Institute of Macromolecular Chemistry and Mongolian Medicine[1], Inner Mongolia University, Department of Pharmacy[2], Hospital of Inner Mongolia Medical College, Huhhot City, Inner Mongolia, China

Hypolipidemic effects of piperlonguminine in HepG2 cells and in Wistar rats

L. Bao[1,2], G. Borjihan[1]

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Prof. Geriletu Borjihan, Institute of Macromolecular Chemistry and Mongolian Medicine, Inner Mongolia University, 010021 Huhhot City, China
borijihan@imu.edu.cn

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A novel thermostable form of piperlonguminine (GB-N) was extracted from medicinal plant *Piper longum* in efforts to explore the bioactive components underlying the mechanism of *Piper longum* in reducing plasma lipids. *In vitro*, HepG2 cells were employed to investigate the effects of GB-N on regulating cellular total cholesterol and low-density lipoprotein (LDL) receptor (LDLR) mRNA abundance, while high-fat and high-cholesterol diet-induced hyperlipidemic Wistar rats were used to investigate *in vivo* effects of GB-N. Cellular total cholesterol assay showed that GB-N dose-dependently reduced cellular total cholesterol in HepG2 cells in the presence and absence of elevated plasma cholesterol levels by 25% and 32%, respectively. Reverse transcription PCR assay showed that LDLR mRNA abundance was up-regulated dose-dependently by 142% via GB-N treatment in HepG2 cells. Animal experiment revealed that GB-N dose-dependently reduced serum total cholesterol by 26%, triglyceride by 47%, LDL cholesterol by 30%, while increased serum high-density lipoprotein (HDL) cholesterol by 524% in diet-induced hyperlipidemic Wistar rats. In conclusion, the results suggest the potential therapeutic uses of GB-N in the prevention and treatment of hyperlipidemia and related diseases.

1. Introduction

Mammalian cells maintain their cholesterol content within a narrow range by controlling the rate of intracellular *de novo* cholesterol biosynthesis and the amount of cholesterol obtained from extracellular lipoproteins by receptor mediated endocytosis (Brown and Goldstein 1997). Mammalian cells can obtain cholesterol either from *de novo* synthesis or uptake from circulating lipoproteins. However, cholesterol is toxic when accumulated in cellular membranes and elaborate pathways have evolved to control its uptake, synthesis and storage (Brown and Goldstein 1998). The low-density lipoprotein (LDL) receptor (LDLR) is the primary pathway for removal of cholesterol from circulation (Slater et al. 1984), and its activity is meticulously governed by intracellular cholesterol levels (Brown and Goldstein 2009). Previous studies have demonstrated that more than 70% LDL cholesterol (LDL-c) is removed from plasma by LDLR mediated uptake in the liver (Brown and Goldstein 1986). An excess of intracellular cholesterol must be avoided as it forms toxic crystals whereas excess plasma cholesterol is deposited in arteries, initiating and accelerating atherosclerosis (Small and Shipley 1974).

Recently, our research group has been focusing on developing hypolipidemic medicines using bioactive agents extracted from traditional medicinal plants, especially from *Piper longum*, which is a flowering vine in the family *Piperaceae*. *Piper longum* owns a long history as condiment in many countries worldwide. Furthermore, the medicinal uses of *Piper longum* have been recognized by medical professionals, being frequently prescribed by traditional Chinese-Mongolian physicians due to its properties to warm the spleen, dispel cold, and repress pain, and etc (Flora of China 2004). Via systematic analyses conducted worldwide, piperlonguminine ($C_{17}H_{19}NO_5$) was identified as one of the bioactive components contained in *Piper longum* (Chatterjee and Dutta 1967). The medicinal uses of piperlonguminine have been investigated by researchers worldwide, suggesting that piperlonguminine has effects in treating and managing cancers, infectious diseases and Alzheimer's disease (Min et al. 2004; Silva et al. 2009; Xia et al. 2007).

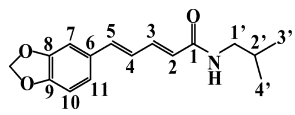

Fig. 1: Chemical structure of GB-N

Inspired by previous studies, we hypothesized that piperlonguminine, as a derivative of piperine, may possess hypolipidemic effects. Our research group has employed modern macromolecular methods to extract a novel thermostable form of piperlonguminine (GB-N) from *Piper longum* fruits. A detailed protocol for the extraction of GB-N is described in our patent application (China patent application number: ZL2004100966117, December 6, 2006), with its chemical structure shown in Fig. 1.

Our research group has developed a three step procedure to search for potential natural hypolipidemic compounds by a modification of screening method proposed previously (Kong et al. 2004). First, the medicinal plant has major applications as traditional medicinal plant in China or in other countries. Second,

Table 1: Effects of GB-N on cellular TC content in HepG2 cells[a]

Treatments	Cellular TC content (nM/mg protein)	
	Without sterols	25-HC plus Chol.
Control	82.04 ± 3.39	112.09 ± 3.50
GB-N30	70.99 ± 2.92*	113.90 ± 2.96*
GB-N10	56.02 ± 2.12*	85.50 ± 2.44*
GB-N1	69.13 ± 2.35*	84.03 ± 3.13*

[a] HepG2 cells were grouped and treated as described in the Experimental section and cellular TC content assay was conducted for two set of cells in the presence and presence of sterols, respectively. Values are expressed as means ± SEM for six wells in duplicate experiments.
* $P < 0.05$ versus control

the chemical structure of the underlying bioactive component has been identified. Third, the extracted compound can reduce cellular total cholesterol (TC) in the presence and absence of elevated plasma cholesterol levels, which implies its potential in the treatment and prevention effects on hypercholesterolemia. In this study, we investigated the effects of GB-N both in HepG2 cells and in Wistar rats. *In vitro*, HepG2 cells are suitable models to study cellular cholesterol homeostasis (Bader et al. 1992; Dunn et al. 1989; Wilkening et al. 2003). We employed cholesterol and 25-hydroxycholesterol (25-HC) with a combination of 1 μg/mL 25-HC plus 10 μg/mL cholesterol to establish hypercholesterolemic models of HepG2 cells, according to a previous study conducted by the Goldstein group (Adams et al. 2004). *In vivo*, Wistar rats fed high fat and high cholesterol (HFHC) diet were employed as hyperlidemic models.

2. Investigations and results

2.1. Effects of GB-N on cellular total cholesterol in HepG2 cells

To evaluate the cellular TC-lowering effect of GB-N in HepG2 cells, two paralleled sets of experiments were designed to evaluate the effect of GB-N on cellular TC content in the presence or absence of elevated plasma cholesterol levels, respectively (Table 1).

On the one hand, when HepG2 cells were treated with GB-N supplemented with sterols (1 μg/mL 25-HC and 10 μg/mL cholesterol), the values of the three GB-N treatment groups suggested that GB-N reduces cellular TC content in a dose-dependent manner, as compared to control. Of the three GB-N groups, GB-N1 (1 μm/L) markedly reduced the cellular TC by 25%. On the other hand, when HepG2 cells were incubated in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Lipoprotein deficient serum (LPDS) without sterols, the values of three GB-N groups indicated that GB-N reduced cellular TC content in a dose-dependent manner as well, as compared to control. Of the three GB-N groups, GB-N10 (10 μm/L) markedly reduced cellular TC content by 32%.

In conclusion, GB-N dose-dependently reduced the cellular total cholesterol content in HepG2 cells in the presence and absence of elevated plasma cholesterol levels, respectively.

2.2. GB-N up-regulates LDLR mRNA abundance in HepG2 cells

Reverse transcription PCR assay demonstrated that GB-N markedly up-regulated LDLR mRNA abundance in HepG2 cells which were exposed to sterols (Fig. 2). The value of the model group indicated that the LDLR mRNA abundance was down-regulated by 48% after cells were treated with sterols

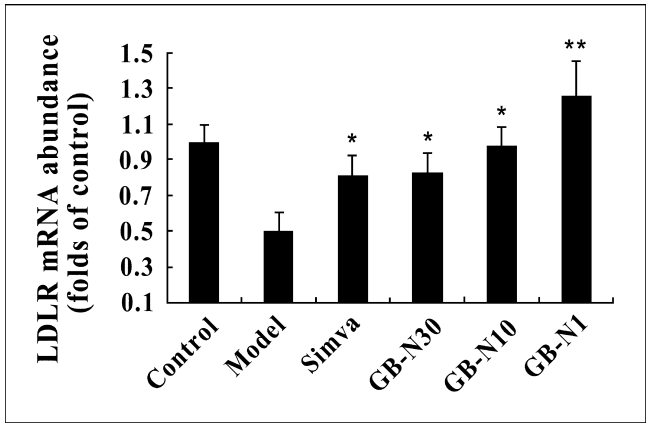

Fig. 2: GB-N up-regulates LDLR mRNA abundance in HepG2 cells. Reverse transcription PCR assay was carried out by the method described in the Experimental section. The level of LDLR mRNA abundance in control group is normalized to 1. The amounts of LDLR mRNA abundance from GB-N-treated HepG2 cells are plotted relative to control. The levels of LDLR mRNA abundance are corrected by measuring GAPDH mRNA levels. The results represent 6 wells in duplicate experiments. Data are expressed as means ± SEM, $^{*}P < 0.05$; $^{**}P < 0.01$

for 24 h. Considering various factors affecting the results, the down-regulation of 48% was comparable to a previous report (Liu et al. 2003). By contrast, the treatment with GB-N dose-dependently reversed the down-regulation of LDLR mRNA abundance caused by sterols. Of the three GB-N groups, GB-N1 (1 μg/mL) up-regulated the LDLR mRNA abundance most significantly, representing an increase of 142% of the model group.

2.3. Effects of GB-N on serum lipid content in Wistar rats

The statistical analyses indicated that the treatment of GB-N dose-dependently reduced serum TC, TG, and LDL-c by 26%, 47%, and 30% respectively, while GB-N remarkably increased serum HDL-c by 524%, in a dose-dependent manner (Table 2). In the first step, serum TC, TG and LDL-c of the model group increased as compared to those of control, indicating that the hyperlipidemic models had been established, thus validating conclusions can be drawn from this experiment. In the second step, GB-N was both effective in reducing serum TC, TG, LDL-c and in increasing HDL-c as well, surpassing the efficacy of simvastatin.

3. Discussion

In recent years, berberine, extracts from medicinal plants *Ginkgo biloba* and *fenugreek* seeds have been reported to possess cholesterol lowering effects (Kong et al. 2004; Xie et al. 2009). Thus, our research group has been dedicating to developing novel drugs in managing hyperlipidemia for years and focusing on investigating extracts from natural medicinal plants.

We also proposed a new screening method for screening cholesterol lowering compounds. Apart from safe records and known chemical structure, we recommend using the cellular TC content in hepatocytes as the indicator of potential efficacy. It is also noteworthy that we test the efficacy GB-N in reducing hepatic TC content with and without sterol influence. In the following step, experiments were conducted to reveal possible mechanisms underlying the cholesterol-lowering effect of the selected compound.

Next, we conducted reverse transcription PCR assay to investigate whether the LDLR activity was up-regulated or not. The expression level of hepatic LDLR directly influences plasma

Table 2: Effects of GB-N on serum lipid content in Wistar rats[a]

	Control	Model	Simvastatin	GB-N2.5	GB-N5	GB-N10
			(mM/L)			
TC	1.86 ± 0.05	17.69 ± 2.17	$13.19 \pm 1.30^{*}$	$13.69 \pm 2.01^{*}$	$14.05 \pm 0.35^{*}$	$13.07 \pm 3.30^{*}$
TG	1.06 ± 0.17	1.52 ± 0.34	$1.51 \pm 0.29^{**}$	$0.84 \pm 0.15^{**}$	$0.81 \pm 0.12^{**}$	$0.93 \pm 0.19^{**}$
LDL-c	3.46 ± 0.06	9.85 ± 1.74	$7.82 \pm 1.32^{*}$	$8.46 \pm 1.01^{*}$	$6.94 \pm 1.47^{*}$	$7.09 \pm 1.05^{*}$
HDL-c	0.86 ± 0.07	0.37 ± 0.12	$0.33 \pm 0.10^{**}$	$1.21 \pm 0.20^{**}$	$1.68 \pm 0.11^{**}$	$2.31 \pm 0.17^{**}$

[a] Sixty adult Wistar rats were divided into six groups and were subject to serum lipid content measurement after respective treatment as described in the Experimental section. Values are expressed as means $\pm$ SEM, $n = 10$

$^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control

LDL-c. LDLR expression is predominantly regulated at the transcriptional level through a negative-feedback mechanism by the intracellular cholesterol pool. Looking ahead, we will investigate the SREBP pathway using transcriptomic profiling method to explore the regulation networks modulated by GB-N.

The third experiment was an animal experiment using Wistar rats. In the fist place, the effect of GB-N on modulating cholesterol homeostasis needed to be further confirmed *in vivo*, and it did. In the second place, the results of animal experiment broadened our knowledge on GB-N *in vivo*. The animal experiment clearly indicated that HDL metabolism played a significant role in the effects of GB-N in modulating serum lipids in Wistar rats. Previous studies have revealed that HDL is taken up in the liver by the LDLR and by scavenger receptor B1 (SRB1), respectively (Krieger 1999). It is also noteworthy that there are significant differences, both quantitatively and qualitatively, in cholesterol turnover between mice and humans (Veniant et al. 2001). Therefore, gene knockout techniques will be employed in further *in vivo* studies. Moreover, it has been reported that phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) are key factors in HDL metabolism and reverse cholesterol transport (Tall 1995).

In summary, pathways regulating the metabolisms of LDL and HDL will be investigated to elucidate the mechanisms underlying the hypolipidemic effects of GB-N.

4. Experimental

4.1. Materials

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Trypsin was purchased from Dingguo Biotech (Beijing, China). Cholesterol and 25-HC were from Sigma (MO, USA). Trizol reagent was from Tiangen Biotech (Beijing, China). Bovine plasma albumin (BSA) and Taq DNA polymerase were from Sangon Biological Engineering Technology and Services (Shanghai, China). M-MLV reverse transcriptase was from Promega (Madison, WI, USA). Oligonucleotides were synthesized by Sangon (Shanghai, China). PCR primers were synthesized by Invitrogen (Invitrogen Inc, Shanghai, China). LPDS was a generous gift from Inner Mongolia University Life Science School (Huhhot, China).

4.2. Cell culture

HepG2 cells were cultured a 75-cm^2 culture flask with high glucose DMEM supplemented with 10% fetal calf 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. The culture media used in this study for cell culture as mentioned above is referred to as FCS-DMEM. All cells were fed fresh 10% FCS-DMEM every 2 d. When reaching 80% confluence, the cells were trypsinized. The suspension was transferred into a tube and the cells were centrifuged at 1000 rpm for 5 min. Supernatant was discarded. The cells were split at 1:3 to 4 every 5–6 d. Cells were then incubated in 10% FCS-DMEM at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air.

4.3. Cellular total cholesterol assay

HepG2 cells were plated in 10% FCS-DMEM in culture dishes (24-well) and incubated for 24 h. Thereafter, all cells were fed 10% LPDS-DMEM for 24 h to up-regulate their LDLR activities. Then all cells were divided into two paralleled experiments with four groups (control group and three GB-N groups) of cells in each set. All cells were re-fed fresh 10% LPDS-DMEM supplemented with GB-N at different concentrations (30, 10, and 1 μm/L) except for the control. After 1 h, sterols (1 μg/mL 25-HC and 10 μg/mL cholesterol) were added to one set of cells to investigate the GB-N effects under influence of sterols, while another set of experiments was to evaluate the GB-N effect without influence of sterols. All cells were incubated under their respective conditions for 24 h at 37 °C, before cellular total cholesterol measurement was conducted by a modification of the method as previously reported (Huang et al. 2006). Briefly, cells were harvested and washed twice with phosphate buffered saline (PBS), followed by resuspension in 1 mL isopropanol and sonication for 20 min at 4 °C. After centrifugation at $9000 \times g$ for 10 min at 4 °C, the supernatant was evaporated under a vacuum and the pellet was resuspended in 20 μL isopropanol for cellular cholesterol measurement. The cholesterol was measured colorimetrically using a Total Cholesterol Detection Kit (Jiemen Biotech, Shanghai, China). Additionally, the sediment fractions were lysed using a Mammalian Cell Extraction Kit (BioVision, CA, USA). Total protein levels were quantified by the Bradford method.

4.4. Reverse-transcription PCR assay

HepG2 cells were trypsinized after reaching 80% confluence. The cell density was adjusted to 1×10^6 cells/mL before they were plated in 60-mm culture dishes (24-well). One mL of cell suspension and 3 mL of 10% FCS-DMEM were added to each well. The cells were separated into 6 groups (control, model, positive control, and 3 treatment groups) and incubated for 24 h. Thereafter, the media were replaced with DMEM supplemented with 10% lipoprotein deficient serum (LPDS-DMEM) in the presence or absence of sterols (1 μg/mL of 25-HC and 10 μg/mL of cholesterol). Then the cells were incubated for an additional 24 h under their respective conditions. Thereafter, three treatment groups exposed to sterols were treated with GB-N at different concentrations (30, 10, and 1 μm/L), respectively. In parallel, the control was treated with vehicle and the positive control was treated with 1% simvastatin which was dissolved in dimethyl sulfoxide (DMSO). After 24 h, the media were removed and the total RNAs were extracted using Trizol reagent (Tiangen Biotech, Beijing, China) according to the manufacturer's protocols. The total RNAs were stored at −70 °C. The total RNA concentration and purity were determined by UV spectrophotometry (260 and 280 nm).

For each reverse transcription, 2 μg of total RNA was used to perform using a GeneAmp RNA PCR Kit (Perkin Elmer, Singapore) according to manufacturer's protocols.

PCR amplification was performed in a final volume of 20 uL using a GeneAmp RNA PCR Kit (Perkin Elmer, Singapore) according to manufacturer's protocols. The housekeeping gene GAPDH served as an internal control. Reverse transcription-PCR products were separated by 1% agarose gel electrophoresis and visualized by UVP BioImaging System (UVP, CA, USA). The primers used in this experiment were as follows: LDLR, forward 5'-TGTCAACCGCTACATTCCT-3' and reverse 5'-GGCACTCGTAGCCGATCTTA-3'; GAPDH, forward 5'-TGCACCACCAACTGCTTAG-3' and reverse 5'-AGTAGATGCAGGGATGATGT-3'.

4.5. Animal experiments

Experimental animals were acquired from Inner Mongolia University Animal Test Center. The animal experiment was approved by the center and was

carried out in accordance with EU Directive 86/609/EEC. On day 0, sixty adult Wistar rats, which were acquired from University of Inner Mongolia Animal Test Center, weighing 160–180 g each, were kept at room temperature (18–22 °C) in a room that was lit from 6:00 AM to 6:00 PM. And they were fed normal diet for 7 d. On day 7, they were randomly separated into 6 groups (control, model, positive control, and 3 GB-N groups) with 10 rats in each group. The control group was fed normal diet while the other 5 groups were fed high-fat and high-cholesterol (HFHC) diet (cholesterol 3%, lard 10%, sodium cholate 0.5%, and normal diet 86.5%). According to our previous experiences, all groups except for the control group were fed 200 g/d of HFHC-diet to achieve desirable effects, and continued throughout the experiment. From day 7 on, three treatment groups were administered GB-N at different concentrations (2.5 mg/kg/d, 5 mg/kg/d, and 10 mg/kg/d) respectively, while the control group and the positive control group was administered vehicle (0.5% CMC-Na) or simvastatin (10 mg/kg/d), respectively. The compound treatment was conducted using oral gavage and lasted 15 d. All groups were observed closely during experiment, and the rats were weighed every 3 d and at the end of the experiment. On day 22, all six groups were fasted for 12 h without limit to drinking before they were decapitated and the blood was taken out. The levels of serum TC, TG, LDL-c and HDL-c were measured at the end of the experiment.

4.6. Statistical analyses

Results are expressed as means ± SEM. All statistical analyses are conducted using one-way ANOVA and Student's t-test. A difference between mean values is considered significant if the p value obtained is less than 0.05.

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