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Original Article

Structure-activity relationship of berberine derivatives for their glucose-lowering activities

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Abstract: Natural product berberine (BBR) has anti-diabetic activity, both in preclinical studies as well as in clinical trials. This study was designed to discover new BBR derivatives with better glucose-lowering efficacy. Thirty-five derivatives were selected from our derivative library and screened for their activities on glucose consumption in HL-7702 cells. Among these derivatives, compound 3e with amide bond and amidogen at 9-position showed an inspiring potency of 33.3-42.1% stronger than BBR when administered at an equal molar concentration. Compound 3f, a pro-drug of berberrubine (M1), which possessed an ester bond at 9-position, showed no activity on glucose consumption *in vitro*, although it had promising glucose-lowering activity in KK-Ay mice. The results suggest that the amide bond at 9-position is essential to maintain the activity of BBR on glucose consumption, the introduction of electron-donating groups such as amidogen at 9-position may be a viable strategy to enhance BBR's potency, and preparation of pro-drugs may be useful to enhance the *in vivo* glucose-lowering efficacies of BBR derivatives.

Keywords: Berberine, glucose consumption, hypoglycemic effect, type 2 diabetes mellitus, structure-activity relationship, pro-drugs

Introduction

Type 2 diabetes mellitus (T2DM) is the most common type of diabetes mellitus (DM) in clinic, its morbidity and mortality is increasing world wide [1]. For decades, despite numerous efforts, the treatment of T2DM and its complications is still a big challenge; the research and development of novel anti-diabetic drugs is of top priority. Berberine (BBR), an isoquinoline natural product extracted from *Coptis chinensis*, has been confirmed to have glucose-lowering and anti-diabetic efficacies, both in preclinical studies as well as in clinical trials [2, 3]. The detailed mechanisms for the glucose-lowering efficacy of BBR are controversial and not fully elucidated. It was reported that BBR could suppress the function of mitochondrial respiratory chain complex I, and then stimulate glycolysis and glucose consumption [4, 5].

However, BBR has a poor oral bioavailability (less than 10%) (6), which may limit its potency when used to treat T2DM. Therefore, a lot of efforts have been made to elevate the potency of BBR, and a BBR derivative library has been established in our laboratory [7-9]. In our previous studies, by using low-density lipoprotein receptor (LDLR) mRNA detection as a screening strategy, a BBR analogue (IMB-Y53) was discovered and showed better activity for LDLR up-regulation [7-9]. However, in the *in vitro* experiments, the activity of IMB-Y53 on glucose consumption was similar to that of BBR, although it had better glucose-lowering efficacy in diabetic animals due to improved pharmacokinetics [10].

To our knowledge, until now, there is no BBR derivative with an improved intrinsic bioactivity for glucose metabolism has been discovered. To resolve this issue, 35 derivatives were selected from our BBR derivative library and evaluated for their activities on glucose consumption. We found that compound 3e pos-

sessing amide bond and amidogen at 9-position had a stronger potency than BBR.

Materials and methods

Chemistry

All compounds were selected from our BBR derivative library. Using commercially available BBR metabolites (berberrubine (M1), thalifendine, jatrorrhizine) (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) as the starting materials, the target compounds (3a-3e) were prepared through a two-step process. The hydroxyl was first reacted with 3-(tert-butoxycarbonylamino) propyl bromide using K_2CO_3 as the base, compounds 3b, 3c and 3e were obtained. And then, compounds 3b and 3c were converted into 3a and 3d using trifluoroacetic acid (TFA) as the de-protection agent. Finally, the desired products were purified *via* flash column chromatography using methanol/dichloromethane as the gradient eluent. And compound 1a-1n, 2a-2k, 4a-4e and pro-drug 3f were designed and synthesized as previously described [8, 11-13].

Cell culture

HL-7702 human liver cells and L6 rat skeletal muscle cells were obtained from the Cell Culture Center of Peking Union Medical College (PUMC) (Beijing, China) or Shanghai Institutes for Biological Sciences (SIBS) of Chinese Academy of Sciences (CAS) (Shanghai, China). Cells were cultured under standard conditions with 10% fetal bovine serum (FBS) and appropriate antibiotics [6, 10], L6 cells were differentiated as described previously [10].

Glucose consumption

BBR and derivatives were dissolved in dimethyl sulphoxide (DMSO) to make stock solutions of a concentration of 10 mg/mL and stored at -20°C in aliquots. Cells were seeded onto 96-well plates with 2×10^4 cells per well and cultured for 24 hrs. Then, culture media were discarded and replaced with serum-free fresh media, and cells were subjected to serum starvation overnight.

The stock solutions of BBR and derivatives were thawed and diluted with serum-free media to desired concentrations and added to cells, with 4 replicate wells for each treatment. After 48 hrs' incubation, culture media were collected and centrifuged at 1000 rpm for 5 min. Glucose levels in the supernatants were assayed with a commercial kit (Beijing Strong Biotechnologies, Inc., Beijing, China) by the glucose oxidase method as previously described [10]. Glucose consumption was calculated as glucose level of the fresh medium minus glucose level of the cultured medium. For the calculation of glucose consumption-stimulation, glucose consumptions of cells treated with the studying compounds were used to minus that of cells treated with DMSO; the stimulating effects were normalized to that of BBR, which was designed as 1.

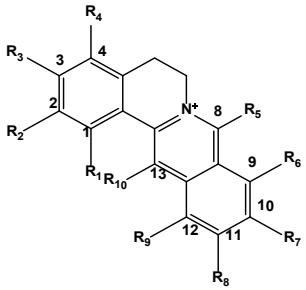
Western blot

After serum starvation and treated with BBR or compound 3e for 18 hrs, cells were rinsed with phosphate-buffered saline (PBS) and lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The lysates were subjected to 10% SDS-PAGE; protein bands were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) through a Wet Transfer Cell (Bio-Rad, Hercules, CA). For the detection of AMP-activated protein kinaseα (AMPKα) and phosphorylated-AMPKα (p-AMPKα) (Thr172) levels, membranes were probed with specific rabbit or mouse monoclonal antibodies (Cell Signaling Technology, Inc., Danvers, MA) with β-actin (ACTB) as an internal control. After incubation with horseradish peroxidase (HRP)-conjugated second antibodies, the bands were visualized with a Chemiluminescent HRP kit (Millipore, Billerica, MA). Gel-Pro Analyzer 4.0 Software was used to quantify the blot signals; the levels of p-AMPKα (Thr172) were normalized to those of AMPKα and plotted as fold of DMSO, which was designated as 1.

Animal experiment

The protocol of the animal experiment was reviewed and approved by the research committee of the Institute of Medicinal Biotechnology. Male KK-Ay (28±3 g) and C57BL/6J (C57) (20±2 g) mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS) (Beijing, China). Animals were cared and fed as described previously [10]. C57 mice were used as a normal control group (n=8). Diabetic KK-Ay

Table 1. Structures of BBR derivatives and their activities on glucose consumption



Code	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	Glucose consumption-stimulation (fold of BBR)[a]
BBR	H	OCH_2O		H	H	OCH_3	OCH_3	H	H	H	1
1a	H	OCH_2O		H	H	OC_3H_7	OC_3H_7	H	H	H	-0.32±0.12
1b	H	H	OH	H	H	OCH_3	OCH_3	H	H	H	0.21±0.05
1c	H	H	OCH_2Ph	H	H	OCH_3	OCH_3	H	H	H	0.24±0.05
1d	H	OH	OCH_3	H	H	OCH_3	OCH_3	H	H	H	-0.09±0.04
1e	H	OCH_2Ph	OCH_3	H	H	OCH_3	OCH_3	H	H	H	0.01±0.02
1f	OCH_3	OCH_3	OCH_3	H	H	OCH_3	OCH_3	H	H	H	0.55±0.13
1g	H	H	OH	H	H	H	OCH_3	OCH_3	H	H	0.18±0.04
1h	H	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	H	H	H	0.35±0.08
1i	H	OCH_3	OH	H	H	H	OCH_3	OCH_3	H	H	-0.15±0.09
1j	H	OH	OCH_3	H	H	H	OCH_3	OCH_3	H	H	0.09±0.08
1k	H	OCH_3	OCH_3	H	H	H	OCH_3	OCH_3	H	H	0.004±0.04
1l	H	OCH_3	OCH_3	H	H	H	OH	H	H	H	-0.13±0.13
1m	H	OCH_3	OH	H	H	OH	OCH_3	H	H	H	-0.13±0.06
1n	H	OCH_3	OH	H	H	H	OCH_3	OCH_3	OCH_3	H	0.07±0.06
2a	H	OCH_2O		H	H	H	OCH_3	OC_2H_5	H	H	0.24±0.05
2b	H	OCH_2O		H	H	H	OCH_3	H	H	H	0.12±0.05
2c	H	OCH_2O		H	H	H	OCH_2CH_2O	H	H	H	0.6±0.05
2d	H	OCH_2O		H	H	Cl	OCH_3	OCH_3	H	H	0.15±0.06
2e	H	OCH_2O		H	H	OH	OCH_3	H	Br	H	0.4±0.04
2f	H	OCH_2O		H	H	F	OCH_3	H	H	H	-0.01±0.05
2g	H	OC_2H_5	OCH_3	H	H	OCH_3	OCH_3	H	H	H	-0.03±0.07
2h	H	OC_2H_5	OCH_3	H	H	H	OCH_3	OCH_3	H	H	0.03±0.06
2i	H	OCH_3	OC_2H_5	H	H	OCH_3	OCH_3	H	H	H	-0.09±0.06
2j	H	OCH_3	OC_2H_5	H	H	H	OCH_3	OCH_3	H	H	-0.1±0.07
2k	H	H	OCH_3	OCH_3	H	H	OCH_3	OCH_3	H	H	0.1±0.05
3a	H	OCH_2O		H	H	H	OAN[2]	H	H	H	0.14±0.07
3b	H	OCH_2O		H	H	H	OANB[1]	H	H	H	0.18±0.06
3c	H	OANB[1]	OCH_3	H	H	H	OCH_3	H	H	H	0.23±0.06
3d	H	OAN[2]	OCH_3	H	H	H	OCH_3	H	H	H	0.17±0.05
3e	H	OCH_2O		H	H	OANB[1]	OCH_3	H	H	H	0.99±0.11
3f	H	OCH_2O		H	H	PA[3]	OCH_3	H	H	H	0.04±0.01
4a	H	OCH_2O		H	Ph-CH_3-*4*	OCH_3	OCH_3	H	H	H	0.22±0.05
4b	H	OCH_2O		H	Ph-CF_3-*3,5*	OCH_3	OCH_3	H	H	H	0.65±0.06
4c	H	OCH_2O		H	H	OCH_3	OCH_3	H	H	n-C_8H_{17}	-0.4±0.07
4d	H	OCH_2O		H	H	OCH_3	OCH_3	H	H	n-C_9H_{19}	-0.36±0.06
4e	H	OCH_2O		H	H	OCH_3	OCH_3	H	H	n-$C_{10}H_{21}$	-0.4±0.05

[1]OANB = $O(CH_2)_3NHBOC$; [2]OAN= $O(CH_2)_3NH_2$; [3]PA = $OCO(CH_2)_{14}CH_3$. [a]HL-7702 cells were left untreated or treated with BBR or its derivatives at 10 μg/mL for 48 hrs. Values are means ± S.D. of 3 repeated experiments and are presented as fold of BBR-treated cells, which is defined as 1.

mice were left untreated or orally administered with BBR or pro-drug 3f at 100 mg/kg/day, respectively, with 8 mice in each group. Body weights and food intakes of the animals were

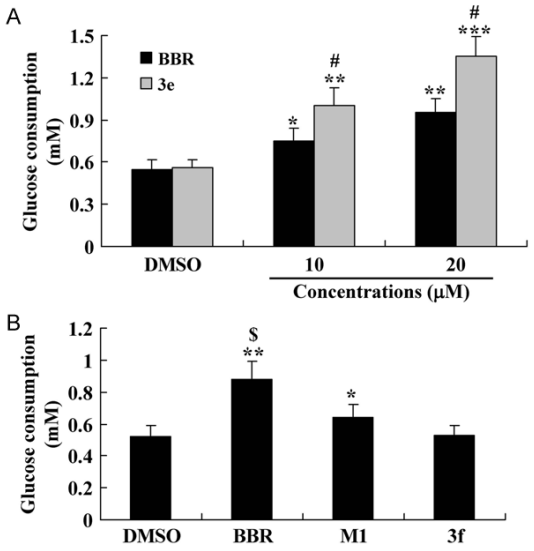


Figure 1. Influences of the studying compounds on glucose consumption in HL-7702 cells. After serum starvation, (A) cells were treated with DMSO, BBR or 3e at indicated concentrations; (B) cells were treated with DMSO, 20 μM of BBR or M1, or 40 μM of 3f, respectively. After 48 hrs of incubation, cellular glucose consumptions were calculated as described in the Materials and methods. Values are means ± S.D. of 3 repeated experiments; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. that of DMSO, $^{\#}P<0.05$ *vs*. that of BBR, $^{\$}P<0.05$ *vs*. that of M1.

measured every other day. After 15 days of treatment and overnight fast, blood samples were harvested by retro-orbital puncture and animals were sacrificed; the fasting blood glucose (FBG) levels were measured by using a commercially available kit.

Statistical analysis

The *in vitro* data were analyzed by the two-tailed unpaired Student's *t*-test. For the animal experiment, 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 to be statistically significant.

Results

Structure-activity relationship (SAR) of BBR derivatives on glucose consumption

Thirty-five BBR derivatives were examined for their activities on glucose consumption in HL-7702 cells. As shown in **Table 1**, when treated at an equal mass concentration (10 μg/mL), compound 3e modified at 9-position had a glucose consumption-stimulating activity similar to that of BBR. Interestingly, compound 3e and 3b had a BOC (tert-butoxycarbonyl) protected 1-amino-n-propyl at the 9- and 10-position in their structures, respectively. But the activity of 3b on cellular glucose consumption was only 0.18±0.06-fold of that of BBR (**Table 1**).

The molecular weight of 3e (515.0) was bigger than BBR (371.8), which implied that when used at a same molecular concentration, 3e should have stronger potency than BBR. To prove that, HL-7702 cells were treated with 3e and BBR at 10 or 20 μM, respectively, and the glucose consumption was determined. When compared to the DMSO control ($P<0.01$ or $P<0.001$), 3e promoted glucose consumption in a dose-dependent manner in HL-7702 cells (**Figure 1A**). And as expected, the potency of 3e increased averagely by 33.3% and 42.1% as compared to BBR at 10 or 20 μM, respectively ($P<0.05$) (**Figure 1A**). Similar results were obtained when equal molar concentrations of 3e and BBR were used to treat L6 skeletal muscle cells (data not shown).

As BBR could activate AMPK, which was a key molecule for cellular energy balance [2, 5, 10, 11], we determined the influence of 3e on AMPK activity by western blot. The results proved that 20 μM of 3e treatment for 18 hrs promoted the activation of AMPK greatly in HL-7702 cells (**Figure 2**), as indicated by the significant up-regulation of p-AMPKα (Thr172) ($P<0.001$ *vs*. DMSO). And when administered at an equal molar concentration, the level of p-AMPKα (Thr172) in 3e-treated cells was averagely 1.47-fold of that of BBR ($P<0.05$) (**Figure 2**). Similar results were obtained in L6 cells (data not shown).

The benefits of the introduction of a lipophilic group at 9-position reminded us of a recently reported compound named 3f, possessing a palmitate (PA) at the 9-position instead [12], which was comparable to the structure of 3e. It was hypothesized that compound 3f with lipophilic group at 9-position should have better glucose-lowering efficacy as compared to BBR. Surprisingly, the results showed that 3f did not have any glucose consumption-stimulating activity (**Table 1**), even when its concentration reached 40 μM (**Figure 1B**).

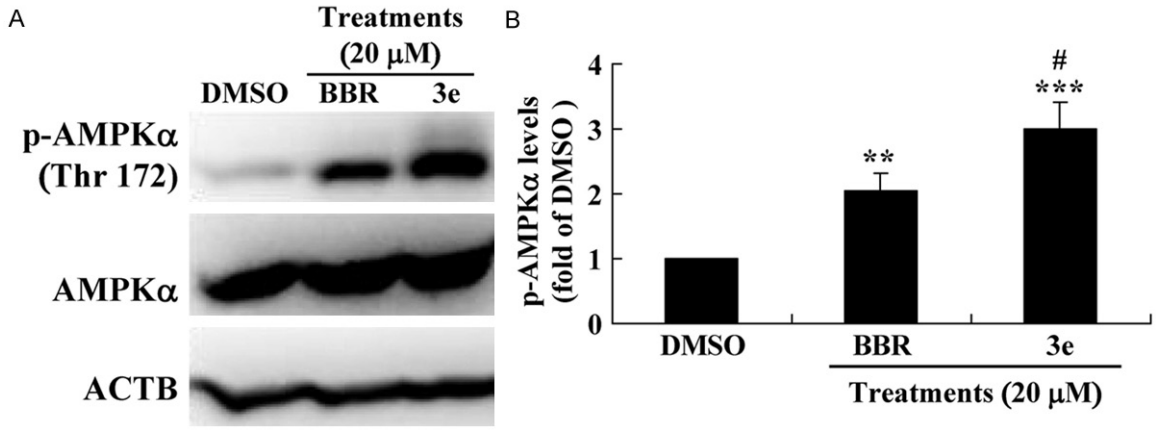


Figure 2. Stimulating activities of BBR and 3e on AMPK. HL-7702 cells were treated with DMSO, BBR or 3e for 18 hrs, p-AMPKα (Thr 172), AMPKα and ACTB protein levels were determined by western blot. Representative blots were presented in (A); the levels of p-AMPKα (Thr 172) were normalized to those of AMPKα and plotted as fold of DMSO (B). The values in (B) were means ± S.D. of 3 repeated experiments; $^{**}P<0.01$, $^{***}P<0.001$ *vs*. that of DMSO, $^{\#}P<0.05$ *vs*. that of BBR.

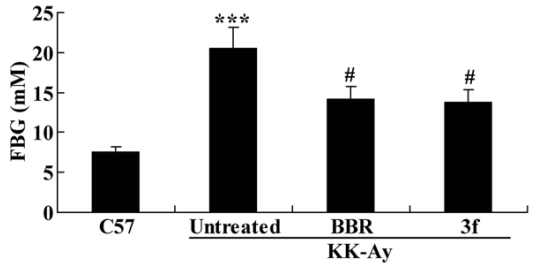


Figure 3. Glucose-lowering efficacies of BBR and 3f in KK-Ay mice. Diabetic KK-Ay mice were left untreated or treated with BBR or 3f at 100 mg/kg/day for 15 day, with C57 mice as a normal control. At the end of the experiment, mice were sacrificed and FBG levels were determined. Values were means ± S.D. of 8 mice in each group; $^{***}P<0.001$ *vs*. that of C57, $^{\#}P<0.05$ *vs*. that of untreated KK-Ay mice.

According to the SAR analysis on glucose consumption, 4 of the derivatives (1f, 1h, 2c and 2e) which bearing 9-,10-dimethoxy, 10-,11-ethylenedioxy and 9-hydroxyl, 10-methoxy fragments on ring D exhibited ideal activities. Introducing appropriate substituents such as 2-,3-methylenedioxy on ring A helped to improve the activity (2c and 2e) (**Table 1**).

Meanwhile, we focused on the modifications of the substituents on position 8 and 13. The large lipophilic groups including 3-,5-(di-trifloromethyl) phenyl was introduced into the position 8 (4b). The glucose consumption-stimulating activity of this compound approached about 65% of that of BBR (**Table 1**), which suggested that large group at position 8 might be helpful for the maintenance of the activity. However, the derivatives with lipophilic substituents at position 13 (4c, 4d and 4e) lost their activities and showed toxicities regardless of the size of the side-chains.

Pro-drug 3f shows promising glucose-lowering efficacy in diabetic mice

Compound 3f was actually a pro-drug of M1 [12], one of BBR metabolites *in vivo* [6]. In our *in vitro* experiments, while 3f was not efficacious, 20 μM of M1 showed a modest but statistically significant stimulating activity on the cellular glucose consumption ($P<0.05$ *vs*. DMSO) (**Figure 1B**), which was in agree with previous reports [6, 11, 14].

The glucose-lowering potency of 3f was examined for the first time in the KK-Ay diabetic mice. The results (**Figure 3**) showed that after 15 days' of treatment, 100 mg/kg/day of 3f lowered the FBG level of the mice significantly (32.7% reduction, $P<0.05$ *vs*. untreated KK-Ay mice). The efficacy of 3f was comparable to that of BBR at a same dose (30.7% reduction) (**Figure 3**). There were no statistical differences on the body weight and food intake among the studying groups of animals (data not shown).

Discussion

In the present study, we determine the glucose-lowering activities of BBR derivatives both *in*

vitro and *in vivo* and conduct SAR analysis. To our knowledge, this is the first time that the SAR of BBR derivatives on glucose consumption has been reported.

Compound 3e had a potent stimulating activity on glucose consumption in our experiments. As far as we know, 3e is the first derivative screened *in vitro* which has a better glucose-lowering effect than BBR itself; we are now synthesizing more amount of 3e which will be used in diabetic animals to evaluate its efficacy *in vivo*.

Compound 3e and 3b had a same lipophilic group in different positions, and their activities on cellular glucose consumption were significantly different. The results indicated that it was helpful to introducing appropriate substituent at the 9-position rather than 10-position, in order to retain the activity of BBR derivatives. In addition, the amidogen at 9-position of 3e was an electron-donating group, indicating that the introduction of electron-donating groups at 9-position might help to increase the potency of BBR on glucose consumption.

In addition to promote glucose metabolism, 3e could stimulate cellular AMPK greatly. Although AMPK might not be involved in the activity of BBR on glucose consumption [5], it was crucial for BBR to inhibit gluconeogenesis and stimulate triglyceride (TG) metabolism [15]. The potent stimulating activity of 3e on AMPK implies that it may also influence gluconeogenesis and TG metabolism, which merits further investigation. It was reported that BBR promoted glucose consumption by stimulating glycolysis [5], whether or not 3e act through the same manner needs further investigation.

Although 3f had a lipophilic group at 9-position, it did not stimulate glucose consumption *in vitro*. 3f had an ester bond at 9-position instead of amide bond as 3e and BBR did, which suggested that the amide bond at 9-position was crucial for the maintenance of the glucose consumption-stimulating activity of BBR and its derivatives.

Pro-drugs were usually effective after the promoiety (PA for 3f) was removed by blood esterases and transformed to their active entities (M1 for 3f) *in vivo*, so as 3f [12]. As reported previously, compound 3f showed a moderate cLog P, hydrolysis rate and a good safety *in vivo* [12]. Considering that in cultured cells, the potency of M1 on glucose consumption was less than that of BBR ($P<0.05$) (**Figure 1B**), but in diabetic mice, 3f showed a comparable glucose-lowering potency, these results suggested that introducing lipophilic groups to 9-position might help to increase the absorption of BBR derivatives.

In summary, through SAR analysis, the amide bond at 9-position is essential to maintain the stimulating activity of BBR on glucose consumption, and the introduction of electron-donating groups such as amidogen at 9-position may be useful to enhance BBR's potency. 3e has improved bioactivity than BBR on stimulating glucose consumption; it may become a promising compound for the development of novel agents for the treatment of T2DM in the future. In addition, preparation of pro-drugs may be useful to enhance the *in vivo* glucose-lowering efficacies of BBR derivatives.

Acknowledgements

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Disclosure of conflict of interest

None.

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