was followed by 72 h of feces collection. BBR and dhBBR in the feces were measured using LC-MS/MS 8040 and GC-MS. Fig. 2b, L shows that dhBBR production was delayed and markedly decreased in the intestine of the PGF rats compared with that in the untreated rats, consistent with the numbers of intestinal bacteria in the PGF rats. In addition, the 72-h-cumulative dhBBR level in the feces of the PGF rats was examined. The accumulated dhBBR was significantly higher in the untreated rats (18.21% of the giving amount) than that in the PGF rats (6.09% of the giving amount, $P < 0.01$); accordingly, the total BBR in the feces of the PGF rats was significantly higher (27.81% of the administered amount) than that in the untreated rats (18.67% of the administered amount, $P < 0.05$, Fig. 2b, M). To evaluate the direct effect of BBR on the gut microbiota, we incubated BBR separately with 17 strains of intestinal bacteria. As shown in Fig. 2b, R, the minimal inhibitory concentration (MIC) values of BBR on the 17 bacteria ranged from 128 to more than 512 μg/ml, which is a concentration close to that of BBR precipitation in the culture medium, indicating that the antibacterial effect of BBR on the gut microbiota was minor. In addition, the antibacterial effect of dhBBR was even weaker than that of BBR in 5 of the 17 intestinal bacteria strains (Fig. 2b, R). Ampicillin was used as the positive control in this experiment.

Figure 2. Generation of dhBBR by the gut microbiota.



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(**a**) BBR was converted *in vitro* into dhBBR by 14 intestinal bacteria strains [*S. aureus* 08-43 (1), *E. faecium* 13-01 (2), *E. faecalis* 13-01 (3), *E. cloacae* 13-12 (4), *E. coli* 06-05 (5), *S. epidermidis* 12-12 (6), *Ps. aeruginosa* 13-10 (7), *K. pneumoniae* 13-14 (8), *P. mirabilis* 13-01 (9), *A. baumannii* 13-02 (10), and *L. casei* (11), *L. acidophilus* (12), *B. longum* (13), and *B. breve* (14)]. L: dhBBR was produced at different levels by the 14 intestinal bacteria strains (*$P < 0.05$ and **$P < 0.01$, n = 6); R: Determination of nitroreductase in the 14 intestinal bacteria strains (***$P < 0.001$, *$P < 0.05$, n = 6). (**b**) Oral treatment with antibiotics generated pseudo germ-free (PGF) rats and resulted in a reduced conversion of BBR to dhBBR in the rat intestinal ecosystem; L: dhBBR production was delayed and decreased in the feces of PGF rats compared with conventional SD rats; L insert: oral treatment with antibiotics for 3 days reduced the bacterial colony numbers by 2 logs in the rat feces (***$P < 0.001$, n = 6); M: the accumulative excretion of BBR and dhBBR in PGF and conventional rats over a period of 72 h (**$P < 0.01$, *$P < 0.05$, n = 6); R: MIC of BBR and dhBBR on the 17 intestinal bacteria (24 h); The Y axis shows the percentage of the administered amount of BBR. (**c**) Molecular docking between BBR and nitroreductase, showing the chemical me**c**hanism of BBR reduction by nitroreductase. (**d**) Nitroreductase-mediated BBR reduction resulted in a BBR-to-dhBBR conversion after 4 h; insert: BBR-to-dhBBR conversion was reduced in the presence of the nitroreductase inhibitor 2-IBA (100 μM) in the nitroreductase-containing cell-free incubation mixture or in the rat gut bacteria cultivation mixture (for 12 h); ND: not detectable.

BBR was converted to dhBBR in a reduction reaction, and nitroreductase and azoreductase are two major reductases that are abundant in the gut microbiota[16]. Because the crystal structures of the reductases have been illustrated and are available in the Protein Data Bank[17], we performed computer-assistant docking in the initial step of analysis, using the AutoDock Vina v.1.0.2 software. The putative chemical mechanism for BBR reduction by nitroreductase is shown in Fig. 2c. BBR exhibited excellent docking performance at docking onto nitroreductase with a binding free energy of −6.5 kcal/mol. BBR anchored into the binding site of nitroreductase through a strong hydrogen bond interaction with the side chain of Arg-225. The distance between the C atom of the C = N group and the N_5 atom of FMN was approximately 5.9 Å, which is within the distance good for electron transfer. After binding to nitroreductase in the pocket, BBR obtained H· from the FMN-$FMNH_2$ system, and its reduction was activated. The H· from $FMNH_2$ moved to the C atom of C = N in BBR, forcing the transfer of one pair of π electrons toward the N atom. The two electrons occupied the orbit of the N atom, forming a lone pair of electrons. The C = N was then transformed into C-N, and BBR was converted into dhBBR (Fig. 2c).

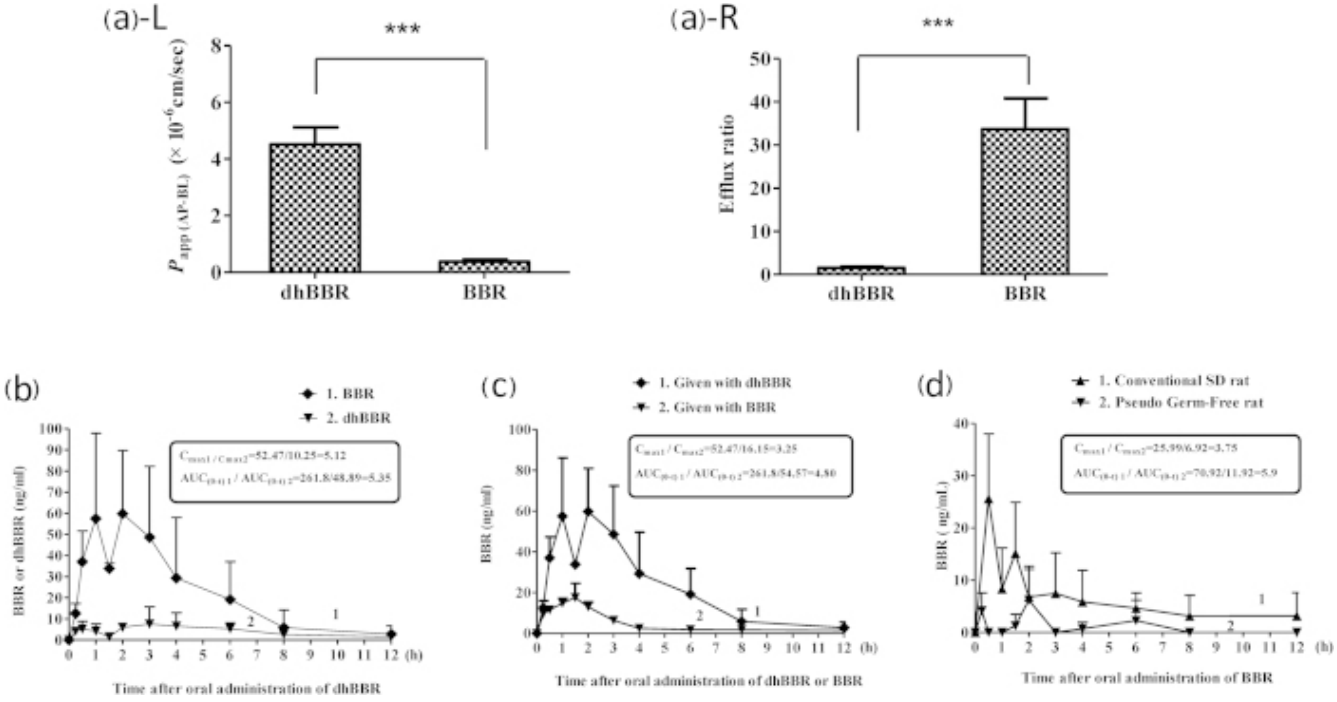
To validate the computational results, the nitroreductase-mediated BBR-to-dhBBR conversion was studied *in vitro*. Following the incubation of BBR with nitroreductase for 0, 2, 4, 6 and 12 h at 37 °C, the amount of dhBBR was examined. As shown in Fig. 2d, dhBBR became detectable after 4 h of incubation of BBR with nitroreductase, and its amount increased over time. Addition of the nitroreductase inhibitor 2-iodosobenzoic acid (2-IBA) into the incubation system significantly inhibited the conversion of BBR to dhBBR, and the inhibition rate reached 88.28% with an increase in the 2-IBA concentration to 100 μM (Fig. 2d, insert), suggesting that the nitroreductase enzymes of the gut bacteria may be an important bacterial enzyme that transforms BBR into dhBBR. Addition of 2-IBA into the incubation system of BBR with the mixture of rat gut bacteria also inhibited dhBBR production by 78.7%, which is in agreement with the results obtained with the cell-free enzymatic reaction. Other bacterial reductases, such as azoreductase (binding free energy of −6.9 kcal/mol), may also be contributors to the conversion. In addition, the correlation between the nitroreductase level in bacteria and BBR-to-dhBBR conversion was examined. In general, as shown in Fig. 2a, L&R, the level of nitroreductase in bacteria was positively correlated with the capability of reducing BBR (into dhBBR), supporting the finding that nitroreductase is at least one of the key enzymes in the conversion of BBR into dhBBR.

DhBBR exhibited an intestinal absorption rate higher than that of BBR

DhBBR in its sulfate form (dihydroberberine sulfate) was previously reported to exhibit better absorption in the intestine as compared with BBR[18]. Here, we focused our investigation on the absorption of the original dhBBR generated by the gut microbiota. First, the absorption of

BBR and dhBBR was investigated in the Caco-2 cell model. In this *in vitro* system, dhBBR displayed significantly improved absorption as compared with BBR. The $P_{app\ (AP\text{-}BL)}$ (apparent permeability coefficient of AP-BL; AP: apical; BL: basolateral) for dhBBR was 11.9-fold higher than that for BBR (4.51×10^{-6} cm/s vs. 0.38×10^{-6} cm/s), suggesting that the bioavailability of dhBBR for absorption was markedly increased (Fig. 3a, L). Furthermore, we found that the efflux ratio (ER) for dhBBR was significantly lower than that for BBR (1.58 versus 32.39), indicating a substantial decrease in the cellular efflux of the compound (Fig. 3a, R). Second, the intestinal absorption of dhBBR was studied via *in vivo* experiments in which dhBBR was orally administered to SD rats at a dose of 200 mg/kg. Interestingly, dhBBR was detected at a level less than 10 ng/ml in the plasma (Fig. 3b); instead, a substantial amount of BBR was detected in the blood with an $AUC_{(0\text{-}t)}$ value of 261.8 ng/ml*h and a maximum concentration (C_{max}) of 52.47 ng/ml (Fig. 3b). The findings suggest that a nearly complete reversion of dhBBR into BBR most likely occurred in the well of the intestine, resulting in a low level of dhBBR and a high level of BBR in blood. The absorption rate of dhBBR (200 mg/kg, oral) was then compared with that of BBR (200 mg/kg, oral). As shown in Fig. 3c, the blood level of BBR in the dhBBR-treated rats exhibited $AUC_{(0\text{-}t)}$ and C_{max} values that were 4.8- and 3.25-fold higher than those of the BBR-treated rats, respectively, indicating a higher intestinal absorption of dhBBR as compared with that of BBR.

Figure 3. DhBBR has a better intestinal absorption than BBR.



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(**a**) Absorption of BBR and dhBBR in the Caco-2 cell model; L: The $P_{app\ (AP-BL)}$ of dhBBR in Caco-2 cells was 11.9-fold higher than that of BBR (***$P < 0.001$); R: The efflux ratio of dhBBR in Caco-2 cells was significantly lower than that of BBR (1.58 vs. 32.39, ***$P < 0.001$). (**b**) Concentration-time curve for BBR or dhBBR in plasma after dhBBR oral administration (200 mg/kg) to rats (n = 3). (**c**) Concentration-time curve of BBR in plasma after the oral administration of dhBBR (200 mg/kg) or BBR (200 mg/kg) (n = 3). (**d**) Concentration-time curve of BBR in pseudo germ-free rats (generated by the oral administration of antibiotics for 3 days, curve 1) or in conventional SD rats (with no antibiotics, curve 2) after the oral administration of BBR (200 mg/kg, n = 3).

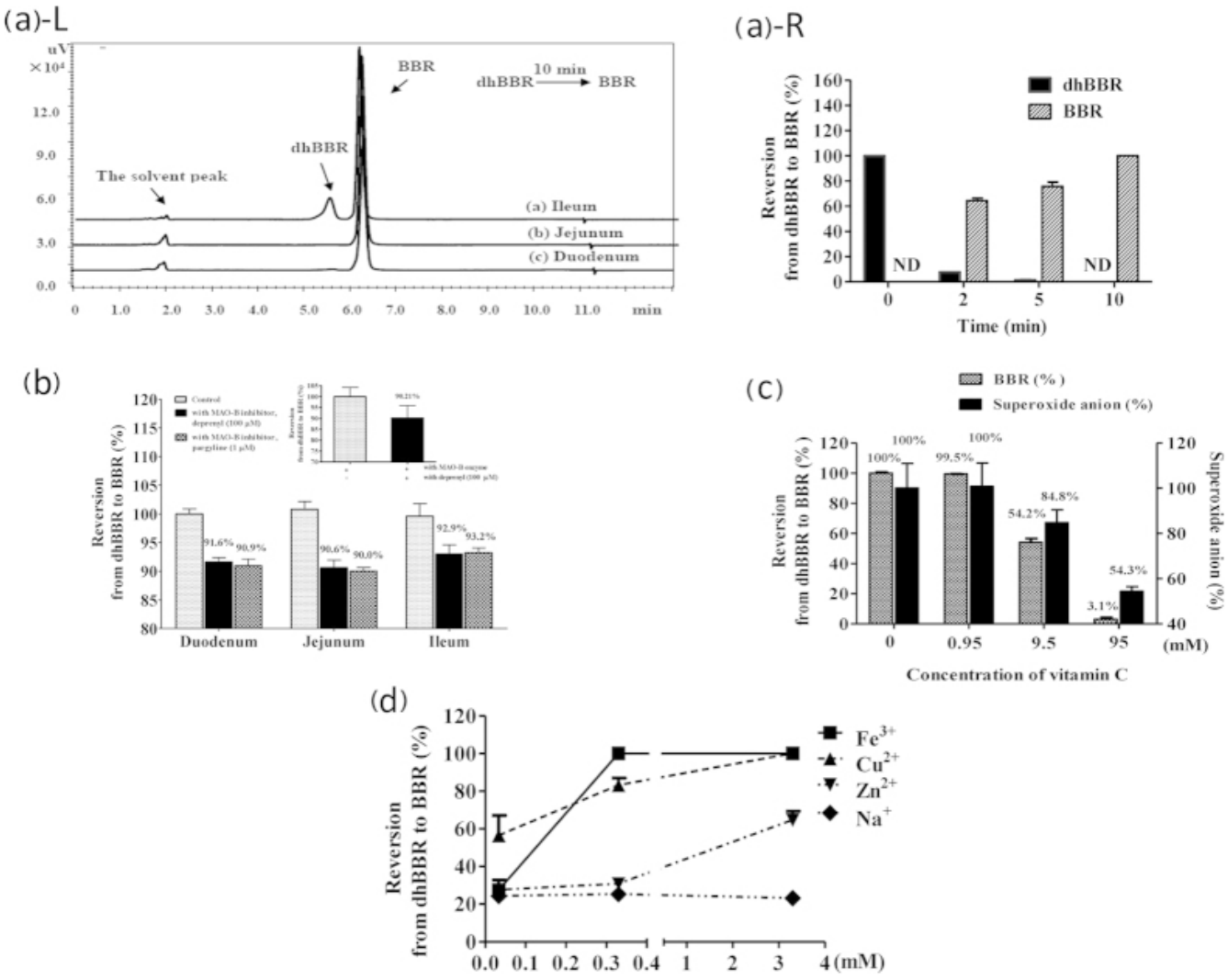
The results were further tested through another approach. We hypothesized that if dhBBR is the absorbable form of BBR, animals with a low intestinal bacteria content should have less plasma BBR because the level of conversion of BBR to dhBBR in the intestine should be low. We orally treated PGF rats (generated by treatment with antibiotics as mentioned above) with BBR (200 mg/kg) and then measured the blood BBR over a period of 12 h, and antibiotic-free rats administered the same dose of BBR served as control. As shown in Fig. 3d, the blood level of BBR in the PGF rats was much lower than that in those without antibiotics, with a C_{max} ratio of 1/3.75 (PGF rats vs. rats with no antibiotics) and an $AUC_{(0\text{-}t)}$ ratio of 1/5.9 (PGF rats vs. rats with no antibiotics), respectively. It seems that the antibiotic-induced decrease in intestinal bacteria resulted in a low level of conversion of BBR into dhBBR in the intestine and thus a low level of BBR in the blood. The results supported the hypothesis presented above. Although dhBBR presented good absorption characteristics, its activity on energy metabolism was lower than that of BBR. As shown in Figure S1 (Supplementary Figure S1), dhBBR

remained active for up- regulating LDLR expression but at a level much lower than that of BBR.

DhBBR reverted to BBR via oxidization in intestine tissues

Because dhBBR was barely detectable in the blood, liver and other organs in rats orally treated with BBR (Fig. 1c), we assumed that dhBBR generated by the gut flora in the intestinal cavity must revert to BBR immediately after it enters intestinal wall tissues. Therefore, we incubated dhBBR with homogenates of the small intestine of rats, including homogenates of the duodenum, jejunum or ileum. After a 10-min incubation, dhBBR reversed to BBR almost completely in the duodenum and jejunum homogenate and at a rate of more than 80% in the ileum homogenate (Fig. 4a, L). Human intestinal microsomes, which were rich in CYP450s, also reversed dhBBR to BBR very rapidly (Fig. 4a, R).

Figure 4. DhBBR reverted to BBR via oxidization in intestine tissue.



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(**a**) dhBBR-to-BBR reversion in the rat small intestine homogenate (L) and in human intestine microsomes (HIMs, R) (the Y axis shows the percentage of administered amount of dhBBR); ND: not detectable. (**b**) DhBBR-to-BBR reversion by monoamine oxidase-B (MAO-B) in a cell-free system (insert) and homogenates of the rat duodenum, jejunum and ileum; the reversion was slightly inhibited by the MAO-B inhibitor deprenyl (100 μM) or pargyline (1 μM) (the Y axis shows the percentage of administered amount of dhBBR). (**c**) Addition of vitamin C into the homogenate almost terminated the oxidization reaction for reverting dhBBR to BBR, and the content of superoxide anion decreased subsequently (the left Y axis shows the percentage of administered amount of dhBBR; the right Y axis shows the percentage of superoxide anion of control). (**d**) Effect of metal ions (Fe^{3+}, Cu^{2+}, and Zn^{2+}) on catalyzing the oxidization of dhBBR to BBR.

Therefore, we investigated the roles of eight subtypes of CYP450 isoforms in the intestine homogenate microsome. The specific CYP450 inhibitors used in the study were furafylline for CYP1A2, ticlopidine for CYP2B6, quercetin for CYP2C8, sulfaphenazole for CYP2C9, nootkatone for CYP2C19, quinidine for CYP2D6, ketoconazole for CYP3A4 and

diethyldithiocarbamate (DDC) for CYP2E1. Although all of the recombinant CYP450 isoforms nearly completed the dhBBR-to-BBR reversion (data not shown), the reversion rate in the presence of CYP450 inhibitors was not different from that obtained in the absence of inhibitors in the experiment using pooled human intestinal microsomes (HIMs). This finding suggests that CYP450 might not be critical for reverting dhBBR to BBR. Our next focus was intestinal oxidases, particularly monoamine oxidase (MAO-B)[19]. We incubated dhBBR with MAO-B in a cell-free system first and found that MAO-B may be responsible for approximately 10% reversion from dhBBR to BBR (Fig. 4b, inert). DhBBR was then incubated for 30 min with intestinal homogenate (including duodenum, jejunum or ileum) in the presence or absence of the MAO-B inhibitor deprenyl or pargyline, and this step was followed by BBR detection. As shown in Fig. 4b, incubation of dhBBR with intestinal homogenate resulted in a complete dhBBR-to-BBR reversion, but this reversion was reduced by less than 10% in the presence of a high concentration of the MAO-B inhibitors, which is consistent with the results obtained with the cell-free reaction. It appeared that tissue MAO-B may play a minor role in the dhBBR oxidization course. We then turned our attention to the other factors.

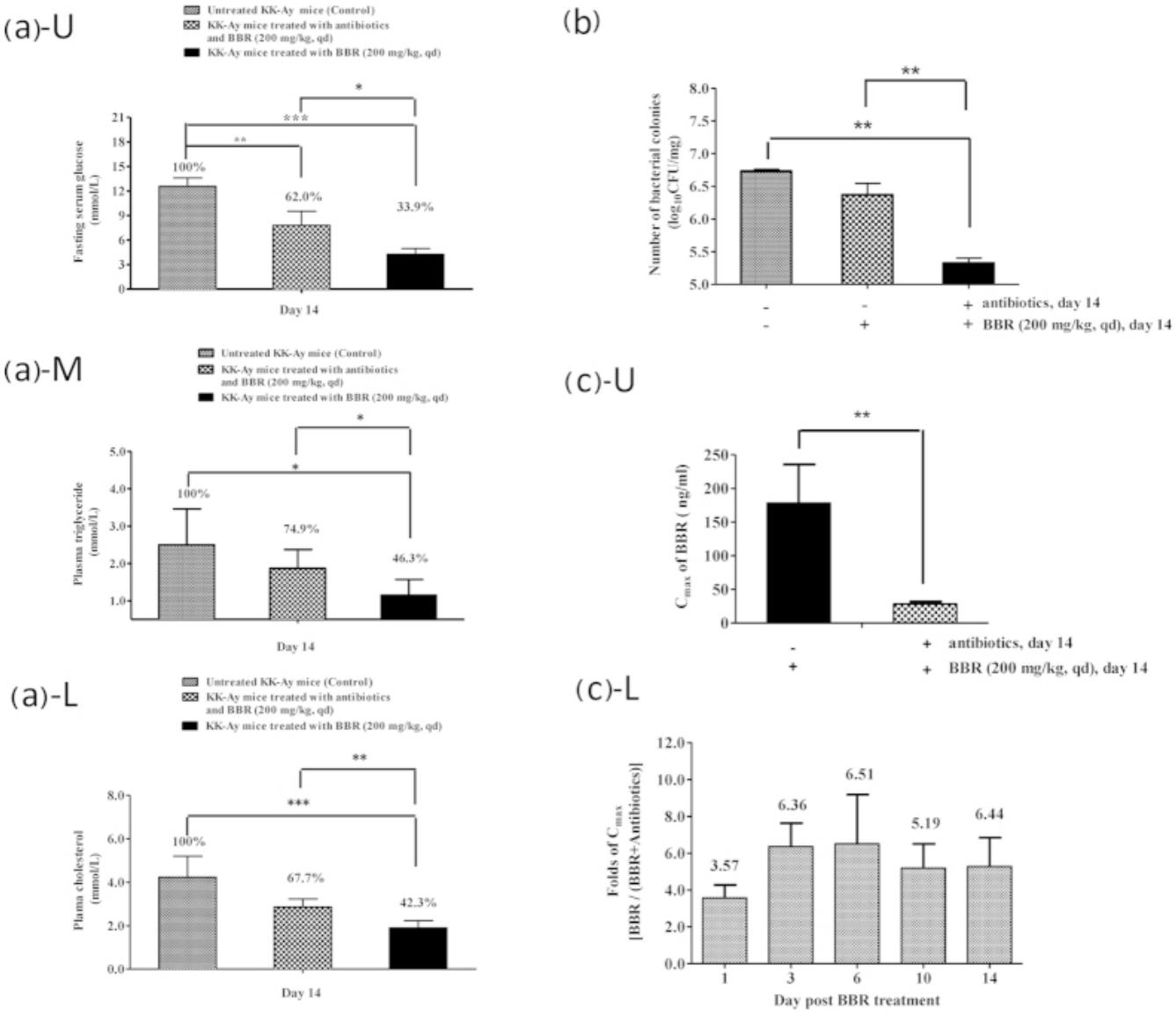
To inactivate proteins within the reaction system, the intestinal homogenate solution was boiled for 2 min. As shown in Figure S2 (Supplementary Figure S2), heat inactivation of the homogenate did not reduce the dhBBR-to-BBR reversion, indicating that the oxidization reaction was mainly a non-enzymatic reaction. Vitamin C, a strong antioxidant, was then added to the reaction solution. As shown in Fig. 4c, the dhBBR-to-BBR reversion was almost completely terminated by vitamin C, suggesting that the oxidization reaction is crucial for the dhBBR-to-BBR reversion in intestinal tissues. Moreover, in this reaction, the content of superoxide anion appeared to be positively correlated with BBR production (Fig. 4c). Metal ions, such as Fe^{3+}, Cu^{2+} and Zn^{2+}, which are a type of oxidant, appeared to play a positive role in catalyzing the oxidization process, whereas Na^{+} had no effect (Fig. 4d). However, the role of these metal ions (such as Fe^{3+}, Cu^{2+} and Zn^{2+}) may be weak in the system because the addition of EDTA-2Na reduced the dhBBR-to-BBR oxidization by only 5%.

Effect of the gut microbiota on the therapeutic efficacy of BBR

To determine whether the gut microbiota alters the therapeutic effects of BBR, we used KK-Ay mice with type 2 diabetes. The mice were either orally pre-treated or untreated with antibiotics for 3 days and then treated with BBR with or without simultaneous treatment with antibiotics. The therapeutic results on day 14 post-BBR treatment are shown in Fig. 5. Compared with the efficacy of BBR in ordinary KK-Ay mice (with no exposure to antibiotics, Group 2), the oral treatment of KK-Ay mice with antibiotics before and during BBR treatment (Group 1) reduced the therapeutic efficacy of BBR on the fasting blood glucose level by 42% [(1−(100–62)/(100–33.9))×100%], the triglyceride level by 53% [(1−(100–74.9)/(100–46.3))×100%] and the

cholesterol level by 44% [(1−(100–67.7)/(100–42.3))×100%] (Fig. 5a U, treated vs. untreated, $P < 0.01$, $P < 0.001$; antibiotics plus BBR vs. BBR, $P < 0.05$; Fig. 5a M, treated vs. untreated, $P < 0.05$; antibiotics plus BBR vs. BBR, $P < 0.05$; Fig. 5a L, treated vs. untreated, $P < 0.001$; antibiotics plus BBR vs. BBR, $P < 0.01$). Accordingly, the number of intestinal bacteria colonies in the PGF KK-Ay mice treated with both BBR and antibiotics (Group 1) was significantly lower than that in KK-Ay mice treated with BBR only (Group 2; $P < 0.01$, Fig. 5b). In addition, mice treated with both antibiotics and BBR had fewer intestinal bacteria colonies compared with the untreated controls (Group 1 vs. untreated controls, $P < 0.01$, Fig. 5b).

Figure 5. Gut microbiota modulates the therapeutic effect of BBR *in vivo*.



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(**a**) Fasting blood glucose (Glu), triglyceride (TG) and cholesterol (CHO) in conventional KK-Ay mice or pseudo germ-free KK-Ay mice (n = 6) treated with (or without) BBR for 14 days; U: plasma glucose; M: plasma triglyceride; L: plasma cholesterol. (**b**) Number of intestinal bacteria colonies (in log scale) on day 14 (n = 6). (**c**) Plasma concentration of BBR in KK-Ay mice treated or untreated with antibiotics; U: C_{max} of BBR on day 14; L: folds of C_{max}[BBR/(BBR+antibiotics)] on days 1, 3, 6, 10 and 14 post-BBR oral administration. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

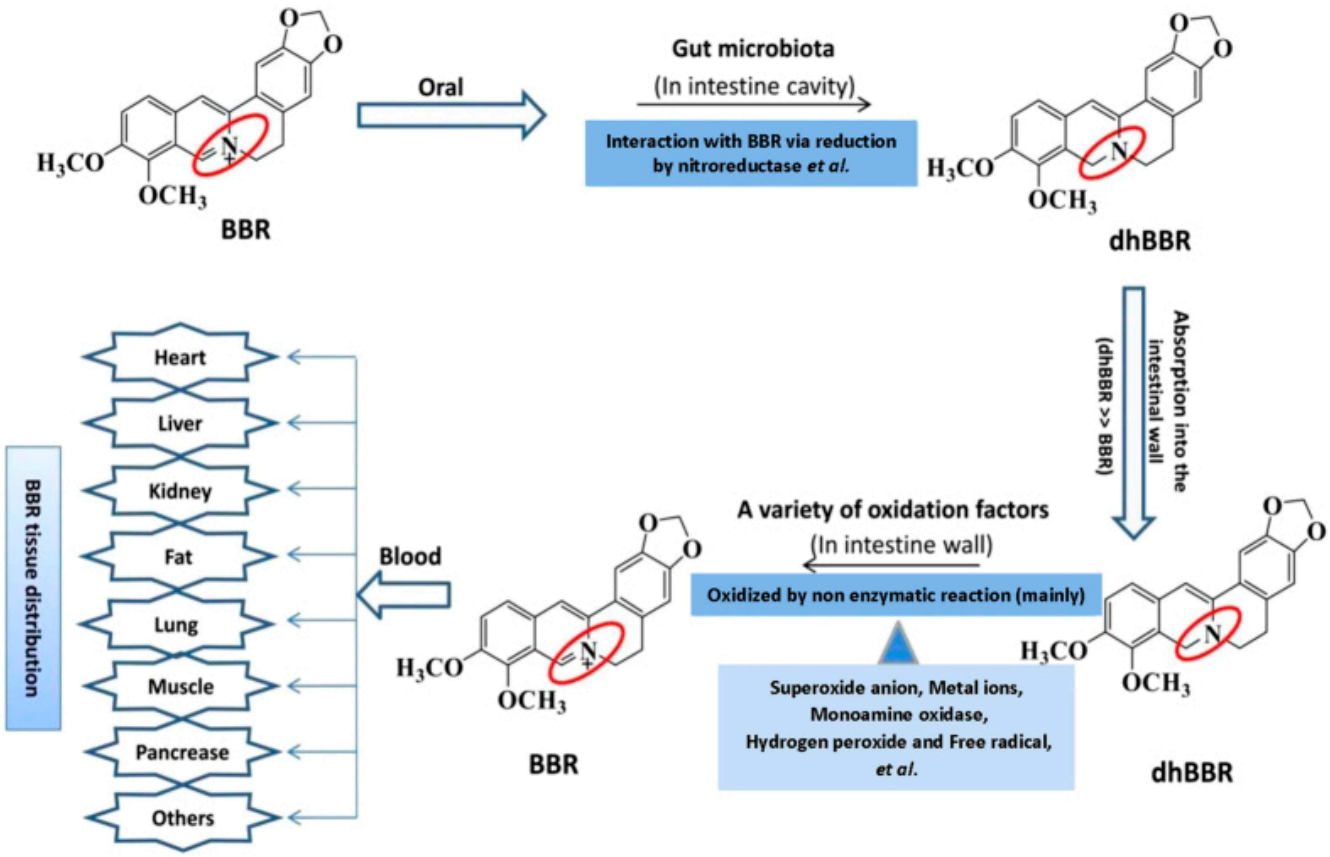
In addition, we measured the BBR plasma concentrations in KK-Ay mice in the BBR-treated groups with or without antibiotics (Group 1 vs. Group 2). As shown in Fig. 5c (U), on day 14, the C_{max} value of BBR in mice treated with antibiotics (Group 1) was 28.32 ± 3.41 ng/ml whereas in those without antibiotics (Group 2) the BBR C_{max} value was 178.15 ± 57.61 ng/ml, which is 6.4-fold higher than that in Group 1 ($P < 0.01$). In fact, the C_{max} value in Group 2 was 3.5-6.5-fold higher than that in Group 1 throughout the BBR treatment course on days 1, 3, 6,

10 and 14 (Fig. 5c, L). It appeared that the oral administration of antibiotics could lower the level of intestinal bacteria and, accordingly, decrease the absorption of BBR into the blood, reducing its therapeutic efficacy in hyperglycemia and hyperlipidemia.

Discussion

This study shows that the gut microbiota can act as an “organ” that converts BBR into dhBBR in the intestine via a reduction reaction mediated through bacterial nitroreductase. DhBBR, which was found to be absorbable by the intestinal epithelia, was reverted to BBR (the active form) immediately after entering the intestinal wall tissues. The reversion of dhBBR to BBR via oxidization occurred mainly through a non-enzymatic reaction in intestinal epithelial tissues, with the participation of multi-faceted factors such as superoxide anion and metal ions. These factors in the intestine ecosystem worked in a coordinated manner to complete the structure transformation between BBR (C = N) and dhBBR (C-N). Because the conversion-absorption-reversion process took place entirely in the intestinal environment, BBR, but not dhBBR, is the main chemical form detectable in the blood. Oral administration of antibiotics decreased the amount of intestinal bacteria and thus suppressed BBR absorption, resulting in a low level of BBR in the blood as well as reduced therapeutic efficacy. This study presents an interesting case to show the importance of the gut microbiota in modifying drug bioavailability and therapeutic efficacy. Figure 6 summarizes the role of the gut microbiota in regulating the conversion-absorption -reversion process of BBR in the intestine system.

Figure 6. Proposed mechanism of BBR absorption in the intestine.



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Nitroreductase in the gut flora plays a very important role in converting BBR into dhBBR. Because this enzyme is abundant in many types of intestinal bacteria[3], the uptake of BBR in the human intestine should be relatively steady among races and individuals. The physiological condition for dhBBR oxidization is so common that dhBBR molecules readily reverse to BBR, which then enters the bloodstream. In fact, the lipid-lowering and hypoglycemia effects of BBR have been verified in patients by independent clinical groups in China, Europe and America, with similar efficacies (reducing the fasting blood glucose level by 21–36%, the LDL-c level by 10–25% and the triglyceride level by 20–35%)[9]. This finding suggests that the bioavailability of BBR in humans is highly stable. Furthermore, nitroreductase in the gut microbiota may be a rate-limiting step for controlling the amount of BBR entering the blood because the intestinal nitroreductase level is approximately constant at a comparatively flat range in humans. Indeed, BBR exhibits good safety in the clinic and is classified as an OTC drug in China. This study also raises an important issue for patients orally receiving drug treatment together with antibiotics because the uptake rate of drugs (such as BBR) may be modified through antibiotic-mediated changes in the gut microbiota, and, accordingly, the clinical therapeutic efficacy may be altered.

Although dhBBR has a higher intestinal absorption rate than that of BBR, its bioactivity on LDLR and bacteria was lower than that of BBR. The finding agrees with our previous analysis of the structure-activity relationship of BBR, which showed that the 7-position quaternary ammonium and planar structure of the compound are required for LDLR upregulation[20,21,22,23]. It appears that dhBBR is a transient form of BBR in the intestinal lumen, with improved physiochemical characteristics for absorption. Although BBR is considered an antibacterial agent, its activity is directed mainly to dysentery bacillus[24]. The MIC values of BBR for the 17 tested strains were all higher than 128 μg/ml, with most of the values being at least 512 μg/ml, a concentration close to that of BBR precipitation in the testing system. The results showed that the antibacterial effect of BBR was very weak for ordinary intestinal bacteria, and the effect of dhBBR was even weaker. The reduction of BBR to dhBBR by nitroreductase enzymes of the gut microbiota may imply the existence of a self-protection mechanism of intestinal bacteria. In fact, because nitroreductase has broad substrate specificity and reduces groups of compounds, the enzyme is thought to play a role in the general detoxification of bacteria[16,25].

The connection between gut microbes and the pathogenesis of energy metabolic disorders and cardiovascular disease has recently piqued research interest in the physiological function of the gut microbiota in the human body[26,27,28,29,30]. This interest has been extended to the interaction between intestinal bacteria and orally administered drugs such as BBR[31,32]. Drug metabolism by the intestinal microflora may potentially generate new metabolites with distorted bioactivities or properties; however, the role of the gut microbiota and its influences on orally administered drugs are much less understood. Studies of the functional connections between the gut microbiota and drug pharmacology are even scarcer. The present study on BBR provides a paradigm showing the role of the gut microbiota in modifying drug structure and altering its absorption and therapeutic efficacy. The results may enrich our understanding and knowledge of drug metabolism *in vivo*, drug-drug interaction, mode of action, and druggability prediction. Our findings regarding the bacteria-caused structural modification of BBR in the intestine, as well as the changes in its absorption and therapeutic effects, lead us to suggest that investigation of drug metabolism by the gut microbiota should be a step in drug development.

Methods

Chemicals and reagents

BBR was obtained from J&K Scientific Ltd (Beijing, China). Tetrahydropalmatine, as an internal standard, was purchased from the National Institute for Food and Drug Control (Beijing, China). Thalifendine (M1), berberrubine (M2), demethyleneberberine (M3),

jatrorrhizine (M4) and dihydroberberine (dhBBR, M17) were obtained from Chengdu Herb Purity Co., Ltd (Chengdu, China). Rutin was purchased from the National Institute for Food and Drug Control (Beijing, China). The purity of the above-mentioned standards was more than 98% (HPLC). HPLC-grade acetonitrile was obtained from J&K Scientific Ltd (Beijing, China). All the other chemicals and reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China) and were of HPLC-grade purity.

Ticlopidine, ketoconazole, β-nicotinamide adenine dinucleotide phosphate (NADP), D-glucose 6-phosphate (G-6-P) and D-glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Furafylline, diethyldithiocarbamate (DDC) and sulfaphenazole were obtained from Santa Cruz Biotechnology Co. Ltd. (Shanghai, China). Quercetin, nootkatone and quinidine were purchased from J&K Scientific Ltd. (Beijing, China).

Nitroreductase (≥90%, recombinant, expressed in *Escherichia coli*), monoamine oxidase-B (recombinant, expressed in baculovirus-infected BTI insect cells), and deprenyl (a monoamine oxidase-B inhibitor) were purchased from Sigma-Aldrich Co. (Shanghai, China). 2-Iodosobenzoic (2-IBA, a nitroreductase inhibitor) and pargyline (another monoamine oxidase-B inhibitor) were obtained from J&K Scientific Ltd. (Beijing, China). Human intestinal microsomes (HIMs) were obtained from the Research Institute for Liver Diseases (Shanghai, China).

Animals

Male Sprague–Dawley (SD) rats (180–200 g) were supplied by the Institute of Laboratory Animal Science, Chinese Academy Medical Sciences (Beijing, China). The animals were housed in cage racks, with a 12-h light/12-h dark cycle (light on from 8:00 AM to 8:00 PM) at ambient temperature (22 °C–24 °C) and 45% relative humidity. The rats were fasted for 12 h before the experiments but during the study had free access to food and water. The research was conducted in accordance with the institutional guidelines and ethics and approved by the Laboratories Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College.

Instruments

A liquid chromatography instrument coupled to an ion trap time-of-flight mass spectrometer (LC/MS^n-IT-TOF) from Shimadzu Corporation (Kyoto, Japan) was used to identify the chemical structures of BBR and its possible metabolites. Liquid chromatography with tandem mass spectrometry (LC–MS/MS 8040 or 8050, Shimadzu Corporation, Kyoto, Japan) and gas chromatography with mass spectrometry (GC-MS QP2010, Shimadzu Cooperation, Kyoto,

Japan) were used for analysis and quantification of BBR and its metabolites in biological samples.

Metabolizing BBR by large intestinal bacteria *in vitro*

The method was reported previously[33]. Briefly, colon contents from six rats were pooled, and 5 g of the sample was transferred into a flask containing the anaerobic medium (100 ml). After thorough mixing, the cultures (which contained intestinal bacteria and anaerobic medium) were pre-incubated under anaerobic conditions with a N_2 atmosphere at 37 °C for 60 min. Rutin (10 μl, 1.0 mg/ml in methanol) was used as a positive reference for intestinal metabolism.

Ten microliters of BBR at different concentrations was added into the fresh human or rat intestinal bacteria cultures (990 μl), with methanol (10 μl) as the negative control. The final concentrations of BBR in the incubation system were 100, 10, and 5 μg/ml. The cultures were incubated at 37 °C for 72 h in the presence or absence of human or rat intestinal bacteria.

After termination of the reaction with acetonitrile (1 ml), 50 μl of the internal standard (tetrahydropalmatine, 2.0 mg/ml in methanol) was added into the BBR samples, which were then mixed for 30 sec and centrifuged at 7,500 g for 15 min. The supernatant was dried under nitrogen flow at room temperature, and the residue was dissolved in 100 μl of methanol and centrifuged at 7,500 g for 15 min. The metabolites were analyzed by LC/MS^n-IT-TOF using a previously reported method[34].

GCMS-QP2010 was also used to analyze the BBR metabolites with low polarity. The GC column was an Alltech capillary column (AT^{TM}-1701, 30 m × 0.25 mm × 0.25 μm) operated in the splitless mode. The helium carrier flow was 39.7 cm/s under a column head pressure of 68.1 kPa. The oven temperature was initially 50 °C for 2 min, gradually increased to 260 °C at a rate of 8 °C/min, and maintained for 25 min. The injector and detector temperatures were set to 280 °C and 230 °C, respectively. The mass spectra were recorded at a scan range of *m/z* 40 to 800. Structure identification of possible metabolites was based on matching with standard mass spectra available in the Shimadzu GC-MS library.

Quantification analysis of BBR metabolites modified by the gut microbiota *in vitro* and *in vivo*

A working solution of BBR was prepared at a series of concentrations by diluting the stock solution (10 mg/ml) with methanol to generate the standard curves. LC-MS/MS 8040 was used to quantify BBR and its metabolites transformed by intestinal bacteria[15].

For the determination of dhBBR obtained after incubation with intestinal bacteria *in vitro*, a working solution of dhBBR was prepared at concentrations of 500, 200, 100, 50, 20, 10 and 1 μg/ml by diluting the stock solution (1 mg/ml) with methanol, which were used to generate the standard curves of dhBBR. Samples from the *in vitro* intestinal bacteria incubation mixture were injected into the GC-MS instrument.

For the analysis of the *in vivo* intestinal metabolites of BBR, six SD rats were orally treated with BBR (200 mg/kg), and their feces were collected 0, 6, 12, 24, 36, 48, and 72 h after treatment. The method used for the preparation of the feces was reported previously[34]. An aliquot of 1 μl was injected into the GC-MS instrument. The level of dhBBR in feces was measured and calculated based on the standard curve obtained with the *in vitro* intestinal bacteria incubation mixture.

To prepare the human samples, we transferred fresh feces (5 g per person) from healthy volunteers (age from 20–25, two males and two females) into a flask containing the anaerobic medium (100 mL)[33]. After thorough mixing, the bacteria were cultured under anaerobic conditions with a N_2 atmosphere at 37 °C for 60 min. The culture solution of intestinal bacteria was then ready for analysis.

BBR metabolism in the rat small intestine bacteria

The small intestine contents from ten sacrificed SD rats were obtained and mixed with anaerobic medium to prepare the cultures. BBR was added into the incubation at final concentrations of 100, 50, and 10 μg/ml, and the reaction was continued for 6, 12 or 24 h. Levels of BBR, dhBBR and other BBR metabolites were determinedusing the LC-MS/MS 8040 and GC-MS instruments according to the above-mentioned method.

Distribution of BBR, dhBBR and other metabolites in the small intestine

Three segments of small intestine (duodenum, jejunum and ileum) were collected 0, 6, 12, 24, 36, and 48 h after the oral administration of BBR in SD rats. The small intestine tissues were washed thoroughly with saline and dried. After being weighed, the samples were homogenized with mixed solution (ethanol: water, 1:1) at a ratio of 1:2 [w(g)/v(ml)]. The samples were centrifuged at 7,500 g for 10 min, and the supernatant was collected and evaporated to dryness in a water bath at 40 °C using a rotary evaporator. The residues of the tissue extraction were dissolved in methanol (250 μl) and vortex-mixed for 5 min. The tissue solution was then centrifuged (at 14,000 g for 5 min), and the supernatant was passed through a 0.22-μm filter. An aliquot of 1 μl of the supernatant was injected into the GC-MS instrument, and a 10-μl aliquot was used for LC-MS/MS analysis. The level of dhBBR was

calculated based on the standard curve, and BBR and other metabolites was quantified using the method described above.

BBR metabolism in the *in vitro* culture of 14 intestinal facultative anaerobes

Ten intestinal facultative anaerobes (clinical isolates)—*Staphylococcus aureus* (*S. aureus*) 08-43, *Enterococcus faecium* (*E. faecium*) 13-01, *Enterococcus faecalis* (*E. faecalis*) 13-01, *Enterobacter cloacae* (*E. cloacae*) 13-12, *Escherichia coli* (*E. coli*) 06-05, *Staphylococcus epidermidis* (*S. epidermidis*) 12-12, *Pseudomonas aeruginosa* (*Ps. aeruginosa*) 13-10, *Klebsiella pneumonia* (*K. pneumoniae*) 13-14, *Proteus mirabilis* (*P. mirabilis*) 13-01 and *Acinetobacter baumannii* (*A. baumannii*) 13-02—were collected from gastrointestinal specimens from patients from hospitals in Beijing between 2006 and 2013. The specimens were identified in the hospitals using the VITEK 2-COMPACT system (bioMerieux, Marcy l'Etoile, France). *Lactobacillus casei (L. casei* ATCC *334), Lactobacillus acidophilus (L. acidophilus* ATCC *4356), Bifidobacterium longum (B. longum* ATCC *15707), and Bifidobacterium breve (B. breve* ATCC *15700)* were purchased from the Microbial Culture Collection Center in Guangdong, China. The bacteria were transferred into a flask containing anaerobic medium. BBR was incubated with the 14 facultative anaerobes at a final concentration of 50 μg/ml at 37 °C for 0, 12, 24, 48 and 72 h. BBR and dhBBR in the culture were analyzed quantitatively using GC-MS and LC-MS/MS 8040.

Determination of nitroreductase

The detection of nitroreductase was performed using the Human Nitroreductase ELISA kit purchased from Beijing Luyuan Dade Biological Science and Technology Co., Ltd (Beijing, China) according to the manufacturer's guidelines.

Pseudo germ-free (PGF) rats and BBR absorption

Male SD rats (180–200 g) were orally treated with cefadroxil (100 mg/kg), terramycin (300 mg/kg) and erythromycin (300 mg/kg) twice a day for 3 days, and pharmacokinetic examination was performed 2 days after final administration. The colon contents of the rats were collected on the first and third days after the final treatment with antibiotics, and the germ-free status was confirmed by culturing fecal samples aerobically on a nutrient agar culture medium. Fecal samples from non-antibiotic-exposed rats served as control samples.

Before oral administration of a single dose of BBR (200 mg/kg), the PGF rats were fasted overnight with free access to water. Blood samples were collected from the posterior orbital venous plexus into a heparinized tube at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 h post-BBR treatment and then subjected to the procedure described above. Fecal samples were also

collected at 0, 6, 12, 24, 36 and 48 h post-BBR treatment. The samples were stored at −20 °C for further analysis.

Anti-bacterial test

Seventeen bacterial strains (see above) were used in the susceptibility test for BBR and dhBBR. The bacterial strains, which belong to different species of bacteria, were collected and identified as described above. The minimum inhibitory concentrations (MICs) of BBR and dhBBR after 24 h were determined through the broth microdilution method according to the CLSI guidelines[35]. BBR and dhBBR were tested at a concentration range of 8 to 512 μg/ml. The MIC was defined as the lowest concentration of an agent that prevents turbidity. The experiment was repeated three times. All of the bacterial strains tested in this study (*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *S. aureus* 08-43, *S. epidermidis* 12-12, *E. faecium* 13-01, *E. faecalis* 13-01, *E. coli* 06-05, *K. pneumoniae* 13-14, *Ps. aeruginosa* 13-10, *A. baumannii* 13-02, *E. cloacae* 13-12, and *P. mirabilis* 13-01) were facultative anaerobes, of which *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 were standard strains from ATCC (USA) and served as quality controls. *L. casei* ATCC 334*, L. acidophilus* ATCC 4356*, B. longum* ATCC 15707, and *B. breve* ATCC 15700 were also tested in this anti-bacterial experiment. Ampicillin was used as the positive control.

Molecular docking between BBR and nitroreductase

AutoDock Vina v.1.0.2 software was used to perform the molecular docking of BBR onto nitroreductase, whose crystal structures are available in the Protein Data Bank[17]. The docking parameters were set to the default values. The grid boxes were 20 Å × 20 Å × 20 Å, encompassing the active site cavities. The binding modes of BBR to enzymes were chosen to further optimize the docking conformation according to their binding free energy, distances from conserved water molecules and the flavin mononucleotide (FMN). The simulation results were visualized using the PyMOL Molecular Graphics System Version 1.3 (Schrödinger LLC, New York, NY, USA) and Discovery Studio Visualizer (Accelrys, Inc., San Diego, CA, USA).

Nitroreductase-mediated BBR reduction

BBR was incubated with nitroreductase in intestinal bacteria cultures. The reaction mixture consisted of BBR (50 μg/ml), an NADPH-regenerating system and nitroreductase (5 μg/ml) in a final volume of 1 ml under the protection of N_2. After incubation for 0, 2, 4, 6 and 12 h at 37 °C, the reaction was terminated by the addition of 1 ml of ice-cold acetonitrile. The samples were then extracted with 1 ml of ethyl acetate after adding 1 ml of 0.5 M sodium hydroxide solution. The organic phase was evaporated to dryness under a nitrogen flow in a water bath at 40 °C. The residue was dissolved with 200 μl of the mobile phase for further analysis. 2-

Iodosobenzoic acid (2-IBA), a specific inhibitor of nitroreductase, was used to verify the role of bacterial nitroreductase.

Absorption of BBR and dhBBR in Caco-2 cells

A Caco-2 cell assay was conducted using a method reported previously[36]. Stock solutions of dhBBR and BBR were prepared in DMSO at 1 mM, and these were then diluted with HBSS buffer (10 mM HEPES, pH 7.4) to a final concentration of 5 μM for both samples. To determine the rate of drug transport in the apical-to-basolateral direction, 200 μl of the compound working solution was added to the filter well (apical compartment), and 800 μl of HBSS was added to the receiver plate (basolateral compartment). Accordingly, to determine the rate of drug transport in the basolateral-to-apical direction, 800 μl of the compound working solution was added to each well of the receiver plate and 200 μl of HBSS was added to the filter well. The plates were incubated for 30 min at 37 °C with shaking at 150 rpm on a rotary shaker. At the end of the transport period, aliquots of 50 μl were removed directly from the apical and basolateral wells and transferred to wells of new plates. Four volumes of cold methanol containing internal standards were added into each well. The samples were centrifuged at 16,000 g for 15 min. Aliquots of 200 μl of the supernatant were used for the LC-MS/MS analysis.

Absorption of dhBBR *in vivo*

Five SD rats were fasted for 12 h and then orally administered 200 mg/kg dhBBR. Blood samples (0.5 ml) were obtained from the posterior orbital venous plexus into a heparinized tube at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 h, and the samples were then centrifuged at 2,000 g for 5 min. The plasma (100 μl) was precipitated with 100 μl of acetonitrile after addition of 10 μl of the internal standard. BBR served as a control in the study. The plasma concentrations of BBR and dhBBR were determined using LC-MS/MS 8040 and LC-MS/MS 8050.

Drug effect on hepatic LDLR gene expression

The HepG2 human hepatoma cell line was obtained from the American Tissue Culture Collection (ATCC, USA) and grown in Eagle's Minimum Essential Medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37 °C. For the drug efficacy test, HepG2 cells were cultured for 24 h and then treated with BBR or dhBBR for 12 h. The compounds were freshly prepared in medium prior to use.

The total cellular RNAs were isolated with the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the vendor's instructions. The total RNAs were reversely

transcribed into cDNAs, and quantitative real-time PCR was performed using a 2-Step RT-qPCR System (Promega, Madison, WI, USA), with GAPDH as an internal control. The normalized LDLR mRNA expression levels were plotted as fold levels compared with the untreated control. The following primers were used: LDLR forward, aggacggctacagctaccc; LDLR reverse, ctccaggcagatgttcacg; GAPDH forward, agccacatcgctcagacac; and GAPDH reverse, gcccaatacgaccaaatcc.

Reversion from dhBBR to BBR

The rat duodenum, jejunum and ileum were collected separately, washed thoroughly with saline and dried. After being weighed, the tissues were homogenized with saline at a ratio of 1: 2 [w (g) /v (ml)]. DhBBR was dissolved in a mixture of methanol and DMSO at a ratio of 1:1 with a final concentration of 5 mg/ml. Ten microliters of dhBBR (5 mg/ml) was mixed with 1 ml of the homogenates. The samples were then centrifuged at 14,000 g for 5 min, and the supernatants were collected and mixed with an equal volume of ice-cold acetonitrile to terminate the reaction. The samples were centrifuged at 14,000 g for 5 min, and the supernatant was used for the HPLC analysis.

Transformation of dhBBR in pooled human intestinal microsomes (HIMs)

The typical incubation mixture contained 0.2 mg of HIMs, 100 mM phosphate buffer (pH 7.4), 100 μM dhBBR (dissolved in DMSO) and an NADPH-regenerating system [at a final concentration of 3.3 mM glucose-6-phosphate (G-6-P), 1.3 mM $NADP^+$, 0.4 unit/ml glucose-6-phosphate dehydrogenase (G-6-PDH) and 3.3 mM $MgCl_2$] in a final volume of 200 μl. After pre-incubation of HIMs with dhBBR for 2 min at 37 °C, the reaction was initiated by the addition of the NADPH-regenerating system. The mixture was incubated at 37 °C with exposure to air for 2, 5 and 10 min and was terminated by the addition of 200 μl of ice-cold acetonitrile. Then, 20 μl of the internal standard was added to the mixture, and the mixture was centrifuged at 14,000 g for 5 min. Fifty microliters of the supernatant was injected into the HPLC instrument for analysis. The control samples were incubated under the same conditions but without HIMs.

Oxidization of dhBBR by monoamine oxidase B (MAO-B)

The reaction system consisted of dhBBR (100 μM), MAO-B (0.2 mg/ml) and phosphate-buffered saline (PBS, pH = 7.4) in a final volume of 200 μl. The MAO-B was pre-incubated via centrifugation at 850 rpm at 37 °C for 5 min, and this step was followed by the addition of dhBBR. The reaction was terminated after 30 min by adding an equal volume of ice-cold acetonitrile and centrifuging at 14,000 g for 5 min. Ten microliters of the supernatant was

injected into the HPLC instrument for analysis. Selective MAO-B inhibitors (deprenyl, 100 μM or pargyline, 1 μM)[37,38] were used in the tests.

Effect of vitamin C on dhBBR-to-BBR transformation

The rats (n = 3) were sacrificed to obtain fresh small intestine homogenate. The homogenate was then boiled for 2 min to inactivate all of the enzymes. After pre-incubation at 37 °C for 2 min, 5 μl of dhBBR (10 mM, dissolved in DMSO) was added into the inactivated homogenate, and fresh homogenate (active one) was used as a reference. The sample was mixed thoroughly and centrifuged at 16,000 g for 5 min. The supernatant was transferred into new tubes, treated with 500 μl of acetonitrile, and then centrifuged. Twenty-microliter aliquots were used for the HPLC analysis.

To examine the oxidative reaction, 100 μl of vitamin C was pre-incubated with the inactivated homogenate; the final concentrations of vitamin C were 95, 9.5 and 0.95 mM. The superoxide anion in the sample was detected with an O_2^- ELISA kit (Beijing Biolab Co., Ltd., Beijing, China; lot: 201404) following the manufacturer's instructions.

Gut microbiota modulates the therapeutic effects of BBR on hyperglycemia and hyperlipidemia

KK-Ay mice with type 2 diabetes (female, 12 ± 1 weeks of age) were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China). The mice were maintained at room temperature (23 ± 2 °C) with 50% moisture and exposed to a 12-h light/12-h dark cycle, and food and water were provided ad libitum. Antibiotics at the doses described above were orally administered to KK-Ay mice for 3 days before the BBR treatment course, and the pseudo germ-free (PGF) state of the experimental mice was confirmed (see above).

Four groups (n = 6 for each group) were used in the experiment: Group1, PGF KK-Ay mice orally treated with antibiotics (qd, at the dosage described above) plus BBR (200 mg/kg, qd) for 2 weeks; Group 2, untreated KK-Ay mice orally treated with BBR (200 mg/kg, qd) for 2 weeks; Group 3, PGF KK-Ay mice orally treated with antibiotics (qd, at a dosage identical to that for Group 1) for 2 weeks; Group 4, untreated KK-Ay mice. The antibiotics were administered in the morning and BBR was administered in the afternoon. All the animals were fed a high-fat diet. The germ-free state of Groups 1 and 3 was confirmed on days 7 and 14 of the treatment course using the same bacterial analytical method described above. All of the mice were euthanized on day 14, and blood samples were taken to measure the fasting serum glucose (Glu), triglyceride (TG) and cholesterol (CHO) using Glu, TG and CHO reagent kits (Biosino Bio-Technology and Science INC, Beijing China), respectively. C57BL/6J mice

were used as a wild-type reference. The body weights of the mice before, during and after treatments were recorded for 14 days.

Blood samples of the mice in Groups 1 and 2 were collected into a heparinized tube 30 min after the oral administration of BBR. The samples were taken at days 1, 3, 6, 10 and 14 of the BBR treatment course. The samples were centrifuged at 10,000 g for 5 min, and the plasma was collected and stored at −80 °C before analysis.

Effect of ions on the dhBBR-to-BBR transformation

dhBBR was added into the reaction solution containing $FeCl_3$, $CuCl_2$, $ZnCl_2$, and NaCl (at concentrations of 0.033, 0.333 and 3.33 mM, respectively). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na, 10 mM), a chelating agent for metal ions, was used in the test.

Statistical analysis

The statistical analyses were conducted using two-way ANOVA and Student's *t*-test with GraphPad Prism Version 5 (GraphPad Software, CA, USA). The data are expressed as the means ± standard deviation, and *p* values less than 0.05 were considered statistically significant.

Additional Information

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Supplementary Material

Supplementary Information

Acknowledgments

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Footnotes

Author Contributions J.-J.D. and W.-Y. conceived of and designed the experiments and contributed to the writing and editing of the manuscript. F.-R., S.-J.W., Z.-Z.X., M.-C., F.-J., H.-C.Y., H.-M., T.-X.S., L.-X.Y., W.-B.Y., C.-X., L.-Y., R.-G. and Y.-X.Y. performed the experiments. J.-J.D. and W.-Y. analyzed the data. J.-J.D., W.-Y., Y.-X.F. and C.-Y.C. contributed to discussions and provided reagents/materials /analysis tools important for completion of the work.

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Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Supplementary Materials

Supplementary Information

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Transforming berberine into its intestine-absorbable form by the gut microbiota

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Abstract

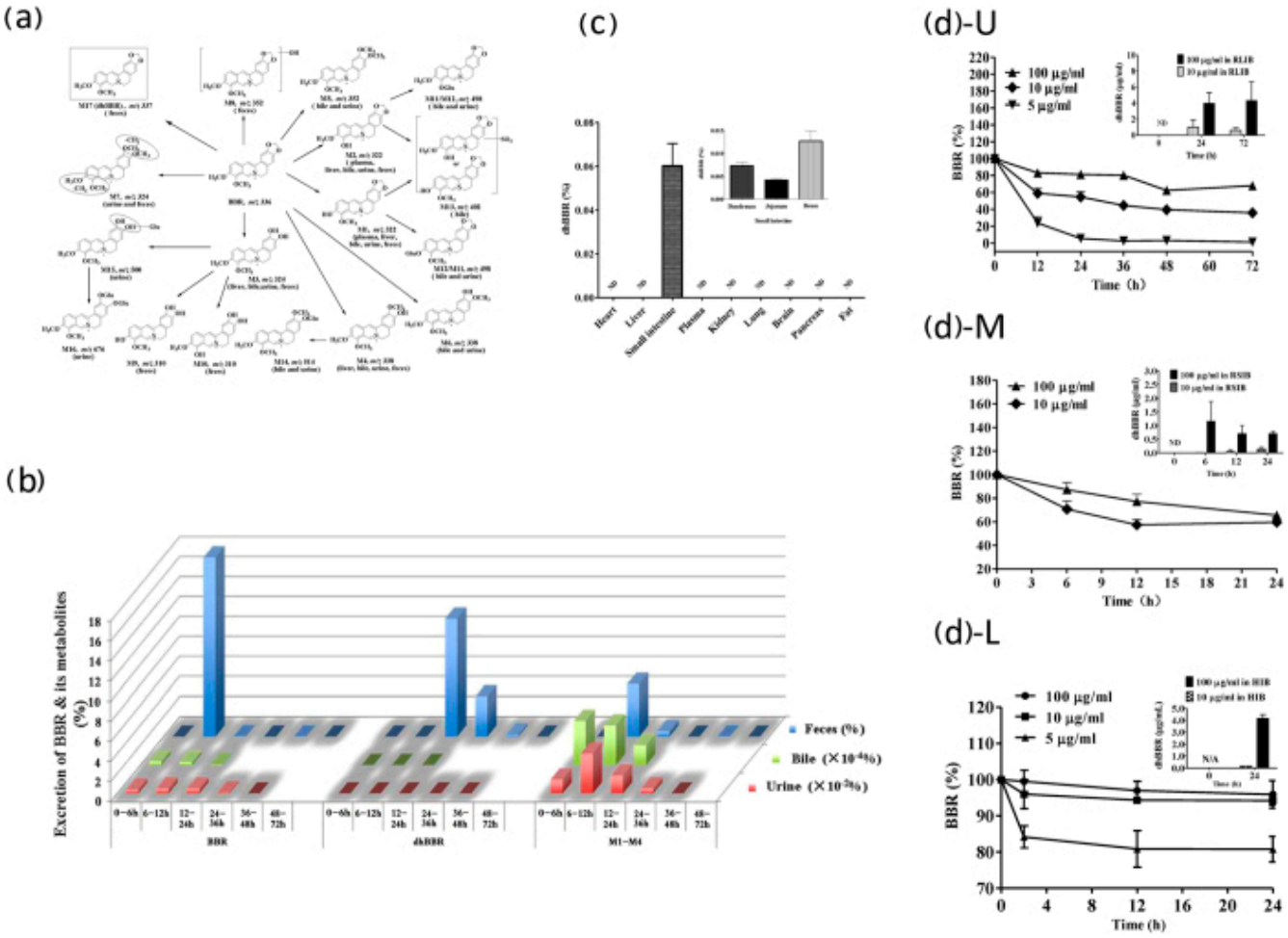
The gut microbiota is important in the pathogenesis of energy-metabolism related diseases. We focused on the interaction between intestinal bacteria and orally administered chemical drugs. Oral administration of berberine (BBR) effectively treats patients with metabolic disorders. However, because BBR exhibits poor solubility, its absorption mechanism remains unknown. Here, we show that the gut microbiota converts BBR into its absorbable form of dihydroberberine (dhBBR), which has an intestinal absorption rate 5-fold that of BBR in animals. The reduction of BBR to dhBBR was performed by nitroreductases of the gut microbiota. DhBBR was unstable in solution and reverted to BBR in intestine tissues via oxidization. Heat inactivation of intestinal homogenate did not inhibit dhBBR oxidization, suggesting the process a non-enzymatic reaction. The diminution of intestinal bacteria via orally treating KK-Ay mice with antibiotics decreased the BBR-to-dhBBR conversion and blood BBR; accordingly, the lipid- and glucose-lowering efficacy of BBR was reduced. Conclusively, the gut microbiota reduces BBR into its absorbable form of dhBBR, which then oxidizes back to BBR after absorption in intestine tissues and enters the blood. Thus, interaction(s) between the gut microbiota and orally administrated drugs may modify the structure and function of chemicals and be important in drug investigation.

The mammalian intestine hosts extremely diverse and vast microorganisms, collectively referred to as the gut microbiota, which mostly comprises four bacterial phyla: the Gram-negative Bacteroidetes and Proteobacteria and the Gram-positive Actinobacteria and Firmicutes[1]. In humans, the gut microbiota consists of more than 10^{14} bacteria and archaea, which cover approximately 1,100 prevalent species[1]. The gut microbiota is considered a “hidden organ” of the body and may be associated with the pathogenesis of diseases such as cardiovascular diseases, obesity, and diabetes[2,3,4]. The microbiota is also an interesting field of research in drug metabolism, particularly with respect to orally administered drugs because

the drug metabolism by gut microbiota may generate metabolites different from those generated by the host organs[5,6]. It is particularly important if the metabolites generated by the gut microbiota possess novel features and bioactivities. Thus, the drug metabolism by intestinal microorganisms is considered attractive in pharmaceutical research. Here, we present berberine (BBR) as an interesting drug example to show the significance of the gut microbiota in drug investigations.

BBR (Fig. 1a) is a medicinal alkaloid isolated from *Coptis chinensis* and has been used orally for decades in China as an over-the-counter (OTC) drug to treat diarrhea with good safety[7]. We and others have previously identified BBR as a new medicine for hyperlipidemia and type 2 diabetes because it reduces blood lipids and glucose in patients[7,8] through a multiple-target mechanism involving low-density-lipoprotein receptor (LDLR), insulin receptor (InsR), AMP-activated protein kinase (AMPK), and proprotein convertase subtilisin kexin 9 (PCSK9), among others[7,8,9,10,11]. The therapeutic effect of BBR on lipid- and glucose-related metabolic disorders was recently confirmed in a large number of patients by independent clinical groups both in and outside China[12], demonstrating the botanic compound to be a promising drug. By contrast, BBR exhibits poor water solubility and is presumably very difficult for intestinal epithelial cells to absorb[13,14]. However, after its oral administration, BBR was detected in nearly all major organs as well as in urine, with the liver exhibiting the highest concentration[15], and these findings raise the question of how BBR is absorbed in the intestine. Our recent study on the metabolism of BBR in the intestine has identified the gut microbiota as the most likely answer, at least in part, to this question, and we consider the finding of general interest in drug discovery and investigation.

Figure 1. BBR is metabolized into dhBBR in the intestine ecosystem.



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(**a**) *In vivo* BBR metabolites in SD rats; M17 (*m/z* 337) was identified to be dihydroberberine (dhBBR), a BBR metabolite detected in the feces only; the parentheses indicate the sample in which the compound was detected. (**b**) Excretion of BBR, dhBBR and other BBR metabolites in rat feces, urine and bile after 72 h. (**c**) Distribution of dhBBR in organs of SD rats orally treated with BBR (200 mg/kg); ND: not detectable. (**d**) Conversion of BBR into dhBBR by the gut microbiota *in vitro*; U & insert: in rat large intestinal bacteria (RLIB); M & insert: in rat small intestinal bacteria (RSIB); L & insert: in human intestinal bacteria (HIB) *in vitro*. The Y axis shows the percentage of the administered amount of BBR (n = 6).

Results

DhBBR is a BBR metabolite generated in the intestinal ecosystem

To advance our understanding of the *in vivo* fate of BBR after oral administration, we recently investigated the BBR metabolites in the urine, bile and feces in rat. The compound detection was performed using both liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and gas chromatography coupled with mass spectrometry (GC-MS). Among the

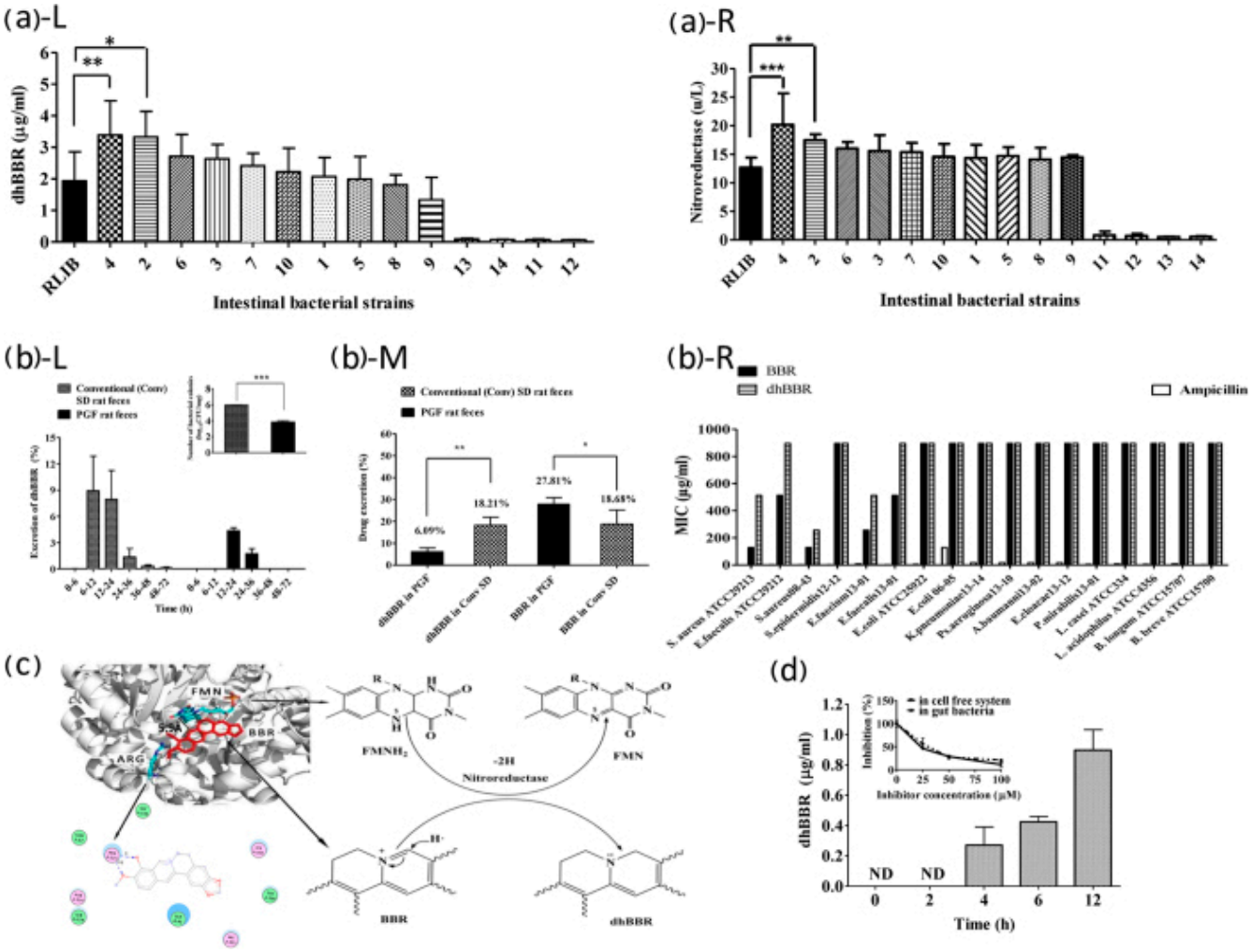
17 BBR metabolites detected in body excretion, a new metabolite, denoted dihydroberberine (dhBBR), caught our attention (Fig. 1a, in frame). First, dhBBR was detectable in feces but not in the bile and urine samples. Second, among these metabolites, dhBBR was the only one that exhibited a change in the backbone structure (Fig. 1a). We assumed that it might be a metabolite generated by the gut microbiota of rats. We then detected BBR, dhBBR and other BBR metabolites in rat excretions over a 72-h period after the oral administration of BBR. As shown in Fig. 1b, although other major BBR metabolites (M1-M4) were detectable in urine, bile and feces, dhBBR was detected only in feces. The amount of dhBBR reached its peak within 12–24 h and then decreased over time. To further confirm that dhBBR is generated within the intestine ecosystem, rat organs were examined for the presence of dhBBR. As shown in Fig. 1c, 24 h after the oral administration of BBR (200 mg/kg) in rats, dhBBR was detected in intestinal tissue only (including duodenum, jejunum and ileum) but not in other organs. Thus, we considered that rat intestinal bacteria might be the “organ” that converts BBR into dhBBR. We then incubated BBR with a large amount of rat intestinal bacteria *in vitro* and found that the amount of BBR decreased over time (Fig. 1d, U) and that, accordingly, dhBBR became detectable within 24 h of incubation (Fig. 1d, U insert). Next, we investigated samples of the rat small intestine by incubating BBR with bacteria isolated from the small intestine of untreated rats. As shown in Fig. 1d (M), the BBR content decreased over time and dhBBR became detectable within 6 h of incubation, suggesting that the bacteria from the small intestine were also active in the conversion of BBR into dhBBR. We also performed the experiment using human gut microbiota, and the results were similar to those obtained with rat gut bacteria (Fig. 1d, L).

Intestinal dhBBR was generated by gut microbiota

To determine whether the gut microbiota is responsible for the BBR-to-dhBBR conversion, we incubated BBR (50 μg/ml) *in vitro* with 14 intestinal bacterial strains (*Staphylococcus aureus, Enterococcus faecium, Enterococcus faecalis, Enterobacter cloacae, Escherichia coli, Staphylococcus, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Acinetobacter baumannii, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium longum, and Bifidobacterium breve*) and then measured the amount of dhBBR in the culture. The first 10 strains were clinical isolates and the last four strains are resident intestinal bacteria. As shown in Fig. 2a–L, all 10 of the clinical isolates produced dhBBR after incubation with BBR for 72 h. *Enterobacter cloacae* and *Enterococcus faecium* produced more dhBBR than did the rat feces, which were used as a positive reference, and *Proteus mirabilis* produced less (Fig. 2a). The four resident intestinal bacteria produced dhBBR at a level markedly lower than that obtained with the clinical isolates. The results suggest that the intestinal microbiota appeared to work as a “tissue” to transform BBR into dhBBR. If this is true, oral treatment of rats with antibiotics should reduce dhBBR production by the intestinal

bacteria. Thus, SD rats were treated orally with cefadroxil (100 mg/kg), terramycin (300 mg/kg) and erythromycin (300 mg/kg), bid, for 3 days. Two days later, the colon contents were collected for bacterial examination. As shown in Fig. 2b (L insert), oral treatment with the antibiotics for 3 days reduced the bacterial colony in the rat feces by 2 logs, indicating a pseudo germ-free (PGF) intestinal environment in the rats after treatment with antibiotics. BBR (200 mg/kg) was then orally administered to the PGF rats using untreated rats as a control; this step was followed by 72 h of feces collection. BBR and dhBBR in the feces were measured using LC-MS/MS 8040 and GC-MS. Fig. 2b, L shows that dhBBR production was delayed and markedly decreased in the intestine of the PGF rats compared with that in the untreated rats, consistent with the numbers of intestinal bacteria in the PGF rats. In addition, the 72-h-cumulative dhBBR level in the feces of the PGF rats was examined. The accumulated dhBBR was significantly higher in the untreated rats (18.21% of the giving amount) than that in the PGF rats (6.09% of the giving amount, $P < 0.01$); accordingly, the total BBR in the feces of the PGF rats was significantly higher (27.81% of the administered amount) than that in the untreated rats (18.67% of the administered amount, $P < 0.05$, Fig. 2b, M). To evaluate the direct effect of BBR on the gut microbiota, we incubated BBR separately with 17 strains of intestinal bacteria. As shown in Fig. 2b, R, the minimal inhibitory concentration (MIC) values of BBR on the 17 bacteria ranged from 128 to more than 512 μg/ml, which is a concentration close to that of BBR precipitation in the culture medium, indicating that the antibacterial effect of BBR on the gut microbiota was minor. In addition, the antibacterial effect of dhBBR was even weaker than that of BBR in 5 of the 17 intestinal bacteria strains (Fig. 2b, R). Ampicillin was used as the positive control in this experiment.

Figure 2. Generation of dhBBR by the gut microbiota.



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(**a**) BBR was converted *in vitro* into dhBBR by 14 intestinal bacteria strains [*S. aureus* 08-43 (1), *E. faecium* 13-01 (2), *E. faecalis* 13-01 (3), *E. cloacae* 13-12 (4), *E. coli* 06-05 (5), *S. epidermidis* 12-12 (6), *Ps. aeruginosa* 13-10 (7), *K. pneumoniae* 13-14 (8), *P. mirabilis* 13-01 (9), *A. baumannii* 13-02 (10), and *L. casei* (11), *L. acidophilus* (12), *B. longum* (13), and *B. breve* (14)]. L: dhBBR was produced at different levels by the 14 intestinal bacteria strains (*$P < 0.05$ and **$P < 0.01$, n = 6); R: Determination of nitroreductase in the 14 intestinal bacteria strains (***$P < 0.001$, *$P < 0.05$, n = 6). (**b**) Oral treatment with antibiotics generated pseudo germ-free (PGF) rats and resulted in a reduced conversion of BBR to dhBBR in the rat intestinal ecosystem; L: dhBBR production was delayed and decreased in the feces of PGF rats compared with conventional SD rats; L insert: oral treatment with antibiotics for 3 days reduced the bacterial colony numbers by 2 logs in the rat feces (***$P < 0.001$, n = 6); M: the accumulative excretion of BBR and dhBBR in PGF and conventional rats over a period of 72 h (**$P < 0.01$, *$P < 0.05$, n = 6); R: MIC of BBR and dhBBR on the 17 intestinal bacteria (24 h); The Y axis shows the percentage of the administered amount of BBR. (**c**) Molecular docking between BBR and nitroreductase, showing the chemical me**c**hanism of BBR reduction by nitroreductase. (**d**) Nitroreductase-mediated BBR reduction resulted in a BBR-to-dhBBR conversion after 4 h; insert: BBR-to-dhBBR conversion was reduced in the presence of the nitroreductase inhibitor 2-IBA (100 μM) in the nitroreductase-containing cell-free incubation mixture or in the rat gut bacteria cultivation mixture (for 12 h); ND: not detectable.

BBR was converted to dhBBR in a reduction reaction, and nitroreductase and azoreductase are two major reductases that are abundant in the gut microbiota[16]. Because the crystal structures of the reductases have been illustrated and are available in the Protein Data Bank[17], we performed computer-assistant docking in the initial step of analysis, using the AutoDock Vina v.1.0.2 software. The putative chemical mechanism for BBR reduction by nitroreductase is shown in Fig. 2c. BBR exhibited excellent docking performance at docking onto nitroreductase with a binding free energy of −6.5 kcal/mol. BBR anchored into the binding site of nitroreductase through a strong hydrogen bond interaction with the side chain of Arg-225. The distance between the C atom of the C = N group and the N_5 atom of FMN was approximately 5.9 Å, which is within the distance good for electron transfer. After binding to nitroreductase in the pocket, BBR obtained H· from the FMN-$FMNH_2$ system, and its reduction was activated. The H· from $FMNH_2$ moved to the C atom of C = N in BBR, forcing the transfer of one pair of π electrons toward the N atom. The two electrons occupied the orbit of the N atom, forming a lone pair of electrons. The C = N was then transformed into C-N, and BBR was converted into dhBBR (Fig. 2c).

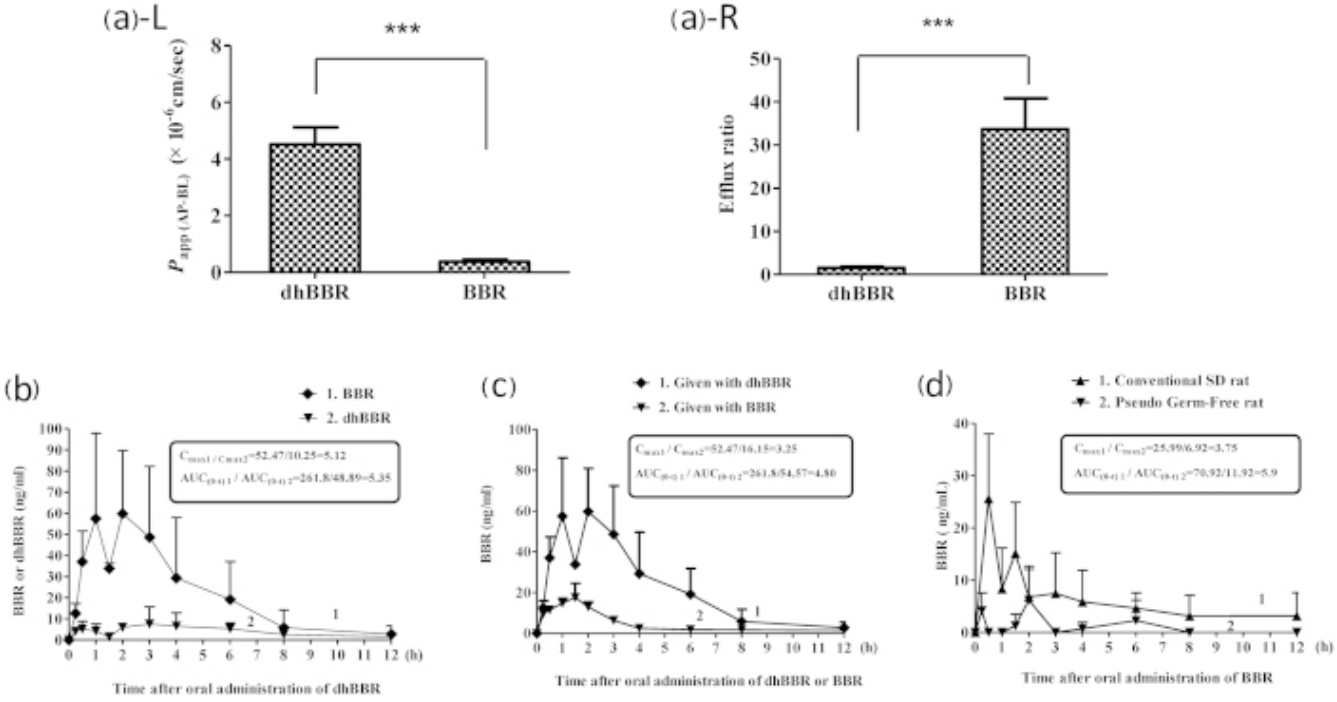
To validate the computational results, the nitroreductase-mediated BBR-to-dhBBR conversion was studied *in vitro*. Following the incubation of BBR with nitroreductase for 0, 2, 4, 6 and 12 h at 37 °C, the amount of dhBBR was examined. As shown in Fig. 2d, dhBBR became detectable after 4 h of incubation of BBR with nitroreductase, and its amount increased over time. Addition of the nitroreductase inhibitor 2-iodosobenzoic acid (2-IBA) into the incubation system significantly inhibited the conversion of BBR to dhBBR, and the inhibition rate reached 88.28% with an increase in the 2-IBA concentration to 100 μM (Fig. 2d, insert), suggesting that the nitroreductase enzymes of the gut bacteria may be an important bacterial enzyme that transforms BBR into dhBBR. Addition of 2-IBA into the incubation system of BBR with the mixture of rat gut bacteria also inhibited dhBBR production by 78.7%, which is in agreement with the results obtained with the cell-free enzymatic reaction. Other bacterial reductases, such as azoreductase (binding free energy of −6.9 kcal/mol), may also be contributors to the conversion. In addition, the correlation between the nitroreductase level in bacteria and BBR-to-dhBBR conversion was examined. In general, as shown in Fig. 2a, L&R, the level of nitroreductase in bacteria was positively correlated with the capability of reducing BBR (into dhBBR), supporting the finding that nitroreductase is at least one of the key enzymes in the conversion of BBR into dhBBR.

DhBBR exhibited an intestinal absorption rate higher than that of BBR

DhBBR in its sulfate form (dihydroberberine sulfate) was previously reported to exhibit better absorption in the intestine as compared with BBR[18]. Here, we focused our investigation on the absorption of the original dhBBR generated by the gut microbiota. First, the absorption of

BBR and dhBBR was investigated in the Caco-2 cell model. In this *in vitro* system, dhBBR displayed significantly improved absorption as compared with BBR. The $P_{app\ (AP\text{-}BL)}$ (apparent permeability coefficient of AP-BL; AP: apical; BL: basolateral) for dhBBR was 11.9-fold higher than that for BBR (4.51×10^{-6} cm/s vs. 0.38×10^{-6} cm/s), suggesting that the bioavailability of dhBBR for absorption was markedly increased (Fig. 3a, L). Furthermore, we found that the efflux ratio (ER) for dhBBR was significantly lower than that for BBR (1.58 versus 32.39), indicating a substantial decrease in the cellular efflux of the compound (Fig. 3a, R). Second, the intestinal absorption of dhBBR was studied via *in vivo* experiments in which dhBBR was orally administered to SD rats at a dose of 200 mg/kg. Interestingly, dhBBR was detected at a level less than 10 ng/ml in the plasma (Fig. 3b); instead, a substantial amount of BBR was detected in the blood with an $AUC_{(0\text{-}t)}$ value of 261.8 ng/ml*h and a maximum concentration (C_{max}) of 52.47 ng/ml (Fig. 3b). The findings suggest that a nearly complete reversion of dhBBR into BBR most likely occurred in the well of the intestine, resulting in a low level of dhBBR and a high level of BBR in blood. The absorption rate of dhBBR (200 mg/kg, oral) was then compared with that of BBR (200 mg/kg, oral). As shown in Fig. 3c, the blood level of BBR in the dhBBR-treated rats exhibited $AUC_{(0\text{-}t)}$ and C_{max} values that were 4.8- and 3.25-fold higher than those of the BBR-treated rats, respectively, indicating a higher intestinal absorption of dhBBR as compared with that of BBR.

Figure 3. DhBBR has a better intestinal absorption than BBR.



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(**a**) Absorption of BBR and dhBBR in the Caco-2 cell model; L: The $P_{app\ (AP-BL)}$ of dhBBR in Caco-2 cells was 11.9-fold higher than that of BBR (***$P < 0.001$); R: The efflux ratio of dhBBR in Caco-2 cells was significantly lower than that of BBR (1.58 vs. 32.39, ***$P < 0.001$). (**b**) Concentration-time curve for BBR or dhBBR in plasma after dhBBR oral administration (200 mg/kg) to rats (n = 3). (**c**) Concentration-time curve of BBR in plasma after the oral administration of dhBBR (200 mg/kg) or BBR (200 mg/kg) (n = 3). (**d**) Concentration-time curve of BBR in pseudo germ-free rats (generated by the oral administration of antibiotics for 3 days, curve 1) or in conventional SD rats (with no antibiotics, curve 2) after the oral administration of BBR (200 mg/kg, n = 3).

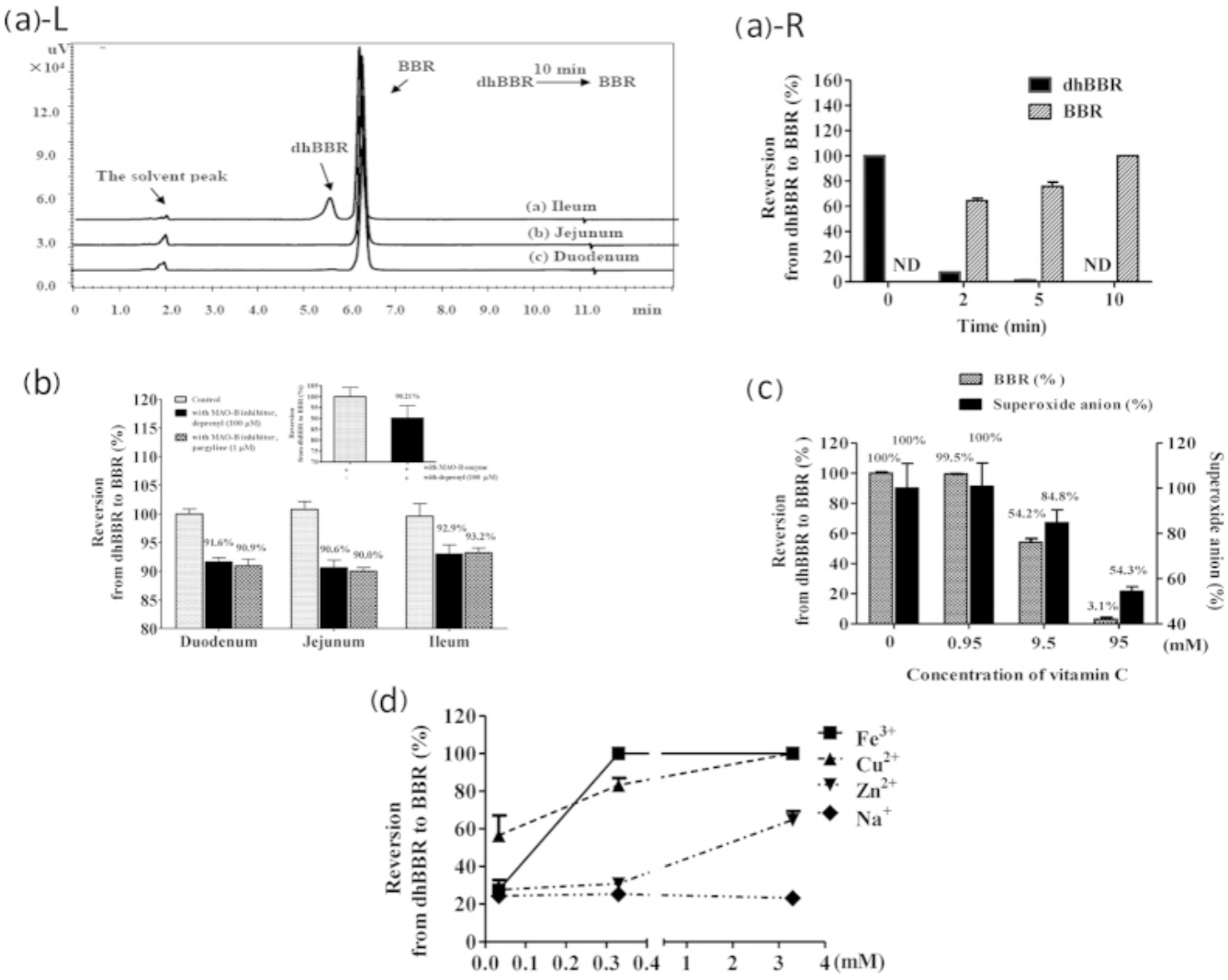
The results were further tested through another approach. We hypothesized that if dhBBR is the absorbable form of BBR, animals with a low intestinal bacteria content should have less plasma BBR because the level of conversion of BBR to dhBBR in the intestine should be low. We orally treated PGF rats (generated by treatment with antibiotics as mentioned above) with BBR (200 mg/kg) and then measured the blood BBR over a period of 12 h, and antibiotic-free rats administered the same dose of BBR served as control. As shown in Fig. 3d, the blood level of BBR in the PGF rats was much lower than that in those without antibiotics, with a C_{max} ratio of 1/3.75 (PGF rats vs. rats with no antibiotics) and an $AUC_{(0\text{-}t)}$ ratio of 1/5.9 (PGF rats vs. rats with no antibiotics), respectively. It seems that the antibiotic-induced decrease in intestinal bacteria resulted in a low level of conversion of BBR into dhBBR in the intestine and thus a low level of BBR in the blood. The results supported the hypothesis presented above. Although dhBBR presented good absorption characteristics, its activity on energy metabolism was lower than that of BBR. As shown in Figure S1 (Supplementary Figure S1), dhBBR

remained active for up- regulating LDLR expression but at a level much lower than that of BBR.

DhBBR reverted to BBR via oxidization in intestine tissues

Because dhBBR was barely detectable in the blood, liver and other organs in rats orally treated with BBR (Fig. 1c), we assumed that dhBBR generated by the gut flora in the intestinal cavity must revert to BBR immediately after it enters intestinal wall tissues. Therefore, we incubated dhBBR with homogenates of the small intestine of rats, including homogenates of the duodenum, jejunum or ileum. After a 10-min incubation, dhBBR reversed to BBR almost completely in the duodenum and jejunum homogenate and at a rate of more than 80% in the ileum homogenate (Fig. 4a, L). Human intestinal microsomes, which were rich in CYP450s, also reversed dhBBR to BBR very rapidly (Fig. 4a, R).

Figure 4. DhBBR reverted to BBR via oxidization in intestine tissue.



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(**a**) dhBBR-to-BBR reversion in the rat small intestine homogenate (L) and in human intestine microsomes (HIMs, R) (the Y axis shows the percentage of administered amount of dhBBR); ND: not detectable. (**b**) DhBBR-to-BBR reversion by monoamine oxidase-B (MAO-B) in a cell-free system (insert) and homogenates of the rat duodenum, jejunum and ileum; the reversion was slightly inhibited by the MAO-B inhibitor deprenyl (100 μM) or pargyline (1 μM) (the Y axis shows the percentage of administered amount of dhBBR). (**c**) Addition of vitamin C into the homogenate almost terminated the oxidization reaction for reverting dhBBR to BBR, and the content of superoxide anion decreased subsequently (the left Y axis shows the percentage of administered amount of dhBBR; the right Y axis shows the percentage of superoxide anion of control). (**d**) Effect of metal ions (Fe^{3+}, Cu^{2+}, and Zn^{2+}) on catalyzing the oxidization of dhBBR to BBR.

Therefore, we investigated the roles of eight subtypes of CYP450 isoforms in the intestine homogenate microsome. The specific CYP450 inhibitors used in the study were furafylline for CYP1A2, ticlopidine for CYP2B6, quercetin for CYP2C8, sulfaphenazole for CYP2C9, nootkatone for CYP2C19, quinidine for CYP2D6, ketoconazole for CYP3A4 and

diethyldithiocarbamate (DDC) for CYP2E1. Although all of the recombinant CYP450 isoforms nearly completed the dhBBR-to-BBR reversion (data not shown), the reversion rate in the presence of CYP450 inhibitors was not different from that obtained in the absence of inhibitors in the experiment using pooled human intestinal microsomes (HIMs). This finding suggests that CYP450 might not be critical for reverting dhBBR to BBR. Our next focus was intestinal oxidases, particularly monoamine oxidase (MAO-B)[19]. We incubated dhBBR with MAO-B in a cell-free system first and found that MAO-B may be responsible for approximately 10% reversion from dhBBR to BBR (Fig. 4b, inert). DhBBR was then incubated for 30 min with intestinal homogenate (including duodenum, jejunum or ileum) in the presence or absence of the MAO-B inhibitor deprenyl or pargyline, and this step was followed by BBR detection. As shown in Fig. 4b, incubation of dhBBR with intestinal homogenate resulted in a complete dhBBR-to-BBR reversion, but this reversion was reduced by less than 10% in the presence of a high concentration of the MAO-B inhibitors, which is consistent with the results obtained with the cell-free reaction. It appeared that tissue MAO-B may play a minor role in the dhBBR oxidization course. We then turned our attention to the other factors.

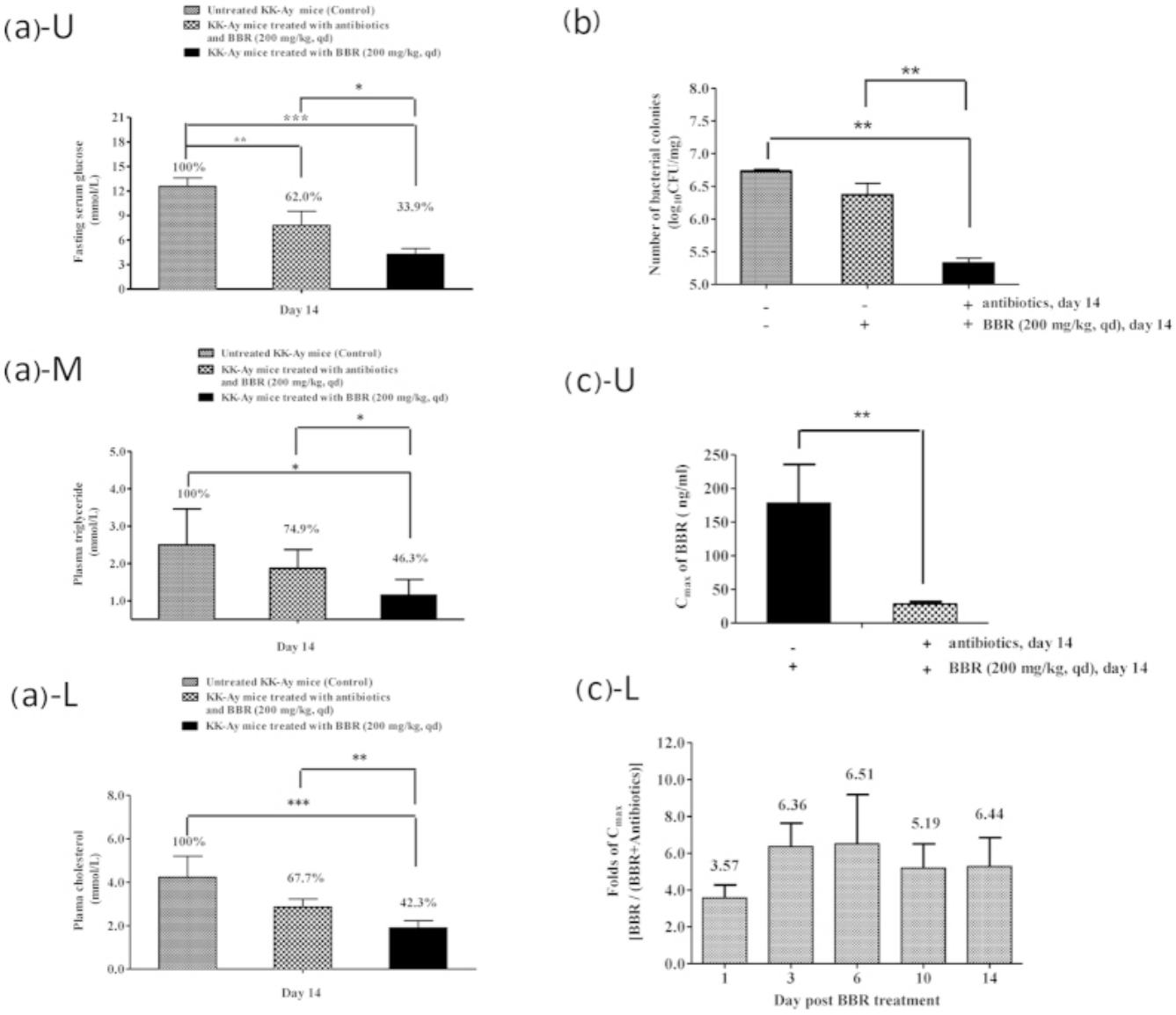
To inactivate proteins within the reaction system, the intestinal homogenate solution was boiled for 2 min. As shown in Figure S2 (Supplementary Figure S2), heat inactivation of the homogenate did not reduce the dhBBR-to-BBR reversion, indicating that the oxidization reaction was mainly a non-enzymatic reaction. Vitamin C, a strong antioxidant, was then added to the reaction solution. As shown in Fig. 4c, the dhBBR-to-BBR reversion was almost completely terminated by vitamin C, suggesting that the oxidization reaction is crucial for the dhBBR-to-BBR reversion in intestinal tissues. Moreover, in this reaction, the content of superoxide anion appeared to be positively correlated with BBR production (Fig. 4c). Metal ions, such as Fe^{3+}, Cu^{2+} and Zn^{2+}, which are a type of oxidant, appeared to play a positive role in catalyzing the oxidization process, whereas Na^{+} had no effect (Fig. 4d). However, the role of these metal ions (such as Fe^{3+}, Cu^{2+} and Zn^{2+}) may be weak in the system because the addition of EDTA-2Na reduced the dhBBR-to-BBR oxidization by only 5%.

Effect of the gut microbiota on the therapeutic efficacy of BBR

To determine whether the gut microbiota alters the therapeutic effects of BBR, we used KK-Ay mice with type 2 diabetes. The mice were either orally pre-treated or untreated with antibiotics for 3 days and then treated with BBR with or without simultaneous treatment with antibiotics. The therapeutic results on day 14 post-BBR treatment are shown in Fig. 5. Compared with the efficacy of BBR in ordinary KK-Ay mice (with no exposure to antibiotics, Group 2), the oral treatment of KK-Ay mice with antibiotics before and during BBR treatment (Group 1) reduced the therapeutic efficacy of BBR on the fasting blood glucose level by 42% [(1−(100–62)/(100–33.9))×100%], the triglyceride level by 53% [(1−(100–74.9)/(100–46.3))×100%] and the

cholesterol level by 44% [(1−(100–67.7)/(100–42.3))×100%] (Fig. 5a U, treated vs. untreated, $P < 0.01$, $P < 0.001$; antibiotics plus BBR vs. BBR, $P < 0.05$; Fig. 5a M, treated vs. untreated, $P < 0.05$; antibiotics plus BBR vs. BBR, $P < 0.05$; Fig. 5a L, treated vs. untreated, $P < 0.001$; antibiotics plus BBR vs. BBR, $P < 0.01$). Accordingly, the number of intestinal bacteria colonies in the PGF KK-Ay mice treated with both BBR and antibiotics (Group 1) was significantly lower than that in KK-Ay mice treated with BBR only (Group 2; $P < 0.01$, Fig. 5b). In addition, mice treated with both antibiotics and BBR had fewer intestinal bacteria colonies compared with the untreated controls (Group 1 vs. untreated controls, $P < 0.01$, Fig. 5b).

Figure 5. Gut microbiota modulates the therapeutic effect of BBR *in vivo*.



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(**a**) Fasting blood glucose (Glu), triglyceride (TG) and cholesterol (CHO) in conventional KK-Ay mice or pseudo germ-free KK-Ay mice (n = 6) treated with (or without) BBR for 14 days; U: plasma glucose; M: plasma triglyceride; L: plasma cholesterol. (**b**) Number of intestinal bacteria colonies (in log scale) on day 14 (n = 6). (**c**) Plasma concentration of BBR in KK-Ay mice treated or untreated with antibiotics; U: C_{max} of BBR on day 14; L: folds of C_{max}[BBR/(BBR+antibiotics)] on days 1, 3, 6, 10 and 14 post-BBR oral administration. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

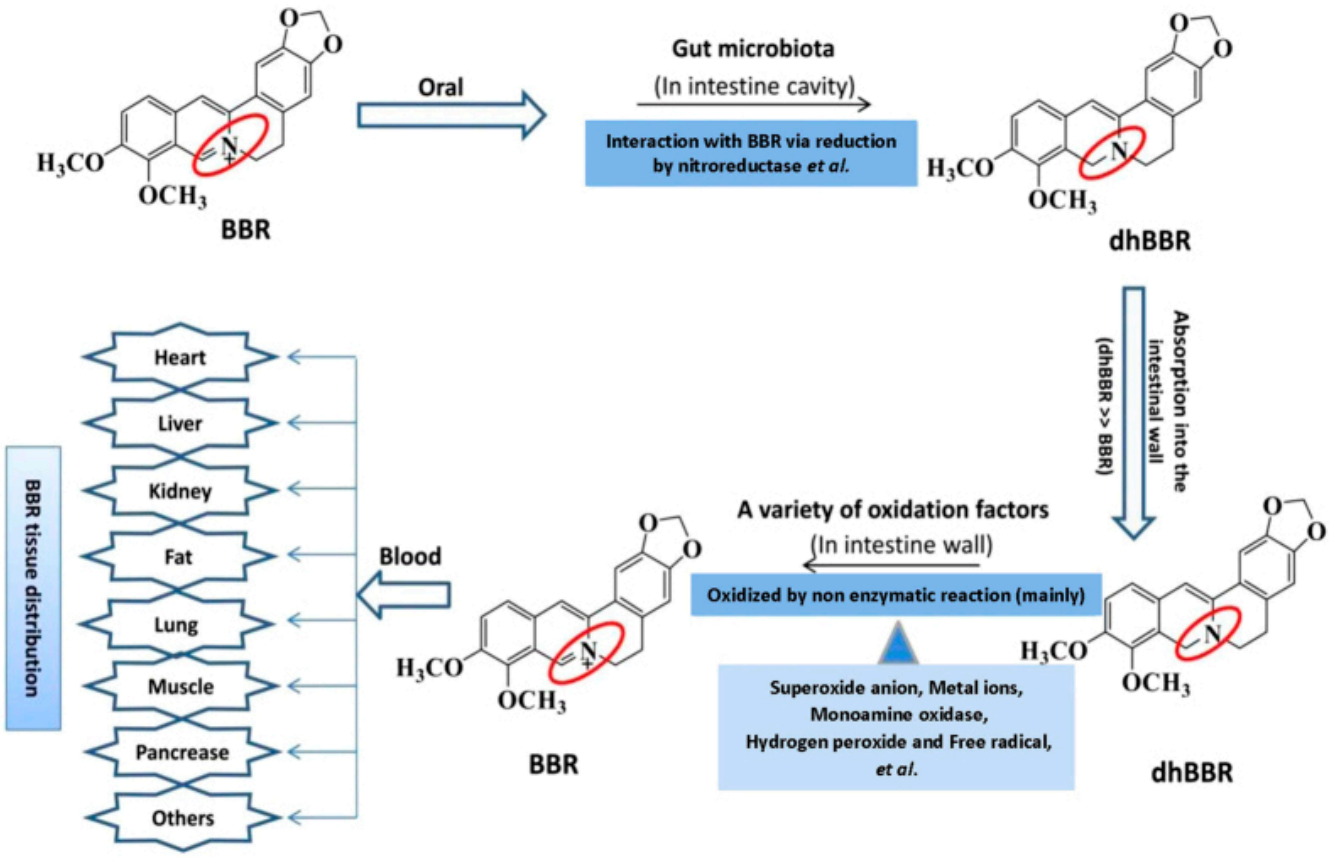
In addition, we measured the BBR plasma concentrations in KK-Ay mice in the BBR-treated groups with or without antibiotics (Group 1 vs. Group 2). As shown in Fig. 5c (U), on day 14, the C_{max} value of BBR in mice treated with antibiotics (Group 1) was 28.32 ± 3.41 ng/ml whereas in those without antibiotics (Group 2) the BBR C_{max} value was 178.15 ± 57.61 ng/ml, which is 6.4-fold higher than that in Group 1 ($P < 0.01$). In fact, the C_{max} value in Group 2 was 3.5-6.5-fold higher than that in Group 1 throughout the BBR treatment course on days 1, 3, 6,

10 and 14 (Fig. 5c, L). It appeared that the oral administration of antibiotics could lower the level of intestinal bacteria and, accordingly, decrease the absorption of BBR into the blood, reducing its therapeutic efficacy in hyperglycemia and hyperlipidemia.

Discussion

This study shows that the gut microbiota can act as an “organ” that converts BBR into dhBBR in the intestine via a reduction reaction mediated through bacterial nitroreductase. DhBBR, which was found to be absorbable by the intestinal epithelia, was reverted to BBR (the active form) immediately after entering the intestinal wall tissues. The reversion of dhBBR to BBR via oxidization occurred mainly through a non-enzymatic reaction in intestinal epithelial tissues, with the participation of multi-faceted factors such as superoxide anion and metal ions. These factors in the intestine ecosystem worked in a coordinated manner to complete the structure transformation between BBR (C = N) and dhBBR (C-N). Because the conversion-absorption-reversion process took place entirely in the intestinal environment, BBR, but not dhBBR, is the main chemical form detectable in the blood. Oral administration of antibiotics decreased the amount of intestinal bacteria and thus suppressed BBR absorption, resulting in a low level of BBR in the blood as well as reduced therapeutic efficacy. This study presents an interesting case to show the importance of the gut microbiota in modifying drug bioavailability and therapeutic efficacy. Figure 6 summarizes the role of the gut microbiota in regulating the conversion-absorption -reversion process of BBR in the intestine system.

Figure 6. Proposed mechanism of BBR absorption in the intestine.



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Nitroreductase in the gut flora plays a very important role in converting BBR into dhBBR. Because this enzyme is abundant in many types of intestinal bacteria[3], the uptake of BBR in the human intestine should be relatively steady among races and individuals. The physiological condition for dhBBR oxidization is so common that dhBBR molecules readily reverse to BBR, which then enters the bloodstream. In fact, the lipid-lowering and hypoglycemia effects of BBR have been verified in patients by independent clinical groups in China, Europe and America, with similar efficacies (reducing the fasting blood glucose level by 21–36%, the LDL-c level by 10–25% and the triglyceride level by 20–35%)[9]. This finding suggests that the bioavailability of BBR in humans is highly stable. Furthermore, nitroreductase in the gut microbiota may be a rate-limiting step for controlling the amount of BBR entering the blood because the intestinal nitroreductase level is approximately constant at a comparatively flat range in humans. Indeed, BBR exhibits good safety in the clinic and is classified as an OTC drug in China. This study also raises an important issue for patients orally receiving drug treatment together with antibiotics because the uptake rate of drugs (such as BBR) may be modified through antibiotic-mediated changes in the gut microbiota, and, accordingly, the clinical therapeutic efficacy may be altered.

Although dhBBR has a higher intestinal absorption rate than that of BBR, its bioactivity on LDLR and bacteria was lower than that of BBR. The finding agrees with our previous analysis of the structure-activity relationship of BBR, which showed that the 7-position quaternary ammonium and planar structure of the compound are required for LDLR upregulation[20,21,22,23]. It appears that dhBBR is a transient form of BBR in the intestinal lumen, with improved physiochemical characteristics for absorption. Although BBR is considered an antibacterial agent, its activity is directed mainly to dysentery bacillus[24]. The MIC values of BBR for the 17 tested strains were all higher than 128 μg/ml, with most of the values being at least 512 μg/ml, a concentration close to that of BBR precipitation in the testing system. The results showed that the antibacterial effect of BBR was very weak for ordinary intestinal bacteria, and the effect of dhBBR was even weaker. The reduction of BBR to dhBBR by nitroreductase enzymes of the gut microbiota may imply the existence of a self-protection mechanism of intestinal bacteria. In fact, because nitroreductase has broad substrate specificity and reduces groups of compounds, the enzyme is thought to play a role in the general detoxification of bacteria[16,25].

The connection between gut microbes and the pathogenesis of energy metabolic disorders and cardiovascular disease has recently piqued research interest in the physiological function of the gut microbiota in the human body[26,27,28,29,30]. This interest has been extended to the interaction between intestinal bacteria and orally administered drugs such as BBR[31,32]. Drug metabolism by the intestinal microflora may potentially generate new metabolites with distorted bioactivities or properties; however, the role of the gut microbiota and its influences on orally administered drugs are much less understood. Studies of the functional connections between the gut microbiota and drug pharmacology are even scarcer. The present study on BBR provides a paradigm showing the role of the gut microbiota in modifying drug structure and altering its absorption and therapeutic efficacy. The results may enrich our understanding and knowledge of drug metabolism *in vivo*, drug-drug interaction, mode of action, and druggability prediction. Our findings regarding the bacteria-caused structural modification of BBR in the intestine, as well as the changes in its absorption and therapeutic effects, lead us to suggest that investigation of drug metabolism by the gut microbiota should be a step in drug development.

Methods

Chemicals and reagents

BBR was obtained from J&K Scientific Ltd (Beijing, China). Tetrahydropalmatine, as an internal standard, was purchased from the National Institute for Food and Drug Control (Beijing, China). Thalifendine (M1), berberrubine (M2), demethyleneberberine (M3),

jatrorrhizine (M4) and dihydroberberine (dhBBR, M17) were obtained from Chengdu Herb Purity Co., Ltd (Chengdu, China). Rutin was purchased from the National Institute for Food and Drug Control (Beijing, China). The purity of the above-mentioned standards was more than 98% (HPLC). HPLC-grade acetonitrile was obtained from J&K Scientific Ltd (Beijing, China). All the other chemicals and reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China) and were of HPLC-grade purity.

Ticlopidine, ketoconazole, β-nicotinamide adenine dinucleotide phosphate (NADP), D-glucose 6-phosphate (G-6-P) and D-glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Furafylline, diethyldithiocarbamate (DDC) and sulfaphenazole were obtained from Santa Cruz Biotechnology Co. Ltd. (Shanghai, China). Quercetin, nootkatone and quinidine were purchased from J&K Scientific Ltd. (Beijing, China).

Nitroreductase (≥90%, recombinant, expressed in *Escherichia coli*), monoamine oxidase-B (recombinant, expressed in baculovirus-infected BTI insect cells), and deprenyl (a monoamine oxidase-B inhibitor) were purchased from Sigma-Aldrich Co. (Shanghai, China). 2-Iodosobenzoic (2-IBA, a nitroreductase inhibitor) and pargyline (another monoamine oxidase-B inhibitor) were obtained from J&K Scientific Ltd. (Beijing, China). Human intestinal microsomes (HIMs) were obtained from the Research Institute for Liver Diseases (Shanghai, China).

Animals

Male Sprague–Dawley (SD) rats (180–200 g) were supplied by the Institute of Laboratory Animal Science, Chinese Academy Medical Sciences (Beijing, China). The animals were housed in cage racks, with a 12-h light/12-h dark cycle (light on from 8:00 AM to 8:00 PM) at ambient temperature (22 °C–24 °C) and 45% relative humidity. The rats were fasted for 12 h before the experiments but during the study had free access to food and water. The research was conducted in accordance with the institutional guidelines and ethics and approved by the Laboratories Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College.

Instruments

A liquid chromatography instrument coupled to an ion trap time-of-flight mass spectrometer (LC/MS^n-IT-TOF) from Shimadzu Corporation (Kyoto, Japan) was used to identify the chemical structures of BBR and its possible metabolites. Liquid chromatography with tandem mass spectrometry (LC–MS/MS 8040 or 8050, Shimadzu Corporation, Kyoto, Japan) and gas chromatography with mass spectrometry (GC-MS QP2010, Shimadzu Cooperation, Kyoto,

Japan) were used for analysis and quantification of BBR and its metabolites in biological samples.

Metabolizing BBR by large intestinal bacteria *in vitro*

The method was reported previously[33]. Briefly, colon contents from six rats were pooled, and 5 g of the sample was transferred into a flask containing the anaerobic medium (100 ml). After thorough mixing, the cultures (which contained intestinal bacteria and anaerobic medium) were pre-incubated under anaerobic conditions with a N_2 atmosphere at 37 °C for 60 min. Rutin (10 μl, 1.0 mg/ml in methanol) was used as a positive reference for intestinal metabolism.

Ten microliters of BBR at different concentrations was added into the fresh human or rat intestinal bacteria cultures (990 μl), with methanol (10 μl) as the negative control. The final concentrations of BBR in the incubation system were 100, 10, and 5 μg/ml. The cultures were incubated at 37 °C for 72 h in the presence or absence of human or rat intestinal bacteria.

After termination of the reaction with acetonitrile (1 ml), 50 μl of the internal standard (tetrahydropalmatine, 2.0 mg/ml in methanol) was added into the BBR samples, which were then mixed for 30 sec and centrifuged at 7,500 g for 15 min. The supernatant was dried under nitrogen flow at room temperature, and the residue was dissolved in 100 μl of methanol and centrifuged at 7,500 g for 15 min. The metabolites were analyzed by LC/MS^n-IT-TOF using a previously reported method[34].

GCMS-QP2010 was also used to analyze the BBR metabolites with low polarity. The GC column was an Alltech capillary column (AT^{TM}-1701, 30 m × 0.25 mm × 0.25 μm) operated in the splitless mode. The helium carrier flow was 39.7 cm/s under a column head pressure of 68.1 kPa. The oven temperature was initially 50 °C for 2 min, gradually increased to 260 °C at a rate of 8 °C/min, and maintained for 25 min. The injector and detector temperatures were set to 280 °C and 230 °C, respectively. The mass spectra were recorded at a scan range of *m/z* 40 to 800. Structure identification of possible metabolites was based on matching with standard mass spectra available in the Shimadzu GC-MS library.

Quantification analysis of BBR metabolites modified by the gut microbiota *in vitro* and *in vivo*

A working solution of BBR was prepared at a series of concentrations by diluting the stock solution (10 mg/ml) with methanol to generate the standard curves. LC-MS/MS 8040 was used to quantify BBR and its metabolites transformed by intestinal bacteria[15].

For the determination of dhBBR obtained after incubation with intestinal bacteria *in vitro*, a working solution of dhBBR was prepared at concentrations of 500, 200, 100, 50, 20, 10 and 1 μg/ml by diluting the stock solution (1 mg/ml) with methanol, which were used to generate the standard curves of dhBBR. Samples from the *in vitro* intestinal bacteria incubation mixture were injected into the GC-MS instrument.

For the analysis of the *in vivo* intestinal metabolites of BBR, six SD rats were orally treated with BBR (200 mg/kg), and their feces were collected 0, 6, 12, 24, 36, 48, and 72 h after treatment. The method used for the preparation of the feces was reported previously[34]. An aliquot of 1 μl was injected into the GC-MS instrument. The level of dhBBR in feces was measured and calculated based on the standard curve obtained with the *in vitro* intestinal bacteria incubation mixture.

To prepare the human samples, we transferred fresh feces (5 g per person) from healthy volunteers (age from 20–25, two males and two females) into a flask containing the anaerobic medium (100 mL)[33]. After thorough mixing, the bacteria were cultured under anaerobic conditions with a N_2 atmosphere at 37 °C for 60 min. The culture solution of intestinal bacteria was then ready for analysis.

BBR metabolism in the rat small intestine bacteria

The small intestine contents from ten sacrificed SD rats were obtained and mixed with anaerobic medium to prepare the cultures. BBR was added into the incubation at final concentrations of 100, 50, and 10 μg/ml, and the reaction was continued for 6, 12 or 24 h. Levels of BBR, dhBBR and other BBR metabolites were determinedusing the LC-MS/MS 8040 and GC-MS instruments according to the above-mentioned method.

Distribution of BBR, dhBBR and other metabolites in the small intestine

Three segments of small intestine (duodenum, jejunum and ileum) were collected 0, 6, 12, 24, 36, and 48 h after the oral administration of BBR in SD rats. The small intestine tissues were washed thoroughly with saline and dried. After being weighed, the samples were homogenized with mixed solution (ethanol: water, 1:1) at a ratio of 1:2 [w(g)/v(ml)]. The samples were centrifuged at 7,500 g for 10 min, and the supernatant was collected and evaporated to dryness in a water bath at 40 °C using a rotary evaporator. The residues of the tissue extraction were dissolved in methanol (250 μl) and vortex-mixed for 5 min. The tissue solution was then centrifuged (at 14,000 g for 5 min), and the supernatant was passed through a 0.22-μm filter. An aliquot of 1 μl of the supernatant was injected into the GC-MS instrument, and a 10-μl aliquot was used for LC-MS/MS analysis. The level of dhBBR was

calculated based on the standard curve, and BBR and other metabolites was quantified using the method described above.

BBR metabolism in the *in vitro* culture of 14 intestinal facultative anaerobes

Ten intestinal facultative anaerobes (clinical isolates)—*Staphylococcus aureus* (*S. aureus*) 08-43, *Enterococcus faecium* (*E. faecium*) 13-01, *Enterococcus faecalis* (*E. faecalis*) 13-01, *Enterobacter cloacae* (*E. cloacae*) 13-12, *Escherichia coli* (*E. coli*) 06-05, *Staphylococcus epidermidis* (*S. epidermidis*) 12-12, *Pseudomonas aeruginosa* (*Ps. aeruginosa*) 13-10, *Klebsiella pneumonia* (*K. pneumoniae*) 13-14, *Proteus mirabilis* (*P. mirabilis*) 13-01 and *Acinetobacter baumannii* (*A. baumannii*) 13-02—were collected from gastrointestinal specimens from patients from hospitals in Beijing between 2006 and 2013. The specimens were identified in the hospitals using the VITEK 2-COMPACT system (bioMerieux, Marcy l'Etoile, France). *Lactobacillus casei (L. casei* ATCC *334), Lactobacillus acidophilus (L. acidophilus* ATCC *4356), Bifidobacterium longum (B. longum* ATCC *15707), and Bifidobacterium breve (B. breve* ATCC *15700)* were purchased from the Microbial Culture Collection Center in Guangdong, China. The bacteria were transferred into a flask containing anaerobic medium. BBR was incubated with the 14 facultative anaerobes at a final concentration of 50 μg/ml at 37 °C for 0, 12, 24, 48 and 72 h. BBR and dhBBR in the culture were analyzed quantitatively using GC-MS and LC-MS/MS 8040.

Determination of nitroreductase

The detection of nitroreductase was performed using the Human Nitroreductase ELISA kit purchased from Beijing Luyuan Dade Biological Science and Technology Co., Ltd (Beijing, China) according to the manufacturer's guidelines.

Pseudo germ-free (PGF) rats and BBR absorption

Male SD rats (180–200 g) were orally treated with cefadroxil (100 mg/kg), terramycin (300 mg/kg) and erythromycin (300 mg/kg) twice a day for 3 days, and pharmacokinetic examination was performed 2 days after final administration. The colon contents of the rats were collected on the first and third days after the final treatment with antibiotics, and the germ-free status was confirmed by culturing fecal samples aerobically on a nutrient agar culture medium. Fecal samples from non-antibiotic-exposed rats served as control samples.

Before oral administration of a single dose of BBR (200 mg/kg), the PGF rats were fasted overnight with free access to water. Blood samples were collected from the posterior orbital venous plexus into a heparinized tube at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 h post-BBR treatment and then subjected to the procedure described above. Fecal samples were also

collected at 0, 6, 12, 24, 36 and 48 h post-BBR treatment. The samples were stored at −20 °C for further analysis.

Anti-bacterial test

Seventeen bacterial strains (see above) were used in the susceptibility test for BBR and dhBBR. The bacterial strains, which belong to different species of bacteria, were collected and identified as described above. The minimum inhibitory concentrations (MICs) of BBR and dhBBR after 24 h were determined through the broth microdilution method according to the CLSI guidelines[35]. BBR and dhBBR were tested at a concentration range of 8 to 512 μg/ml. The MIC was defined as the lowest concentration of an agent that prevents turbidity. The experiment was repeated three times. All of the bacterial strains tested in this study (*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *S. aureus* 08-43, *S. epidermidis* 12-12, *E. faecium* 13-01, *E. faecalis* 13-01, *E. coli* 06-05, *K. pneumoniae* 13-14, *Ps. aeruginosa* 13-10, *A. baumannii* 13-02, *E. cloacae* 13-12, and *P. mirabilis* 13-01) were facultative anaerobes, of which *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 were standard strains from ATCC (USA) and served as quality controls. *L. casei* ATCC 334*, L. acidophilus* ATCC 4356*, B. longum* ATCC 15707, and *B. breve* ATCC 15700 were also tested in this anti-bacterial experiment. Ampicillin was used as the positive control.

Molecular docking between BBR and nitroreductase

AutoDock Vina v.1.0.2 software was used to perform the molecular docking of BBR onto nitroreductase, whose crystal structures are available in the Protein Data Bank[17]. The docking parameters were set to the default values. The grid boxes were 20 Å × 20 Å × 20 Å, encompassing the active site cavities. The binding modes of BBR to enzymes were chosen to further optimize the docking conformation according to their binding free energy, distances from conserved water molecules and the flavin mononucleotide (FMN). The simulation results were visualized using the PyMOL Molecular Graphics System Version 1.3 (Schrödinger LLC, New York, NY, USA) and Discovery Studio Visualizer (Accelrys, Inc., San Diego, CA, USA).

Nitroreductase-mediated BBR reduction

BBR was incubated with nitroreductase in intestinal bacteria cultures. The reaction mixture consisted of BBR (50 μg/ml), an NADPH-regenerating system and nitroreductase (5 μg/ml) in a final volume of 1 ml under the protection of N_2. After incubation for 0, 2, 4, 6 and 12 h at 37 °C, the reaction was terminated by the addition of 1 ml of ice-cold acetonitrile. The samples were then extracted with 1 ml of ethyl acetate after adding 1 ml of 0.5 M sodium hydroxide solution. The organic phase was evaporated to dryness under a nitrogen flow in a water bath at 40 °C. The residue was dissolved with 200 μl of the mobile phase for further analysis. 2-

Iodosobenzoic acid (2-IBA), a specific inhibitor of nitroreductase, was used to verify the role of bacterial nitroreductase.

Absorption of BBR and dhBBR in Caco-2 cells

A Caco-2 cell assay was conducted using a method reported previously[36]. Stock solutions of dhBBR and BBR were prepared in DMSO at 1 mM, and these were then diluted with HBSS buffer (10 mM HEPES, pH 7.4) to a final concentration of 5 μM for both samples. To determine the rate of drug transport in the apical-to-basolateral direction, 200 μl of the compound working solution was added to the filter well (apical compartment), and 800 μl of HBSS was added to the receiver plate (basolateral compartment). Accordingly, to determine the rate of drug transport in the basolateral-to-apical direction, 800 μl of the compound working solution was added to each well of the receiver plate and 200 μl of HBSS was added to the filter well. The plates were incubated for 30 min at 37 °C with shaking at 150 rpm on a rotary shaker. At the end of the transport period, aliquots of 50 μl were removed directly from the apical and basolateral wells and transferred to wells of new plates. Four volumes of cold methanol containing internal standards were added into each well. The samples were centrifuged at 16,000 g for 15 min. Aliquots of 200 μl of the supernatant were used for the LC-MS/MS analysis.

Absorption of dhBBR *in vivo*

Five SD rats were fasted for 12 h and then orally administered 200 mg/kg dhBBR. Blood samples (0.5 ml) were obtained from the posterior orbital venous plexus into a heparinized tube at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 12 h, and the samples were then centrifuged at 2,000 g for 5 min. The plasma (100 μl) was precipitated with 100 μl of acetonitrile after addition of 10 μl of the internal standard. BBR served as a control in the study. The plasma concentrations of BBR and dhBBR were determined using LC-MS/MS 8040 and LC-MS/MS 8050.

Drug effect on hepatic LDLR gene expression

The HepG2 human hepatoma cell line was obtained from the American Tissue Culture Collection (ATCC, USA) and grown in Eagle's Minimum Essential Medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37 °C. For the drug efficacy test, HepG2 cells were cultured for 24 h and then treated with BBR or dhBBR for 12 h. The compounds were freshly prepared in medium prior to use.

The total cellular RNAs were isolated with the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the vendor's instructions. The total RNAs were reversely

transcribed into cDNAs, and quantitative real-time PCR was performed using a 2-Step RT-qPCR System (Promega, Madison, WI, USA), with GAPDH as an internal control. The normalized LDLR mRNA expression levels were plotted as fold levels compared with the untreated control. The following primers were used: LDLR forward, aggacggctacagctaccc; LDLR reverse, ctccaggcagatgttcacg; GAPDH forward, agccacatcgctcagacac; and GAPDH reverse, gcccaatacgaccaaatcc.

Reversion from dhBBR to BBR

The rat duodenum, jejunum and ileum were collected separately, washed thoroughly with saline and dried. After being weighed, the tissues were homogenized with saline at a ratio of 1: 2 [w (g) /v (ml)]. DhBBR was dissolved in a mixture of methanol and DMSO at a ratio of 1:1 with a final concentration of 5 mg/ml. Ten microliters of dhBBR (5 mg/ml) was mixed with 1 ml of the homogenates. The samples were then centrifuged at 14,000 g for 5 min, and the supernatants were collected and mixed with an equal volume of ice-cold acetonitrile to terminate the reaction. The samples were centrifuged at 14,000 g for 5 min, and the supernatant was used for the HPLC analysis.

Transformation of dhBBR in pooled human intestinal microsomes (HIMs)

The typical incubation mixture contained 0.2 mg of HIMs, 100 mM phosphate buffer (pH 7.4), 100 μM dhBBR (dissolved in DMSO) and an NADPH-regenerating system [at a final concentration of 3.3 mM glucose-6-phosphate (G-6-P), 1.3 mM $NADP^+$, 0.4 unit/ml glucose-6-phosphate dehydrogenase (G-6-PDH) and 3.3 mM $MgCl_2$] in a final volume of 200 μl. After pre-incubation of HIMs with dhBBR for 2 min at 37 °C, the reaction was initiated by the addition of the NADPH-regenerating system. The mixture was incubated at 37 °C with exposure to air for 2, 5 and 10 min and was terminated by the addition of 200 μl of ice-cold acetonitrile. Then, 20 μl of the internal standard was added to the mixture, and the mixture was centrifuged at 14,000 g for 5 min. Fifty microliters of the supernatant was injected into the HPLC instrument for analysis. The control samples were incubated under the same conditions but without HIMs.

Oxidization of dhBBR by monoamine oxidase B (MAO-B)

The reaction system consisted of dhBBR (100 μM), MAO-B (0.2 mg/ml) and phosphate-buffered saline (PBS, pH = 7.4) in a final volume of 200 μl. The MAO-B was pre-incubated via centrifugation at 850 rpm at 37 °C for 5 min, and this step was followed by the addition of dhBBR. The reaction was terminated after 30 min by adding an equal volume of ice-cold acetonitrile and centrifuging at 14,000 g for 5 min. Ten microliters of the supernatant was

injected into the HPLC instrument for analysis. Selective MAO-B inhibitors (deprenyl, 100 μM or pargyline, 1 μM)[37,38] were used in the tests.

Effect of vitamin C on dhBBR-to-BBR transformation

The rats (n = 3) were sacrificed to obtain fresh small intestine homogenate. The homogenate was then boiled for 2 min to inactivate all of the enzymes. After pre-incubation at 37 °C for 2 min, 5 μl of dhBBR (10 mM, dissolved in DMSO) was added into the inactivated homogenate, and fresh homogenate (active one) was used as a reference. The sample was mixed thoroughly and centrifuged at 16,000 g for 5 min. The supernatant was transferred into new tubes, treated with 500 μl of acetonitrile, and then centrifuged. Twenty-microliter aliquots were used for the HPLC analysis.

To examine the oxidative reaction, 100 μl of vitamin C was pre-incubated with the inactivated homogenate; the final concentrations of vitamin C were 95, 9.5 and 0.95 mM. The superoxide anion in the sample was detected with an O_2^- ELISA kit (Beijing Biolab Co., Ltd., Beijing, China; lot: 201404) following the manufacturer's instructions.

Gut microbiota modulates the therapeutic effects of BBR on hyperglycemia and hyperlipidemia

KK-Ay mice with type 2 diabetes (female, 12 ± 1 weeks of age) were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China). The mice were maintained at room temperature (23 ± 2 °C) with 50% moisture and exposed to a 12-h light/12-h dark cycle, and food and water were provided ad libitum. Antibiotics at the doses described above were orally administered to KK-Ay mice for 3 days before the BBR treatment course, and the pseudo germ-free (PGF) state of the experimental mice was confirmed (see above).

Four groups (n = 6 for each group) were used in the experiment: Group1, PGF KK-Ay mice orally treated with antibiotics (qd, at the dosage described above) plus BBR (200 mg/kg, qd) for 2 weeks; Group 2, untreated KK-Ay mice orally treated with BBR (200 mg/kg, qd) for 2 weeks; Group 3, PGF KK-Ay mice orally treated with antibiotics (qd, at a dosage identical to that for Group 1) for 2 weeks; Group 4, untreated KK-Ay mice. The antibiotics were administered in the morning and BBR was administered in the afternoon. All the animals were fed a high-fat diet. The germ-free state of Groups 1 and 3 was confirmed on days 7 and 14 of the treatment course using the same bacterial analytical method described above. All of the mice were euthanized on day 14, and blood samples were taken to measure the fasting serum glucose (Glu), triglyceride (TG) and cholesterol (CHO) using Glu, TG and CHO reagent kits (Biosino Bio-Technology and Science INC, Beijing China), respectively. C57BL/6J mice

were used as a wild-type reference. The body weights of the mice before, during and after treatments were recorded for 14 days.

Blood samples of the mice in Groups 1 and 2 were collected into a heparinized tube 30 min after the oral administration of BBR. The samples were taken at days 1, 3, 6, 10 and 14 of the BBR treatment course. The samples were centrifuged at 10,000 g for 5 min, and the plasma was collected and stored at −80 °C before analysis.

Effect of ions on the dhBBR-to-BBR transformation

dhBBR was added into the reaction solution containing $FeCl_3$, $CuCl_2$, $ZnCl_2$, and NaCl (at concentrations of 0.033, 0.333 and 3.33 mM, respectively). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na, 10 mM), a chelating agent for metal ions, was used in the test.

Statistical analysis

The statistical analyses were conducted using two-way ANOVA and Student's *t*-test with GraphPad Prism Version 5 (GraphPad Software, CA, USA). The data are expressed as the means ± standard deviation, and p values less than 0.05 were considered statistically significant.

Additional Information

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Supplementary Material

Supplementary Information

Acknowledgments

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Footnotes

Author Contributions J.-J.D. and W.-Y. conceived of and designed the experiments and contributed to the writing and editing of the manuscript. F.-R., S.-J.W., Z.-Z.X., M.-C., F.-J., H.-C.Y., H.-M., T.-X.S., L.-X.Y., W.-B.Y., C.-X., L.-Y., R.-G. and Y.-X.Y. performed the experiments. J.-J.D. and W.-Y. analyzed the data. J.-J.D., W.-Y., Y.-X.F. and C.-Y.C. contributed to discussions and provided reagents/materials /analysis tools important for completion of the work.

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Associated Data

This section collects any data citations, data availability statements, or supplementary materials included in this article.

Supplementary Materials

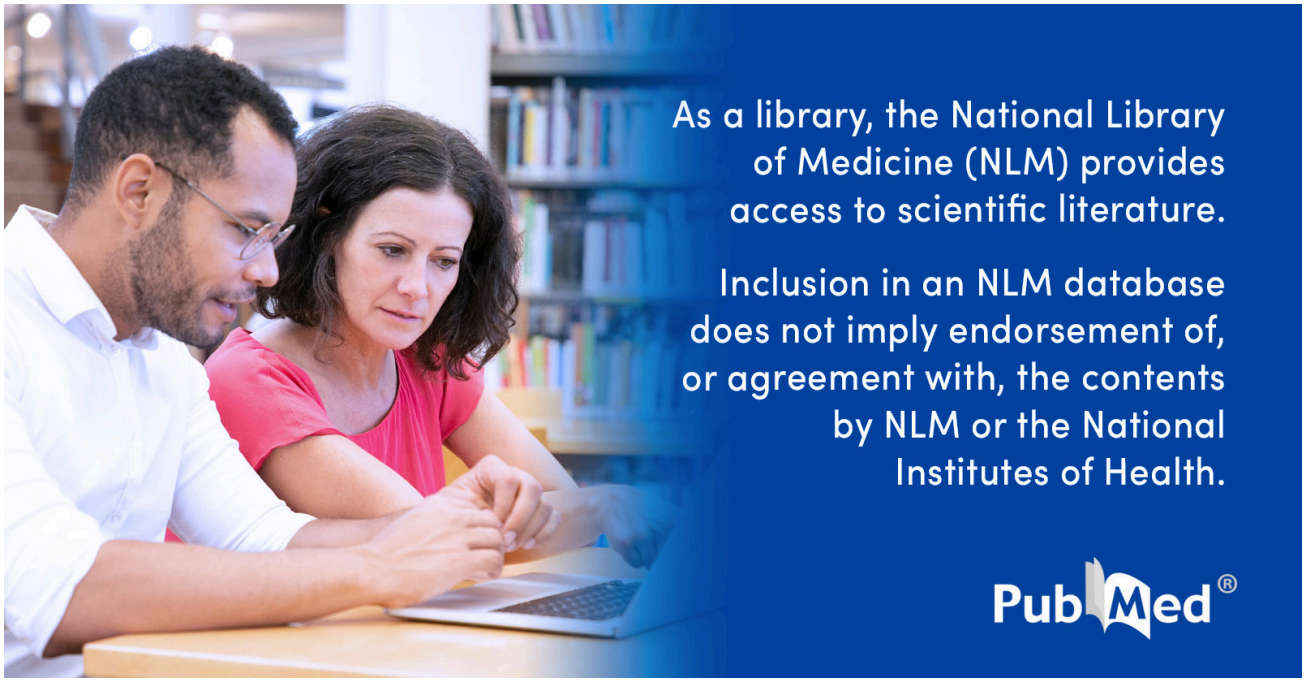
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Berberine and Its Role in Chronic Disease

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Abstract

Berberine is a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids. It is found in such plants as Berberis [e.g. Berberis aquifolium (Oregon grape), Berberis vulgaris (barberry), Berberis aristata (tree turmeric)], Hydrastis canadensis (goldenseal), Xanthorhiza simplicissima (yellowroot), Phellodendron amurense [2] (Amur corktree), Coptis chinensis (Chinese goldthread), Tinospora cordifolia, Argemone mexicana (prickly poppy) and Eschscholzia californica (Californian poppy). In vitro it exerts significant anti-inflammatory and antioxidant activities. In animal models berberine has neuroprotective and cardiovascular protective effects. In humans, its lipid-lowering and insulin-resistance improving actions have clearly been demonstrated in numerous randomized clinical trials. Moreover, preliminary clinical evidence suggest the ability of berberine to reduce endothelial inflammation improving vascular health, even in patients already affected by cardiovascular diseases. Altogether the available evidences suggest a possible application of berberine use in the management of chronic cardiometabolic disorders.

Keywords: Anti-inflammatory; Antioxidant; Berberine; Cardiovascular disease; Depression; Type 2 diabetes.

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