Bioorganic & Medicinal Chemistry 18 (2010) 6422–6428

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design, synthesis, and cholesterol-lowering efficacy for prodrugs of berberrubine

Ying-Hong Li †, Yi Li †, Peng Yang, Wei-Jia Kong, Xue-Fu You, Gang Ren, Hong-Bin Deng, Yue-Ming Wang, Yan-Xiang Wang, Jian-Dong Jiang *, Dan-Qing Song *

Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

ARTICLE INFO

Article history:
Received 25 May 2010
Revised 29 June 2010
Accepted 30 June 2010
Available online 30 July 2010

Keywords:
Berberine
Metabolite
Lipid-lowering
Prodrug
Bioavailability

ABSTRACT

In order to enhance oral bioavailability of berberine (BBR) for its cholesterol-lowering efficacy in vivo, a series of ester or ether prodrugs of berberrubine (M1), which is an active metabolite of BBR after first-pass metabolism, were designed, semi-synthesized, and evaluated. Among these M1 prodrugs, compound **5g** possessing palmitate at the 9-position showed a moderate Log *P* value and esterase hydrolysis rate for releasing M1 in blood. Its cholesterol-lowering efficacy in vivo was evaluated in hyperlipidemic SD rats. Compound **5g** (100 mg/kg/d) reduced blood CHO and LDL-c by 35.8% and 45.5%, respectively, similar to that by BBR. It also exhibited a good safety in rats with no side-effect on liver and kidney function. Therefore, the design of M1 prodrug appears to be an effective strategy to improve pharmacokinetic feature of BBR for its lipid-lowering efficacy in vivo.

1. Introduction

Berberine (BBR, Fig. 1A) extracted from *Coptis chinensis* has been extensively used for decades as a nonprescription drug to treat diarrhea in China with good safety.[1] As the absorption rate of BBR in gut is poor, the compound concentrates in intestine.[1,2] This distribution nature of BBR is good for the treatment of diarrhea. In the past few years, we have identified BBR as a new agent for hyperlipidemia causing reduction of cholesterol and triglyceride in hyperlipidemic patients.[3,4] The results were confirmed by other groups as well.[5–7] BBR increases low-density-lipoprotein receptor (LDLR) expression by stabilization of LDLR mRNA through activation of extracellular signal-regulated kinase (ERK) pathway.[3,8,9] Furthermore, the structure–activity relationship (SAR) of BBR for up-regulating LDLR mRNA expression was elucidated systemically in our pervious reports.[10] Theoretically, its lipid-lowering efficacy in vivo might be significantly improved if oral bioavailability of BBR is increased. Prodrug approach is one of the strategies in our investigation.

It has been documented that BBR has four major metabolites after its first-pass metabolism in liver, berberrubine (M1), thalifendine (M2), demethyleneberberine (M3), and jatrorrhizine (M4) (Fig. 1A).[11] In order to construct prodrugs, the four metabolites were obtained for the evaluation of their activity on LDLR mRNA up-regulation. As shown in Figure 1B, among the four metabolites M1 afforded the best up-regulatory effect for LDLR mRNA expression, although its activity was lower than that of BBR.

As M1 can be easily prepared into ester or ether prodrugs through the hydroxyl at the 9-position, the resultant analogs through this design might have improved lipophilicity and oral bioavailability. In the strategy, the prodrug pro-moieties (in the red circle in Fig. 3) should be removed by esterase after entering into blood, followed by releasing the active structure M1 for the subsequent lipid-lowering action. Based on this strategy, 14 prodrugs for M1 including alkyl esters (**5a**–**j**), aryl ester (**5k**), double ester (**5l**), and hybrid ethers (**5m**–**n**) were designed, semi-synthesized and evaluated biologically. Among the prodrugs, compound **5g** bearing a palmitate at the 9-position exhibited a moderate Log *P* value and hydrolysis rate in blood, and thus was selected as a representative agent for lipid-lowering efficacy study in vivo.

2. Chemistry

The metabolite M4 (purity, 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). M2 was synthesized using 2,3-methylenedioxyphenethylamine and 2-methoxy-3-hydroxyl benzaldehyde as starting materials with methods reported previously.[10a,b,12]

* Corresponding authors. Tel.: +86 10 63188423; fax: +86 10 63017302 (J.-D.J.); tel./fax: +86 10 63165268 (D.-Q.S.).
E-mail addresses: jiang.jdong@163.com (J.-D. Jiang), songdanqingsdq@hotmail.com (D.-Q. Song).
† These authors made equal contribution to this work.

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doi:10.1016/j.bmc.2010.06.106

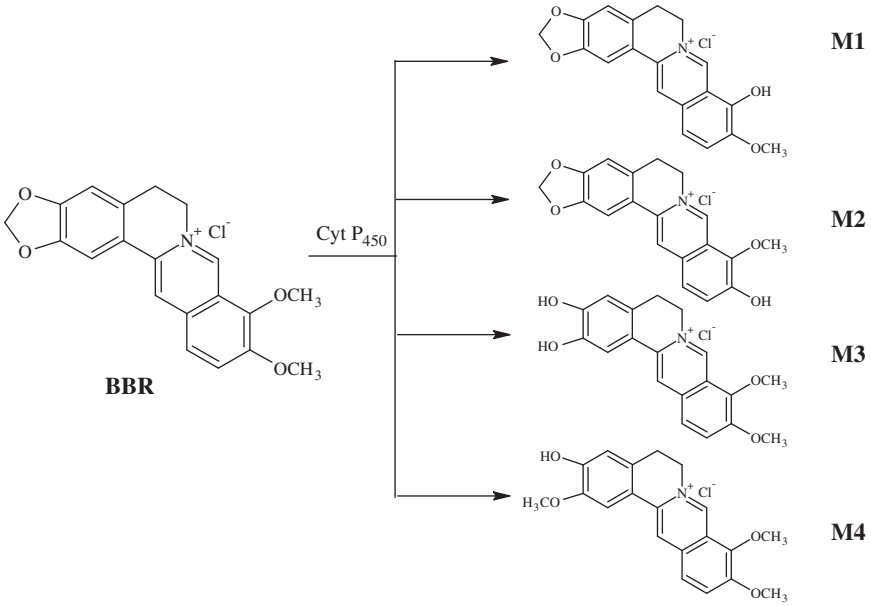


Figure 1A. Structures of BBR and its four metabolites (M1–M4).

M1, M3, and prodrugs (**5a**–**n**) were semi-synthesized as described in the Scheme 1 with BBR as start material.[10c,13] The ester prodrugs for phenol **5a**–**l** (Route A) were obtained by esterification of M1 with acyl chloride (**1**), using acetonitrile as solvent and pyridine as the base with a yield of 37–67%.[14] The hybrid ether prodrugs for phenol **5m**–**n** (Route B) were gained using esterified alkyl iodide (**4**) as the electrophile and K_2CO_3 as the base. The key intermediate **4** was prepared through an additional reaction of **1** and polyformaldehyde (**2**), followed by an exchange reaction with NaI.[15] The final products (**5a**–**n**) were purified through recrystallization in ethanol.

3. Results and discussion

3.1. Up-regulating LDLR expression and Log *P* values

HepG2 cells were respectively treated with each of the study prodrugs (**5a**–**n**) for 12 h. Cellular RNA was extracted for analysis. As anticipated, none of the prodrugs showed up-regulating activity on LDLR mRNA expression in vitro (data not shown), and the results were consistent with the SAR results reported previously.[10]

For most of the drugs lipophilicity is an important determinant of properties. The lipophilicity of a compound is commonly estimated using Log *P* from octanol/water partitioning. Here, the Log *P* values of the study prodrugs (**5a**–**n**) were calculated with Chemoffice ultra 2005 (Cambriage Soft, Table 1). The results showed that Log *P* or lipophilicity of M1 could be largely increased by introducing lipophilic groups at the 9-hydroxyl side chain. The prodrugs (**5a**–**d**, **5i**–**n**) with a lower Log *P* (<2) have more polarity and poorer permeability for the lipid bilayer. The prodrugs (**5e**–**h**) with a moderate Log *P* (2–7) are generally considered optimal for oral dosing. We deduce that increase of lipophilicity might lead to an improvement of oral bioavailability.

3.2. Enzymatic hydrolysis rate in blood

An ideal prodrug should be resistant to hydrolysis during the absorption phase in intestine, but hydrolyzed to parent rapidly and quantitatively after entering into blood.[16] The enzymatic hydrolysis rate in blood of the prodrugs **5a**–**n** was then examined, and the dissociative concentration of M1 was tested, respectively, at the 10 min and 60 min after incubation. As shown in Figure 2, for most of the ester prodrugs including aliphatic ester prodrugs

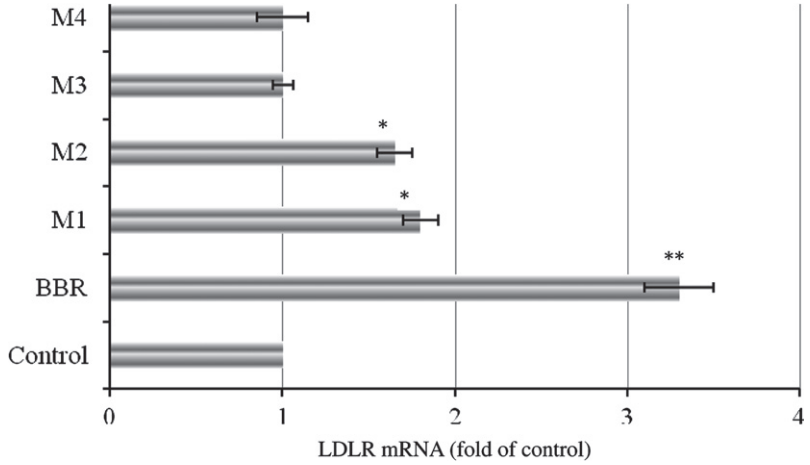
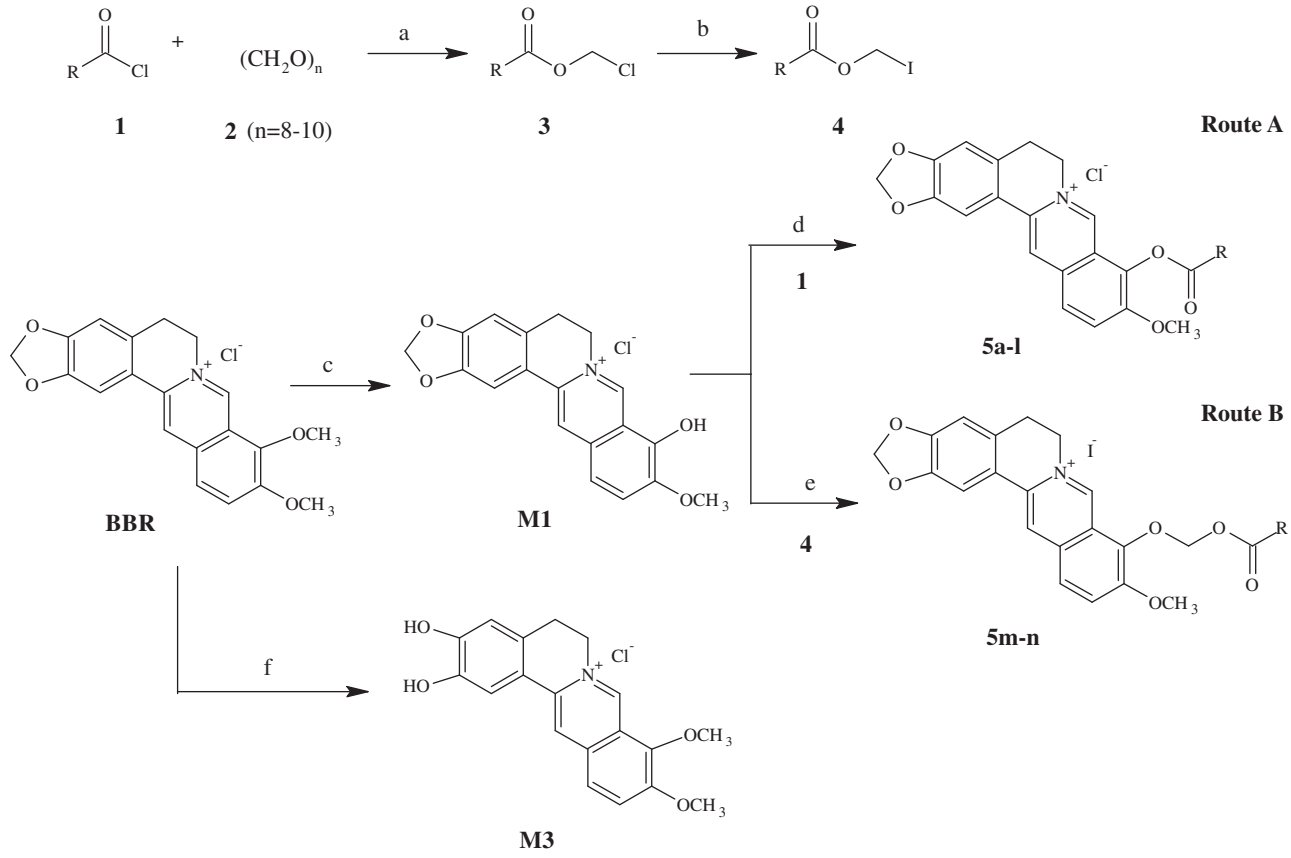
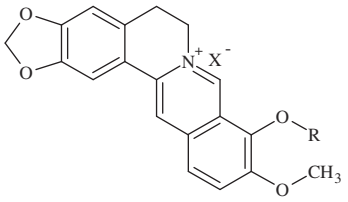


Figure 1B. Effect of BBR and M1–M4 on LDLR gene expression. HepG2 cells were treated with the compounds (15 μg/mL, 8 h), followed by LDLR mRNA analysis using real-time RT-PCR. LDLR mRNA in the treated cells was normalized to that of control. $^{*}p$ <0.05, $^{**}p$ <0.01, versus control.



Scheme 1. Synthetic routes of M1, M3, and prodrugs **5a**–**n**. Reagents and conditions for the chemical synthesis: (a) 100 °C, 5 h; (b) NaI, CH_3CN, rt, 1 h; (c) 30–40 mmHg, 195–210 °C, 15 min; (d) pyridine, CH_3CN, reflux, 4 h; (e) RX, K_2CO_3, CH_3CN, 60 °C, 5 h; (f) phloroglucinol, 60% H_2SO_4, 90–95 °C, 10–15 min.

Table 1
Structures and calculated Log *P* value for study prodrugs (**5a**–**n**)



Compound	R	X	Log *P*[a]
BBR	CH_3	Cl	−0.92
M1	H	Cl	−1.06
5a	$CO(CH_2)_2CH_3$	Cl	−0.43
5b	$CO(CH_2)_3CH_3$	Cl	0.10
5c	$CO(CH_2)_5CH_3$	Cl	1.16
5d	$CO(CH_2)_6CH_3$	Cl	1.69
5e	$CO(CH_2)_8CH_3$	Cl	2.74
5f	$CO(CH_2)_{10}CH_3$	Cl	3.80
5g	$CO(CH_2)_{14}CH_3$	Cl	5.91
5h	$CO(CH_2)_{16}CH_3$	Cl	6.98
5i	$COC(CH_3)_3$	Cl	−0.25
5j	COCH=CHPh	Cl	1.03
5k	COPh	Cl	0.58
5l	$COOCH_2CH_3$	Cl	−1.18
5m	$CH_2OCOC(CH_3)_3$	I	−0.06
5n	CH_2OCOPh	I	0.97

[a] Chemoffice ultra 2005 (Cambriage Soft).

(**5a**–**f**, **5i**), aromatic ester prodrugs **5k** and double ester prodrug **5l** were hydrolyzed rapidly, with over 60% hydrolyzed in 10 min and 100% in 1 h. Prodrugs **5g**–**h**, **5j** and **5m**–**n** displayed a release delay on certain levels. The chemical mechanism is illustrated in Figure 3.

After overall evaluation, aliphatic ester prodrug **5g** showed a moderated Log *P* and hydrolysis rate in the blood. Therefore, this compound was selected for further evaluation in vivo for its lipid-lowering effect.

3.3. Lipid-lowering efficacy of 5g in hyperlipidemic animals

As the pro-moiety of **5g** has been extensively used in clinic and shows no toxicity, the safety of **5g** should be relatively good. It encouraged us to test its cholesterol-lowering efficacy in vivo. The rats were fed with a high-fat and high-cholesterol (HFHC) diet orally for 7 weeks before treatment, with a normal diet group as reference. LDL-c and total cholesterol (CHO) in blood were detected after a 28-day treatment with BBR or **5g** at 100 (mg/kg/d). As shown in Figure 4A, while BBR reduced blood CHO and LDL-c by 27.4% and 38.0% (p <0.01), respectively, **5g** reduced CHO and LDL-c by 35.8% and 45.5% (p <0.01), respectively. Considering that the molecular weight of **5g** was larger than that of BBR, the in vivo cholesterol-lowering efficacy of **5g** seemed to be better than that of BBR.

Compound **5g** was safe and well tolerated in the rats. We did not observe weight loss, food intake reduction, or liver/kidney function abnormalities (Fig. 4B and C) in the rats treated with **5g**. The results of **5g** agreed with that of BBR,[10c] and suggest prodrug **5g** to be safe one in vivo.

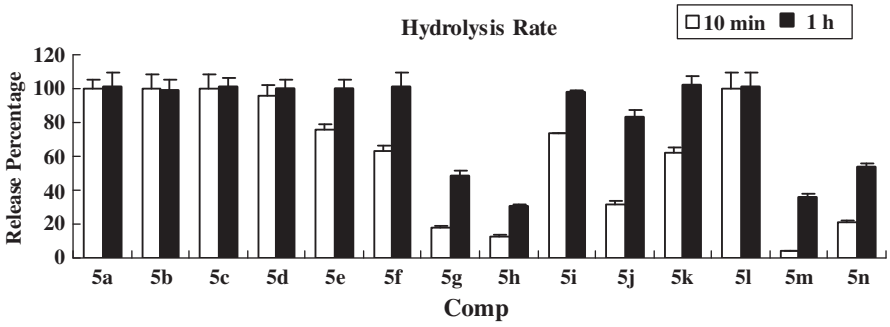


Figure 2. The concentration of M1 cleaved from prodrugs.

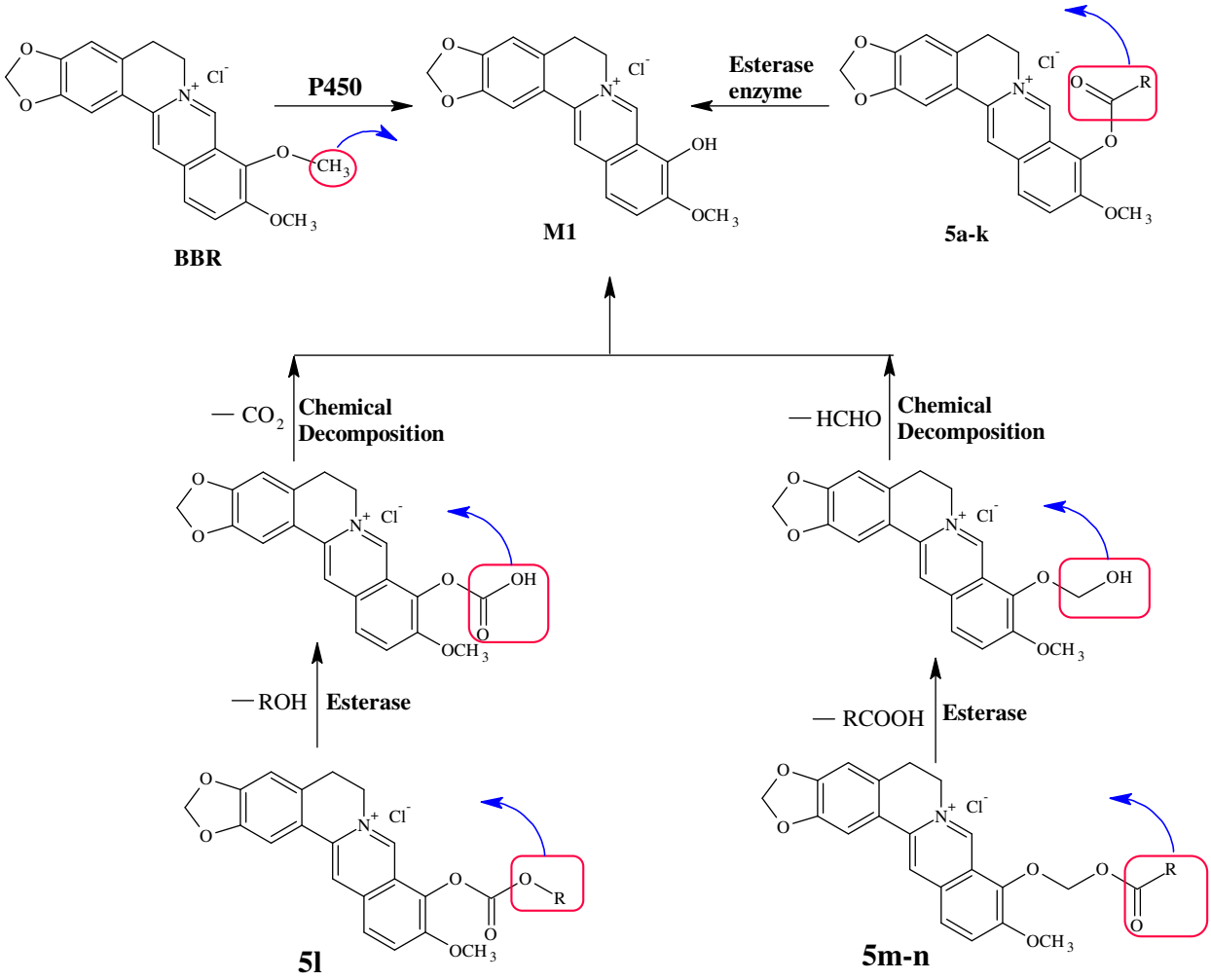


Figure 3. Mechanisms of prodrugs (**5a**–**n**) used to increase lipophilicity.

4. Conclusions

To enhance the oral availability of BBR, four major metabolites (M1–M4) of BBR were obtained and evaluated for their up-regulatory effect on LDLR mRNA expression, in which M1 bearing a hydroxyl at the 9-position exhibited the best activity. Therefore, a series of ester or ether prodrugs of M1 were designed and synthesized, and their M1 releasing rates were examined in blood. Among these prodrugs, compound **5g** possessing palmitate ester at position 9 showed an enhanced lipid-lowering efficacy in vivo as compared to that of BBR. The increased effect of **5g** might result from the higher lipophilicity in respect to that of BBR. As **5g** shows an improved lipid-lowering activity with a good safety in vivo, it merits further investigation. We consider ester prodrug strategy of M1 an effective approach to improve oral bioavailability of BBR.

5. Experimental section

5.1. Chemical methods

Melting point (mp) was obtained with YRT-3 melting point apparatus and uncorrected. ^{1}H NMR spectra was performed on a Varian Inova 400 MHz spectrometer (Varian, CA) in DMSO-d_6, with Me_4Si as the internal standard. FAB high-resolution mass spectra

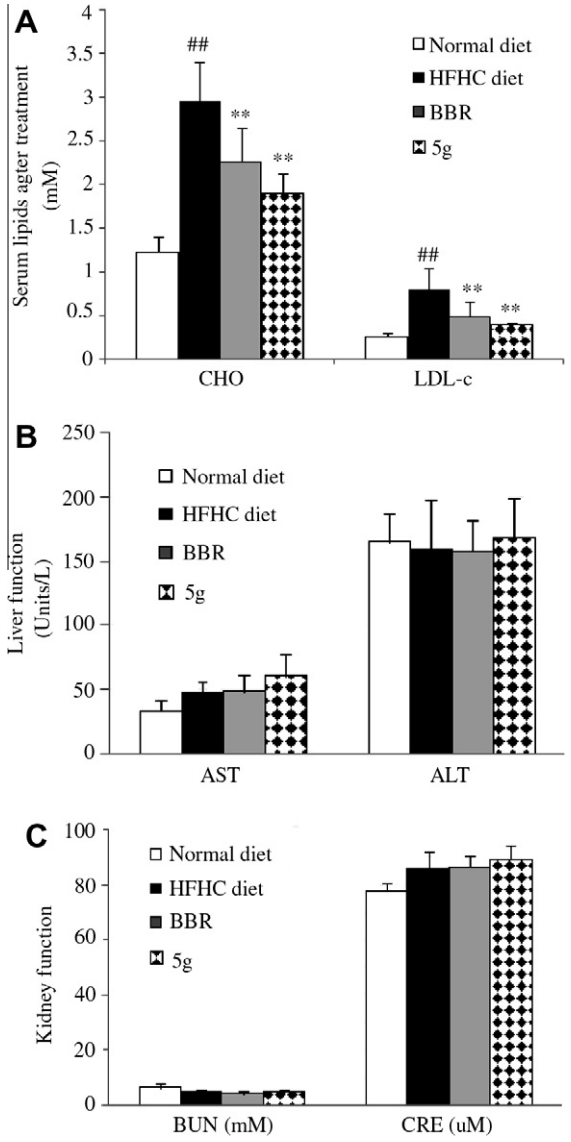


Figure 4. Lipid-lowering effect of BBR and **5g** in the hyperpilidemic SD rats. (A) End-point blood lipid levels. At the end of the treatment course, blood samples were taken from animals, and CHO, LDL-c levels were determined. Presented are mean and sd values. Values are the mean ± SE of all of the animals in each group: $^{\#\#}p$ <0.01 versus that of the normal diet group (n = 8); $^{**}p$ <0.01 versus that of the HFHC diet group by one-way ANOVA and multiple comparisons. (B) Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the treatment with compounds BBR and **5g** in rats. Hyperlipidemic rat were treated with the study compound (100 mg/kg/d, oral) for 28-day. Blood samples were taken before and after the treatment, and their AST and ALT levels were measured. Presented are means and sds. (C) Blood urea nitrogen (BUN) and creatinine (CRE) levels in the treatment with compounds BBR and **5g** in rats. Hyperlipidemic rat were treated as mentioned above. Blood samples were assayed for their BUN and CRE levels. Presented are means and sds.

(HSMS) was recorded on an Autospec Ultima-TOF mass spectrometer (Waters, USA).

Solvent and reagent abbreviations used are $SOCl_2$ = sulfoxide dichloride, CH_3CN = acetonitril, K_2CO_3 = potassium carbonate, DMSO = dimethylsulfoxide.

5.2. 2,3-Dihydroxy-9,10-dimethoxyprotoberberine chloride (M3)

To a stirred solution of 60% H_2SO_4 (100 mL), phloroglucin (5.0 g, 40 mmol) was added portionwise to form a colorless solution. BBR (5.0 g, 0.013 mol) was added portionwise and the resulting system was stirred at 90–95 °C for 10–15 min. Then the mixture was poured into brine (100 mL) immediately with violent stirring, and the resulting mixture was stirred at room temperature for 2 h and cooled down to precipitate completely. The crude product was re-crystallized with methanol twice to afford the title compound M3 (2.50 g, 54%) as a yellow needle, mp 220–222 °C. ^{1}H NMR δ: 3.10 (t, 2H, J = 6.0 Hz), 4.05 (s, 3H), 4.06 (s, 3H), 4.88 (t, 2H, J = 6.0 Hz), 6.80 (s, 1H), 7.50 (s, 1H), 8.04 (d, 1H, J = 9.2 Hz), 8.16 (d, 1H, J = 9.2 Hz), 8.74 (s, 1H), 9.36 (br, 1H), 9.82 (s, 1H), 10.16 (br, 1H); HRMS: calcd for $C_{19}H_{18}NO_4Cl$ $(M-Cl)^+$ 324.1236, found 324.1245.

5.3. 2,3-Methylenedioxy-9-hydroxy-10-methoxyprotoberberine chloride (M1)

BBR (2.0 g, 5.38 mmol) was heated at 195–210 °C for 10–15 min under vacuum (20–30 mmHg) to afford dark wine solid which was re-crystallized with anhydrous ethanol twice, and then ethanol/concentrated HCl (95:5) to afford the title compound M1 (1.15 g, 60%) as a yellow needle. mp 195–196 °C. ^{1}H NMR δ: 3.17 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.88 (t, 2H, J = 6.0 Hz), 6.15 (s, 2H), 7.06 (s, 1H), 7.68 (d, 1H, J = 9.2 Hz), 7.78 (s, 1H), 8.08 (d, 1H, J = 9.2 Hz), 8.82 (s, 1H), 9.89 (s, 1H), 11.23 (br s, 1H); HRMS: calcd for $C_{19}H_{16}NO_4Cl$ $(M-Cl)^+$ 322.1079, found 322.1077.

5.4. General procedure for the synthesis of compounds 5a–i (Route A)

To a solution of $SOCl_2$ (20 mL, 268 mmol) was added corresponding carboxyl acid (20.0 mmol) with stirring. The mixture was stirred at 50–60 °C for 4 h, and the extra $SOCl_2$ was distilled under air condition, then the residue was distilled under vacuum to collect yellow or red major component acyl chloride **1**.

To a solution of CH_3CN (70 mL) was added M1 (2.06 g, 5.8 mmol) at room temperature, then heated to form a dark-red solution, to which was then added anhydrous pyridine (0.5 mL) and **1**. The resulting mixture was stirred at 50–60 °C for 2–4 h and cooled to precipitate completely. The resulting filtrated was then purified by flash chromatography over silica gel using dichloromethane/methanol (40:1) and recrystallization in ethanol to give **5a**–**i**. Compounds **5j**–**l** were gained following the same procedure using purchased acyl chloride **1** as material.

5.4.1. 2,3-Methenedioxy-9-butyloyloxy-10-methoxyprotoberberine chloride (5a)

Using the previous procedure, yield: 46%; Yellow solid; mp 204–206 °C; ^{1}H NMR δ: 0.88 (t, 3H, J = 7.2 Hz,), 1.73 (m, 2H), 2.86 (t, 2H, J = 7.2 Hz), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.92 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.02 (s, 1H), 9.86 (s, 1H); HRMS: calcd for $C_{23}H_{22}NO_5Cl$ $(M-Cl)^+$ 392.1498, found 392.1486.

5.4.2. 2,3-Methenedioxy-9-pentanoyloxy-10-methoxyprotoberberine chloride (5b)

Using the previous procedure, yield: 43%; Yellow solid; mp 206–207 °C; ^{1}H NMR δ: 0.88 (t, 3H, J = 7.2 Hz,), 1.44 (m, 2H), 1.73 (m, 2H), 2.86 (t, 2H, J = 7.2 Hz), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.92 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.02 (s, 1H), 9.86 (s, 1H); HRMS: calcd for $C_{24}H_{24}NO_5Cl$ $(M-Cl)^+$ 406.1654, found 406.1655.

5.4.3. 2,3-Methenedioxy-9-heptanoyloxy-10-methoxyprotoberberine chloride (5c)

Using the previous procedure, yield: 45%; Yellow solid; mp 208–210 °C; ^{1}H NMR δ: 0.88 (t, 3H, J = 7.2 Hz,), 1.35 (m, 4H), 1.44

(m, 2H), 1.73 (m, 2H), 2.86 (t, 2H, J = 7.2 Hz), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.92 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.02 (s, 1H), 9.86 (s, 1H); HRMS: calcd for $C_{26}H_{28}NO_5Cl$ $(M-Cl)^+$ 434.1967, found 434.1954.

5.4.4. 2,3-Methenedioxy-9-capryloyloxy-10-methoxyprotoberberine chloride (5d)

Using the previous procedure, yield: 48%; Yellow solid; mp 210–212 °C; ^{1}H NMR δ: 0.88 (t, 3H, J = 7.2 Hz,), 1.35 (m, 6H), 1.44 (m, 2H), 1.73 (m, 2H), 2.86 (t, 2H, J = 7.2 Hz), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.92 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.02 (s, 1H), 9.86 (s, 1H); HRMS: calcd for $C_{27}H_{30}NO_5Cl$ $(M-Cl)^+$ 448.2124, found 448.2117.

5.4.5. 2,3-Methenedioxy-9-decoyloxy-10-methoxyprotoberberine chloride (5e)

Using the previous procedure, yield: 53%; Yellow solid; mp 222–224 °C; ^{1}H NMR δ: 0.86 (t, 3H, J = 7.2 Hz), 1.36 (m, 10H), 1.42 (m, 2H), 1.73 (m, 2H), 2.84 (m, 2H,), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.92 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 H), 9.02 (s, 1H), 9.86 (s, 1H); HRMS: calcd for $C_{29}H_{34}NO_5Cl$ $(M-Cl)^+$ 476.2437, found 476.2443.

5.4.6. 2,3-Methenedioxy-9-lauroyloxy-10-methoxyprotoberberine chloride (5f)

Using the previous procedure, yield: 52%; Yellow solid; mp 230–232 °C; ^{1}H NMR δ: 0.85 (t, 3H, J = 7.2 Hz), 1.31 (m, 14H), 1.42 (m, 2H), 1.73 (m, 2H), 2.86 (m, 2H), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H,), 4.95 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.05 (s, 1H), 9.95 (s, 1H); HRMS: calcd for $C_{31}H_{38}NO_5Cl$ $(M-Cl)^+$ 504.2750, found 504.2751.

5.4.7. 2,3-Methenedioxy-9-palmitate-10-methoxyprotoberberine chloride (5g)

Using the previous procedure, yield: 55%; Yellow solid; mp 215–216 °C; ^{1}H NMR δ: 0.85 (t, 3H, J = 7.2 Hz), 1.33 (m, 22H), 1.42 (m, 2H), 1.73 (m, 2H), 2.84 (m, 2H), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.91 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.02 (s, 1H), 9.85 (s, 1H); HRMS: calcd for $C_{35}H_{46}NO_5Cl$ $(M-Cl)^+$ 560.3376, found 560.3376.

5.4.8. 2,3-Methenedioxy-9-stearate-10-methoxyprotoberberine chloride (5h)

Using the previous procedure, yield: 56%; Yellow solid; mp 228–230 °C; ^{1}H NMR δ: 0.84 (t, 3H, J = 7.2 Hz), 1.31 (m, 26H), 1.42 (m, 2H), 1.73 (m, 2H), 2.83 (t, 2H, J = 7.2 Hz), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.95 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.19 (d, 1H, J = 9.2 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.05 (s, 1H), 9.95 (s, 1H); HRMS: calcd for $C_{37}H_{50}NO_5Cl$ $(M-Cl)^+$ 588.3689, found 588.3638.

5.4.9. 2,3-Methenedioxy-9-pivaloyloxy-10-methoxyprotoberberine chloride (5i)

Using the previous procedure, yield: 56%; Yellow solid; mp 218–220 °C; ^{1}H NMR δ: 1.46 (s, 9H), 3.21 (t, 2H, J = 6.0 Hz), 4.02 (s, 3H), 4.96 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.10 (s, 1H), 7.81 (s, 1H), 8.19 (d, 1H, J = 8.8 Hz), 8.27 (d, 1H, J = 9.2 Hz), 9.04 (s, 1H), 9.52 (s, 1H); HRMS: calcd for $C_{24}H_{24}NO_5Cl$ $(M-Cl)^+$ 406.1654, found 406.1648.

5.4.10. 2,3-Methenedioxy-9-cinnamate-10-methoxyprotoberberine chloride (5j)

$C_{28}H_{22}NO_5Cl$; Yield: 50%; Yellow solid; mp 214–216 °C; ^{1}H NMR δ: 3.20 (t, 2H, J = 6.0 Hz), 4.04 (s, 3H), 4.92 (t, 2H, J = 6.0 Hz), 6.18 (s, 2H), 7.06 (d, 1H, J = 16.0 Hz), 7.08 (s, 1H), 7.50 (s, 1H), 7.51–8.13 (m, 5H), 7.99 (d, 1H, J = 16.0 Hz), 8.23 (d, 1H, J = 9.2 Hz), 8.32 (d, 1H, J = 9.2 Hz), 9.05 (s, 1H), 9.95 (s, 1H); HRMS: calcd for $C_{28}H_{22}NO_5Cl$ $(M-Cl)^+$ 452.1498, found 452.1490.

5.4.11. 2,3-Methenedioxy-9-benzoxy-10-methoxyprotoberberine chloride (5k)

Using the previous procedure, yield: 67%; Yellow solid; mp 210–212 °C; ^{1}H NMR δ: 3.20 (t, 2H, J = 6.0 Hz), 4.03 (s, 3H), 4.91 (t, 2H, J = 6.0 Hz), 6.19 (s, 2H), 7.09 (s, 1H), 7.50–7.60 (m, 2H), 7.69–7.73 (m, 2H), 8.15–8.36 (m, 4H), 9.09 (s, 1H), 10.00 (s, 1H); HRMS: calcd for $C_{26}H_{20}NO_5Cl$ $(M-Cl)^+$ 426.1341, found 426.1337.

5.4.12. 2,3-Methenedioxy-9-ethyloxyformyl-10-methoxyprotoberberine chloride (5l)

Using the previous procedure, yield: 37%; Yellow solid; mp 218–220 °C; ^{1}H NMR δ: 1.36 (t, 3H, J = 7.2 Hz), 3.21 (t, 2H, J = 6.0 Hz), 4.06 (s, 3H), 4.36 (q, 2H, J = 7.2 Hz), 4.93 (t, 2H, J = 6.0 Hz), 6.17 (s, 2H), 7.09 (s, 1H), 7.80 (s, 1H), 8.21 (d, 1H, J = 9.2 Hz), 8.30 (d, 1H, J = 9.2 Hz,), 9.03 (s, 1H), 9.97 (s, 1H); HRMS: calcd for $C_{22}H_{20}NO_6Cl$ $(M-Cl)^+$ 394.1291, found 394.1281.

5.5. General procedure for the synthesis of compounds 5m–n (Route B)

To a solution of **2** (0.56 mmol), one molar equivalent of relevant **1** was added, the resulting system wad heated to 95–100 °C for 4 h. Anhydrous CH_3CN (12 mL) and NaI (0.84 g, 0.56 mmol) was then added and stirred at rt overnight to generate the intermediate **4**.

To a solution of CH_3CN (70 mL) was added M1 (0.10 g, 0.28 mmol) at rt, and then heated to form a dark-red solution, to which was then added anhydrous K_2CO_3 (0.77 g, 0.56 mmol) and intermediate **4**. The resulting mixture was stirred at 60 °C for 4 h and cooled to precipitate completely. The residue filtrated was then purified by flash chromatography over silica gel using dichloromethane/methanol (40:1) to give **5m–n**.

5.5.1. 2,3-Methenedioxy-9-pivalyloxymethylenoxy-10-methoxyprotoberberine iodide (5m)

Using the previous procedure, yield: 34%; Yellow solid; mp 220–222 °C; ^{1}H NMR δ: 1.05 (s, 9H, J = 7.2 Hz), 3.18 (t, 2H, J = 6.0 Hz), 4.06 (s, 3H), 4.93 (t, 2H, J = 6.0 Hz), 5.94 (s, 2H), 6.17 (s, 2H), 7.09 (s, 1H), 7.79 (s, 1H), 7.99 (d, 1H, J = 9.2 Hz), 8.19 (d, 1H, J = 9.2 Hz), 8.94 (s, 1H), 9.93 (s, 1H); HRMS: calcd for $C_{25}H_{26}NO_6I$ $(M-I)^+$ 436.1760, found 436.1779.

5.5.2. 2,3-Methenedioxy-9-phenacyloxymethylenoxy-10-methoxyprotoberberine iodide (5n)

Using the previous procedure, yield: 36%; Yellow solid; mp 220–222 °C; ^{1}H NMR δ: 3.13 (t, 2H, J = 6.0 Hz), 4.06 (s, 3H), 4.89 (t, 2H, J = 6.0 Hz), 6.15 (s, 2H), 6.20 (s, 2H), 7.07 (s, 1H, 13), 7.50–7.54 (m, 5H), 7.79 (s, 1H), 7.99 (d, 1H, J = 9.2 Hz), 8.19 (d, 1H, J = 9.2 Hz), 8.96 (s, 1H), 9.98 (s, 1H); HRMS: calcd for $C_{27}H_{22}NO_6I$ $(M-I)^+$ 456.1447, found 456.1438.

5.6. Biological methods

5.6.1. Cell culture

HepG2 cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% antibiotics at 37 °C with 5% CO_2. Twenty-four hours before drug treatment, the cells were trypsinized and grown in RPMI-1640 containing 10% lipoprotein-

deficient serum (LPDS, Sigma). When the cells reached to about 80% confluence, they were switched to the culture with a mediums of RPMI-1640 containing 0.5% LPDS and supplemented with BBR (Sigma) or M1–M4 at the concentrations indicated. The cells were treated for 12 h before harvest for RNA extraction.

5.6.2. RNA extraction and real-time RT-PCR

Total RNAs were isolated from cells or animal livers using the Ultraspec RNA lysis solution (Biotecxs Laboratory, Houston, TX) according to the supplier's protocol. For reverse transcription, 1 μg of sample RNA was used as template in a 20 μL reaction system (Promega, Madison, WI). The reverse transcription reactions were conducted at 42 °C for 30 min and then inactivated at 95 °C for 5 min. Real-time RT-PCR was performed with these cDNAs using the Applied Biosystems 7500 real-time RT-PCR System and the 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). In each of the 20 μL real-time RT-PCR reaction, 2 μL of cDNA, 10 μL of the Universal PCR Master Mix and 1 μL of the 20 × TaqMan Gene Expression Assay Reagent were used. Each experiment was repeated at least three times. The comparative threshold cycle (C_T) method was used in relative gene expression quantification with the GAPDH as an endogenous control.

5.6.3. Blood esterase hydrolysis rate for prodrugs of M1

Male Wistar rats (160 ± 10 g) were obtained from the Institute of Laboratory Animal Science (Beijing, China). Whole blood was taken from the rats into anti-coagulate tubes, then the resulting homogenate was centrifuged at 16,000*g* for 10 min at 4 °C. The plasma supernatant was stored at −20 °C prior to use.

The concentration of M1 released from prodrugs **5a**–**n** was examined in rat plasma. The reactions were initiated by adding 5 μL of stock solution of study prodrugs (0.2 M in methanol) to 500 μL of preheated plasma to give an initial concentration about 2×10^{-3} M. The reaction mixture was kept in a water bath at 37 ± 0.5 °C and aliquot samples (200 μL) were taken at 10 and 60 min and deproteinised with ice-cold acetonitrile (800 μL) respectively. The mixture was immediately vortexed and after centrifugation at 16,000*g* for 15 min at 4 °C, the supernatant was analyzed by LC–MS/MS for the concentration of M1.

5.6.4. Animal experiments

Male Sprague–Dawley rats (180 ± 10 g) were from the Institute of Laboratory Animal Science (Beijing, China). Animals were cared according to the institutional guidelines of the Chinese Academy of Medical Sciences. Animals were housed in an air-conditioned room with 3–5 rats/mice per cage, and with a 12 h-light and 12 h-dark cycle. After 1 week of accommodation period, all of the animals were fed with HFHC diet containing 2% cholesterol, 10% yolk powder, 15% lard and 0.2% of sodium cholate for 7 weeks. Five of the rats were fed with regular rodent chow serving as normal diet controls. The HFHC as well as the regular rodent chow was also from the Institute of Laboratory Animal Science (Beijing, China).

Hyperlipidemic rats were untreated ($n = 8$) or treated with 100 mg/kg/d of BBR ($n = 8$) or **5g** ($n = 8$) orally for 28 days. Study compounds were suspended in saline (1 mL/rat) prior to use. Compound BBR or **5g** was orally administered to the animals using gavage twice a day at 8 am and 5 pm, respectively. Before, after and during the treatment, blood samples were taken by posterior orbital venous plexus after fast. CHO, LDL-c levels as well as liver and kidney functions were assayed using commercially available kits.

5.6.5. Statistical analysis

The in vitro gene expression data were analyzed by the Student's *t*-test. For animal experiments, after validation of the test for homogeneity of variance, results were examined by one-way ANOVA followed by the Newman–Keuls test for multiple comparisons. $p < 0.05$ was considered as statistically significant.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (90913002) and National S&T Major Special Project on Major New Drug Innovation (2009ZX09301-003).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.106. These data include MOL files and InChiKeys of the most important compounds described in this article.

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