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Synthesis and antihyperlipidaemic activity of a new piperine derivative

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ABSTRACT

Long pepper (*Piper longum* Linn.) is widely used as a medicinal substance in traditional Ayurvedic medicine. Its major alkaloid piperine is the main active constituent with various therapeutic activities and has low solubility in water. In this study, a soluble new derivative of a piperine alkaloid, named N-leucinylpiperamide was synthesized. The animal experiment showed that N-leucinylpiperamide has more hypolipidemic effects than commercially available simvastatin and piperine in modulating serum lipids in Wistar male rats. At the amount of 10 mg/kg bw, it significantly reduced TC (-52.4%), TG (-61.7%), and LDL-C (-27.8%), respectively, and increased HDL-C (+147.4%) in the serum of the high-lipid model group. Furthermore, the synthesized N-leucinylpiperamide had no noticeable cytotoxicity against HepG2 cell line *in vitro*. Thus, our study shows that N-leucinylpiperamide has an ability to improve serum lipid profile in hyperlipidemic model rats and could be a valuable promising agent for the preventing hyperlipidemia.

Keywords: Piperine, alkaloid, cholesterol, hyperlipidemia, amino acid, solubility

INTRODUCTION

The Piper species have a long history as a condiment in many countries, but they have been more valued for their therapeutic properties and broadly used in Ayurvedic medicine. The plant contains many alkaloids, such as piperine, chavicin, and piperamine; the most abundant of which is piperine, 4-5% [1, 2].

Piperine alkaloid is a non-soluble piperic acid amide, predominantly found in *Piper longum* L. (long pepper) and *Piper nigrum* L. (black pepper) of the Piperaceae family. Its content is 3-5 percent on a dry weight basis [3, 4]. By systematic pharmacological studies, piperine showed having diverse pharmacological activities [5]. As a bio-active component, it has the potency to reduce blood cholesterol and triglycerides [6, 7]. In addition, it reduces low-density lipoprotein, very low-density lipoprotein, and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in the tissues, while increases lipoprotein lipase (LPL) and lecithin:cholesterol acyltransferase (LCAT) [8] as well possesses hypolipidemic activity in diet-induced experimental hyperlipidemic rats [9]. Thus, piperine and its derivatives may significantly modulate lipid parameters and lead the compound to develop new potential antihyperlipidemic drug candidates.

However, its biological applications are denied due to its low aqueous solubility [6, 10], which is a significant barrier to drug development. Therefore, one main concern is the development of the formulations to increase its solubility [11, 12].

Many studies have shown that improving the solubility of piperine affects its bioavailability and increases pharmacological activity [10]. Introduction of amino acids is one way to increase the water solubility of non-soluble compounds. Amino acids are widely used as carriers of poorly absorbed therapeutic agents via increasing their water solubility [13].

By the research on piperine as an inhibitor of Leishmania, the combination of piperonic acid, which is a piperine hydrolyzed product, and amino acid methyl esters showed better anti-Leishmanial activity than natural piperine alkaloid [14].

Thus, we hypothesised that such a combination of naturally occurring piperine and amino acid ester

might possess more anti-hypolipidemic effects and low cytotoxicity. In this study, we report synthesis and evaluation of the antidyslipidemic activity of a new form of piperine, N-leucinylpiperamide.

EXPERIMENTAL

Chemical reagents: The *Piper longum* L. fruits were purchased from Yikan Chinese Herbal Medicine Ltd. and Hainan Zun Ao Technology Development Co. Ltd., China, respectively. The specimen was identified according the method accepted previously [15]. Piperic acid was prepared at the Institute of Mongolian Medicine Chemistry, Inner Mongolia University with a purity rate >98%. The commercially available simvastatin was provided by Hai Zheng Pharmaceutical Co. Ltd, China. The kits for determining the total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were supplied by the BioSino Bio-Technology and Science Inc., China. All other chemicals were analytical grade from Sigma-Aldrich.

Equipment: Ultra-performance liquid chromatography (UPLC), Waters acquity UPLC, PDA detector, USA with a mobile phase of acetonitrile-water was used for the determination of the purity of synthesized compounds. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 500 MHz nuclear magnetic resonance (NMR) spectrometer at 25 °C. Mass spectrometry (MS) analysis was performed on LCQ ADVANTAGE MAX Mass Spectrometer System. Serum lipid profile was measured by commercial kit with a Pronto Evolution Biochemistry Analyser, Italy.

Cell culture: HepG2 cells obtained from the Chinese Academy of Sciences Cell Bank were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FCS, HyClone, Utah, USA), 4 mM/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. When reaching 80% confluence, the medium was discarded; the cells were washed 3 times with phosphate-buffered saline (PBS) and digested with 0.25% trypsin. The cells were re-suspended in DMEM, containing 10% fetal bovine serum (FBS), and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and a proper amount of DMEM containing 10% FBS was added into the cells to prepare a fine suspension. Cell number was counted and seeded into the 96-well plates at a density of 1×10^4/ml, 100 µL per well, and cultured at 37 °C with 5% CO_2 and saturated humidity for 24 h.

Animals: Animal experiments were carried out with 50 male adult Wistar rats with the weight of 190±10 g, provided by the Research Center for Laboratory Animals Science, China. According to the Institutional Guidelines for the Care and Use of Laboratory Animals, all procedures were performed following the ethical standards, after approval from the Institutional Animal Care and Use Committee (Inner Mongolia University, China).

After initial acclimatization for a week, animals were randomly divided into five different groups as described in the experimental design. The rats were housed in plastic cages under the controlled conditions of the ambient temperature of 24 ± 0.5 °C and 40-70% relative humidity with a 12 h light/dark cycle for 15 days. For the experiment, rats were fed with either a normal or freshly prepared high-fat diet (15 g/rat/d) or water ad libitum.

Chemistry of N-leucinylpiperamide: Extraction of piperine from *Piper longum* L. fruits and the following synthesis of piperic acid were carried out according to literature methods described [16, 17]. Then, the synthesized piperic acid (0.218 g) and L-leucine methyl ester (0.181 g) were dissolved in 3 mL anhydrous dimethylformamide (DMF) in a dry three-necked flask equipped with a reflux condenser and connected to the line of N_2 gas. The solution was placed in the ice bath for 30-60 min for instant cooling and 0.221 g of phosphonium hexafluorophosphate (BOP) was added. Then, 0.5 mL of N,N-diisopropylethylamine (DIEA) was added to the mixture slowly stirring at room temperature for 24 h. After that DMF was removed by evaporation, a viscous solid was dissolved in ethyl acetate (150 mL). Subsequently, the solution was washed with saturated sodium chloride (NaCl) solution, 5% citric acid, 5% NaOH, and then twice by distilled water in a separatory funnel. Afterward, ethyl acetate was removed by evaporating and the water was freeze-dried, subsequently a yellow solid was obtained (0.191 g). The solid was dissolved in methanol (10 mL) and sodium methoxide (10x) was added afterward. Then, it was stirred at room temperature for 24 h, and methanol was removed through evaporation. The mixture was dissolved in 60 mL of 5% sodium bicarbonate ($NaHCO_3$) solution followed by the extraction with methylene chloride (CH_2Cl_2) 3 times. After all, the reaction mixture was poured into 200 mL of 30% HCl to produce a yellow precipitate and was recrystallized to give 0.153 g of a new compound, N-leucinylpiperamide.

Experimental design: To test the anti-hyperlipidemic activities, the rats were divided into the following groups:

Group I - Normal diet control;
Group II - High-fat diet control;
Group III - High-fat diet + simvastatin (10 mg/kg bw);
Group IV - High-fat diet + piperine (10 mg/kg bw);
Group V - High-fat diet + N-leucinylpiperamide (10 mg/kg bw);

The normal diet group (I) was fed with a standardized laboratory chow diet, containing the standard composition of necessary macro- and micronutrients (56% carbohydrate, 18.5% protein, 8% fat, 12% fiber, and adequate levels of minerals and vitamins). While others were fed with high-fat diet recipe with the following nutrition: cholesterol (3%), sodium cholate (0.5%), lard (10%), wheat flour (17.3%), millet (8.65%), vitamins (0.0865%), salt (0.7785%), bran (21.685%), fish (1.73%), bone meal (1.73%) and beans material

(17.3%) to provide hyperlipidemic model.

Treatment groups (III, IV, and V) were given a daily oral administration of each compound sample (4 mL/kg) for 14 days, which were prepared as a suspension of sodium carboxymethyl cellulose (0.5% in water).

Biochemical analyses: The body weights were measured daily and the daily food intake was recorded. After 14 days of oral administration of treatments, the animals have fasted for 12 h. The blood samples were collected and centrifuged at 3000 rpm for 15 minutes at 10 °C to obtain serum. Freshly prepared serum samples were frozen at -80 °C until biochemical analyses.

Serum TC, TG, LDL-C, and HDL-C were determined with a biochemistry analyzer using standard kits.

In vitro cytotoxicity: For performing the MTT cell viability assay, the medium was replaced with fresh media, containing various concentrations of each compound (0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 µM) prepared in DMSO. After 24 h incubation, 50 µL of MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) reagent was added into each well and incubated at 37 °C for 4 h. After that medium was discarded and 1 mL of DMSO was added to dissolve the formazan crystals. The plates were gently shaken and absorbance at 490 nm was obtained in the microplate reader. In the blank control and the untreated wells, cell viability was 100%.

Statistical analysis: All results are expressed as the mean ± SD of animals in each group. Statistical analyses are conducted using Student's t-test [2]. Significance levels at $p < 0.05$ and 0.001 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

A piperine-amino acid new derivative, N-leucinyl-piperamide was prepared with the synthetic route as illustrated in Fig. 1.

As described, in the first step, piperine (1) was hydrolyzed giving a piperic acid, which is an efficient and cheap way for the preparation of piperic acid (3) with a yield of 86.6%. In the second step, piperic acid was reacted with L-leucine methyl ester to afford N-leucinyl methyl ester piperamide (N-leucinylpiperamide methyl ester, 4). Here, carboxylic group of piperic acid (3) is the main site to link with amino acid ester via amide bond formation. In the third step, the N-leucinylpiperamide methyl ester is the critical intermediate product to obtain the final expected product, N-leucinylpiperamide (5) with a yield of 80.1%

The purity of synthesized N-leucinylpiperamide was assessed by using UPLC with a mobile phase of acetonitrile:water, 75:35. In addition, the structure of N-leucinylpiperamide was determined using mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance (NMR).

A new derivative of piperine alkaloid, N-leucinyl-piperamide, was tested in male Wistar rats. Male rats were chosen in order to avoid the estrogen effects on lipid metabolism [18].

During the experiment, four critical biomarkers of hyperlipidemia showed an indicative change: the levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) were significantly increased, while the level of high-density lipoprotein cholesterol (HDL-C) was decreased in serum of the model male rats. Herein, the anti-hyperlipidemic activities of samples were expressed as the differences of lipid parameters TC, TG, LDL-C, and HDL-C in the rat serum of experimental and control groups.

Results showed that the lipid-lowering activity of N-leucinylpiperamide was comparable to piperine and simvastatin (Table 1).

In an animal experiment, the new derivative N-leucinyl-piperamide showed noticeable hypolipidemic effects on hyperlipidemia in rats, showing a substantial activity in modulating serum lipids in Wistar rats. At the dose of 10 mg/kg bw, it significantly reduced TC (-52.4%), TG (-61.7%), and LDL-C (-27.8%), respectively, and increased HDL-C (+147.4%) in the serum of the hyperlipidemic model animals. Consistent with

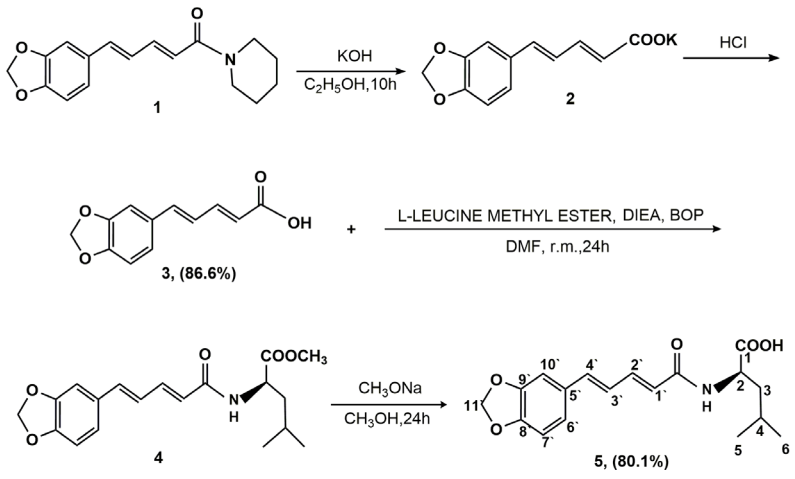

Fig. 1. Synthetic route of N-leucinylpiperamide synthesis

Table 1. Effect of antihyperlipidemic compounds on serum lipid content

Groups[a]		Serum lipid profile[b], mM			
		TC	TG	LDL-C	HDL-C
I	Control	1.46 ± 0.51	0.67 ± 0.26	2.39 ± 1.64	0.30 ± 0.10
II	HFD model	9.41 ± 1.97	2.27 ± 2.10	3.99 ± 0.62	0.19 ± 0.05
III	Simvastatin	7.06 ± 1.74	1.01 ± 0.54	3.34 ± 1.04	0.23 ± 0.04
IV	Piperine	7.32 ± 1.31	0.70 ± 0.12	3.47 ± 0.63	0.26 ± 0.02
V	N-leucinylpiperamide	4.48 ± 1.24	0.87 ± 0.13	2.88 ± 1.24	0.47 ± 0.31

[a] Fifty Wistar rats were divided into 5 groups and were subject to serum lipid content measurement after treatment as described in the Experimental section.
[b] Values are the mean of SD of 10 rats.

other literature values [9, 17], these results indicate a significant difference between the serum parameters of the treatment group and the control group. In addition, these results suggest that the development of hyperlipidemia in experimental animals was successful showing its reliability.

In our study, commercially available simvastatin was chosen to compare the antihyperlipidemic activity. Simvastatin reduced the corresponding TC, TG, and LDL-C lipid profiles by 25.0%, 55.5%, and 16.3%, respectively, and increased the HDL-C level by 21% up at the same amount as the N-leucinylpiperamide. Simvastatin, lovastatin, and pravastatin are the main members of the statin drug class widely used to reduce lipids in the blood [19, 20]. However, as reported, these drugs have unfavourable side effects, like severe myopathy, memory loss [21], and liver damage [22].

Piperine given at the same dose reduced the TC, TG, and LDL in serum levels by 22%, 61.2%, and 13.0%, respectively. Then, the serum HDL level was increased by 36.84% in the group of treated with piperine.

In comparison, the lipid-lowering activity of N-leucinyl-piperamide with simvastatin, the level of serum TC, TG, and LDL-C was decreased by 52%, 10%, and 42%, respectively, and increased the serum HDL-C level by 86% than simvastatin in the hyperlipidemic model animals.

Relevant studies have shown that the piperlongiminine from the *P. longum* extract decreased the TC and TG by 1.5-2.5 times, LDL-C by 1.3-1.5 times, while increased HDL-C by 1.9-3 times after oral administration at a dose of 10 mg/kg in high-fat diet rats for 14 days [2,9].

In our study, the hyperlipidemic model group had a significant reduction in the fat profile compared to the control group, in particular, N-leucinylpiperamide reduced TC in the plasma of rats by 1.5 times, TG and LDL-C by 1.2 times. The amount of HDL-C was increased by 2 times, resulting in the synthesis of a highly active functional compound of N-leucinylpiperamide against hyperlipidemia.

As a result, N-leucinylpiperamide significantly modulated lipid parameters in animal blood and its effect was markedly higher than piperine and simvastatin.

A major concern in developing novel drugs from plants is toxicity level. Based on the study, the literature has shown that the piperine may cause liver toxicity in CF-1 albino mice via an increase of serum alanine aminotransferase (ALT), serum aspartate aminotransferase (ASAT), and alkaline phosphatase (ALP) [23]. Another study on determining the LD_{50} values for a single intraperitoneal (IP), intravenous (IV), intramuscular (IM), intragastric (IG), and subcutaneous (SC) administration of piperine to adult male mice showed that piperine is acutely toxic to mice, rats and hamsters [24].

In our study, cytotoxicity of all tested compounds was investigated against HepG2 cell line at concentrations, ranging from 0.5-8 μM. The HepG2 cells were chosen as a suitable model to study cellular cholesterol homeostasis due to their high degree of morphological and functional differentiation *in vitro* [23, 25]. All substances were evaluated in three independent experiments, in triplicate. Results are shown in Table 2. Cell viability decreased with the increase in concentrations of compounds. The IC_{50} of N-leucinyl-piperamide was evaluated according to its concentration and cell viability. Results of experiments exhibited that the IC_{50} values of N-leucinylpiperamide, piperine, and simvastatin were 7.7 μM, 5.6 μM, and 3.2 μM, respectively, indicating that the cell viability in all testing concentrations of N-leucinylpiperamide was higher than the others. The IC_{50} values for compounds that inhibit cell viability by more than 50% are plotted graphically from the concentration-effect curve, shown in Fig. 2. The results showed that N-leucinylpiperamide reduced cell viability of HepG2 by 50.57%, while

Table 2. Effect of antihyperlipidemic compounds on cell cytotoxicity

Groups[a]		Cell viability (%)[b] at various concentrations, mM					
		0.5	1	2	4	6	8
III	Simvastatin	79.03	66.74	55.71	46.15	31.68	28.07
IV	Piperine	89.02	79.47	67.78	56.26	48.68	41.92
V	N-leucinyl-piperamide	92.53	83.61	72.64	63.44	54.21	49.43

[a] HepG2 cells were divided into 3 groups and were subject to treatment with compounds as described in the Experimental section.
[b] Values are the average of at least three tests.

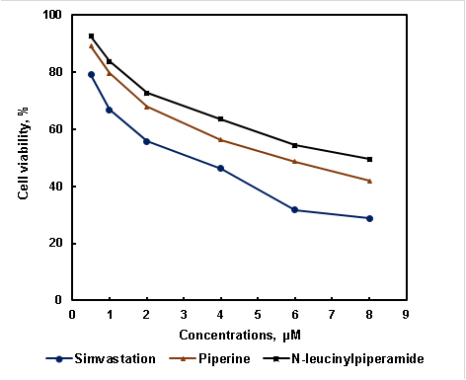

Fig. 2. The IC_{50} values of antihyperlipidemic compounds with various concentrations on the cell viability

simvastatin and piperin inhibited the cell growth by 71.93% and 62.9%, respectively, at the same dose. In particular, N-leucinylpiperamide showed twice-lower cytotoxicity to the cell line than the simvastatin at the 8 μM concentration.

Piperine, as an alkaloid with poor solubility, requires modifications for its increased pharmaceutical activities [10, 11]. The structure of piperine is suitable for chemical modifications; therefore, it can present an advantage in developing novel derivatives with various therapeutic activities [26]. Accordingly, several research studies were conducted to alter piperine to be effective to deliver in a targeted manner, such as fabricated nanoliposomal complex of piperine and an anti-CD133 monoclonal antibody [27], piperine-loaded mono-disperse chitosan nanoparticles [28] as well as a self-emulsifying drug delivery system of piperine to enhance its solubility and bioavailability [11].

While, introduction of amino acids to parent drugs is the one way to overcome the limited therapeutic applications of non-soluble drug compounds [29, 30]. Accordingly, formulating piperine with L-leucine methyl ester improved the solubility, bioavailability, and thereby its therapeutic activity of piperine.

CONCLUSIONS

In this work, piperine-amino acid derivative, N-leucinyl-piperamide, was synthesized using L-leucine methyl ester, and results indicate that N-leucinylpiperamide possesses a higher lipid-lowering activity over its parent compound piperine and simvastatin in high-fat diet model animals. Such behaviour is attributed to the formation with L-leucine methyl ester since the application of amino acids in the structural modification of natural products can improve many properties, including solubility and bioavailability of therapeutic drugs. Therefore, this finding suggests the application of amino acid ester could be a potential system to oral delivery of piperine and newly synthesized N-leucinylpiperamide could be a valuable promising anti-hyperlipidemic candidate.

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