Mangiferin Inhibits Renal Urate Reabsorption by Modulating Urate Transporters in Experimental Hyperuricemia

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Mangiferin, a natural glucosyl xanthone from the leaves of Mangifera indica L., was previously shown to exert potent hypouricemic effects associated with inhibition of the activity of xanthine dehydrogenase/oxidase. The present study aimed to evaluate its uricosuric effect and possible molecular mechanisms underlying the renal urate transporters responsible for urate reabsorption in vivo. Mangiferin (1.5–24.0 mg/kg) was administered intragastrically to hyperuricemic mice and rats induced by the intraperitoneal injection of uric acid and potassium oxonate, respectively. The uricosuric effect was evaluated by determining the serum and urinary urate levels as well as fractional excretion of uric acid (FEUA). The mRNA and protein levels of renal urate-anion transporter 1 (URAT1), organic anion transporter 10 (OAT10), glucose transporter 9 (GLUT9), and PDZ domain-containing protein (PDZK1) were analyzed. The administration of mangiferin significantly decreased the serum urate levels in hyperuricemic mice in a dose- and time-dependent manner. In hyperuricemic rats, mangiferin also reduced the serum urate levels and increased the urinary urate levels and FEUA. These results indicate that mangiferin has uricosuric effects. Further examination showed that mangiferin markedly inhibited the mRNA and protein expression of renal URAT1, OAT10, and GLUT9 in hyperuricemic rats, but did not interfere with PDZK1 expression. Taken together, these findings suggest that mangiferin promotes urate excretion by the kidney, which may be related to the inhibition of urate reabsorption via downregulation of renal urate transporters.

Key words: mangiferin; hyperuricemia; uricosuric effect; urate reabsorption; renal urate transporter

Hyperuricemia is characterized by a persistent increase of serum urate concentrations above 400 μmol/L, in which monosodium urate monohydrate crystals may be deposited in tissues causing several pathological conditions, including acute gouty arthritis, uric stone formation, and obstructive uropathy. Uric acid (urate), the final breakdown product of purines in humans, is created from the oxidation of hypoxanthine and xanthine by xanthine oxidase in the liver. Its homeostasis depends on the balance of the production and excretion of urate. The kidney, as a main regulator of serum urate level, is responsible for more than 70% of urate excretion. Renal insufficient excretion is the primary cause of hyperuricemia in about 90% of affected individuals. Nowadays, increasing clinical reports have shown that hyperuricemia associated with a risk of not only gout, but also chronic nephritis, renal dysfunction, cardiovascular diseases, hypertension, diabetes, and metabolic syndrome. Therefore, improvement of the insufficient excretion of urate is very important in the prevention and treatment of these diseases.

It is commonly accepted that urate reabsorption plays a key role in renal urate excretion. Increasing investigations have indicated that multiple transporters in the proximal tubule are involved in regulating renal urate reabsorption. The filtered urate is transported into the proximal tubule cells by the apical exchanger proteins urate-anion transporter 1 (URAT1) (SLC22A11), whereas the intracellular urate is released through glucose transporter 9 (GLUT9) (SLC2A9), a basolateral transporter. Recently, it is found that organic anion transporter 10 (OAT10) (SLC22A13) localized in brush border membranes of proximal tubule cells also participate in the urate reabsorption. The altered expressions of the urate transporters are suggested to be associated with hyperuricemia. Moreover, PDZ domain-containing protein 1 (PDZK1), a scaffolding protein, has been identified to participate in the process of urate transport in kidney.

Current antihyperuricemic agents in clinical use are very limited, including xanthine oxidase inhibitors, such as allopurinol and febuxostat, and uricosuric agents. Uricosuric agents such as probenecid and benzbromarone act on the urate transport pathway and inhibit renal proximal tubular urate reabsorption. However, the existing antihyperuricemic agents owing to possess some undesirable effects such as hypersensitivity toward allopurinol and hepatotoxicity associated with benzbromarone always limit their clinical uses. Therefore, it is very urgent and important to search for alternative antihyperuricemic agents with a more favorable toxicological profile. Plant products may provide an option for the treatment of the patients with hyperuricemia and gout.

Mangiferin (Fig. 1), 1,3,6,7-tetrahydroxyxanthone-C-2-β-D-glucoside, is a natural glucosyl xanthone from the leaves of Mangifera indica L. (Anacardiaceae), which is a tropical tree that was cultivated in many areas of the Old and the New World for its edible fruit. In previous study, we found that mangiferin had the potent hypouricemic effect associated with inhibiting the activity of xanthine dehydrogenase/oxidase, at a dose of 1.5–6.0 mg/kg, as well as oral safety with a dose of 25.0 g/kg in mice, exhibiting the potential to be developed as a new therapeutic agent for treatment of hyperuricemia and gout.
the method described previously. Benz bromarone was isolated from the leaves of *Mangifera indica* according to the method described previously. Benz bromarone was from Sano Arzneimittelfabrik GmbH. Potassium oxonate and uric acids were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Creatinine and urate assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. RNA-seq whole Total RNA Kit was purchased from Tiangen Biotech (BEIJING) Co., Ltd. RevertAid First Strand cDNA Synthesis Kit was purchased from Thermo Fisher Scientific. Goat anti-SLC22A12 antibody and donkey anti-goat immunoglobulin-G complexed with horseradish peroxidase (IgG-HRP) were got from Santa Cruz Biotechnology, Inc. Rabbit anti-SLC2A9, anti-SLC22A13, anti-PDZK1 antibodies and goat anti-rabbit IgG were obtained from Sigma-Aldrich.

**Animals** Adult Sprague-Dawley rats weighing 180–200 g and Kunming mice weighing 18–22 g were obtained from the Chengdu Dashuo Biotechnology Company, Sichuan, China (Certificate No. SCXK(chuan)2011-24). The animals were housed on a constant 12-h light/dark cycle in a temperature- and humidity-controlled room, and given free access to solid food and tap water for study duration. Animals were allowed 3–7 d to adapt the environment before experiments. All of the procedures were performed in accordance with the Institute Ethical Committee for Experimental Animal Use.

**Materials** Mangiferin (purity >90% by HPLC) was isolated from the leaves of *Mangifera indica* L. according to the method described previously. Benz bromarone was from Sano Arzneimittelfabrik GmbH. Potassium oxonate and uric acids were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Creatinine and urate assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. RNA-seq whole Total RNA Kit was purchased from Tiangen Biotech (BEIJING) Co., Ltd. RevertAid First Strand cDNA Synthesis Kit was purchased from Thermo Fisher Scientific. Goat anti-SLC22A12 antibody and donkey anti-goat immunoglobulin-G complexed with horseradish peroxidase (IgG-HRP) were got from Santa Cruz Biotechnology, Inc. Rabbit anti-SLC2A9, anti-SLC22A13, anti-PDZK1 antibodies and goat anti-rabbit IgG were obtained from Sigma-Aldrich.

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**Effects of Mangiferin on Serum Urate Levels in Uric Acid-Induced Hyperuricemic Mice** The hyperuricemic mice were induced by an intraperitoneal injection of uric acid. Male mice were divided into several groups. The normal control mice and hyperuricemic control mice were treated with 0.5% sodium carboxymethylcellulose (CMC-Na), whereas other hyperuricemic mice were administrated with mangiferin and benz bromarone dissolved in 0.5% CMC-Na, respectively. All drugs were given intragastrically twice daily for five doses or once daily for 1, 3, and 5 d. The last dosage was given at 30 min before injection intraperitoneally with uric acid (250 mg/kg) to increase the serum urate level. Sixty minutes later, blood samples were collected from the mice via orbit vein bleeding after exposure to ethyl ether. Serum urate levels were determined by the method of phosphotungstic acid using commercially available kits.

**Effects of Mangiferin on Fractional Excretion of Urate in Potassium Oxonate-Induced Hyperuricemic Rats** The hyperuricemic rats were induced by an intraperitoneal injection with uricase inhibitor potassium oxonate (200 mg/kg) at 08:00 in the morning for 5 d. Rats were randomly divided into 7 groups: the normal control group receiving vehicle only; the hyperuricemic group receiving potassium oxonate and vehicle; the mangiferin groups receiving potassium oxonate and mangiferin with different doses (1.5, 3.0, 6.0, and 12 mg/kg); the benz bromarone group receiving potassium oxonate and benz bromarone 12.5 mg/kg. All test compounds were dissolved in 0.5% CMC-Na and orally administered twice a day (at 09:00 and 21:00) for nine times.

On the forth day of experiment, all rats were kept in the metabolic cages and urine was collected for 24 h. On the fifth day, 2 h after receiving vehicle or drugs, the rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), and blood was sampled by abdominal aortic puncture. Blood was centrifuged at 3600×g for 10 min to separate serum. The serum and urine samples were stored at −20°C for determination of urate levels by high performance liquid chromatography (HPLC) and creatinine concentrations by commercially available kits. The fractional excretion of urate was then calculated as follows to assess the uricosuric effect of the test compound: Fractional excretion of urate (FEUA) = ([urate in urine]/[urate in serum])/([urate in creatinine]/[urate in creatinine]). In addition, the kidney cortex tissues were rapidly and carefully separated on ice-plate, and then stored at −80°C for subsequent assays.

**Determination of Urate Levels by HPLC** Urate level was analyzed by HPLC method using a reverse phase C18 column (Xterra® RP 18 4.6×250mm Column, Waters) on a Binary HPLC system, and the column temperatures were kept at 25°C. After suitable dilution, the plasma and urine samples were filtered by 0.45 μm microfiltration membrane. Twenty microliter of the filtered sample was injected into the column and eluted with the mobile phase, triethylamine, pH 6.0, at a flow rate of 1 mL/min. The eluate was monitored for absorbance at 290 nm.

**Measurement of mRNA Expressions of Renal URAT1, GLUT9, OAT10 and PDZK1 in Rats by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** The cortex tissues of kidney (60–80 mg) in rats were homogenized in 1 mL Trizol reagent (Invitrogen) to extract total RNA. RNA integrity was then evaluated by electrophoresis in 1.0% agarose gel. The samples of RNA were stored at −80°C. An equal amount of total RNA (2 μg) was used for cDNA synthesis following the manufacturer’s protocol. The reverse transcription reaction products were stored at −20°C for further amplification.

PCR amplification was carried out using gene-specific PCR primers. The primer sequences were designed according to their mRNA sequences in the gene bank [rURAT1 (NM_001034943.1), rGLUT9 (NM_001191551.1), rOAT10 (NM_001126285.1), rPDZK1 (NM_031712.1), rGAPDH (NM_017008.4)] and synthesized by Sangon Biotechnology Co., Ltd. All primer sequences were checked in GenBank (National Center for Biotechnology Information, Bethesda, MD, U.S.A.). The annealing temperatures and production sizes used in the experiments are summarized in Table 1.
PCR products were separated by electrophoresis in 1.5% agarose gel and visualized by staining with gelview. The intensities of PCR-product bands were determined using Bio-Rad ChemiDoc XRS. The intensities of target genes of rURAT1, rGLUT9, rOAT10, and rPDZK1 were expressed by their ratios to the intensities of rGPADH.

**Measurement of Protein Expressions of Renal URAT1, GLUT9, OAT10 and PDZK1 in Rats by Western Blotting Method** After liquid nitrogen grounding, kidney cortex samples from each treatment group were homogenized in radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime) containing phenylmethanesulfonyl fluoride (PMSF) (Sigma) and bathed on ice for 30 min. Then the lysate was centrifuged at 12000 rpm, 4°C for 10 min to isolate total proteins and the amount of the proteins were determined by bicinecinonic acid (BCA) method. The total proteins were incubated with 1/5 volume 5% sodium dodecyl sulfate (SDS) loading buffers in the boiling water for 10 min. Equal amount of total proteins were then separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in TBST (Tris-buffered saline (TBS) containing 0.1% Tween-20) containing 5% skimmed milk powder overnight at 4°C. After washing the membrane three times with TBST, immunoreactive bands were detected using HRP conjugated donkey anti-goat IgG (Santa Cruz) or goat anti-rabbit IgG (Invitrogen) as the secondary antibody diluted 1:1000 in TBST containing 5% skimmed milk powder overnight at 4°C. After washing the membrane three times with TBST, immunoreactive bands were detected using HRP conjugated donkey anti-goat IgG (Santa Cruz) or goat anti-rabbit IgG (Invitrogen) as the secondary antibody diluted 1:5000 in TBST for 1 h at room temperature. The proteins were visualized using ECL detection reagent (Bio-Rad). The density of bands was analyzed by ChemiDoc XRS+ and normalized to β-actin.

**Statistical Analysis** All data were present as the mean±standard error of the mean (S.E.M.) and statistical analysis was performed using an ANOVA followed by the Dunnett’s multiple comparison tests to determine the levels of significance. A value of *p*<0.05 was considered statistically significant and the figures were obtained by the GraphPad Prism version 5.0 software.

**RESULTS**

**Effects of Mangiferin on Serum Urate Levels in Uric Acid-Induced Hyperuricemic Mice** An intraperitoneal injection of uric acid caused a marked increase in serum urate level, and this increase could be maintained for 4 h after the injection (data not shown). As reported in Fig. 2, mangiferin given intragastrically at the dose of 3.0, 6.0, 12.0, and 24.0 mg/kg twice daily for five times significantly attenuated the serum urate levels in uric acid-induced hyperuricemic mice, when compared to the untreated hyperuricemic mice (*p*<0.05). The hypouricemic action of mangiferin was as potent as that of benzbromarone (*p*<0.05). Table 2 illustrated that the serum urate levels were time-dependently decreased after the hypouricemic mice administered with mangiferin. Three doses of 6.0 mg/kg were able to reduce serum urate level (*p*<0.05). Although its onset was slower than that of benzbromarone, the hypouricemic effect of mangiferin was as potent as that of benzbromarone.

**Effects of Mangiferin on Excretion of Urate in Potassium Oxonate-Induced Hyperuricemic Rats** As reported in Fig. 3, intraperitoneal injection of potassium oxonate significantly increased the urate levels in serum and 24-h urine as well as decreased the fractional excretion of urate (FEUA) in rats, compared with the normal control rats (*p*<0.01). Administration of mangiferin significantly reduced the serum urate levels in a dose-dependant manner, and enhanced the urate quantity in 24-h urine and FEUA at the dose of 6.0 and 12.0 mg/kg, compared with the hyperuricemic rats (*p*<0.01), exhibiting the promotion of urate excretion in kidney. As a positive control, benzbromarone (12.5 mg/kg) also showed a remarkable reduction of serum urate and the enhancement of urate excretion in the hyperuricemic rats. However, there were no significant changes of creatinine levels in serum and 24-h
urine, and 24-h volume of urine in normal and experimental rats.

**Effects of Mangiferin on mRNA Expression of Renal Urate Transporters and PDZK1**

Effects of mangiferin on mRNA expression of renal urate transporters and PDZK1 are shown in Fig. 4, from which it can be seen that potassium oxonate-induced hyperuricemic rats showed the up-regulation of rURAT1, rGLUT9 and rOAT10 mRNA expression in kidney, compared with the normal control rats (p<0.05). Mangiferin remarkably decreased the mRNA expression of urate transporters in a dose-dependent manner, exhibiting the down-regulation of rURAT1 mRNA expression at a dose of 12.0 mg/kg, rGLUT9 at doses of 6.0 and 12.0 mg/kg and rOAT10 at all four doses of mangiferin. Similarly, benzbromarone significantly attenuated the potassium oxonate-induced elevation in renal rURAT1, rGLUT9, and rOAT10 mRNA expressions in rats (p<0.05). However, no changes of the mRNA expression of rPDZK1 were observed in normal and hyperuricemic rats.

**Effects of Mangiferin on Protein Expression of Renal Urate Transporters and PDZK1**

Effects of mangiferin and benzbromarone on protein expressions of rURAT1, rGLUT9, rOAT10, and rPDZK1 in hyperuricemic rats are shown in Fig.
5, from which it can be seen that the protein expression levels of renal rURAT1, rGLUT9, and rOAT10 were elevated significantly in potassium oxonate-induced hyperuricemic rats, compared with the normal control rats \( (p<0.05) \). Mangiferin significantly reduced the protein expression of urate transporters in a dose-dependent manner, exhibiting the down-regulation of rURAT1 protein expression at doses of 3.0, 6.0, and 12.0 mg/kg, as well as rGLUT9 and rOAT10 protein expression at doses of 6.0 and 12.0 mg/kg of mangiferin. Similarly, benzbromarone markedly inhibited the potassium oxonate-induced elevation in renal rURAT1, rGLUT9 and rOAT10 protein expression in rats \( (p<0.05) \). However, there was no significant alteration in renal rPDZK1 protein levels in normal and hyperuricemic rats.
DISCUSSION

In the previous study, mangiferin at a dose of 1.5–6.0 mg/kg significantly decreased the serum urate levels in hyperuricemic mice by inhibiting the activities of xanthine dehydrogenase/oxidase in liver, indicating that mangiferin could attenuate the production of uric acid.\textsuperscript{12} In the current study, it was demonstrated that mangiferin was able to significantly reduce the serum urate levels in uric acid-induced hyperuricemic mice, in a dose- and time-dependent manner, exhibiting a uricosuric effect. The uricosuric action was as potent as that of benzbromarone, although the onset of mangiferin was not as fast as that of benzbromarone with the onset time being less than 1 h after the administration. These findings suggested that the hypouricemic action of mangiferin might be related to not only the inhibition of urate production, but also the enhancement of urate excretion. The dual actions of mangiferin provide an explanation for its potent hypouricemic action \textit{in vivo}.

To further elucidate the uricosuric effect observed was associated with the excretion of urate in the kidney, we investigated the effect of mangiferin on the FEUA in hyperuricemic rats induced by potassium oxonate. Potassium oxonate is an inhibitor of uricase, which converts uric acid to the soluble allantoin, and often used to make an animal model of hyperuricemia in study.\textsuperscript{17} As expected, an intraperitoneal injection of potassium oxonate with a dose of 200 mg/kg for 5 d markedly induced the increases of serum urate levels in rats. Administration of mangiferin significantly decreased the serum urate levels and increased the urine urate levels and parameter FEUA in hyperuricemic rats, at the doses of 6.0 mg/kg and 12.0 mg/kg, similar to benzbromarone. Those results illustrated that mangiferin has the ability of promoting renal urate excretion and is a potent uricosuric agent. Hu \textit{et al.} reported that mangiferin at the doses of 50.0, 100.0, and 200.0 mg/kg also enhanced the renal urate excretion in hyperuricemic mice,\textsuperscript{18} although the dosages were much higher than that used in our investigation.

The process of urate handling in kidney involves glomerular filtration followed by a complex array of reabsorptive and secretory mechanisms taking place in the proximal tubules. Normally, more than 90% of the filtered urate is reabsorbed in mature humans,\textsuperscript{19} only about 10% is eventually excreted in the urine, suggesting that urate reabsorption has played an important role in renal urate excretion. In the body, urate exists as an organic anion (pK\textsubscript{a} value 5.75) and needs membrane transporters to permeate into the cell across the plasma membrane. Documented evidences exhibit that multiple specific urate transporters in kidney mediates this process. It was reported that a host of urate transporters expressed in renal proximal tubule cells functioned in urate reabsorption. URAT1 and GLUT9 are two major transporters involving in urate reabsorption in kidney. URAT1, a member of the major facilitator superfamily, was first identified by Enomoto \textit{et al.} in 2002.\textsuperscript{5} It was an anion exchanger localized in brush border membranes of renal proximal tubule cells which could transport urate in exchange for Cl\textsuperscript{−} or other organic anions such as lactate and pyrazinoic acid.\textsuperscript{20} It is also an important determinant charging of urate reabsorption from the lumen to the cytosol in kidney tubules, and a therapeutic target inhibited by some uricosuric agents, such as probenecid and benzbromarone.\textsuperscript{21} GLUT9, localized in basolateral membranes of renal proximal tubule cells, is another urate transporter involved in urate reabsorption from the cytosol kidney tubules to the interstitiumin a voltage-dependent manner.\textsuperscript{22} The presence of GLUT9 mutations lead to losing the function of the protein, and in subsequent to hypouricemia, as well as increased FEUA.\textsuperscript{7} More recently, OAT10 from OAT family was shown to mediate urate-nicotinamide exchange in brush border membranes of renal proximal tubule cells.\textsuperscript{5,23} In the current study, to elucidate the underlying molecular mechanisms of uricosuric effects of mangiferin, the mRNA and protein expressions of the transporters responsible for urate reabsorption were examined. The results showed that the mRNA and protein expressions of renal URAT1, GLUT9, and OAT10 increased in hyperuricemic rats induced by potassium oxonate. Mangiferin significantly decreased the mRNA and protein levels of URAT1 and GLUT9 in kidney, which was consistent with the results in hyperuricemic mice reported by Hu.\textsuperscript{19} Notably, the protein levels of renal OAT10 increased by 241.2% in hyperuricemia, while URAT1 and GLUT9 enhanced only by 111.3 and 130.0%, suggesting that OAT10 might play an important role in urate reabsorption in hyperuricemia. Of interest was that the protein levels of OAT10 decreased by 48.3% in hyperuricemic rats treated with mangiferin at the dose of 12.0 mg/kg. This is the first report that mangiferin has the ability of inhibiting the protein expression of OAT10. Those findings indicated that the uricosuric effect of mangiferin was related to inhibiting the reabsorption of urate in kidney by targeting the urate transporters not only URAT1 and GLUT9 but also OAT10.

Additionally, serum urate level is affected by variation in the gene encoding the subapical apical scaffolding protein PDZK1, one of several PDZ domain proteins that tether and regulate the apical complex of reabsorptive urate transporters.\textsuperscript{24} The protein can interact with URAT1, OAT10 and some other proteins\textsuperscript{10,25,26} to increase urate transport. In the present study, we also investigated the mRNA and protein expressions of renal PDZK1 in normal and hyperuricemic rats. The results showed that no changes of PDZK1 expressions were observed in all experimental rats, suggesting that the uricosuric effect of mangiferin might not be related to PDZK1.

It is well known that uric acid is also excreted from the body extra-renally, although urinary excretion is predominant. It has been suggested that one-third to one-fourth of uric acid is recovered in feces, indicating that biliary and/or intestinal secretion is an important alternative pathway(s) of uric acid excretion.\textsuperscript{25} Recently, it has been reported that ABCG2, a member of the ATP-binding cassette transporter superfamily, plays a key role in the regulation of urate homeostasis. It is highly expressed on the renal proximal tubular cells, the apical membranes the intestinal epithelium and the liver hepatocytes, mediating the renal and/or extra-renal urate excretion, as a high-capacity urate exporter,\textsuperscript{26,27} and its dysfunction has an association with serum uric acid levels and gout/hyperuricemia risk. In addition, unlike humans, urate degradation in liver affects the levels of serum urate in rodents. Hepatic uric acid is partially exported to the blood, and partially is degraded by uricase within hepatic peroxisomes to the more aqueous allantoin, which is then excreted by the kidney and intestine. A major fraction of extracellular urate is transported into hepatocytes where it is transformed into allantoin. GLUT9 and
ABCG2 might be contributed to the transport of urate in and out of the kidney.\(^{10,30,31}\) Whether the hypouricemic action of mangiferin is related to the activities of uricase in the liver and extra-renal urate excretion of urate via ABCG2 is still not elucidated. Further research need to be done for more evidence.

In conclusion, mangiferin has the uricosuric action in hyperuricemic animals, which was associated to inhibiting reabsorption of urate by down-regulating the mRNA and protein expressions of urate transporters in kidney.

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Conflict of Interest The authors declare no conflict of interest.

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