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Design, synthesis and biological evaluation of a hybrid compound berberine and magnolol for improvement of glucose and lipid metabolism

Yan Li,[a] Xiao Yuan,[b] Xianglu Rong,[c] Ying Gao,[a] Zhibin Qiu,[a] Zhipeng Zhang,[c] Dongbin Zhou,[b] and Weimin Li[a*]

Abstract

The discovery and structure optimization of lead compounds is the main task in new drug research and development. In order to search new compounds with greater activities and lower toxicity, the synthesis of two or more drug basic structure stitched together were designed on the basis of combination principles. Here, a hybrid compound consisting of berberine (Ber) and magnolol (Mag) was synthesized, and its biological activities were evaluated. We name the hybrid compound Huanghousu (HHS), and its size and structure were confirmed by spectroscopic (^{1}H NMR, ^{13}C NMR and HRMS spectra). The LD_{50} of HHS was determined in NIH mice by intraperitoneal injection according to Modified karber's method. Treatment of transgenic aP2-SREBP-1c mice with HHS markedly reduced blood triglycerides (TG) and improved the sugar tolerance. Additionally, we also evaluated the effects of berberine, magnolol and HHS on the proliferation and differentiation of 3T3-L1 preadipocytes and to explore the underlying the mechanism. The adipocyte differentiation-related gene, PPARγ and C/EBPα, were evaluated using Real-time Quantitative polymerase chain reaction (Real-time PCR). Additionally, FAS, UCP2 and adiponectin mRNA, which were related to adipocyte adipogenesis, were also measured in mature 3T3-L1 adipocytes induced by differentiation medium. The efficacy of HHS in preventing obesity was somewhat more effective than magnolol or berberine. Taken together, these results indicated that HHS was effectively improving disorders of glucose and lipid metabolism in vivo and regulating lipid metabolism-related gene expression in vitro.

1. Introduction

Diabetes mellitus (DM) is a complex endocrine and metabolic disorder characterized by high levels of glucose and lipid in the blood.[1,2] Recent study finds that the estimated prevalence of diabetes in Chinese adults is 11.6% and the prevalence of prediabetes is 50.1%.[3] It is predicted that approximated 4% of the population worldwide suffer from diabetes and there is expected to increase by 5.4% in 2025.[4] Obesity is a key risk factor for type 2 diabetes as it desensitizes glucose recipient organs to the action of insulin.[2] Growing evidence shows that obesity is characterized by intraabdominal visceral fat accumulation, which depends on adipocyte proliferation and differentiation of preadipocytes.[5-7] Lipogenic gene expression in adipocytes is known to be mainly regulated by CCAAT-enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor-γ (PPAR-γ).[8-10] It has long been known that transient expression of C/EBPβ and C/EBPδ gene are manifested in the early stage of

[a]*School of Chinese Material Medical, Guangzhou University of Chinese Medicine, Guangzhou 510006, People's Republic of China. E-mail addresses: 13925023915@139.com; Fax: +86 20 39358290.*

[b]*Guangzhou Pi & Pi Technology Inc, Guangzhou 510006, People's Republic of China*

[c]*Center Laboratory, Guangdong Pharmaceutical University, Guangzhou 510006, People's Republic of China*

† Electronic supplementary information (ESI) available: The ^{1}H NMR, ^{13}C NMR and HRMS spectra for synthesized compounds; HPLC profile for HHS; The oligonucleotide primer sequence used for real-time PCR. See DOI: 10.1039/x0xx00000x

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cell differentiation, and the C/EBPα is expressed in the late stage. Once C/EBPα is activated, it can trigger the expression of several genes that influence intracellular lipid droplets deposition, insulin resistance, metabolism of adipose and balance of energy. It has been reported that activation of PPAR-γ up-regulates FAS and UCP2 expression, resulting in augmentation of glucose uptake and lipogenesis.

Traditional Chinese medicine (TCM) is based on herbal extracts containing natural active components. In TCM, the herb couples, as the basic composition units of Chinese herbal formulae, have specific clinical significance.[11,12] Coptis Magnoliae Decoction is a representative formula composed of *Rhizoma coptidis* and *Magnolia officinalis* in mass prescriptions, which is obtained from The Complete Record of Holy Benevolence, a traditional Chinese medical classic compiled in the Song dynasty. Berberine and magnolol are as the major active components, which can be used as lead compounds of chemical drug for the corresponding structure modification. Berberine is an isoquinoline alkaloid isolated from Chinese herb Rhizoma coptidis, which mainly exists in ranunculaceae, rutaceae and berberidaceae. The high medicinal value of berberine and the physiological activeties of berberine derivatives have recently attracted remarkable attention for its extensive pharmacological effects and usefulness in biomedical applications.[13,14] Studies have shown that berberine possesses the anti-inflammatory,[15] hypotensive, anti-platelet aggregative,[16-18] hypoglycemic[19,20] and hypolipidemic[21,22] activities. Also, derivatization of berberine core structure has resulted in the development of molecules with positively enhanced biological effects.[23,24] Magnolol is a polyphenolic binaphthalne compound from Chinese medicinal herb *Magnolia officinalis*, which has been reported to have multiple biological effects, including anti-inflammatory,[25] anti-oxidant, [26,27] anti-angiogenic[28] and antidiabetic[29] activities. Moreover, *Rhizoma coptidis* and *Magnolia officinalis* are widely used for the treatment of diabetes and hyperlipidemia in Chinese traditional herbal medicines and prescriptions.

Although the researches on the synthesis and structure modification of berberine and magnolol have been well done recently, little information has been available on the synthesis of a hybrid compound consisting of berberine and magnolol. Therefore, this study aims to investigate the structure combination process for berberine and magnolol and to synthesize a new compound with the pharmacodynamic activity. In this study, the structure of the new compound was confirmed by spectroscopic (^{1}H NMR, ^{13}C NMR and HRMS spectra). We investigated hypoglycemic and hypolipidemic activities of the new compound using transgenic aP2-SREBP-1c mice in vivo. Furthermore, we determined the anti-obesity effects using 3T3-L1 in vitro, which measured mRNA expression of adipogenic transcription factors using Real-time PCR.

2. Rusults and discussion

2.1 Design of berberine and magnolol hybrid compound (HHS)

The synthesis route of HHS was conveniently undertaken as outlined in Scheme 1. With berberine hydrochloride as a starting material, berberrubine **1** was synthesized by 9-position demethylation reaction under microwave irradiation in excellent yields (92%). Afterwards, berberrubine **1** on reaction with 1, 2-dibromoethane in acetonitrile as the solvent, followed by the heating reflux reaction gives 9-(2-ethyl bromide) berberine hydrochloride 2 in good yields (56%). Finally, the hybrid compound 3 (HHS) could be obtained by the reaction of 9-(2-ethyl bromide) berberine hydrochloride **2** with magnolol and Na_2CO_3 (1:1.5:6) in acetonitrile under reflux in moderate yields (24%). After crystallization and purification, the structure characterizations of those products were analyzed by ^{1}H NMR, ^{13}C NMR and HRMS spectra, and the profile for HHS was established by HPLC (ESI†).

Previous studies have suggested that berberrubine was usually synthesized by vacuum pyrolysis, with low yield and purity due to uneven heating.[30] In the preliminary experiment, we adopted microwave synthesis method as described by references,[31,32] but no chemical transformation occurred. After that, we used DMF with a

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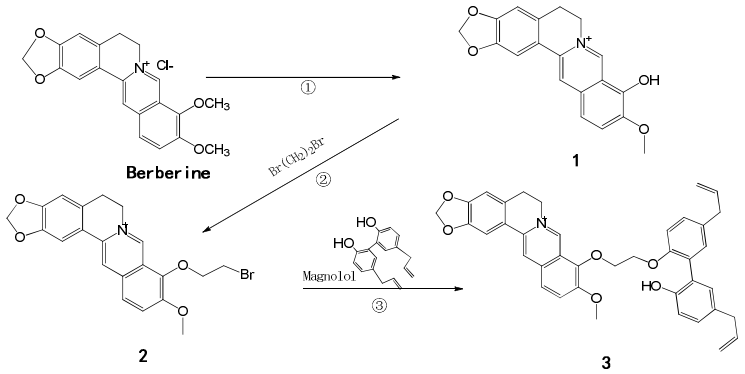
high boiling point and good stability as reaction medium under microwave irradiation to afford berberrubine in 92% yield. 9-(2-ethyl bromide) berberine hydrochloride was synthesized by a solvent acetonitrile. There have been few studies on using two natural active molecules stitched together through chemical bond to synthesize a hybrid compound.



Scheme 1 Synthesis of HHS. Reagents and conditions: ① DMF, microwave power (400 W), reflux, 15 min, 92%; ② acetonitrile, 1,2-dibromoethane, reflux, 3 h, 56%; ③ acetonitrile, anhydrous sodium carbonate, reflux, 8 h, 24%.

2.2 Acute toxicity study

After intraperitoneal injection of HHS, the various situations of mice were observed strictly in the first 4 h. The mice administered with HHS at a dose of 132.1 mg/kg showed no significant changes in behavior, breathing and postural abnormalities. However, the significant changes occurred in the mice of other groups, including unsteady walking, difficult breathing, and death, even with exsistence of moderate organ damages. Moreover, the mortality of treated mice at dose of 188.7 and 385.0 mg/kg was 0.2 and 0.8 in a dose-dependent manner. The LD_{50} value (lethal dose 50) was 279 mg/kg, and 95% confidence limit was 234.6~332.5 mg/kg (Table 1). The results obtained from this study demonstrate that, after single intraperitoneal injection, the Acute toxicity of HHS was lower than the berberine, while the toxicity of berberine in the previous reports was of the LD_{50s} ranging between 38.8 and 85.5 mg/kg[33] and LD_{50} being 50 mg/kg.[34]

Table 1 The results of acute toxicity testing of HHS in NIH mice administered by intraperitoneal injection within 14 d.

Group	Dose (mg/kg)	Mortality	LD_{50} (mg/kg)	95% confidence limit (mg/kg)
1	550.0	10/10	279.3	234.6～332.5
2	385.0	8/10		
3	269.5	4/10		
4	188.7	2/10		
5	132.1	0/10		
control	0	0		
		$\sum p=2.4$		

These data were calculated according to Modified karber's method.

2.3 Effect of HHS on glucose and lipid metabolism in aP2-SREBP-1c mice

It has been known for excessive amounts of white tissue (obesity) are the crucial factors in insulin resistance.[35-37] In contrast to all other mouse models of insulin resistance,[38,39] aP2-SREBP-1c mice were not obese, which exhibit a distinct syndrome that includes lipodystrophy, insulin resistance, diabetes mellitus and fatty liver.[40,41] In the

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present study, we examined the effect of HHS on glucose and lipid metabolism disorder. OGTT was performed at end of 9 weeks. As shown in Fig. 1A, the blood glucose level in model group was much higher than that in control group. However, the mice treated with HHS showed a significant decrease in blood glucose level compared with model group at 20 and 60 min. Moreover, the AUC was significantly lower in the HHS group than that in the model group (Fig. 1B). As shown in Fig. 1C, at the end of 8 and 13 week, the plasma TG level in model group was significantly higher than that in control group, while HHS group showed significantly decreased TG level compared to model group. According analysis of TG level, compared with 0 week (0.85±0.43), the TG level at 8 and 13 weeks in control group was no significant change, and the level in model group was increased significantly. Conversely, after HHS treatment for 8 and 13 weeks, plasma TG level significantly decreased compared with 0 week. These data indicated that HHS improved glucose and lipid metabolism in aP2-SREBP-1c mice.

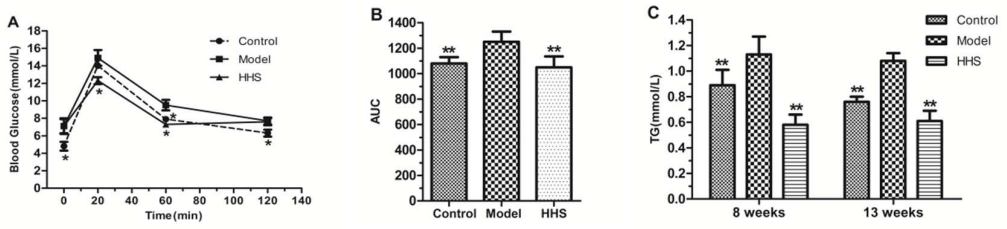
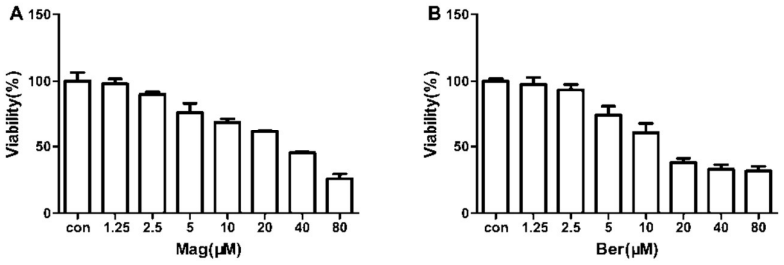


Fig. 1 In vivo effects of HHS on the glucose and lipid metabolism in aP2-SREBP-1c mice. Mice at 12 weeks of age were divided into 3 groups. HHS was suspended in 5% CMC-Na aqueous solution and administered orally for 13 weeks. (A) After 9 weeks after final dosing, following an overnight fast, an oral gavage of 50% glucose (2 g/kg body weight) was performed on each animal. Blood glucose levels were estimated from the blood samples just prior to glucose administration (0 min) and 20, 60 and 120 min post-glucose administration. (B) By oral glucose tolerance test (OGTT), area under blood glucose concentration curve (AUC) was calculated from the formula: AUC=[1/4 × 0 min (mmol/L) + 1/2 × 20 min (mmol/L) + 3/4 × 60 min (mmol/L) + 120 min (mmol/L)]. (C) At the end of 8 and 13 weeks, mice were anesthetized with diethyl ether after fasting for 12 h, plasma triglyceride levels were measured. Each group was composed of 8 mice. Data are presented as means ± SD. $^{*}P<0.05$, $^{**}P<0.01$ (one-way ANOVA) compared with model group.

2.4 Cytotoxicity of HHS in 3T3-L1 preadipocyte

In the present study, the cytotoxicity of berberine, magnolol and HHS towards 3T3-L1 preadipocyte was investigated using CCK-8. The results are shown in Fig. 2. Within the range of 0-80 μM, the cell viability of Mag group decreased with increasing concentration, which had a dose-dependent manner (Fig. 2A). When the concentration is more than 20 μM, Berberine had an obvious inhibitory effect toward 3T3-L1 preadipocyte (Fig. 2B). However, the result showed that HHS could significantly reduced cell viability when the concentration reached 10 μM (Fig. 2C). Meanwhile, IC_{50} values of berberine, magnolol and HHS were 19.43±0.56, 27.93±0.63 and 11.54±0.52 μM. These observations suggested that HHS exerted stronger effect than berberine, magnolol on the viability of 3T3-L1 preadipocyte.



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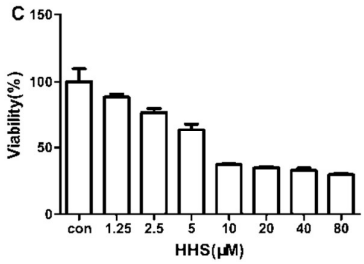

Fig. 2 The cytotoxic activity of Mag (A), Ber (B) and HHS (C). Cells were seeded in 96-well-plate at the density of 5000-8000 cells per well and incubated for 24h. Then various concentrations of berberine, magnolol and HHS (1.25, 2.5, 5, 10, 20, 40 and 80 μM) were added to the medium and incubated for 48 h. The cells treated with 0.1% DMSO served as control (con). Values are means ± SD (n=3 in each group).

2.5 Differentiation of 3T3-L1 preadipocytes and Oil Red O staining

The cultured 3T3-L1 preadipocytes were in a fibroblast-like from with long fusiform or polygon shape (Fig. 3A). And the cells could successfully be trans-differentiated into adipocytes with an appreciable amount of accumulated lipid droplets after 10 days of induction, which were confirmed by using Oil Red-Ostaining (Fig. 3B and 3C).[42] Lipid and Oil Red O were extracted using isopropanol, and absorbance was measured at 520nm. the result of oil red O staining shown in Fig. 3D. The OD_{520} value was significantly increased in model group compared with the control group.

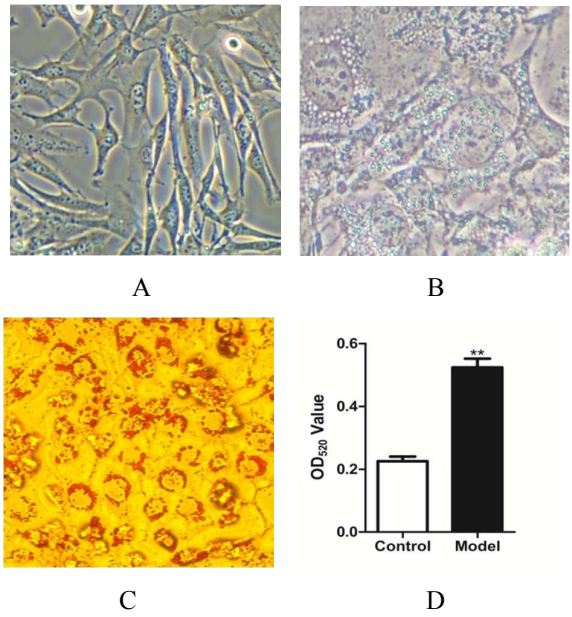

Fig. 3 3T3-L1 preadipocytes were successfully differentiated into adipocytes. Differentiated 3T3-L1 cells were identified using Oil Red O staining. (A) Control (preadipocytes), (B) differentiated adipocytes, (C) Model (differentiated adipocytes, using Oil Red O staining), (D) Oil Red O was extracted from the cells using isopropyl alcohol, and the absorbance was measured at 520 nm. Three independent experiments were performed and data are shown as mean ± SD. $^{**}P<0.01$ compared with Model group.

2.6 Effects of HHS on expression of differentiation-related gene in 3T3-L1 adipocytes

Research shows that PPARγ and C/EBPα play major roles in the process of adipocyte differentiation, which are also critical in the regulation of lipid metabolism and glucose homeostasis.[43-46] To quantitatively describe the effect

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of berberine, magnolol and HHS at different concentrations on adipocyte differentiation, we examined the expression of PPARγ2 and C/EBPα using Real time PCR. As shown in Fig. 4A and 4B, the PPARγ2 and C/EBPα mRNA levels were significantly increased in MD group compared to the NC group, and HHS was effective in reducing their levels. Ten days after initiating treatment, the Mag and Ber treated group at 5 μM had a significant effect on PPARγ2 and C/EBPα mRNA levels compared with MD group, which were consistent with previous studies.[47-49] And the Mag treated group at 2.5 μM did not induce efficient change in PPARγ2 and C/EBPα mRNA levels. HHS treatment significantly decreased PPARγ2 mRNA level in a dose-dependent manner. However, surprisingly, the HHS treated group at 5 μM was less effective in decreasing C/EBPα mRNA level than the Ber group at 5 μM. Although the HHS treated group had a significant decrease in C/EBPα mRNA level, it showed no dose-dependent manner. Through the comparison of the effects of both doses HHS on PPARγ2 and C/EBPα, we found that the HHS at 2.5 μM had a higher reduction in the mRNA expression than berberine or magnolol. Thus, we concluded that HHS to some extent showed stronger inhibitory effect on PPARγ2 and C/EBPα mRNA levels than berberine or magnolol.

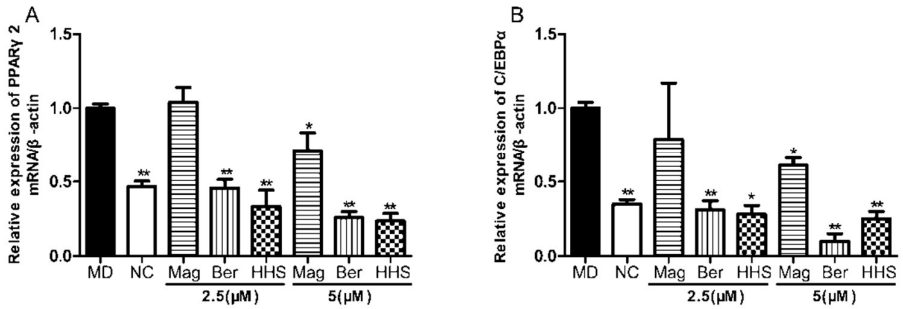

Fig. 4 Effects of HHS on mRNA expression levels of PPARγ2 and C/EBPα in 3T3-L1 cells. Three independent experiments were performed and data are shown as mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ compared with DM group. NC as control group: the cells treated with 0.1% DMSO, MD as model group: 3T3-L1 mature adipocytes.

2.7 Effects of HHS on expression of adipogenesis-related gene in 3T3-L1 adipocytes

Adipocyte differentiation is a complex process that is controlled and regulated by various adipocyte-specific genes, such as FAS, UCP2, adiponectin and others, which participate in creating the adipocyte phenotype.[50] Here we focused more on downstream genes of PPARγ2, C/EBPα and studied how their mRNA expression is influenced by HHS. When 3T3-L1 preadipocyte cells differentiated into adipocytes, we tested mRNA levels of FAS, UCP2 and adiponectin in all groups. And as shown in Fig. 5A, 5B and 5C, a significant increase in the mRNA expression of FAS and UCP2 were observed in the MD group compared to the NC group, which confirmed the presence of lipid accumulation. After treatment with the berberine, magnolol and HHS, it was found that 5 μM HHS-treated cells showed greater reductions in the mRNA expression of FAS and UCP2 than those of berberine, magnolol-treated; while, it increased the mRNA expression of adiponectin more obviously which demonstrated the good anti-obesity effect.[51]

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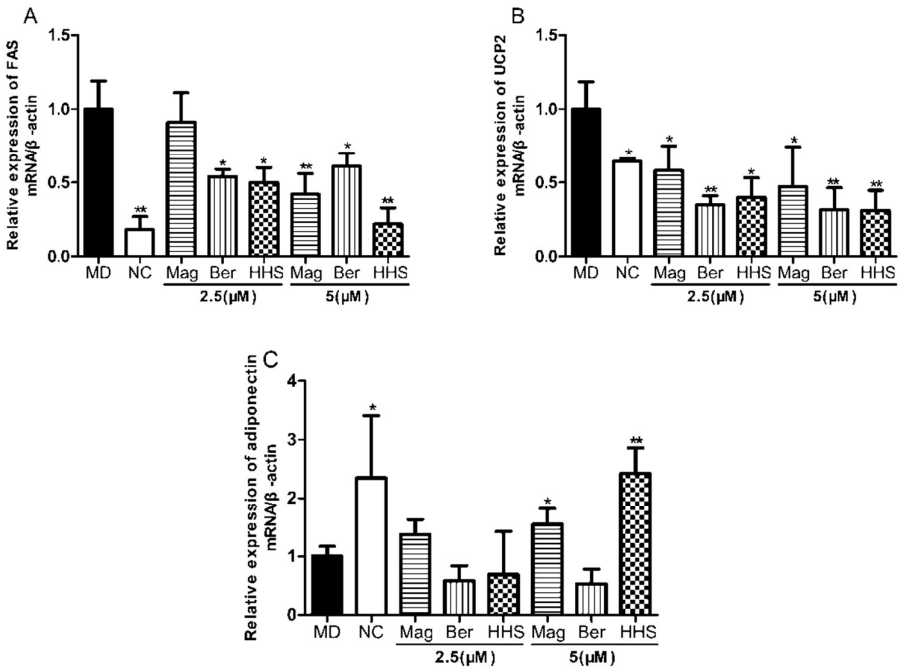

Fig. 5 Effects of HHS on mRNA expression levels of FAS, UCP2 and adiponectin in 3T3-L1 cells. Three independent experiments were performed and data are shown as mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ compared with MD group. NC as control group: the cells treated with 0.1% DMSO, MD as model group: 3T3-L1 mature adipocytes.

3. Experimental

3.1 Reagents and chemicals

Berberine and Magnolol (purity ≥ 98%) were purchased from the China Drugs and Biological Products Inspection Institute (Beijing, China). Dulbecco's Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Insulin (INS), 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX) and Oil Red O were purchased from Sigma Aldrich (St Louis, MO). RNAiso Plus, PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), SYBR® *Premix Ex* Taq™ II (Tli RNaseH Plus) were purchased from Takara (Tokyo, Japan). All other chemicals and reagents were of analytical grade and purchased from Aldrich Chemical Co. (Beijing, China). ^{1}H- and^{13}C-NMR spectra were recorded on Bruker AVANCE 400 at room temperature with tetramethylsilane (TMS) as an internal standard and chloroform-d_3 ($CDCl_3$) as solvents. Mass spectra were recorded with a DSQ mass spectrometer (Thermo, USA).

3.2 Synthesis of HHS

3.2.1 General procedure for the synthesis of berberrubine **(1)**

Berberine (1g) was weighted and dissolved in DMF (25ml), and then added several zeolites. The reaction mixture was refluxed for 15 min at the 400 W microwave power. After completion, the reaction mixture was diluted with 40 ml of water and refrigerated until well crystallization. The combined extracts were washed with petroleum ether and evaporated in vacuum. The crude product obtained was purified by PiPo-02 macroporousresin resin to get pure compound **1** (**berberrubine**) in yields (92%).

Berberrubine 1

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Red needle crystal; Mp 281.6～282.4 ℃. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.35 (1H, s, H-1), 6.76 (1H, s, H-4), 3.04 (1H, t, J = 5.8, H-5), 4.53 (1H, t, J = 5.8, H-6), 9.20 (1H, s, H-8), 6.83 (1H, d, J = 9.1, H-11), 7.46 (1H, d, J = 9.1, H-12), 7.97 (1H, s, H-13), 5.95 (1H, s, H-15), 3.81 (1H, s, H-16); ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 106.27 (C-1), 121.81 (C-1a), 151.42 (C-2), 149.97 (C-3), 109.14 (C-4), 131.16 (C-4a), 57.04 (C-6), 143.72 (C-8), 134.21 (C-8a), 147.84 (C-9), 147.84 (C-10), 120.21 (C-11), 123.21 (C-12), 131.16 (C-12a), 124.22 (C-13), 136.23 (C-14), 103.68 (C-15), 55.94 (C-16); LC-MS (ESI^+, m/z) $[C_{19}H_{16}NO_4]^+$: 322. 2 $[M\text{-}Cl]^+$.

3.2.2 General procedure for the synthesis of 9-(2-ethyl bromide) berberine hydrochloride **(2)**

Compound **1** (3.23 mmol) was refluxed in acetonitrile (200 ml), with stirring and heating to 85 ℃. Then, 1, 2-Dibromoethane (20 ml) was added and the reaction mixture was refluxed for 3 h. The combined extract continued to refrigerate until well crystallization. After completion, the crude product obtained was diluted with acetonitrile and petroleum ether and evaporated in vacuum to get pure compound **2** [**9-(2-ethyl bromide) berberine hydrochloride**] in yields (56%).

9-(2-ethyl bromide) berberine hydrochloride 2

Yellow crystal; Mp 273.6～274.4 ℃. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.68 (1H, s, H-1), 6.97 (1H, s, H-4), 3.87 (2H, t, J = 5.8, H-5), 4.74 (2H, t, J = 5.8, H-6), 9.87 (1H, s, H-8), 8.04 (1H, d, J = 9.1, H-11), 8.15(1H, d, J = 9.1, H-12), 8.74 (1H, s, H-13), 6.11 (2H, s, H-15), 3.30 (3H, s, H-16), 4.65 (2H, t, J = 5.6, H-17), 4.12 (2H, t, J = 5.6, H-18); ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 106.43 (C-1), 151.64 (C-2), 149.39 (C-3), 109.29 (C-4), 131.77 (C-4a), 27.59 (C-5), 57.53 (C-6), 143.27 (C-8), 134.68 (C-8a), 146.24 (C-9), 146.24 (C-10), 121.57 (C-11), 124.94 (C-12), 131.77 (C-12a), 127.53 (C-13), 139.26 (C-14), 121.34 (C-14a), 103.31 (C-15), 56.88 (C-16), 57.53 (C-17), 74.49 (C-18); LC-MS (ESI^+, m/z) $[C_{21}H_{19}BrNO_4]^+$: 429.1 $[M\text{- }Cl]^+$.

3.2.3 General procedure for the synthesis of HHS **(3)**

To a solution of compound **2** (1.21mmol) in acetonitrile (150ml), anhydrous sodium carbonate (10.85mmol) and mgnolol (1.81mmol) were added, stirred and heated to 85℃. The reaction mixture was refluxed for 8 h. The reaction mixture was immediatly filtered and then dissolved in dimethyl sulfoxide (DMSO). The solution mixture obtained was purified by C18 column and washed with 30%, 40%, 50%, 60% methanol. The concentrated solution obtained was purified by a gel silica column and washed with petroleum ethe-ethyl acetate (1:1). The crude product obtained was evaporated in vacuum to get pure compound **3** (**HHS**) in yields (24%).

HHS (3)

{9-(2'-((5',5"-diallyl-2"-hydroxy-[1',1"-biphenyl]-2'-yl)oxy)ethoxy)-10-methoxy-5,6-dihydro-[l,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium}.

Yellow solid; Mp 145.2～146.1 ℃ ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.64 (1H, s, H-1), 6.91 (1H, s, H-4), 3.87 (2H, t, J = 5.8, H-5), 4.74 (2H, t, J = 5.8, H-6), 9.38 (1H, s, H-8), 7.90 (1H, d, J = 9. 1, H-11), 8.04 (1H, d, J = 9.1, H-12), 8.56 (1H, s, H-13), 6.11 (2H, s, H-15), 3.30 (3H, s, H-16), 4.65 (2H, t, J = 5. 6, H-17), 4.12 (2H, t, J = 5.6, H-18), 7.00 (1H, d, H-21), 7.11 (1H, d, J = 8.45, H-23), 6.69 (1H, d, J = 8.45, H-24), 3.00 (1H, d, H-25), 6.39 (1H, d, H-30), 5.99-5.86 (1H, m, J = 9.6, 16.9, H-26), 5.81-5.67 (1H, m, J = 9.6, 16.9, H-27), 7.04 (1H, s); ^{13}C NMR ($CDCl_3$, 300 MHz) δ: 106.81 (C-1), 152.38 (C-2), 150.21 (C-3), 109.65 (C-4), 131.76 (C-4a), 28.39 (C-5), 57.83 (C-6), 143.27 (C-8), 134.13 (C-8a), 146.70 (C-9), 152.06 (C-10), 123.88 (C-12), 145.30 (C-12a), 127.65 (C-13), 121.34 (C-14a , 121.75 (C-11), 103.97 (C-15), 56.88 (C-16), 69.31 (C-17), 75.19 (C-18), 155.69 (C-19), 125.04 (C-20), 132.70 (C-21), 129.66 (C-22), 129.25 (C-23), 113.69 (C-24), 40.49 (C-25), 131.76 (C-26), 116.09 (C-27), 153.83 (C-28), 126.51 (C-29), 129.92 (C-30), 133.57 (C-31), 129.25 (C-32), 115.86 (C-33), 40.65 (C-34), 131.93 (C-35), 116.59 (C-36); LC-MS (ESI^+, m/z) $[C_{39}H_{36}NO_6]^+$: 615.3 $[M\text{- }Cl]^+$.

3.3 Acute toxicity study

The mice strain used in this study was NIH, which was bred originally by the National Institutes of Health

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(NIH). Two sexes of SPF (Specific Pathogen Free) NIH mice were investigated by regular methods for toxicological evaluation. NIH mice weighing 18-22g [No. SCXK (Yue) 2008-0002], with half male and female, were purchased from The Medical Laboratory Animal Center of Guangdong (Guangdong, China). Mice were housed in temperature-controlled animal facility with 12-hour light and dark cycle and free to access water and food. Procedures for animal care and handling were in strict accordance with the Chinese Legislation on the Care and Use of Laboratory Animals. The Animal Experiment Center of Guangdong Pharmaceutical University approved the experimental procedures and protocols conducted during this study. After acclimating for 1 week, ten mice were treated with each dose level of HHS (132.1, 188.7, 269.5, 385.0 and 550.0 mg/kg). After intraperitoneal injection of HHS according to Modified karber's method, mice were observed for 14 days, recording signs of toxicity and mortality.

3.4 In vivo pharmacologic test

Transgenic aP2-SREBP-1c mice were introduced from Jackson Lab (USA) at 6 weeks of age, and commissioned Model Animal Research Center of Nanjing University to purification [NO. J003393 SCXK(Su)2010-0001]. Mice were housed in temperature-controlled animal facility with 12-hour light and dark cycle and free to access water and food. Procedures for animal care and handling were in strict accordance with the Chinese Legislation on the Care and Use of Laboratory Animals. The Animal Experiment Center of Guangdong Pharmaceutical University approved the experimental procedures and protocols conducted during this study. After the processes of animal reproduction and genotype identification, transgenic mice at 12 weeks of age were divided into model group and HHS group, and the wild-type littermates as control group (8 mice in each group). Then, animals were treated with 40 mg/kg HHS, or 0.5% CMC-Na aqueous solution orally once daily by gavage for 13 weeks. At the end of 8 and 13 weeks, the animals were anesthetized with diethyl ether after fasting for 12 h, and blood samples were collected by retro-orbital venous plexus puncture. Serum triglyceride levels were determined using enzymatic kits. At the end of 9 weeks, following an overnight fast, an oral gavage of 50% glucose (2 g/kg body weight) was performed on each animal. Blood glucose levels were estimated from the blood samples just prior to glucose administration (0 min) and 20, 60 and 120 min post-glucose administration. By oral glucose tolerance test (OGTT), area under blood glucose concentration curve (AUC) was calculated from the formula: AUC= [1/4 × 0 min (mmol/L) + 1/2 × 20 min (mmol/L) + 3/4 × 60 min (mmol/L) + 120 min (mmol/L)]. All samples were stored in –80°C until further analysis.

3.5 In vitro cell culture and biological studies

3.5.1 Cell culture

3T3-L1 preadipocyte was obtained from the Cell bank of The Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM and supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were cultured at 37 °C and under a humidified atmosphere of 5% CO_2 in a cell culture incubator.

3.5.2 CCK-8 assay

Cell viability was assessed using a Cell Counting kit (CCK-8). Cells were seeded in 96-well-plate at the density of 5000-8000 cells per well and incubated for 24 h. Then various concentrations of berberine, magnolol and HHS were added to the medium and incubated for 48 h. Berberine, magnolol and HHS was respectively dissolved in DMSO with a concentration of 100 mM and stored at -20℃. The final concentrations of berberine, magnolol and HHS were 1.25, 2.5, 5, 10, 20, 40 and 80 μM, respectively. The subsequently diluted solution was prepared in the medium with a final DMSO concentration of 0.1%. The control group always treated with the same amount of DMSO as used in the corresponding experiments. CCK-8 solution was added to each well and the plate was incubated in an incubator for 2-4 h, and then the absorbance was measured at 450 nm with a microplate reader

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(Thermo Fisher, USA). IC_{50} was taken as the concentration that caused 50% inhibition of cell viabilities and calculated by the Logit method.

3.5.3 Differentiation of 3T3-L1 adipocytes

For 3T3-L1 adipocytes differentiation, the cells were grown in 6-well plates to full confluence for two days. Then cells were induced in differentiation DMEM containing 10% FBS, 0.5 mmol/L IBMX, 1.0 μmol/L DEX and 10 μg/ml insulin. After two days of incubation, the cell culture differentiation medium was changed to DMEM containing 10% FBS and 10 μg/ml insulin. After two days, the cell were grown in DMEM containing 10% FBS for an additional ten days. When 80–90% of cells exhibited adipocyte morphology, 3T3-L1 adipocytes were used in experiments. The methods of differentiation of 3T3-L1 adipocytes was performed as previously described.[52]

3.5.4 Oil red O staining and Determination of Lipid Content

The cells were washed twice with phosphate-buffered saline (PBS), and then fixed for 30 min with 10% formaldehyde at room temperature. Then the cells were washed twice with PBS and stained for 1 h in freshly filtered Oil Red O solution (0.5% in 60% isopropanol). After washing three times with distilled water, the cells were imaged using a Microscope. Lipid and Oil Red O were extracted using isopropanol, and absorbance was measured at 520nm using a spectrophotometer.

3.5.5 Quantitative real-time PCR

Total RNA was extracted from 3T3-L1 adipocytes using RNAiso Plus according to the manufacturer's instructions. Then 1ug total RNA from each sample was reverse-transcribed to cDNA according to the protocol of the reverse transcript system. RNA was treated with gDNA Eraser at 42□ for 2 min to remove genomic DNA contamination. Then the cDNA was highly efficient synthesized at 37□ for 15 min. After cDNA synthesis, the gene expression levels were analyzed by Quantitative real-time PCR conducted using the CFX96^{TM} Real-Time PCR Detection system (Bio-Rad). The relative amount of each gene was calculated using the $2^{-\Delta\Delta CT}$. All results were obtained from at least three independent experiments. The mRNA levels of all genes were normalized using β-actin as internal control. The oligonucleotide primers used in the experiments are shown in the ESI.

3.6 Statistical analysis

Data were presented as means ± SD. Statistical analysis was done using SPSS 20.0 statistical software by one-way analysis of variance (ANOVA) followed by Fisher's LSD tests. Graphical representations were performed using GraphPad Prism 5. Differences were considered significant when $P < 0.05$.

4. Conclusion

In the present study, based on association principle, we designed and synthesized a hybrid compound berberine and magnolol, as a mutual drug, possessing potential biological activities as well as safety and low toxicity. The method of piecing the two molecules together is carried out to obtain the target product based on chemical bonding. The results of in vivo study demonstrated that, administration with 40 mg/kg HHS orally to aP2-SREBP-1c mice reduced plasma glucose and lipid levels. Additionally, HHS could obviously down-regulate the expression of PPARγ2, C/EBPα, FAS and UCP2 mRNA and up-regulate the expression of adiponectin mRNA in 3T3-L1 adipocytes. Although the underlying action mechanism for HHS is not currently well understood, the HHS induced reduction of TG in mouse model is remarkable. For further studies, as the 1, 9, 10, 13-position of berberine are used to structural modification, the association of these positions with magnolol can be considered.

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