SHORT COMMUNICATION

Antihyperuricemic effect of mangiferin aglycon derivative J99745 by inhibiting xanthine oxidase activity and urate transporter 1 expression in mice

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Abstract A mangiferin aglycon derivative J99745 has been identified as a potent xanthine oxidase (XOD) inhibitor by previous in vitro study. This study aimed to evaluate the hypouricemic effects of J99745 in experimental hyperuricemia mice, and explore the underlying mechanisms. Mice were orally administered 600 mg/kg xanthine once daily for 7 days and intraperitoneally injected 250 mg/kg oxonic acid on the 7th day to induce hyperuricemia. Meanwhile, J99745 (3, 10, and 30 mg/kg), allopurinol (20 mg/kg) or benzbromarone (20 mg/kg) were orally administered to mice for 7 days. On the 7th day, uric acid and creatinine in serum and urine, blood urea nitrogen (BUN), malondialdehyde (MDA) content and XOD activities in serum and liver were determined. Morphological changes in kidney were observed using hematoxylin and eosin (H&E) staining. Hepatic XOD, renal urate transporter 1 (URAT1), glucose transporter type 9 (GLUT9), organic anion transporter 1 (OAT1) and ATP-binding cassette transporter G2 (ABCG2) were detected by Western blot and real time polymerase chain reaction (PCR). The results showed that J99745 at doses of 10 and 30 mg/kg significantly reduced serum urate, and enhanced fractional excretion of uric acid (FEUA). H&E staining confirmed that J99745 provided greater nephroprotective effects than allopurinol and benzbromarone. Moreover, serum and hepatic XOD activities and renal URAT1 expression declined in J99745-treated hyperuricemia mice. In consistence with the ability to inhibit XOD, J99745 lowered serum MDA content in hyperuricemia mice. Our results...
suggest that J99745 exerts urate-lowering effect by inhibiting XOD activity and URAT1 expression, thus representing a promising candidate as an anti-hyperuricemia agent.

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1. Introduction

Hyperuricemia characteristic with excessive uric acid in blood leads to gout, and increases the risk of cardiovascular diseases, metabolic disorder and chronic renal disease1,2. It is widely regarded relevant to the progress and prognosis of gout, nephrosis and cardiovascular diseases. Adequate control of hyperuricemia contributes to prevention and treatment of these diseases3.

Uric acid is a product of the metabolic breakdown of purine nucleotides. The enzyme xanthine oxidase (XOD) catalyzes the oxidation of hypoxanthine or xanthine to uric acid. The renal excretion of uric acid is mainly dependent on the kidney urate transport system including glomerular filtration, as well as reabsorption into and secretion from proximal tubular cells. Urate transporter 1 (URAT1) located at the luminal membrane and glucose transporter type 9 (GLUT9) at the apical membranes of the renal proximal tubules constitute the main pathway of urate reabsorption in the kidney4,5. However, 90% of urate reabsorption is obtained through renal URAT1. Moreover, renal organic anion transporter 1 (OAT1) localized in the basolateral membrane and ATP-binding cassette transporter G2 (ABCG2) located at the apical membrane have been identified as main secretory urate transporters6,7.

Currently, there are a few urate-lowering drugs in clinical use while most of them have severe side effects or are insensitive to kidney function impairment8,9. XOD inhibitors and uricosuric drugs are two major therapies to reduce blood urate levels. Allopurinol and febuxostat are two major XOD inhibitors used

Figure 1

Chemical structure of J99745 (A) and its effect–concentration curve on XOD activity and superoxide anion scavenging in vitro (D). The effect–concentration curves of allopurinol (B) and TBHq (C) are also showed. Data are mean ± SD (n = 3). XOD, xanthine oxidase; TBHq, tert-butylhydroquinone.
clinically. Both drugs must be administered at an initial low dose and are not suitable for patients with kidney function impairment. On the other hand, renal damage is a main side effect of uricosuric drugs including benzibromarone and probenecid13. A new uricosuric named lesinurad was approved by U. S. Food and Drug Administration (FDA) in 2015. It is a specific URAT1 inhibitor which promotes urate excretion, but not recommended for patients with moderate to severe renal impairment either. Therefore, the discovery and development of a novel urate-lowering drug with better effect and safety profiles is still in urgent demand.

Mangiferin is a natural glucosyl xanthone enriched in the leaves of Mangifera indica L. and has been previously identified with a variety of potential pharmacological actions including antimicrobial and antioxidant activities, improving glycometabolism and lipid metabolism, and anti-diabetic effects14–15. Moreover, some studies showed that mangiferin exerted potent hypouricemic effects through modulation of uric acid production and excretion16–18. J99745 is a synthesized derivative from mangiferin aglycon (Fig. 1A) and has been identified as a potent XOD inhibitor by screening over 40,000 compounds using the method described previously17. The present study aimed to evaluate the urate-lowering effects of J99745 and explore the underlying mechanisms in experimental hyperuricemia mice.

2. Materials and methods

2.1. Chemicals and reagents

J99745 (purity > 95%, by HPLC) was synthesized by Department of New Drug Research & Development, Institute of Materia Medica (Beijing, China), and determined by electronic spray ion-mass spectrometry (ESI-MS) and 1H NMR spectroscopy (Supplementary Information Fig. S1). m.p.: > 250 °C, ESI-MS (m/z): 351.31 [M+H]+. 1H NMR (400 MHz, DMSO-d6) δ: 13.03 (s, 1 H), 10.89 (s, 1 H), 9.83 (s, 1 H), 7.62 (t, J = 4 Hz, 1 H), 7.45 (m, 2 H), 7.42 (s, 1 H), 7.31 (3, 1 H), 7.27 (t, J = 4 Hz, 1 H), 6.34 (s, 1 H), 6.17 (s, 1 H), 5.23 (s, 2 H).

XOD (SLBB1570V), xanthine, oxonic acid, allopurinol, tert-butylhydroquinone (TBHQ, 112941) and benzobromarone were purchased from Sigma–Aldrich, St. Louis, USA. The XOD assay kit (A002), uric acid (UA) assay kit (C012), malondialdehyde (MDA) assay kit, blood urea nitrogen (BUN) assay kit (C013-2), and creatinine assay kit (C001-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Water-soluble tetrazolium (WST-1) (2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulphophenyl]-2H-tetrazolium monosodium salt, EQ. 756) was purchased from Dojindo Laboratory (Kumamoto, Japan).

2.2. Animals

All the animal experiments were carried out in accordance with the Institutional Guidelines for the Care of Laboratory Animals of the Chinese Academy of Medical Science, and were approved by the Institutional Ethics Committee for Research on Laboratory Animal Use. Healthy male Kunming strain mice of SPF grade, weighing 18–22 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (approval number SCXK (BJ) 2012-0001) and housed in air-conditioned room at 25 ± 2 °C on a 12 h light/dark cycle with free access to food and tap water.

2.3. Dual assay of xanthine oxidase activity and superoxide anion scavenging

The dual assay targeting XOD activity and superoxide anion scavenging ability was carried out according to the method described previously19. Briefly, WST-1 works as the probe for the superoxide anion. The superoxide anion (O2•−) accompanying with generation of uric acid reacts with WST-1 to form formazan, which has maximum absorption at the wavelength of 450 nm, indicating the level of O2•− in the assay system. Uric acid (which has maximum absorption at the wavelength of 295 nm) works as an indicator of xanthine oxidase activity. The 50 μL reaction system is composed of 4 U/L XOD, 250 μmol/L xanthine, 100 μmol/L WST-1, and the test compounds at various concentration in the reaction buffer (0.1 mol/L sodium phosphate, 0.3 mmol/L ethylene diamine tetra acetate acid (EDTA), pH 8.3). All reaction components were mixed in 384-well UV plate (Corning, USA). At last, XOD was added to initiate the reaction. The optical density at 295 nm (OD295) of the reaction system was monitored dynamically for 15 min at 37 °C with microplate reader SpectraMax M5, and the optical density at 450 nm (OD450) was just recorded once at the end of dynamic study. The initial velocity was calculated from the slope of the OD295-time curve which reflects the activity of XOD enzyme, and the slope of the OD450-time curve represents the level of O2•−. The effect-concentration curve was obtained and the IC50 value of each compound was calculated. XOD inhibitor allopurinol and antioxidant TBHQ were used as references. By comparing the IC50 value, the compound could be determined to scavenge O2•− or not20. If the IC50 value of one compound on XOD inhibition is equal to the IC50 value on the level of O2•−, this compound could be regarded as just an XOD inhibitor. If the IC50 value of one compound on XOD inhibition is higher than the IC50 value on the level of O2•−, the compound could be thought to possess the ability to scavenge O2•− in addition to XOD inhibition.

2.4. Induction of hyperuricemia and drug administration

Hyperuricemia was induced in mice by xanthine and oxonic acid according to the previous studies with some modifications18,19. Male mice were randomly allocated into 7 groups. The normal group was only treated orally with vehicle (0.5% carboxymethyl cellulose sodium, CMC-Na). In other groups, xanthine (600 mg/kg) was administered by gavage once daily for 7 days followed by single intraperitoneal injection of oxonic acid (250 mg/kg) to induce hyperuricemia. The hyperuricemia group received vehicle in addition to xanthine and oxonic acid treatment. J99745 (3, 10, and 30 mg/kg) group received hyperuricemia induction and oral administration of 3, 10, and 30 mg/kg J99745, respectively. Allo (20 mg/kg) group received hyperuricemia induction and oral administration of 20 mg/kg allopurinol. Benz (20 mg/kg) group received hyperuricemia induction and oral administration of 20 mg/kg benzobromarone. J99745, allopurinol and benzobromarone were dissolved in 0.5% CMC-Na and administered 30 min after xanthine administration each day during the experiment. The dosage of J99745 was set at doses of 3, 10, and 30 mg/kg according to the preliminary studies. On the 7th day, single intraperitoneal injection of oxonic acid (250 mg/kg) was administered immediately after the last dosage (Supplementary Information Fig. S2). Two hours later, animals were euthanized to collect blood and tissues.
2.5. Urine, blood, and tissues collection

On the 6th day, the mice were transferred to metabolic cages to collect 24 h urine samples for each mouse. Urine samples were then centrifuged at 2000 × g for 10 min to obtain the supernatant for uric acid and creatinine analysis. Whole blood samples were collected after final administration on the 7th day and centrifuged at 10,000 × g for 5 min to obtain the serum. Simultaneously, liver tissues were excised and stored at −80 °C until analysis. Kidney tissues were quickly and carefully dissected on an ice plate and some parts were immediately fixed for H&E staining. Other parts were stored at −80 °C for PCR and Western blot analysis.

2.6. Determination of levels of uric acid, creatinine, MDA, and XOD activities

Uric acid levels in serum (Sur) and urine (Uur), creatinine levels in serum (Scr) and urine (Ucr), as well as BUN were determined using commercially available kits according to the manufacturers’ instructions. Fractional excretion of uric acid (FEUA) was calculated...
as follows: FEUA (%) = \frac{\text{Uur/Scr}}{\text{Ucr/Scr}} \times 100^{22,23}.
Serum MDA content was determined using commercially available colorimetric kits according to the manufacturer's instruction\(^24,25\). Hepatic and serum XOD activities were determined using commercially available colorimetric kits according to the manufacturer's instruction\(^26\).

**2.7. Histopathological examination**

Renal tissues were rapidly fixed with 4% paraformaldehyde, and then embedded in paraffin and sectioned at 5 μm for H&E staining. The prepared sections were visualized under light microscopy.

**Figure 3** Effects of J99745 on serum MDA content (A), BUN (B) and renal morphological changes in mice (C). Serum MDA content and BUN was expressed as mean ± SD (n = 6). Kidney sections for HE staining (n = 4) were presented at a magnification of 200 × . Scale bar, 20 μm. J99745 (3, 10 and 30 mg/kg), allopurinol (Allo, 20 mg/kg) or benzbromarone (Benz, 20 mg/kg) were respectively administrated to mice in addition to xanthine and oxonic acid treatment. The hyperuricemia group was administrated with vehicle. \( P < 0.05, P < 0.01 \) vs. the normal group; \(^* P < 0.05, ^{**} P < 0.01 \) vs. the hyperuricemia group.
2.8. RNA isolation and real-time PCR analysis

Liver and kidney were extracted to prepare total RNA using Trizol reagent, followed by the synthesis of cDNA. 10 μg of total RNA isolated were subjected to reverse transcription-PCR using SuperScript III First Strand (Invitrogen, USA). Then 20–50 ng of cDNA was amplified by real-time PCR. The used primers were listed as below:

**Xod**, 5′-TCAGAAGCCAAGAAGGTG-3′ and 5′-ATGGCTGGGGTGTCAGC-3′;
**Urat1**, 5′-AGCTTTGGACCCCAATG-3′ and 5′-CTTCAGAGCGTGAGAGTCACACA-3′;
**Gapdh**, 5′-GCTGAGTATGTGGAGT-3′ and 5′-GTTCACACCCATCACAAAC-3′;
**Abcg2**, 5′-TGCCAGATAAGAGGGGTTAGGT-3′ and 5′-TGCTTGCAGTGGAGTTGAGA-3′;
**Glut9**, 5′-TCTCAGTTGCTTGGGAGCAG-3′ and 5′-AGCTAAAGCAAGCTCCCTGG-3′;
**Oat1**, 5′-GCTGGTACTCCTCCTCTGGA-3′ and 5′-TCCATGACCCCGTAGTA-3′.

2.9. Western blot analysis

Liver or renal tissues were homogenized in ice-cold RIPA buffer supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and then centrifugated at 12,000 × g for 20 min. The protein concentration of supernatant was determined by bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Nanjing, China). Protein were loaded by a SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The PVDF membranes were blocked with 5% skim milk and incubated with the primary antibodies for XOD (ab109235), URAT1 (ab198791), GLUT9 (ab82910), OAT1 (ab131087), ABCG2 (ab108312) or GAPDH (ab9984) at 4°C overnight. The membranes were incubated with secondary antibody at room temperature. The bands were visualized by a gel imaging system using enhanced chemiluminescence detection kit (Beijing Kangwei Century Biological Technology, Beijing, China) and densitometric analysis were performed by Image J software (National Institutes of Health, USA).

2.10. Statistical analysis

All data were presented as the mean ± standard deviation (SD). The significant differences were statistically assessed by one-way analysis of variance (ANOVA) followed by Dunnett’s test for post hoc
analysis using the GraphPad Prism 6.0 software, and the difference was considered statistically significant when $P < 0.05$.

### 3. Results

#### 3.1. J99745 exhibited potent XOD inhibition in vitro

In the dual assay, allopurinol inhibited the activities of XOD enzyme in a dose-dependent manner with an $IC_{50}$ of 3.902 μmol/L while the $IC_{50}$ on the level of $O_2^\cdot-$ was 3.966 μmol/L (Fig. 1B). In contrast, TBHQ slightly increased the XOD activity, this may be due to the decline in the level to the product $O_2^\cdot-$ shifted the reaction equilibrium to the right side (Fig. 1C). The $IC_{50}$ of TBHQ on the level of $O_2^\cdot-$ was 2.575 μmol/L. J99745 presented a valid XOD inhibition ($IC_{50} = 3.297$ μmol/L). J99745 also exhibited an inhibition on the level of $O_2^\cdot-$ with an $IC_{50}$ of 3.353 μmol/L (Fig. 1D). Like allopurinol, the $IC_{50}$ on XOD inhibition is almost equal to the $IC_{50}$ on the level of $O_2^\cdot-$, indicating that J99745 did not show the ability to scavenge $O_2^\cdot-$ in addition to XOD inhibition. The results demonstrated that J99745 is a potent XOD inhibitor.

#### 3.2. J99745 reduced serum urate levels and enhanced excretion of urate in hyperuricemia mice

Oral administration of xanthine for 7 days and single oxonic acid injection significantly increased serum urate in the hyperuricemia mice compared to those in the normal mice (Fig. 2A). J99745 at 3 mg/kg failed to alter serum urate levels, while J99745 at 10 and 30 mg/kg effectively reduced serum urate in hyperuricemic mice after 7 days of gavage administration. Moreover, animals receiving allopurinol and benzbromarone exhibited significant decline in serum urate levels. Nevertheless, the effect of benzbromarone seemed to be weaker than that of J99745 at 30 mg/kg. Under hyperuricemia induction, the hyperuricemia group presented a
significant increase in urine urate level, which was aggravated by J99745 at 10 and 30 mg/kg or benzbromarone at 20 mg/kg. In addition, all test compounds showed no significant impact on the serum and urinary creatinine as well as urine volume over 24 h in mice (Fig. 2C–E). On the other hand, a remarkable reduction in FEUA was observed in the hyperuricemia mice, which seemed to be restored in 10 and 30 mg/kg J99745-treated mice or benzbromarone-treated mice (Fig. 2F).

3.3. J99745 improved renal function and histopathological changes

Compared to the normal mice, the hyperuricemia mice showed increased BUN level, but no significant change in serum creatinine, indicating mild renal function damage. J99745 at doses of 10 and 30 mg/kg reduced BUN compared to the hyperuricemia group, while allopurinol and benzbromarone treatment failed to reduce BUN (Fig. 3B). H&E staining on kidney sections from the hyperuricemia group showed obvious vacuolization in renal tissue, irregular cell arrangement, and renal tubular dilatation, which could be significantly alleviated following the treatment of J99745 at 10 and 30 mg/kg (Fig. 3C). Dispersed vacuolization and slightly dilated renal tubule were observed in the 3 mg/kg J99745-treated group as well as the benzbromarone group and the allopurinol group.

3.4. J99745 lowered serum MDA content

To investigate the effects of J99745 on oxidative stress injury, serum MDA content was checked, which is an important readout of lipid and protein peroxidation. Serum MDA increased significantly in the hyperuricemia group. J99745 at 10 and 30 mg/kg and allopurinol dramatically decreased serum MDA content, indicating that oxidative stress was relieved under J99745 treatment (Fig. 3A).

3.5. J99745 decreased XOD activity with no impact on XOD expression

J99745 exhibited potent XOD inhibition in vitro. Thus, its effect on XOD activity was evaluated in the hyperuricemia mice. Serum and hepatic XOD activities were significantly increased in the hyperuricemic mice (Fig. 4A and B). In contrast, J99745 with two high doses and allopurinol at 20 mg/kg significantly lowered the XOD activities in serum and liver. However, J99745 and allopurinol exerted no impact on the mRNA and protein levels of XOD (Fig. 4B). Treatment with 20 mg/kg benzbromarone showed no influence on serum and hepatic XOD activities as well as mRNA and protein levels. These data indicated that J99745 inhibited XOD activity to reduce uric acid production.

3.6. J99745 downregulated URAT1 expression

Renal mRNA and protein levels of URAT1 and GLUT9 increased remarkably in the hyperuricemic mice (Fig. 5A–C). J99745 at doses of 10 and 30 mg/kg downregulated the mRNA and protein levels of URAT1, but failed to alter GLUT9 expression (Fig. 5D and E). Compared to the normal group, renal mRNA as well as protein levels of OAT1 and ABCG2 in hyperuricemic mice did not alter significantly. None of the treatments of J99745, allopurinol or benzbromarone exerted impact on OAT1 and ABCG2 expression (Fig. 5B, C, and F). These results indicated that J99745 downregulated renal URAT1 expression to promote the excretion of uric acid.

4. Discussion

This study is the first to demonstrate that xanthone flavone J99745 analogous to mangiferin aglycon exerts urate-lowering and nephroprotective effects in hyperuricemia mice. Furthermore, the underlying mechanism of J99745 might be through inhibiting hepatic XOD activity and renal URAT1 expression.

In the present study, hyperuricemia was reproduced in mice using xanthine gavage for 7 days and single injection of potassium oxonic acid at the last day, in accordance with some reports. In contrast, consecutive administration of potassium oxonic acid for 7 days was adopted in some other reports to stimulate the hyperuricemia. Our protocol induced milder renal function impairment indicated by decreased BUN without the alteration in serum creatinine. H&E staining in kidney sections confirmed the mild impairment. Interestingly, some kidney injuries were also observed following allopurinol or benzbromarone treatment. The renal damage observed may be partially attributable to the fact that benzbromarone, as a uricosuric drug, promotes renal urate excretion and elevates urinary urate concentration; thereby increasing the excretory burden of kidney and induces damage.

XOD as a typical oxidant-producing enzyme and a major source of reactive oxygen species (ROS) is upregulated in experimental hyperuricemia. The uric acid conversion from hypoxanthine and xanthine by XOD is accompanied by the generation of O₂⁻. In patients with gout, the overproduction of ROS causes oxidative stress and increases the risks of diseases secondary to hyperuricemia including cardiovascular events. Moreover, the evidence that uric acid within the cell may function as a pro-oxidant instead of an antioxidant (primarily in plasma) and play a contributory role in the pathogenesis of many diseases has been established. Therefore, antioxidant could be of therapeutic value in retarding these pathogenic processes. It was demonstrated that XOD inhibitors could play a role as antioxidant to reduce the ROS level and subsequent oxidative stress injury, and protected against ischemia–reperfusion injury, endothelial injury, and heart failure. In the study, the dual in vitro assay demonstrated that J99745 potently inhibited the activity of XOD enzyme, although it did not exhibit the ability to scavenge O₂⁻. In hyperuricemic mice, J99745 also exerted effective inhibition on serum and hepatic XOD activities as well as relieved the oxidative stress injury indicated by decline in serum MDA content. These observations might explain why J99745 performed with better profile than benzbromarone, a uricosuric drug without antioxidant action, in hyperuricemic mice.

Under physiological conditions, a fine balance exists between urate production and excretion in humans. Hence, any pathological process stimulating urate overproduction due to increased hepatic XOD activity or decreased renal excretion could result in hyperuricemia. Recent studies also exhibited that insufficient renal excretion of uric acid contributed to the majority of hyperuricemia. In the present study, the hyperuricemia group demonstrated insufficient excretion of uric acid as compared to it overproduction although the urine urate level was elevated. J99745 treatment at doses of 10 and 30 mg/kg further increased urine uric acid excretion and FEUA as well. The beneficial effects of J99745 may result from efficient reduction in serum urate levels through...
not only blocking overproduction but also reversing underexcretion of uric acid. Some previous studies have suggested that the urate handling was mainly dependent on the renal urate transport system, and OAT1, URAT1, GLUT9, and ABCG2 were regarded as the potential therapeutic targets for the treatment of hyperuricemia\(^{1,2}\). Our results demonstrated that xanthine- and oxonic acid-treatment remarkably increased renal URAT1 and GLUT9 protein levels but not OAT1 and ABCG2 protein levels in mice. J99745 downregulated renal URAT1 expression in hyperuricemic mice, but failed to alter the expression of GLUT9, OAT1 and ABCG2. Actually, the effect of J99745 on uric acid transfer across cell membrane has been checked on manipulating HEK293 cell line stably overexpressing human URAT1 combined HPLC method for supernatant uric acid detection, and found that J99745 had no significant influence on human URAT1 function (data not shown). J99745 treatment showed no such injury in renal function and morphology compared to the benzbromarone treated mice. Maybe the phenomenon that J99745 exerted merely inhibited urate on URAT1 promoted uric acid excretion so moderately as not to induce the subsequent kidney damage. The mechanisms by which J99745 showed specificity on URAT1 expression need to be elucidated in future study.

5. Conclusions

In summary, this study showed that J99745 significantly decreased the serum urate level and protected against hyperuricemia-associated renal damage in xanthine- and oxonic acid-induced hyperuricemia mice. The mechanism by which J99745 lowered urate might be inhibiting XOD activity and URAT1 expression. Therefore, J99745 may represent a promising candidate as an anti-hyperuricemia drug candidate.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2017.05.004.

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