Discovery of C-9 Modified Berberine Derivatives as Novel Lipid-Lowering Agents

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Introduction

Berberine (BBR), a kind of quaternary ammonium benzylisoquinoline alkaloids with multiple pharmacological activities, has been regarded as a promising lipid-lowering agent in the field of drug repurposing. Particularly, the chemical modification at the C-9 position of BBR can remarkably improve its lipid-lowering efficacy. In this study, thirteen novel BBR derivatives were rationally designed, synthesized, and evaluated by preliminary pharmacological tests. The results showed that most compounds exhibited more potent hypolipidemic activities when compared with BBR and simvastatin. Among these compounds, compound 2h-1 and 2h-2 exhibited better activity profiling in these four tests involving with inhibition of total cholesterol (TCHO), triglyceride (TG), and low-density lipoprotein cholesterol (LDLC) and the increase of high-density lipoprotein cholesterol (HDLC). Correspondingly, the BBR analogs with 9-O-cinnamic moiety probably exhibited potent lipid-lowering activity, and should be exploited as an important versatile template for the development of BBR-like lipid-lowering agents.

Key words berberine; natural product; total cholesterol; triglyceride; low-density lipoprotein cholesterol; high-density lipoprotein cholesterol

Results and Discussion

Chemistry

The synthesis of compounds berberrubine and 2a-2k were prepared in the way according to Charts 1, 2, and 2k. The synthetic pathway of berberrubine was depicted in Chart 1. Synthesis of Berberrubine 2

Chart 1. Synthesis of Berberrubine 2

Reagents and conditions: (i) 30–40 mmHg, 190–210 °C, 30 min.
3. BBR was stirred at 190–210 °C for 15–20 min under the 30–40 mmHg atmospheric pressure to afford berberrubine 2 (Chart 1). Treatment of berberrubine with each corresponding acyl chloride (derived from organic acids) in CH₃CN in the presence of pyridine or K₂CO₃ could produce the BBR derivatives 2b, 2c, 2d, 2f, 2g, and 2h as shown in Chart 2. In addition, berberrubine 2 reacted with bezafibrate under the catalysis of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 4-dimethylaminopyridine (DMAP) to produce 2a. Compound 2e was prepared by linking berberrubine and kaempferol with 1,4-dibromobutane (Chart 2). The chemical structures of all new compounds were characterized by ¹H-NMR spectra and MS. What is more, some compounds as representative products of berberine derivatives were selected to give IR and MS spectra.

**Assay for Cytotoxicity to HepG2 Cells**

The cytotoxic activities of all these compounds were tested by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) method using HepG2 cells and 3T3L cells cultured in a Dulbecco's modified Eagle medium (DMEM) medium. The cells monolayer was incubated with berberine analogs (2a–2g) at the concentration of 12.5 μM for 72 h. As shown in Fig. 1, it is obvious that most of these compounds exhibit very low toxicity in both HepG2 cells and 3T3L cells when compared with the blank control (only cells sap added group) and the two positive molecules (BBR and simvastatin). Nevertheless, in 3T3L cell model, compound 2k displayed slight cytotoxicity, comparable to that of BBR’s. In HepG2 cell model, compound 2g exhibited modest cytotoxicity, which lead to nearly 40% of the loss of cell survival rate. Consequently, considering both two cells models showed good tolerant degree to most compounds at higher drug concentration, the following test on lipid lowering activity would be conducted at the concentration of 10 μM.

**Lipid-Lowering Activity**

3T3L cells and HepG2 cells can be regarded as two kinds of main cells models for the development of lipid lowering agents. In this study, these two types of cells were employed as screening tools to evaluate compounds 2a–2k. The expression level of total cholesterol (TCHO), TG, LDLc, and HDLC in these two kinds of cells was measured by commercial reagent kits according to the standard protocol provided by the manufacturer (see the Biological evaluation section). The drug concentration was cautiously set at 10 μM, whereas the cells viability; the obtained results are presented in Figs. 2 to 5.

With regard to TCHO (Fig. 2), all the compounds have to some extent potency for the reduction of TCHO, no matter whether in 3T3L cell model or in HepG2 cell model. Compared with the parent compound BBR in 3T3L cells, most
compounds except 2j exhibited more potent inhibitory activity of TCHO expression. Three compounds (2h-2, 2k, and 2h-1) in order were in the top three of hypolipidemic activity in 3T3L cells. Interestingly, compound 2d exhibited the most inhibitory activity in HepG2 cells while displayed no activity in 3T3L cells. In comparison with compound 2h-1 or 2h-2, compound 2d has little difference in structure, but has a striking gap for hypolipidemic activity in 3T3L cells. This result still need to be discussed further. By and large, the inhibitory activities of these fifteen compounds (two positives plus thirteen BBR derivatives) showed the same trend in 3T3L cells and HepG2 cells. Compound 2c, 2f, 2h-1, 2h-2, and 2k, all can display hypolipidemic activities both in these two cells. Compound 2c had the moiety of nicotinic acid; compound 2f had the moiety of acrylic acid; compound 2h had the moiety of cinnamic acid; compound 2k was obtained by reduction reaction. It is obviously seen that one common feature in physicochemical property of these compounds is the increase of lipophilicity when compared with BBR.

BBR should be superior to simvastatin in the inhibition of TG, mainly due to the latter belongs to HMG CoA reductase inhibitors related with cholesterol production. Hence, most of berberine derivatives can inhibit the expression of triglyceride in two cell models (Fig. 3). In 3T3L cells, the best two compounds were 2c and 2f, the inhibitory activities of which can both reach 60%. Compound 2c, in particular, not only displayed potent activity in 3T3L cells, but also had higher lipid-lowering activity in HepG2 cells. In HepG2 cells, compounds 2a, 2c, 2e, 2h-1, and 2h-2, all exhibited more potency in reducing triglycerides when compared with other compounds. It was noteworthy that the inhibition rate of compounds 2h-1 and 2h-2 achieved nearly 70%. As well as compound 2c that
contained the moiety of nicotinic acid, compound 2a with the pharmacophore of fibrates displayed significant inhibition against TG, which may be closely related to the action of these fragments (fibrates and nicotinic acid). In general, these compounds performed better in HepG2 cells than in 3T3L cells. It is somewhat arbitrarily speculated that this difference may be related to the anti-tumor mechanism of BBR-like compounds. On the contrary, compound 2k as a prominent candidate in TCHO inhibition experiment performed not well in reducing TG. Accordingly, it can be inferred that the structural reduction of quaternary amine in BBR may not significantly reduce triglyceride in cells, and that can also be observed in compounds 2i and 2j.

In the ordinary way the decrease of LDLC should be consistent with the increase of HDLC. Therefore, an assumption can be given that the decrease of LDLC caused by compounds may increase the expression of HDLC in cells. Comparing Fig. 4 with Fig. 5, except for compounds 2c and 2i, the other compounds showed the same trend between LDLC and HDLC. For instance, compounds 2d, 2f, 2g, and 2j displayed very low LDLC inhibitory activity; so correspondingly the increase of HDLC was almost zero. In addition, it was strange that compound 2b-2 showed almost no response in these two activity tests while compound 2b-1 with the same skeleton of salicylic acid exhibited obvious activity, implying that different substituents on the salicylic acid probably had a significant impact on the lipid-lowering activity. Moreover, it was worth noting that the aforementioned four compounds 2a, 2b-1, 2h-2, and 2k still performed well in LDLC and HDLC activity tests, comparable to the parent compound BBR and superior to simvastatin. Compound 2a contained the fragment of bezafibrate; compounds 2h-1 and 2h-2 contained the group of cinnamic acid; compound 2k belonged to dihydriodroprotobberines. These important modifications of BBR can really led to the rapid increase of hypolipidemic activity. The last compound 2e contained a flavonoid linked by short carbon chain at the C-9 position. However, none of activity in 3T3L cells and high activity in HepG2 cells was observed for compound 2e. Based on its molecular structure, it can be very difficult to determine the role of flavonoid scaffold or BBR scaffold in the decrease of LDLC and the increase of HDLC. This is being worthy of further discussion.

In a word, the above results can be concluded as follows: 1) the introduction of fibrates at the C-9 position (2a) can significantly improve the lipid-lowering activity of BBR, especially in reducing LDLC; 2) the introduction of salicylic acid (2b-1 and 2b-2) can cause the change of lipid-lowering activity, but different substituents on salicylic acids might play a key role in the design of novel BBR analogs; 3) the introduction of nicotinic acid (2c) is the same as that of fibrates: it can obviously improve the lipolowering activity, especially in TCHO and TG; 4) Compared with BBR, the introduction of flavonoid (2e) can not increase the inhibitory activity against TCHO and TG, but it has a very important impact on the decrease of LDL and the increase of HDL. The detailed mechanism is not clear whether BBR plays a vital role or flavonoid plays an essential role in hypolipidemic activity; 5) most importantly, the obtained results indicated that the introduction of cinnamic acid and the reduction of N-7 position (2h-1, 2h-2, and 2k) performed better than other modifications in terms of hypolipidemic activity. These two kinds of structural optimization methods should be discussed more specifically in the further study, for example selecting substituents on cinnamic acid.

**Conclusion**

In summary, thirteen new BBR derivatives containing various natural moieties at the C-9 position were designed, synthesized, and evaluated by a series of biological tests. Based on two common cell models (3T3L cells and HepG2 cells), the biological tests included the experiments on inhibition of total cholesterol (TCHO), triglyceride (TG), and low-density lipoprotein cholesterol (LDLC), together with the increase assay of high-density lipoprotein cholesterol (HDLC). The results showed that most compounds exhibited more potent hypolipidemic activities when compared with BBR. Although compounds 2a, 2b-1, 2c, and 2e displayed good activity profiling in these four tests, their performances were still slightly inferior to that of compounds 2h-1 and 2h-2. Therefore, the
BBR analogs with 9-O-cinnamic moiety probably exhibited potent lipid-lowering activity, and should be exploited as an important versatile template for the development of BBR-like lipid-lowering agents. Different substituents on 9-O-cinnamic moiety should be further discussed to study SAR of this novel scaffold.

**Experimental Chemistry**

Reagents and General Methods

1H-NMR spectra were recorded on Bruker AM 600 MHz spectrometers with tetramethylsilane (TMS) as the internal standard. Electrospary ionization (ESI)-MS were recorded by Agilent 1100 series LC/MSD ion trap mass spectrometer. FT-IR spectra were carried out on Nicolet 380 Fourier transformation IR spectrometer using KBr pellets in the 400–4000 cm⁻¹ range. Column chromatography (CC): silica gel (200–300 mesh; Qingdao Makall Group Co., Ltd.; Qingdao; China). Analytical HPLC was performed on a Agilent 1260 HPLC system using a Chromolith SpeedROD RP-18 column (4.6 × 50 mm). A linear gradient elution was performed with eluent A (H₂O/TFA, 100 : 0.1) containing 0% of solvent B (CH₃CN/H₂O/TFA, 90 : 10 : 0.1) rising to 100% of B during 20 min with a flow rate of 1.0 mL/min. All reactions were monitored using TLC on silica gel plates. Reaction reagents containing anhydrous pyridine (0.5 mL) at 0 °C. After the addition was evaporated to obtain high reaction mixture, which was stirred initially for 30 min at 80 °C, and the mixture was cooled and evaporated to obtain high reactivity scaffold.

**Synthesis of the Title Compounds**

9-(2-Hydroxybenzoyloxy)-10-methoxy-5,6-dihydro-[1,3]dioxolo[4,5-d]isoquinolino[3,2-a]isoquinolin-7-ium chloride (2b-1). A yellow solid was obtained. ESI-MS m/z: 442.10 (M+Cl)⁺. mp 174–176 °C. IR KBr (cm⁻¹): 3606 (N-H), 3363 (Ar-H), 1615, 1579, 1506, 1482, 1393, 1367, 1339, 1281, 1219 (C=O), 1164, 1106 (C-O), 1065, 1039 (C-N). 1H-NMR (600 MHz, CDCl₃) δ: 9.08 (s, 1H), 7.99 (s, 1H), 7.62 (s, 1H), 7.22 (d, J = 7.8 Hz, 1H), 6.96 (s, 1H), 6.36 (d, J = 7.8 Hz, 1H), 6.10 (s, 2H), 4.63–4.36 (m, 2H), 3.73 (s, 2H), 3.32 (s, 3H), 3.17 (d, J = 5.2 Hz, 1H), 3.12–2.98 (m, 2H). Purity by anal. HPLC: 96% (254 nm).

General Procedure for the Preparation of 2a-2g

The starting material bezafibrate should be activated in the first procedure: bezafibrate (1 mmol, 362 mg) were mixed with SOCl₂ (5 mL), and the mixture was stirred at reflux 80 °C for 2 h. After completion of the reaction as monitored by TLC, the mixture was cooled and evaporated to obtain high reactive acyl chloride. The acyl chloride was then dissolved in anhydrous CH₂CN (10 mL). Finally, a solution of acyl chloride in anhydrous CH₂CN (10 mL) was added dropwise to berberine (0.8 mmol, 257 mg) in anhydrous CH₂CN (10 mL) containing anhydrous pyridine (0.5 mL) at 0 °C. After the addition of acyl chloride, the reaction solution was heated to 50–60 °C with constant stirring overnight. The mixture was cooled and filtered. The filter cake was purified by flash chromatography with methyl alcohol/dichloromethane (1:20–1:30, v/v) to obtain these two compounds. The yields were around 50%.

9-(2-(4-(2-Chlorobenzamido)ethyl)phenoxy)-2-methylpropanoyloxy)-10-methoxy-5,6-dihydro-[1,3]dioxolo[4,5-g]isoquinolino[3,2-a]isoquinolin-7-ium chloride (2a). A faint yellow solid was obtained. ESI-MS m/z: 665.17 (M+Cl)⁺. mp 198–200 °C. IR KBr (cm⁻¹): 3358 (N-H), 3243 (Ar-H), 3058 (C-H), 2990 (–CH₃), 2922 (–CH₂), 2852 (–CH), 1759, 1715 (C=O), 1607, 1544, 1503, 1479, 1389, 1366, 1338, 1279, 1225 (C=C), 1146, 1115 (C-O), 1095, 1036 (C-N). 1H-NMR (600 MHz, DMSO-d₆) δ: 9.29 (s, 1H), 9.06 (s, 1H), 8.32 (d, J = 9.3 Hz, 1H), 8.25 (d, J = 9.2 Hz, 1H), 7.84 (d, J = 2.9 Hz, 2H), 7.85–7.80 (m, 2H), 7.26 (d, J = 8.5 Hz, 2H), 7.14–7.08 (m, 2H), 7.02 (d, J = 8.5 Hz, 2H), 6.75 (d, J = 8.5 Hz, 1H), 6.19 (s, 2H), 4.85 (t, J = 6.5 Hz, 2H), 4.06 (s, 3H), 3.52–3.45 (m, 2H), 3.43 (td, J = 8.0, 7.5, 5.8 Hz, 2H), 3.21 (t, J = 6.5 Hz, 2H), 2.83 (t, J = 7.5 Hz, 2H), 1.85 (s, 6H). Purity by anal. HPLC: 96% (254 nm).
(3H), 3.22–3.18 (m, 2H). Purity by anal. HPLC: 97% (254 nm).

Synthesis of the Title Compound 2d

Compound 2d was prepared according to the procedure described for compound 2e, starting from compound 2d-1 (E)-3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)acrylic acid, 1 mmol, 206 mg).

The yield was 68%.

(E)-9-((3-(2,3-Dihydrobenzo[b][1,4]dioxin-6-yl)acryloyloxy)-10-methoxy-6,6-dihydro-1,3]dioxole[4,5-g]-
siquinolinolino[3,2-al snaguinolin-7-ium chloride (2d). Faint yellow solid was obtained. ESI-MS m/z: 510.12 (M–Cl)+, m p 234–236 °C. IR KBr (cm−1): 3371 (N–H), 3034 (Ar–H), 2985 (=C–H), 2938 (–CH3), 2896 (–CH2), 2839 (–CH), 1730 (C=O), 1628, 1601, 1579, 1503, 1480, 1431, 1391, 1365, 1332, 1281, 1220 (C=O), 1168, 1111 (C–O), 1059, 1033 (C–N). 1H-NMR (600 MHz, DMSO-d6): δ: 9.93 (s, 1H), 9.05 (s, 1H), 8.32 (d, J = 9.3 Hz, 1H), 8.23 (d, J = 9.2 Hz, 1H), 7.88 (d, J = 16.0 Hz, 1H), 7.82 (s, 1H), 7.45 (d, J = 2.1 Hz, 1H), 7.38 (dd, J = 8.4, 2.1 Hz, 1H), 7.09 (s, 1H), 6.98 (d, J = 8.3 Hz, 1H), 6.89 (d, J = 16.0 Hz, 1H), 6.18 (s, 2H), 4.93 (t, J = 6.4 Hz, 2H), 4.35–4.31 (m, 2H), 4.32–4.28 (m, 2H), 4.04 (s, 3H), 3.21 (t, J = 6.4 Hz, 2H). Purity by anal. HPLC: 97% (254 nm).

Synthesis of the Title Compound 2e

The mixture of kaempferol (1 mmol, 286 mg) and K2CO3 (1 g) was stirred in anhydrous DMF (10 mL) at room temperature for 30 min. 1,4-dibromobutane (2 mmol, 240 μL) in anhydrous DMF (5 mL) was added dropwise to the mixture solution, which was heated to 120°C for 2 h. After completion of the reaction monitored by TLC, the reaction mixture was cooled and extracted by ethyl acetate (3 x 10 mL). The organic phase was dried over MgSO4 and purified by flash chromatography with methyl alcohol/dichloromethane (1:10, v/v) to give the intermediate compound 2e-1. In the next step, compound 2e-1 in anhydrous CH2CN (10 mL) was added dropwise to the mixture of berberrubine (1.2 mmol, 386 mg) and K2CO3 (1 g) in anhydrous CH2CN (10 mL) at 0°C, and then heated to 50–60°C overnight. The reaction mixture was cooled and filtered. The filter cake was purified by flash chromatography with methyl alcohol/dichloromethane (1:20–1:30, v/v) to give the target compound 2e. The yield was 47%.

7-(4-Bromobutoxy)-3,5-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (2e-1). A yellow solid was obtained. ESI-MS m/z: 419.00 (M–H)−. 1H-NMR (600 MHz, DMSO-d6): δ: 12.46 (s, 1H), 10.13 (s, 1H), 9.52 (s, 1H), 8.08 (d, J = 8.9 Hz, 2H), 6.93 (d, J = 8.9 Hz, 2H), 6.74 (d, J = 2.1 Hz, 1H), 6.34 (d, J = 2.1 Hz, 1H), 4.13 (t, J = 6.4 Hz, 2H), 3.62 (t, J = 6.7 Hz, 2H), 1.97 (dt, J = 14.5, 6.7 Hz, 1H), 1.86 (dt, J = 13.2, 6.5 Hz, 2H). Purity by anal. HPLC: 96% (254 nm).

9-(4-(3,5-Dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl)oxy)butyloxy)-10-methoxy-6,6-dihydro-1,3]dioxole[4,5-g]-
siquinolinolino[3,2-al snaguinolin-7-ium chloride (2e). A yellow solid was obtained. ESI-MS m/z: 662.17 (M–Cl)+, m p 256–258 °C. IR KBr (cm−1): 3437 (N–H), 2959 (Ar–H), 2917 (–CH3), 2875 (–CH2), 2849 (–CH), 1660 (C=O), 1589, 1503, 1435, 1399, 1357, 1305, 1258, 1221 (C=O), 1169 (C–O), 1086 (C–N). 1H-NMR (600 MHz, DMSO-d6): δ: 12.46 (s, 1H), 10.13 (s, 2H), 9.52 (s, 2H), 8.08 (d, J = 8.1 Hz, 3H), 6.93 (d, J = 8.1 Hz, 3H), 6.75 (s, 2H), 6.34 (d, J = 0.6 Hz, 2H), 4.13 (t, J = 6.0 Hz, 3H), 3.62 (t, J = 6.3 Hz, 2H), 3.35 (s, 4H), 2.05–1.76 (m, 6H). Purity by anal. HPLC: 96% (254 nm).

Synthesis of the Title Compound 2f–2h

Compounds 2f–2h were prepared according to the procedure described for compounds 2e.
206.40, 149.47, 146.84, 146.29, 143.08, 137.85, 128.65, 127.39, 124.96, 123.11, 118.14, 112.19, 107.80, 103.89, 100.90, 94.85, 60.08, 55.81, 53.81, 46.58, 45.69, 30.13, 29.45. Purity by anal. HPLC: >95% (254 nm).

BBR (322 mg, 1 mmol) was added into the DMSO solution containing sodium hydroxide (500 mg) at room temperature, and then heated to 50–60 °C for overnight. The TLC method was used to detect the reaction. After reaction completion, the reaction mixture was extracted by ethyl acetate for three times. The organic phase was evaporated and purified by silica gel column chromatography (ethyl acetate : petroleum ether = 1:1) to obtain the light yellow solid 2j.

9,10-Dimethoxy-5,6-dihydro-8H-1,3dioxolo[4,5-g]-isoquinolino[3,2-a]isoquinolin-8-one (2j). Yield: 30%. ESI-MS m/z: 352.08 (M + H)+. mp 135–137°C. 1H-NMR (500 MHz, DMSO-d6) δ: 7.52 (d, J = 8.2 Hz, 1H), 7.48 (s, 1H), 7.40 (d, J = 8.4 Hz, 1H), 7.09 (s, 1H), 6.92 (s, 1H), 6.07 (s, 2H), 4.11 (s, 2H), 3.87 (s, 3H), 3.77 (s, 3H), 2.87 (s, 2H). 13C-NMR (126 MHz, DMSO-d6) δ: 158.72, 150.87, 148.31, 147.98, 146.93, 134.97, 131.81, 129.88, 123.14, 122.55, 119.12, 118.52, 107.86, 106.45, 101.34, 100.84, 60.13, 56.35, 42.10, 27.72. Purity by anal. HPLC: >95% (254 nm).

At room temperature, BBR (322 mg, 1 mmol) was slowly added into the mixture solution including pyridine (10 mL) and sodium borohydride (100 mg, 4 mmol). After the addition operation, the reaction solution continued to be stirred for 2 h. The TLC method was used to detect the reaction. After reaction completion, the filter cake was washed with distilled water for 3 times to get dark yellow solid 2k.

9,10-Dimethoxy-5,8-dihydro-6H-1,3dioxolo[4,5-g]-isoquinolino[3,2-a]dioxolone (2k) Yield: 70%. ESI-MS m/z: 338.10 (M + H)+. mp 107–109°C. 1H-NMR (600 MHz, DMSO-d6) δ: 7.29 (s, 1H), 6.82 (d, J = 8.3 Hz, 1H), 6.75 (s, 1H), 6.69 (d, J = 8.3 Hz, 1H), 6.05 (s, 1H), 6.00 (s, 2H), 4.21 (s, 2H), 3.76 (s, 3H), 3.72 (s, 3H), 3.06 (t, J = 5.9 Hz, 2H), 2.80 (t, J = 5.9 Hz, 2H). 13C-NMR (150 MHz, DMSO-d6) δ: 149.86, 146.90, 146.38, 143.93, 140.99, 128.57, 128.30, 123.91, 121.56, 118.36, 111.70, 107.84, 103.48, 100.93, 95.93, 60.13, 55.71, 48.75, 48.29, 28.93. Purity by anal. HPLC: >95% (254 nm).

Biological Evaluation

Reagents and Cells Culture

HepG2 cells and 3T3-L1 cells were from Nanjing University. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from Sigma (U.S.A.). Six-well, 96-well plates and BCA protein assay kit (P0010) were from Beyotime biotechnology (China). Total cholesterol assay kit (TCHO, A111-1), triglyceride assay kit (TG, A110-1), low-density lipoprotein cholesterol assay kit (LDLC, A112-1) were purchased from Nanjing Jiancheng bioengineering institute. 3-Isobutyl-1-methylxanthine (IBMX), insulin (INS) and dexamethasone (DEX) were purchased from Sigma-Aldrich. HepG2 cells and 3T3-L1 cells were grown in High glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100 µg/mL streptomycin and propagated at 37°C in a humidified atmosphere containing 5% CO2 in air.

Compounds 2a–2k, BBR, and simvastatin were dissolved in dimethyl sulfoxide to make stock solutions kept at −20°C. The final concentration of the vehicle in the solution never exceeded 0.1% and had no effects on cells viability.

Assay for Cytotoxic Activity

Non-toxic concentrations of compounds 2a–2g were determined according to MTT test31) and concentration of 12.5 µM were chosen to test the effects of BBR analogs. MTT was dissolved at 4 mg/mL in phosphate buffered saline (PBS) and used essentially as previously described. Briefly, cell lines in logarithmic phase were seeded at a density of 3 × 105 cells/well in 100 µL of DMEM into 96-well microtiter plates. After 6 h, exponentially growing cells were exposed to the indicated compounds at the concentration of 12.5 µM. After 72 h in final volumes of 200 µL, cells survival was determined by the addition of an MTT solution (20 µL of 4 mg/mL MTT in PBS) for 4 h. After carefully removing the medium, the precipitates were dissolved in 200 µL of DMSO, shaken mechanically for 10 min, and then absorbance values at a wavelength of 540 nm were taken on a SpectraMax 190 microplate reader (Molecular Devices, U.S.A.). The survival rate was expressed in percentages with respect to untreated cells.

Protein Extraction and 3T3-L1 Cells Differentiation

The experiment of protein extraction was performed as follows.31) The HepG2 cells were seeded at a density of 1 × 104 cells/well in 100 mL of DMEM into 6-well plates. After 24 h, exponentially growing cells were exposed to the indicated compounds at the concentration of 10 µM. After another 48 h, the HepG2 cells on 6-well plates were rinsed twice with cold PBS and centrifuged (4°C, 6000 rpm, 30 s) to obtain the protein samples, then were lysed in 100 µL RIPA lysis buffer (10 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 1 mM PMSF) on ice for 30 min. The protein concentrations were measured using the BCATM protein quantification kit.

The protocol of 3T3-L1 cells differentiation was as follows. Briefly, 3T3-L1 cells were seeded at a density of 1 × 105 cells/well in 6-well plates with 1 mL of DMEM per well. The cells shape became round after two days. The culture medium was replaced with differentiation medium (DMEM, 10% FBS, 0.5 mM/L IBMX, 1.0 µmol/L DEX and 10 mg/L insulin) and the cells would be cultured for 3d. Subsequently, the cells were maintained in differentiation medium containing only 10 mg/L insulin for 2 d. The cells were replenished with DMEM every other day. On day 10, over 80% of the 3T3-L1 cells had differentiated into mature adipocytes. The procedure for protein extraction and quantification of 3T3-L1 was similar with the HepG2 cells.

The Quantification of Total Cholesterol, Triglyceride, LDLC and HDLC

The HepG2 or 3T3L cell lysates were used to test the levels of TCHO and TG by assay kit directly. 2.5 µL of cell lysates and 250 µL of working fluid were mixed together at 37°C for 10 min. The absorbance was quantified at 510 nm with a spectrophotometer. The TCHO and TG values (mmol/L) were calculated using the eq. [(OD experimental group − ODblank)/(OD standard group − ODblank)] × Cstandard group/Cprotein. The OD experimental group, OD standard group and ODblank are mean absorbance of experimental group, standard group and only ultrapure water added group, respectively. Cstandard group and Cprotein are the concentrations of standard group and protein, respectively. The results were determined through at least three independent experiments.

The HepG2 or 3T3L cell lysates were used to test the levels of LDLC and HDLC by assay kit directly. Two and half microliters of cell lysates and 180 µL of working fluid R1 were
mixed together at 37 °C for 5 min. The absorbance OD1 was quantified at 546 nm with a spectrophotometer. Then 60 μL of working fluid R2 was added and mixed together at 37 °C for 5 min. The absorbance OD2 was quantified at 546 nm with a spectrophotometer. The LDLc and HDLc values (mmol/l gprot) were calculated using the equation: 

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\Delta OD = (\Delta OD_{standard group} - \Delta OD_{blank}) / (\Delta OD_{standard group} - \Delta OD_{blank}) \times C_{standard group} / C_{protein} \]

The ΔOD = OD1 − OD2, ΔOD experimental group, ΔOD standard group, and ΔOD blank are mean absorbance of experimental group, standard group and only ultrapure water added group, respectively. C standard group and C protein are the concentrations of standard group and protein, respectively. The results were determined through at least three independent experiments.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

References
1) Luo L. J., Experience of berberine in the treatment of diarrhea. (1955).
2) Cicero A. F., Rovati L. C., Sartori M., Arzneimittelforschung, 57, 26–30 (2007).
3) Zhang S., Wang X., Yin W., Liu Z., Zhou M., Xiao D., Liu Y., Peng D., Bioorg. Med. Chem. Lett., 26, 4799–4803 (2016).
4) Liu C. S., Zheng Y. R., Zhang Y. F., Long X. X., Fitoterapia, 109, 274–282 (2016).
5) Kheir M. M., Wang Y., Hua L., Hu J., Li L., Lei F., Du L., Food Chem. Toxicol., 48, 1105–1110 (2010).
6) Feng P., Zhao L., Gao F., Zhang B., Fang L., Zhan G., Xu X., Fang Q., Liang Z., Li B., Chem. Biol. Interact., 293, 115–123 (2018).
7) Liu D. L., Xu L. J., Hong J., Wu P., Ren G., Ning G., J. Lipid Res., 57, 2559–2565 (2008).
8) Luo L. J., Zheng Y. R., Zhang Y. F., Long X. X., Fitoterapia, 26, 4799–4803 (2016).
9) Feng P., Zhao L., Gao F., Zhang B., Fang L., Zhan G., Xu X., Fang Q., Liang Z., Li B., Chem. Biol. Interact., 293, 115–123 (2018).
10) Liu D. L., Xu L. J., Hong J., Wu P., Ren G., Ning G., J. Lipid Res., 57, 2559–2565 (2008).
11) Nechepeurenko I. V., Boyarskikh U. A., Khvostov M. V., Baev D. S., Komarova N. I., Filipenko M. L., Tolstikova T. G., Salakhutdinov N. F., Chem. Nat. Compd., 51, 916–922 (2015).
12) Xia X., He K., Zhou Z., Zhang B., Chen X., Yi J., Li X., Med. Chem. Res., 21, 1353–1362 (2012).
13) Ye X., He K., Zhou Z., Zhang B., Chen X., Yi J., Li X., Med. Chem. Res., 21, 1353–1362 (2012).
14) Zhou X., Ren F., He K., Zhou Z., Shen T., Xu S., Wei J., Ren J., Ni H., Lipids Health Dis., 16, 239 (2017).
15) Abidi P., Zhou Y., Jiang J. D., Liu J., Arterioscler. Thromb. Vasc. Biol., 25, 2170–2176 (2005).
16) Liu D. L., Xu L. J., Dong H., Chen G., Huang Z. Y., Zou X., Wang K. F., Luo Y. H., Lu F. E., Chin. J. Integr. Med., 21, 132–138 (2015).
17) Brusq J. M., Ancellin N., Grondin P., Guilhard R., Martin S., Santillan Y., Issandou M., J. Lipid Res., 47, 1281–1288 (2006).
18) Wang Y. X., Kong W. J., Li Y. H., Tang S., Li Z., Li Y. B., Shan Y. Q., Bi C. W., Jiang J. D., Song D. Q., Bioorg. Med. Chem., 20, 6552–6558 (2012).
19) Wan Q., Liu Z., Yang Y., Cui X., Int. J. Mol. Med., 41, 1939–1948 (2018).
20) Baron S. H., Diabetes Care, 5, 64–71 (1982).
21) Baltazar M., I. Dinis-Oliveira R., A. Duarte J., L. Bastos M., Carvalho F., Curr. Med. Chem., 18, 3252–3264 (2011).
22) Xiao X., Weng J., J. Diabetes Care, 38(1), 933–956 (2015).
23) de Vries-van der Weij J., Toet K., Zadelaar S., Wielinga P. C., Koolstra T., Arterioscler. Thromb. Vasc. Biol., 20, 6552–6558 (2012).
24) Fang V. S., Arch. Int. Pharmacodyn. Ther., 178, 315–323 (1969).
25) Rice-Evans C. A., Miller N. J., Paganga G., Free Radic. Biol. Med., 20, 933–956 (1996).
26) Wei B. L., Weng J. R., Chiu P. H., Chung C. F., Wang J. P., Lin C. N., J. Agric. Food Chem., 53, 3867–3871 (2005).
27) Ye X., He K., Zhou Z., Shen T., Xu S., Wei J., Ren J., Ni H., Lipids Health Dis., 16, 239 (2017).
28) Bao L., Hu L., Zhang Y., Wang Y. L., Exp. Ther. Med., 11, 1417–1424 (2016).
29) Yu Z., Xu H., Yu X., Sui D., Lin G., Chin. J. Integr. Med., 21, 315–323 (1969).
30) Ferraroni M., Bazzicalupi C., Papi F., Fiorillo G., Guamán-Ortiz L., Bioorg. Med. Chem., 20, 6552–6558 (2012).
31) Yu P., Xia C. J., Li D. D., Ni J. J., Zhao L. G., Ding G., Wang Z. Z., Bioorg. Med. Chem. Lett., 26, 4799–4803 (2016).