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8,8-Dimethyldihydroberberine with improved bioavailability and oral efficacy on obese and diabetic mouse models

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ABSTRACT

The clinical use of the natural alkaloid berberine (BBR) as an antidiabetic reagent is limited by its poor bioavailability. Our previous work demonstrated that dihydroberberine (dhBBR) has enhanced bioavailability and in vivo efficacy compared with berberine. Here we synthesized the 8,8-dimethyldihydroberberine (Di-Me) with improved stability, and bioavailability over dhBBR. Similar to BBR and dhBBR, Di-Me inhibited mitochondria respiration, increased AMP:ATP ratio, activated AMPK and stimulated glucose uptake in L6 myotubes. In diet-induced obese (DIO) mice, Di-Me counteracted the increased adiposity, tissue triglyceride accumulation and insulin resistance, and improved glucose tolerance at a dosage of 15 mg/kg. Administered to *db/db* mice with a dosage of 50 mg/kg, Di-Me effectively reduced random fed and fasting blood glucose, improved glucose tolerance, alleviated insulin resistance and reduced plasma triglycerides, with better efficacy than dhBBR at the same dosage. Our work highlights the importance of dihydroberberine analogs as potential therapeutic reagents for type 2 diabetes treatment.

1. Introduction

The isoquinoline alkaloid berberine (Fig. 1A) is the main active constituent of Rhizoma coptidis, a traditional Chinese herb for the medication of diabetes with a history that can be traced to as early as A.D. 500.[1] The beneficial metabolic effects of berberine have been reported by several groups in clinical studies with type 2 diabetes mellitus patients since 1988.[2–5] Improved glucose and lipid utility and increased insulin sensitivity were observed on several diabetic rodent models treated with berberine, for example, *ob/ob* mice,[6] *db/db* mice,[6,7] diet-induced obese rats,[7–9] and alloxan-[8,10] or streptozotocin-[11–13] induced diabetic rats.

Several mechanisms were proposed to explain the anti-hyperglycemic effects of berberine. Berberine inhibits α-glucosidase, an enzyme that digests polysaccharides and disaccharides into monomers, leading to reduced intestinal absorption of carbohydrates.[14,15] Besides, berberine suppresses adipocyte differentiation probably through PPARγ and C/EBPα inhibition,[16] and reduces cholesterol through elevating the mRNA level of low-density lipoprotein receptor.[17] In HepG2 cells, berberine inhibits lipid synthesis[18] and enhances glucose consumption in an insulin-independent manner.[19] In 2006, we reported that in L6 myotubes, berberine stimulates glucose uptake by activating AMP-activated protein kinase (AMPK), a fuel gauge protein sensitive to cellular energy status, through raising cellular AMP:ATP ratio.[20] Similar results were later demonstrated by several other groups on C2C12 myotubes[9] and 3T3-L1 adipocytes.[21,22] In the mechanism investigations, we proved that the inhibition of complex I in the mitochondrial respiration chain[23] is a key process for the activation of AMPK by berberine. AMPK activation results in increased glucose absorption by peripheral tissues via promoting GLUT4 translocation,[24] repressed transcription of gluconeogenesis enzyme genes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase,[25] and reduced fatty acid and cholesterol synthesis by inhibiting ACC[26] and HMG-CoA reductase.[27] Among these in vitro schemes for berberine's antidiabetic mechanism, AMPK activation is proved to contribute at least partially to the anti-hyperglycemic and anti-lipidemic properties of berberine in vivo.[6,7]

Nevertheless, the disadvantage of poor bioavailability[28] made high dosage (100–560 mg/kg day) necessary for berberine to yield its efficacy in vivo.[7,8,11,13] In DIO rats, 125 mg/kg twice a day was necessary for berberine to reduce random fed and fasting blood glucose,[9] and 380 mg/kg/day was required for berberine to reduce plasma triglyceride and relieve insulin resistance.[7] For *db/db* mice, 560 mg/kg berberine was administered to reduce blood glucose and triglyceride and relieve glucose tolerance.[7] P-glycoprotein

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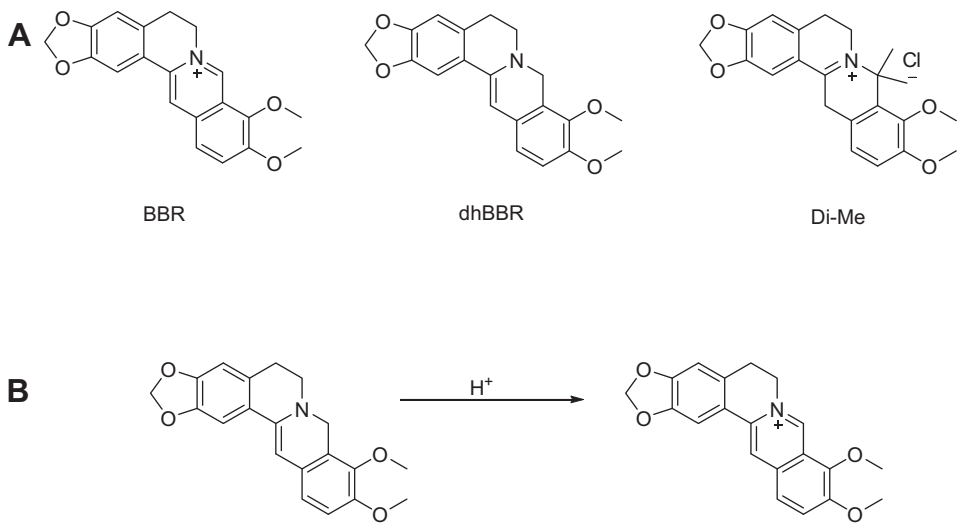


Figure 1. (A) Structures of BBR, dhBBR, and Di-Me. (B) dhBBR converts into BBR via acid-catalyzed aromatization.

inhibitors[29] and sodium caprate[30] were administered together with berberine on rodent models to improve its intestinal absorption. Although berberine analogs were synthesized and evaluated by several groups[23,31–35] in order to find more potent derivatives, efforts aiming to improve its oral absorption and pharmacokinetic profile are relatively few. In our previous work, we designed a number of berberine derivatives and found that dhBBR (Fig. 1A) had markedly improved in vivo efficacy in the treatment of insulin-resistant rodents.[23] In this regard, we previously developed a series of berberine derivatives, and found that dhBBR preserved the ability to activate AMPK and had a higher bioavailability of 2.65% compared to berberine which was not detectable in the plasma.[23] In DIO mouse and rat models, dhBBR effectively counteracted the increased insulin resistance and triglyceride accumulation in adipose tissue at a much lower dosage (100 mg/kg/day). Interestingly, a majority (90%) of absorbed drug form in rat plasma was berberine,[23] suggesting that dhBBR may undergo acid-catalyzed aromatization after absorption. Actually, dhBBR easily converted to BBR in acid condition in the in vitro stability study (Data not shown). This may be also the reason why the bioavailability of dhBBR is limited since certain part of dhBBR may convert back to BBR in the stomach (Fig. 1B) and which hinders its the in vivo absorption.

Here we proposed to block aromatization of dhBBR by 8,8-disubstitution with alkyl groups to further optimize dhBBR into the more aqueous soluble and acid stable 8,8-dialkyldihydroberberine hydrochlorides.[36] 8,8-Dimethyl-13,13a-dihydroberberine (Di-Me) (Fig. 1A) was identified as a promising compound. With improved aqueous solubility and acid stability, it has significantly higher bioavailability and is not converted back to berberine in vivo. Similar to BBR and dhBBR, Di-Me potently activates AMPK pathway in a mitochondria-oriented manner, with beneficial metabolic effects in L6 myotubes. Furthermore, Di-Me improves glucose and lipid metabolism in diet-induced obese (DIO) mice at a relatively low dosage, and behaves more effectively than dhBBR in *db/db* mice. Our work highlights the potential importance of dihydroberberine analogs as new candidates for type 2 diabetes therapy.

2. Results and discussion

2.1. Chemistry

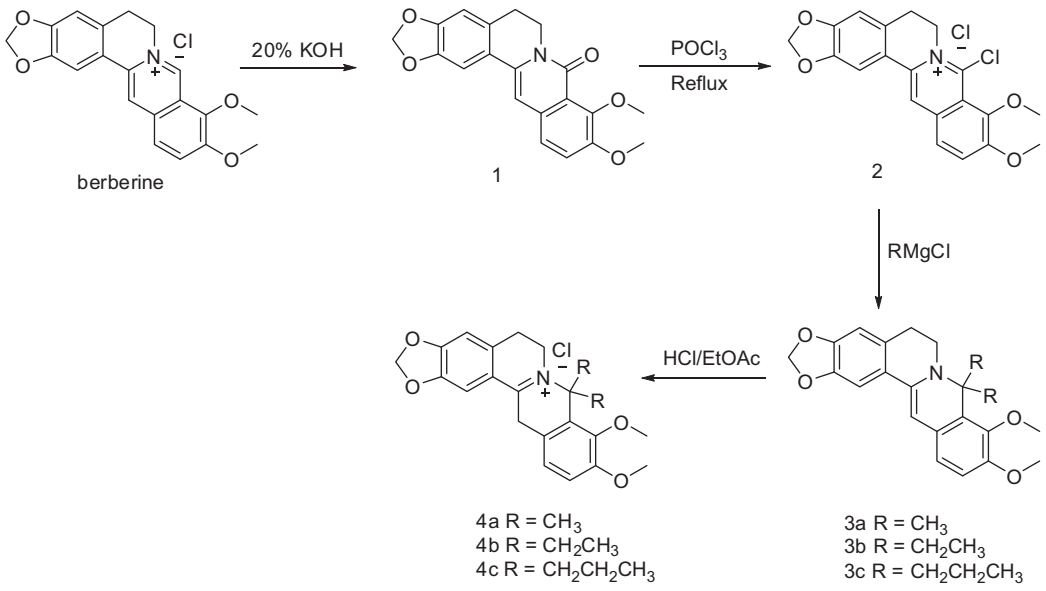
The synthesis of the products **4a–c** is depicted in Scheme 1. The lactam **1** was prepared by refluxing of berberine with 20% solution of KOH. Treatment of **1** in the presence of $POCl_3$ at 110 °C for 2 h afforded 8-chloroberberine **2**, which was reacted with different Grignard reagents to yield the 8,8-dialkyldihydroberberines **3a–c**. Finally, salification of the **3a–c** with the solution of hydrochloric acid in EtOAc provided the products **4a–c**.

2.2. Active dhBBR derivatives identified

Our previous study[23] suggests that dhBBR is readily transformed into BBR after oral administration via acid-catalyzed aromatization (Fig. 1B) under the acid conditions in the stomach. To prove this hypothesis, we designed and synthesized three 8,8-dialkyldihydroberberine derivatives (Scheme 1) in order to improve the chemical stability of dhBBR, and tested their cellular efficacy on L6 myotubes. As shown in Figure 2A, from 2.5 to 10 μM range, Di-Me induced a stronger dose-dependent AMPK/ACC phosphorylation than Di-Pr, while the effect of Di-Et was the strongest. Di-Me dose-dependently stimulated glucose uptake (37–85%) similarly with Di-Pr (63–62%), while the promotion effect of Di-Et declined (Fig. 2B). While Di-Me and Di-Pr had almost no cell toxicity at up to 40 μM after 24 h treatment, Di-Et was obviously toxic at as low as 10 μM (Fig. 2C). So Di-Me was selected for further pharmacological analysis.

2.3. Di-Me activates AMPK and promotes glucose uptake via inhibition of complex I of mitochondria respiration chain

Thus, the effects of Di-Me on L6 myotubes were investigated in details. In 1 h, Di-Me dose-dependently increased AMPK/ACC phosphorylation from 1.25 to 10 μM (Fig. 3A). This effect reached climax in as short as 10 min, and sustained for at least 3 h (Fig. 3B). In 1 h, Di-Me dose-dependently enhanced glucose uptake from 2.5 (48%) to 20 μM (67%, Fig. 3C), and the stimulation was observed from 15 min (28%) to 2 h (84%) (Fig. 3D). The enhanced glucose transport was effectively abolished by a selective AMPK inhibitor compound C[37] but not PI-3 kinase inhibitor wortmannin (Fig. 3E). In previous studies, BBR and dhBBR activated AMPK by increasing cellular AMP:ATP ratio through blocking complex I of the mitochondria respiration chain.[20,23] Acute dose-dependent increase of intracellular AMP:ATP ratio by Di-Me (2.5–10 μM) was observed after 1 h incubation in L6 myotubes (Fig. 3F). In isolated rat liver mitochondria, Di-Me dose-dependently inhibited respiration (2.5–40 μM) by up to 40% with pyruvate and malate as



Scheme 1. Synthesis of 8,8-dialkyldihydroberberine hydrochloride.

substrates (Fig. 3G). These results indicate that Di-Me activates AMPK through a similar mitochondria-oriented mechanism with BBR[9,20,23,38–40] and dhBBR,[23] suggesting Di-Me a good candidate for oral administration with diabetic animal models.

2.4. Di-Me displays higher aqueous solubility, stability, and bioavailability compared to dhBBR

Next, we examined the stability of Di-Me and dhBBR under acid condition (pH 2). In 6 h, the content of dhBBR significantly declined by 17%, while Di-Me stably kept its structure (Fig. 4A). Moreover, Di-Me had a better aqueous solubility (81 mg/mL) over dhBBR (<1 mg/mL). Orally administered at 10 mg/kg to SD-rats, Di-Me was rapidly detected in the plasma at 15 min (Fig. 4B), displaying a half-life ($t_{1/2}$) of 2.9 ± 0.2 h, and a maximum concentration (C_{max}) of 17.8 ± 4.4 ng/mL. The bioavailability of Di-Me was 10.03%, much higher than dhBBR (2.65%, administered at 20 mg/kg[23]). The plasma form of absorbed Di-Me was mostly its original structure. No detectable BBR or dhBBR was found in plasma. These results proved that 8,8-substituted methyl groups protect dhBBR from aromatization and ameliorate its in vivo absorption. Moreover, the in vivo stability of Di-Me increases the reliability of in vitro cellular assays to understand its pharmacological mechanism.

2.5. Di-Me displays improved oral efficacy on DIO-mouse model

In obesity, adipose tissue releases increased amounts of hormones, inflammatory cytokines and non-esterified fatty acid that may lead to the development of insulin resistance.[41] With maintained cellular activity and more efficient oral absorption, Di-Me was evaluated on DIO mice with a dosage of 15 mg/kg by daily gavage for 6 weeks. During the whole process, body weight and food intake change of the Di-Me group were observed with no significant difference with the vehicle group. In ipGTT test, metformin decreased blood glucose at 60 and 120 min time points, and Di-Me significantly improved glucose tolerance at 30 and 120 min time points (Fig. 5A), decreasing the area under curve by 15% (Fig. 5B). In ITT test, Di-Me alleviated insulin resistance at 60 and 90 min time points (Fig. 5C). After 6-week treatment, the plasma triglyceride level declined for 26% (Fig. 5E, 33% for metformin), the liver triglyceride reduced for 36% (Fig. 5F), the subcutaneous fat proportion reduced for 36% (Fig. 5G) and the plasma insulin decreased for 45% (Fig. 5H, 43% for metformin). These results demonstrate that Di-Me exert beneficial effects on DIO mice at a dosage much lower than dhBBR.[23] Notably, these effects were accompanied with significantly decreased plasma insulin level, indicating the alleviation of insulin-resistant state. The reduced plasma glucose levels may also be attributed to the possible augmentation of glucose absorption in peripheral tissues by Di-Me.

2.6. Di-Me displays improved oral efficacy on *db*/*db*-mouse model

The diabetic symptom of *db*/*db* mice is characterized by increased body weight, blood glucose, plasma insulin, plasma TG, etc.[42] We evaluated both dhBBR and Di-Me on *db*/*db* mice at the same dosage of 50 mg/kg. During the whole process, body weight and food intake change of both dhBBR and Di-Me groups were observed with no significant difference with the vehicle group. At 3 weeks, dhBBR significantly reduced random fed blood glucose (by 25%) and Di-Me achieved a similar result (by 24%). At 4 weeks, the decrease scope in random fed blood glucose shrunk for dhBBR (by 18%), but the effects of Di-Me was much more potent (by 40%) (Fig. 6A). dhBBR decreased fasting blood glucose (FBG) by 17% at 3 weeks, but fluctuated at 4 weeks. In contrast, Di-Me more potently reduced FBG at 3 weeks (24%) and the reduction sustained at 4 weeks (24%). The positive control metformin decreased FBG at 5 weeks (Fig. 6B). While dhBBR failed to alleviate glucose tolerance capacity, Di-Me significantly improved glucose tolerance at 0, 15, 90, and 120 min, decreasing the area under curve by 16% (Fig. 6C and D). dhBBR, Di-Me and metformin alleviated insulin resistance in ITT at multiple time points (Fig. 6E), while Di-Me behaved more effectively, and potently reduced the area under curve (28% for Di-Me, 12% for dhBBR, and 14% for metformin, Fig. 6F). Furthermore, Di-Me greatly reduced plasma TG level by 38% while dhBBR was ineffective (Fig. 6G, 23% for metformin). After 6 weeks, we sacrificed the mice and found that dhBBR reduced liver weight by 11% and increased subcutaneous fat by 14%, while Di-Me did not alter the ratio of liver or fat to body weight. These results indicate that Di-Me is more potent than dhBBR on *db*/*db* mice. This dosage is obviously lower than the effective oral dosage of BBR reported by previous study with *db*/*db* mice (560 mg/kg).

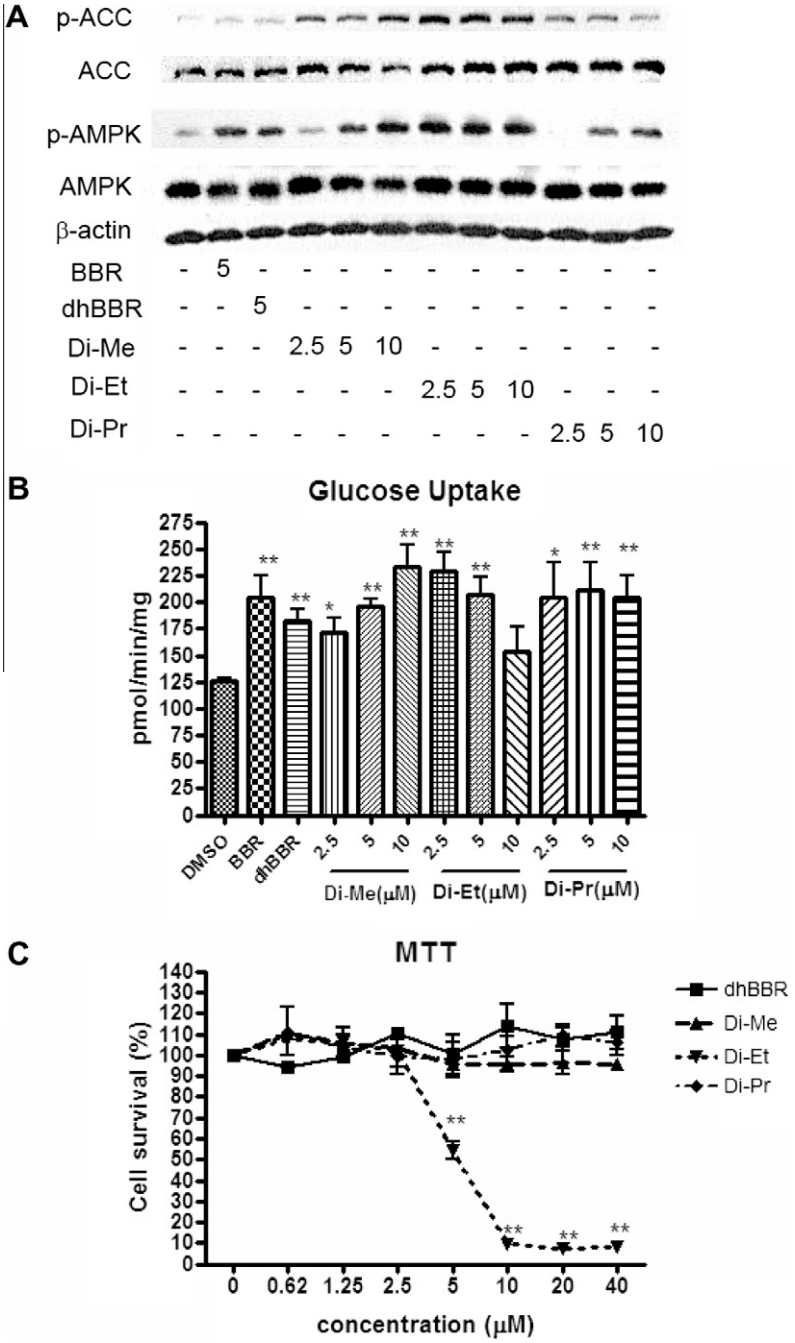


Figure 2. Comparison of Di-Me, Di-Et, and Di-Pr. Fully differentiated L6 myotubes were serum-starved for 2 h before various treatments. (A) Effects of BBR, dhBBR, Di-Me, and Di-Et on AMPK and ACC phosphorylation in L6 myotubes after 1 h incubation. (B) Effects of BBR, dhBBR, Di-Me, and Di-Et on glucose uptake in L6 myotubes after 1 h incubation. (C) Cell toxicity of dhBBR, Di-Me, and Di-Et. Data are the mean results of three independent experiments ± SE ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with DMSO group.

Interestingly, unlike BBR, Di-Me did not alter the adipose tissue mass or body weight of *db/db* mice, suggesting that the peripheral glucose absorption might be crucial for the anti-hyperglycemic effects of Di-Me.

3. Conclusion

By further structural modification, we found Di-Me, a more aqueous soluble and chemical stable dhBBR derivative. The beneficial effects on both obese and diabetic mouse models indicate that the improved aqueous solubility, stability and bioavailability, at least partially, enhanced oral efficacy of Di-Me in vivo. Our work suggests that dihydroberberine analogs are an attractive direction for development of potential therapeutic reagents for the treatment of type 2 diabetes and metabolic syndrome.

4. Experimental

4.1. General experimental procedure

^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian Mercury-VX300 Fourier transform spectrometer. The

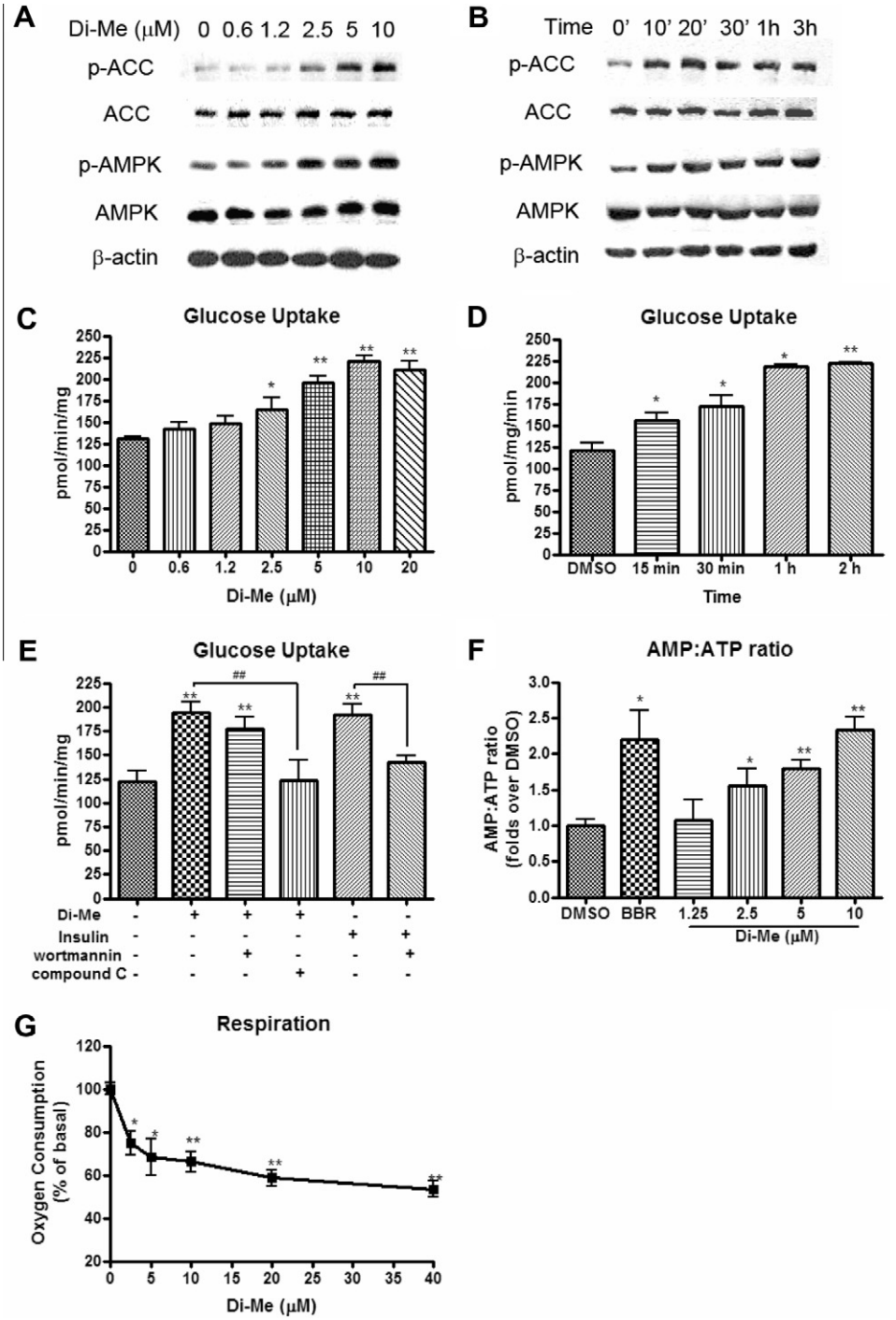


Figure 3. Effects of Di-Me on AMPK activation and glucose uptake in L6 myotubes. Fully differentiated L6 myotubes were serum-starved for 2 h before various treatments with Di-Me. (A) Di-Me dose-dependently raised AMPK and ACC phosphorylation in L6 myotubes after 1 h incubation. (B) Di-Me (10 μM) time-dependently stimulates AMPK and ACC phosphorylation in L6 myotubes during 3 h incubation. (C) Di-Me dose-dependently promotes glucose uptake in L6 myotubes after 1 h treatment. (D) Di-Me time-dependently promotes glucose uptake in L6 myotubes within 2 h. (E) Effects of wortmannin (100 nM) and compound C (10 μM) on glucose uptake induced by Di-Me (10 μM) and insulin (100 nM) in L6 myotubes. (F) After 1 h incubation with increasing doses of Di-Me, intracellular AMP, ADP, and ATP levels were measured and AMP:ATP ratio was calculated. Berberine (5 μM) was employed as a positive control. (G) Effect of Di-Me on oxygen consumption of mitochondria isolated from rat liver. Data are the mean results of three independent experiments ± SE (n = 5–7). $^{\#\#}P < 0.01$ between groups; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with DMSO group.

chemical shifts were reported in δ (ppm) using the δ 7.26 signal of $CDCl_3$ (^{1}H NMR), the δ 77.23 signal of $CDCl_3$ (^{13}C NMR), the δ 2.50 signal of DMSO-d_6 (^{1}H NMR), the δ 39.51 signal of DMSO-d_6 (^{13}C NMR), the δ 4.80 signal of D_2O (^{1}H NMR) as internal standards. HR-ESI-MS was run on a Bruker Atex III spectrometer in CH_3CN, respectively. All commercially available reagents were used without further purification. The solvents used were all AR grade and were redistilled under positive pressure of dry nitrogen atmosphere in the presence of proper desiccant when necessary. The progress of the reactions was monitored by analytical thin-layer chromatography (TLC) on $HSGF_{254}$ precoated silica gel plates.

Western blotting detection kits [enhanced chemiluminescence (ECL)] were purchased from Amersham Biosciences (Uppsala, Sweden). Radiochemical 2-deoxy-[^{3}H]-D-glucose was purchased

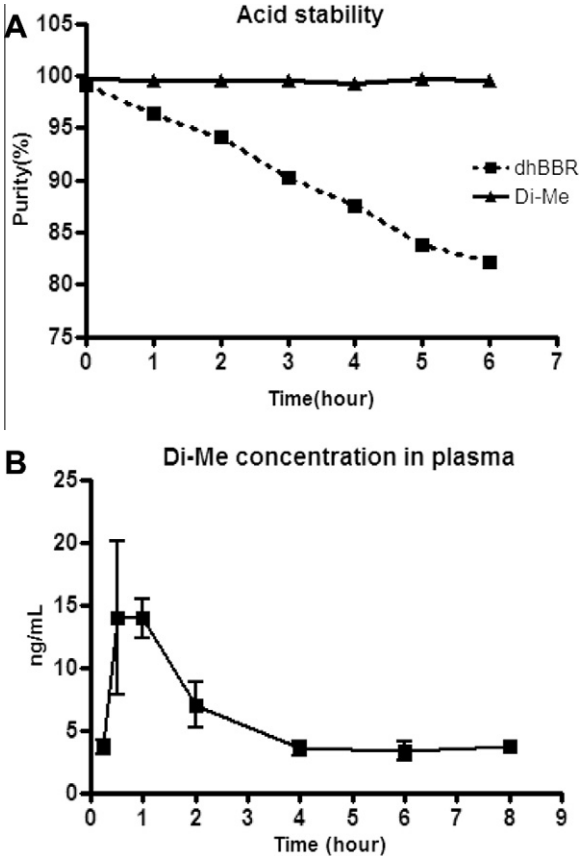


Figure 4. Stability and bioavailability of Di-Me. (A) Comparison of the purity of the DHBBR and Di-Me under acid conditions (pH 2). (B) Pharmacokinetics of Di-Me. Sprague–Dawley rats were gavaged with Di-Me, and blood samples were collected at 0 (pre-dosing), 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h post administration. Di-Me, BBR, and dhBBR concentrations were determined. The absolute bioavailability reaches 10.03%.

from PerkinElmer (Waltham, USA). Compound C were from Calbiochem and wortmannin were from Sigma. Anti-AMPKα, anti-acetylcoenzyme A carboxylase (ACC), antiphospho-ACC (Ser79), antiphospho-AMPK (Thr172) primary antibodies, antimouse, and antirabbit IgG HRP-linked antibodies were from Cell Signal Technology. Rodent diet with 60 kcal% fat (D12492i) was from Research diets, Inc.

L6 cell culture and differentiation, Western blot, glucose uptake, AMP:ATP ratio tests and mitochondrial were conducted as previously described.[20]

4.2. Preparation of 8,8-dialkyldihydroberberine

4.2.1. Preparation of lactam (1)

Berberine (370 mg, 1.0 mmol) was dissolved in 10 mL of the solution of 20% KOH and refluxed for 8 h. The mixture was then cooled to room temperature and extracted with EtOAc (3× 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give the crude product, which was purified using silica gel column chromatography ($CHCl_3$/MeOH = 50:1) to give the lactam (**1**) (210 mg, 59%).

IR (KBr) V_{max} 1641; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.30 (d, J = 8.7 Hz, 1H, H-11), 7.26 (d, J = 8.7 Hz, 1H, H-12), 7.19 (s, 1H, H-1), 6.70 (s, 1H, H-4), 6.68 (s, 1H, H-13), 5.98 (s, 2H, –OCH_2O–), 4.27 (t, J = 6.0 Hz, 2H, H-6), 3.99 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 2.89 (t, J = 6.0 Hz, 2H, H-5); ^{13}C NMR (75 Hz) δ: 160.1, 151.4, 149.4, 148.4, 147.3, 135.6, 132.3, 130.0, 123.7, 122.4, 119.3, 118.8, 107.9, 104.7, 101.5, 101.3, 61.6, 56.8, 39.3, 28.7; ESIMS m/z 352.2 ([M+H]$^+$).

4.2.2. Preparation of the 8-chloroberberine (2)

The lactam (**1**) (5.0 g, 14 mmol) was dissolved in $POCl_3$ and refluxed for 2 h. The mixture was then cooled to room temperature and filtrated. The precipitate was washed with $CHCl_3$ and Et_2O successively to give crude (**2**) (4.5 g, 78%), which was used for the next step without further purification.

4.2.3. Preparation of the hydrochloride of 8,8-dimethyldihydroberberine (4a)

The 8-chloroberberine (**2**) (1 g, 2.47 mmol) was dissolved in anhydrous Et_2O (15 mL), to the suspension were added slowly 5 mL (15 mmol) of MeMgCl (3.0 M in THF) under Ar. When the addition was completed, the mixture was refluxed for 1 h, the mixture was then allowed to room temperature. The reaction was quenched with saturated solution of NH_4Cl at 0 °C, extracted with dichloromethane, the organic layers were basified with aqueous ammonia, dried over anhydrous Na_2SO_4, concentrated under reduced pressure to give the crude product, which was purified using silica gel column chromatography (petroleum ether/ethyl acetate = 30: 1) to give the compound 8,8-dimethyldihydroberberine (**3a**).

The 8,8-dimethyldihydroberberine (**3a**), gained from the above reaction, was dissolved in EtOAc immediately, the solution of hydrochloric acid in EtOAc was then added dropwise. When the addition was completed, the resulting mixture was stirred for 1 h and filtrated, the precipitate was washed with EtOAc and dried in high vacuum to give the compound (**4a**), the hydrochloride of 8,8-dimethyldihydroberberine (500 mg, 46%).

^{1}H NMR (DMSO-d_6, 300 MHz) δ: 7.78 (s, 1H), 7.20 (d, J = 8.7 Hz, 1H), 7.17 (s, 1H), 7.02 (d, J = 8.7 Hz, 1H), 6.25 (s, 2H), 4.69 (s, 2H), 4.15 (t, J = 6.9 Hz, 2H), 3.90 (s, 3H), 3.85 (s, 3H), 3.06 (t, J = 6.9 Hz, 2H), 1.92 (s, 6H); ^{13}C NMR (75 Hz) δ: 169.9, 153.5, 151.9, 147.3, 144.6, 136.0, 128.8, 122.4, 120.5, 117.3, 114.0, 108.9, 107.8, 103.0, 66.9, 60.5, 56.1, 45.7, 32.0, 26.2, 25.7; HRESIMS m/z 366.1707 (Calcd for $C_{22}H_{24}NO_4$, 366.1700).

4.2.4. 8,8-Diethyldihydroberberine (4b)

The 8,8-diethyldihydroberberine (**4b**) and 8,8-dipropyldihydroberberine (**4c**) were prepared with the similar method.

8,8-Diethyldihydroberberine (**4b**): ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 7.78 (s, 1H), 7.23 (d, J = 8.4 Hz, 1H), 7.21 (s, 1H), 7.06 (d, J = 8.4 Hz, 1H), 6.26 (s, 2H), 4.89 (s, 2H), 4.13 (t, J = 6.9 Hz, 2H), 3.85 (s, 3H), 3.84 (s, 3H), 3.06 (t, J = 6.9 Hz, 2H), 2.54 (m, 2H), 2.30 (m, 2H), 0.57 (t, J = 6.9 Hz, 6H); ^{13}C NMR (75 Hz) δ: 172.9, 154.1, 151.8, 147.4, 143.8, 136.4, 123.8, 122.6, 120.0, 119.7, 114.2, 109.2, 107.9, 103.1, 76.5, 60.2, 56.0, 44.2, 31.9, 28.6, 25.9, 8.6; HRESIMS m/z 394.2020 (Calcd for $C_{24}H_{28}NO_4$, 394.2013).

4.2.5. 8,8-Dipropyldihydroberberine (4c)

8,8-Dipropyldihydroberberine (**4c**): ^{1}H NMR (D_2O, 300 MHz) δ: 7.48 (s, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.10 (d, J = 8.4 Hz, 1H), 6.97 (s, 1H), 6.15 (s, 2H), 4.14 (t, J = 6.9 Hz 2H), 3.91 (s, 3H), 3.90 (s, 3H), 3.11 (t, J = 6.9 Hz, 2H), 2.56 (m, 2H), 2.22 (m, 2H), 1.03 (m, 2H), 0.84 (m, 2H), 0.79 (t, J = 6.6 Hz, 3H), 0.77 (t, J = 6.6 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 Hz) δ: 171.9, 155.4, 152.5, 148.6, 144.3, 136.5, 125.0, 123.4, 119.8, 118.1, 113.9, 109.2, 108.5, 103.3, 76.4, 60.6, 56.0, 45.7, 38.6, 32.9, 26.8, 18.1, 13.8; HRESIMS m/z 422.2334 (Calcd for $C_{26}H_{32}NO_4$, 422.2326).

4.3. Aqueous solubility measurements

The solubility of Di-Me in distilled H_2O was determined from undersaturation by adding excess solid phase in water. The suspension was stirred with magnetic stirrers for 24 h at constant temperature (25 ± 0.5 °C) maintained with a water bath. The

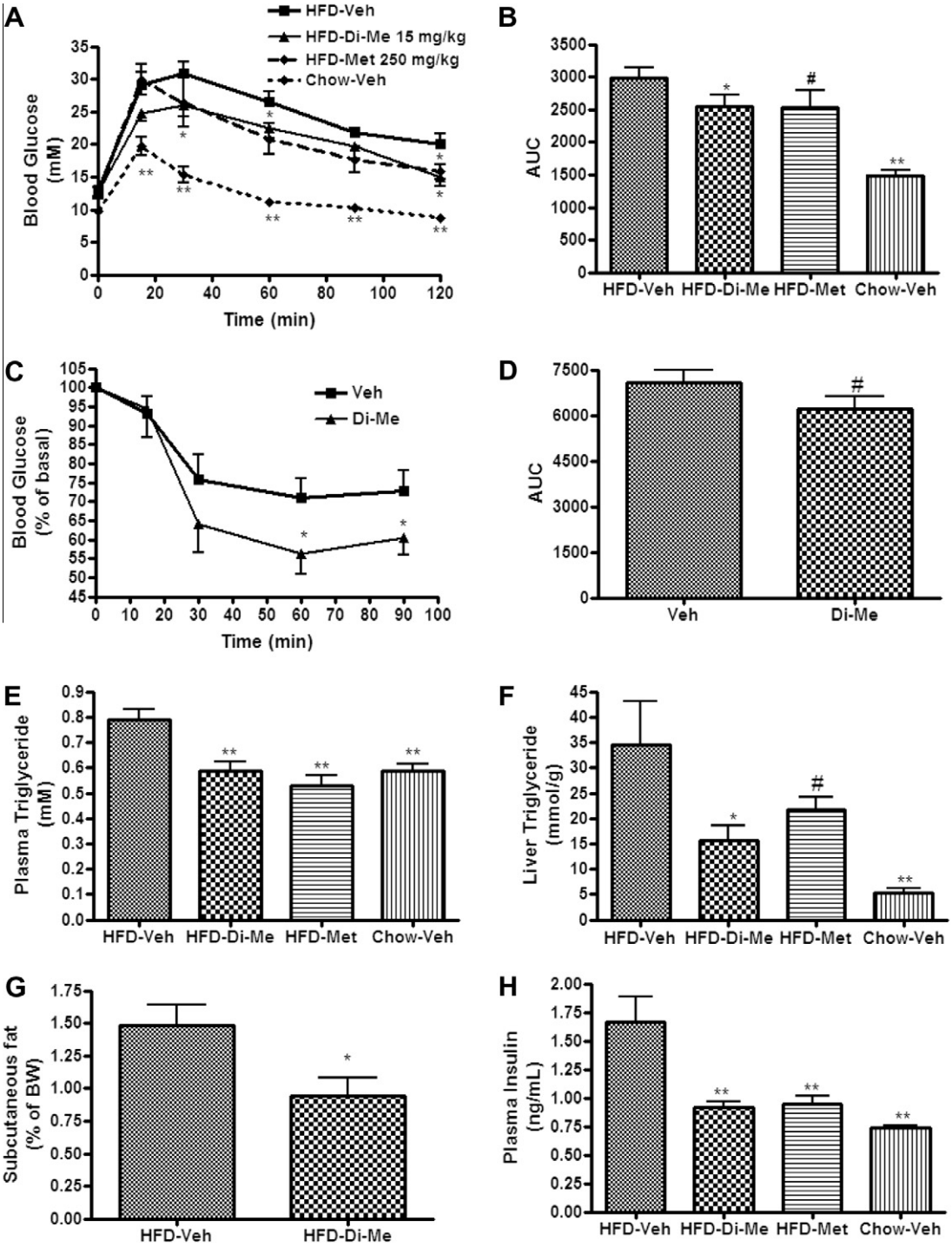


Figure 5. Oral efficacy of Di-Me during 6-week treatment with DIO mice with metformin as positive control. (A) Blood glucose levels in the intraperitoneal glucose tolerance test (ipGTT) after 4 weeks of treatment. (B) Area under curve for the ipGTT after 4 weeks of treatment. (C) Blood glucose levels in the insulin tolerance test (ITT) after 4 weeks of treatment. (D) Area under curve for ITT after 4 weeks of treatment. (E) Plasma triglyceride content after 4-week treatment. (F) Liver triglyceride content after 6-week treatment. (G) Subcutaneous fat mass expressed as a percentage of body mass after 6-week treatment. (H) Plasma insulin content after 4-week treatment. Data are the mean results ± SE (n = 5–8). $^{\#}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle group.

resulting solution was clarified through filter. Concentration was measured by HPLC.

4.4. Stability determination

The solution of the dhBBR (0.5 mg/mL) in acetonitrile, which its pH value was adjusted to 2 with H_3PO_4, was analyzed by HPLC, using a 5 μm TEDAChrom. Kromasil C18 250 × 4.6 mm column, the chromatogram was recorded at 320 nm. The temperature of the column oven was set at 25 °C. The elution was 58% acetonitrile in the solution of 0.05 mol/L aqueous KH_2PO_4 and 0.005 mol/L SDS. The solution of the Di-Me (0.5 mg/mL) in acetonitrile, which its pH value was adjusted to 2 with H_3PO_4, was analyzed by HPLC, using a 5 μm TEDAChrom. Kromasil C18 250 × 4.6 mm column. The

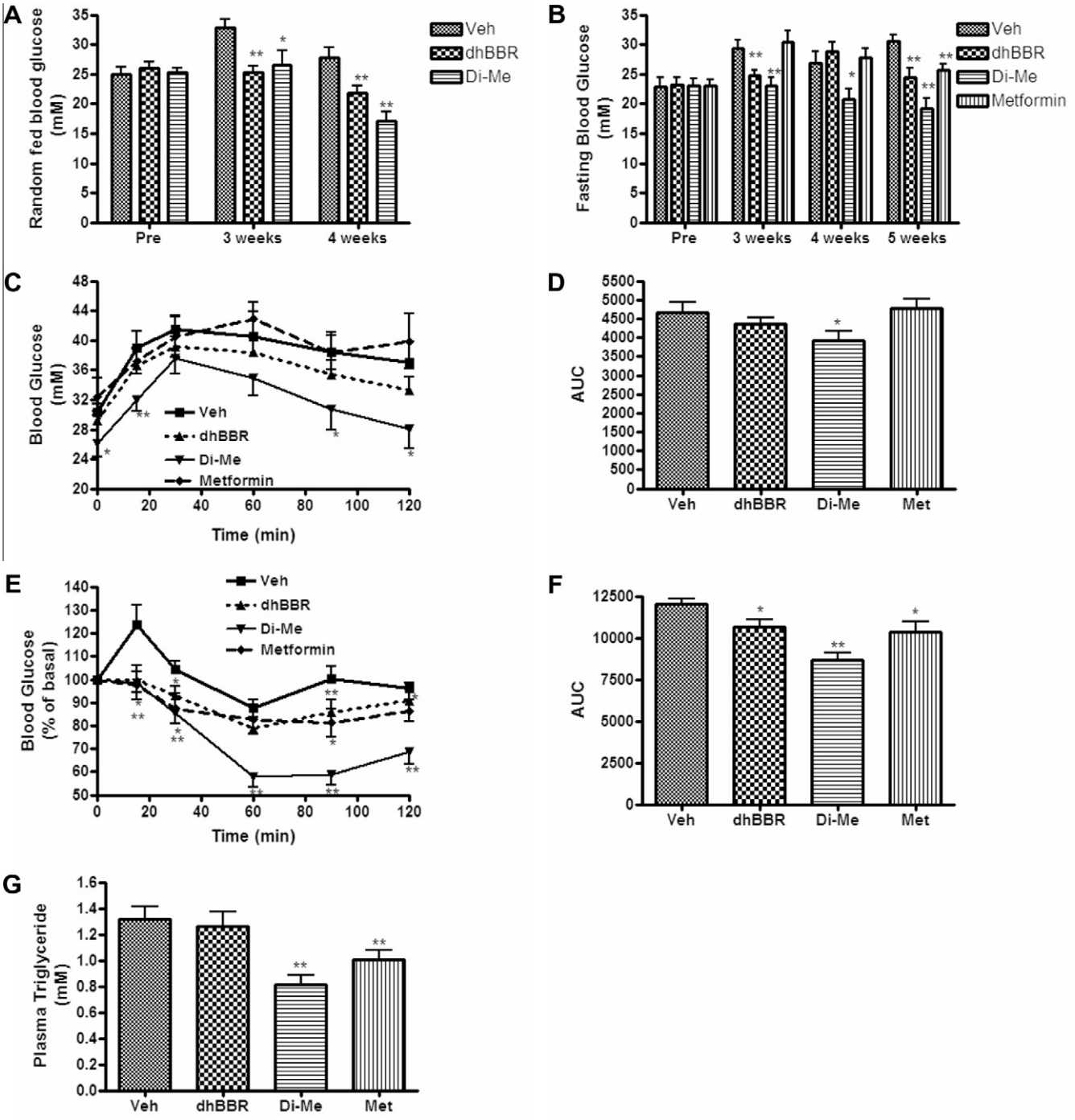


Figure 6. Oral efficacy of Di-Me and dhBBR during 6-week treatment with *db*/*db* mice. C57BLKS/J-*db*/*db* male mice was subjected to 50 mg/kg dhBBR or Di-Me, and positive metformin treatment for 6 weeks as described in Section 4. (A) Random fed blood glucose levels after 3 and 4 weeks of dhBBR or Di-Me treatment. (B) Fasting blood glucose levels after 3 and 4 weeks of treatment. (C) Blood glucose levels in the intraperitoneal glucose tolerance test (ipGTT) after 3 weeks of treatment. (D) Area under curve for the ipGTT after 3 weeks of treatment. (E) Blood glucose levels in the insulin tolerance test (ITT) after 4 weeks of treatment. (F) Area under curve for ITT after 4 weeks of treatment. (G) Plasma triglyceride content after 5-week treatment. Data are presented as mean ± SE (n = 9–12). $^{*}P < 0.05$, $^{**}P < 0.01$.

elution of the Di-Me was 50% acetonitrile in the solution of 0.05 mol/L aqueous KH_2PO_4 and 0.005 mol/L SDS. Both of the flow rates was 1 mL/min. An auto injector was used to inject 5 μL of the test solution into the HPLC system.

4.5. Pharmacokinetic analysis

After 12 h fasting, male Sprague–Dawley rats were gavaged with 10 mg/kg Di-Me, and blood samples were collected from retro-orbital puncture into tubes with heparin anticoagulant at 0 (pre-dosing), 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h post administration. Plasma concentrations of berberine analogs were determined by LC–MS/MS.[43]

4.6. Cytotoxicity assay

L6 myoblast cells were cultured into 96-well plates. After differentiation, a range of concentrations of compounds were added, and

the plate was incubated at 37 °C for 24 h. Then MTT was added to a final concentration of 5 mg/mL. After 3 h incubation, the medium was removed and 100 μL DMSO was added. The absorbance at 550 nm was measured with a reference wavelength of 690 nm.

4.7. Mitochondria isolation and respiration measurements

Mitochondria were isolated from rat liver with a protocol reported previously.[44] The respiration medium contained: 225 mM mannitol, 75 mM sucrose, 10 mM Tris–HCl, 10 mM KH_2PO_4, 10 mM KCl, 0.8 mM $MgCl_2$, 0.1 mM EDTA, 0.3% fatty acid free BSA, pH 7.0, and the respiratory control ratios (state III/state IV respiration) determined with 5 mM pyruvate as substrate, were approximately 5, indicating well coupled mitochondria. Dose–response effects of the different compounds on mitochondrial respiration were determined in the presence of excess ADP (2.4 mM), using substrate combinations targeting Complex I (5 mM pyruvate plus 2 mM malate) of the respiratory chain.

4.8. Animal studies

All animal experiments were approved by the Animal Ethics Committee of the Shanghai Institute of Materia Medica.

Male C57BL/6J mice at the age of 6 weeks were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. After 8 weeks of high fat feeding, mice were randomized into various treatment groups by body weight and fed glucose levels. Treatment groups were as follows: DIO vehicle (0.5% microcrystalline cellulose and 1.6% lactose), Di-Me (15 mg/kg), metformin (250 mg/kg) as positive control and chow fed vehicle. Mice were orally administered, body weights and food intake were measured once daily. Intraperitoneal glucose tolerance test (ipGTT) was performed by intraperitoneal injection of D-glucose (2 g/kg). For the insulin tolerance test (ITT), mice were fasted for 6 hr, then injected intraperitoneally with 0.75 U insulin/kg body weight. Glucose levels were measured at 0, 15, 30, 60, 90, and 120 min. Both the ipGTT and the ITT tests were carried out at 4 weeks post administration. After 6 weeks of treatment, mice were sacrificed after a final dose and tissues were collected for further analysis.

C57BLKS/J-*db*/*db* mice (50% male, 50% female) were introduced from Jackson Laboratories. At the age of 6 weeks, *db*/*db* mice were randomized into the various treatment groups by body weight and fed glucose levels. Treatment groups were as follows: *db*/*db* vehicle (0.5% microcrystalline cellulose, 1.6% lactose), dhBBR (50 mg/kg), Di-Me (50 mg/kg), and metformin (250 mg/kg as positive control). Mice were orally administered once daily. Body weights and food intake were measured daily, and random fed blood glucose and fasting blood glucose (FBG) were determined once a week. ITT and ipGTT tests were performed as described above at 3 weeks and 4 weeks, respectively. After 6 weeks of treatment, mice were sacrificed after a final dose and tissues were collected for further analysis.

4.9. Metabolite analysis

Blood glucose was determined with a free-style blood glucose monitoring system (Abbott Laboratories). Plasma and liver triglycerides were determined with a triglyceride assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Plasma insulin was determined by ELISA (Linco Research, St. Louis, MO).

4.10. Statistical analysis

One-way ANOVA followed by Student's *t*-tests were applied for multiple comparisons. All data are expressed as means ± SEM. The significance threshold employed was at a level of 5% ($P < 0.05$).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.085. These data include MOL files and InChiKeys of the most important compounds described in this article.

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