The Dual Actions of Morin (3,5,7,2′,4′-Pentahydroxyflavone) as a Hypouricemic Agent: Uricosuric Effect and Xanthine Oxidase Inhibitory Activity

Zhifeng Yu, Wing Ping Fong, and Christopher H. K. Cheng
Department of Biochemistry, The Chinese University of Hong Kong, Shatin, Hong Kong, China
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ABSTRACT
Hyperuricemia is associated with a number of pathological conditions such as gout. Lowering of elevated uric acid level in the blood could be achieved by xanthine oxidase inhibitors and inhibitors of renal urate reabsorption. Some natural compounds isolated from herbs used in traditional Chinese medicine have been previously demonstrated to possess xanthine oxidase inhibitory activities. In the present investigation, morin (3,5,7,2′,4′-pentahydroxyflavone), which occurs in the twigs of Morus alba L. documented in traditional Chinese medicinal literature to treat conditions akin to gout, was demonstrated to exert potent inhibitory action on urate uptake in rat renal brush-border membrane vesicles, indicating that this compound acts on the kidney to inhibit urate reabsorption. Lineweaver-Burk transformation of the inhibition kinetics data demonstrated that the inhibition of urate uptake was of a competitive type, with a $K_i$ value of 17.4 $\mu$M. In addition, morin was also demonstrated to be an inhibitor of xanthine oxidase. Lineweaver-Burk analysis of the enzyme kinetics indicated that the mode of inhibition was of a mixed type, with $K_i$ and $K_{H_{2}O}$ values being 7.9 and 35.1 $\mu$M, respectively. Using an oxonate-induced hyperuricemic rat model, morin was indeed shown to exhibit an in vivo uricosuric action, which could explain, in part at least, the observed hypouricemic effect of morin in these rats. The potential application of this compound in the treatment of conditions associated with hyperuricemia was discussed.

Gout is a common systemic rheumatic disease that affects at least 1% of adult male in Western countries (Lawrence et al., 1998; Arromdee et al., 2002). Recent epidemiologic data indicated that the incidence of primary gout has increased by 2-fold over the past 20 years (Arromdee et al., 2002). This disease is also rapidly rising in China (Li et al., 1997), due probably to recent changes in dietary habits. Hyperuricemia is a hallmark of gout (Rott and Agudelo, 2003). A serum uric acid level of more than 9 mg/dl is associated with a gouty arthritis incidence of 4.9% (Campion et al., 1987). Pathogenic mechanisms of hyperuricemia include uric acid overproduction or underexcretion caused by aberrations in renal uric acid handling (Liote, 2003). In the general population, 80 to 90% of gout patients are underexcreters (Rott and Agudelo, 2003).

Uric acid is the end product of purine metabolism in humans. It is a weak organic acid with a poor pH-dependent solubility in aqueous solutions. Unlike other mammals, humans have a high serum uric acid level due to the lack of uricase, which converts uric acid to the more soluble allantoin. Uric acid production is catalyzed by xanthine oxidase in the liver, and ~70% of the daily output of uric acid is excreted through the kidney (Marangella, 2005). A three-component model has been proposed for uric acid excretion in humans. This includes filtration, reabsorption, and secretion. Recently, a urate-anion transporter (URAT1) was identified in the brush-border membrane of the proximal tubule in human kidney (Enomoto et al., 2002). This transporter is believed to play a significant role in regulating serum uric acid level through reabsorption of urate from the lumen to the cytosol in kidney tubules.

The treatment of gout entails several approaches. Apart from the symptomatic relief of pain and swelling associated with inflammation, drugs that could help to reduce the elevated uric acid level in the blood are employed as therapeutic agents. These include xanthine oxidase inhibitors, of which allopurinol is the most often prescribed. Inhibitors of renal urate reabsorption such as probenecid and benz bromarone...
are also employed as hypouricemic agents. However, these existing antihyperuricemic agents possess some undesirable effects such as hypersensitivity toward allopurinol (Bomalaski and Clark, 2004) and hepatotoxicity associated with benz bromarone (Schlesinger, 2004). Thus, the search for alternative antihyperuricemic agents with a more favorable toxicological profile is highly warranted. In China, natural herbs have been used for a long time in the treatment of gout and hyperuricemia-related disorders (Chou and Kuo, 1995). We have previously reported the identification of some natural compounds from these herbs possessing xanthine oxidase inhibitory activities (Kong et al., 1999, 2000). We also have reported that some natural compounds might exert their hypouricemic action via mechanisms other than xanthine oxidase inhibition (Kong et al., 2002). In the present investigation, we report that morin (Fig. 1), which occurs in a herb used to treat gouty conditions in traditional Chinese medicine, is a potent inhibitor of urate reabsorption in the kidney, in addition to its inhibitory effects on xanthine oxidase. The dual actions of this compound provide an explanation for its in vivo hypouricemic effect.

Materials and Methods

Materials

Unless otherwise stated, all chemical reagents and compounds were obtained from Sigma-Aldrich (St. Louis, MO). [8-14C]Urate (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Mixed cellulose membrane filters (diameter, 25 mm; pore size, 0.45 μm) and 30,000 mol. wt. cut-off centrifugal filters (catalog number UFC3LTK00) were purchased from Millipore Corporation (Billerica, MA). HI-SAFE II scintillant was supplied by PerkinElmer Life and Analytical Sciences (Boston, MA). The Creatinine Kit (catalog number 04290-250) was purchased from Stanbio Laboratory (Boerne, TX). Metabolic cages and experimental animals were provided by the Laboratory Animal Services Center of The Chinese University of Hong Kong.

In Vitro Action of Morin on Urate Uptake in Rat Renal Brush-Border Membrane Vesicles

Preparation of BBMVs. Brush-border membrane vesicles (BBMVs) were prepared from rat kidney cortex by the magnesium aggregation method (Dan and Koga, 1990). Briefly, adult male Sprague-Dawley rats (300 g body weight) were first anesthetized by i.p. injection of pentobarbital. The kidneys were removed rapidly, by housing the rats in metabolic cages (Dan et al., 1994). Rats were obtained from Millipore Corporation (St. Louis, MO). Metabolic cages and experimental animals were purchased from Sigma-Aldrich (St. Louis, MO). Mixed cellulose membrane filters (diameter, 25 mm; pore size, 0.45 μm) and 30,000 mol. wt. cut-off centrifugal filters (catalog number UFC3LTK00) were purchased from Millipore Corporation (Billerica, MA). HI-SAFE II scintillant was supplied by PerkinElmer Life and Analytical Sciences (Boston, MA). The Creatinine Kit (catalog number 04290-250) was purchased from Stanbio Laboratory (Boerne, TX). Metabolic cages and experimental animals were provided by the Laboratory Animal Services Center of The Chinese University of Hong Kong.

In Vivo Action of Morin on Plasma and Urine Urate Levels in Oxonate-Induced Hyperuricemic Rats

Treatment of Animals. A hyperuricemic rat model was adopted by the i.p. injection of oxonate (Yonetani and Iwaki, 1983; Yamada et al., 1999; Kong et al., 2002). The in vivo actions of morin were studied by Housing the rats in metabolic cages (Dan et al., 1994). Rats were divided into five groups: the normal group receiving vehicle only; the hyperuricemic control group receiving potassium oxonate only; and three morin groups, each receiving potassium oxonate and in addition different amounts (5, 20, and 80 mg/kg) of morin. All rats (200 g centrifuged at 2400g for 20 min. The supernatant obtained was carefully transferred to a new tube and centrifuged twice at 2400g for 20 min. The final supernatant was centrifuged again at 28,000g for 20 min. The pellet obtained was suspended in a small volume of 50 mM potassium phosphate buffer, pH 7.5, containing 150 mM mannitol and 2 mM MgSO4 at a final protein concentration of 15 to 20 mg/ml. The purity of BBMV was assessed by the fold enrichment of the brush-border membrane marker enzyme alkaline phosphatase, which was quantitated by a spectrophotometric method using p-nitrophenylphosphate as the substrate (Winchester et al., 1999). Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

Urate Uptake into BBMV. [14C]Urate uptake into BBMV was determined at room temperature by the rapid filtration method (Edwards et al., 1996) in the presence of an OH− gradient. Urate uptake was initiated by adding 10 μl of the freshly prepared BBMV (in the pH 7.5 buffer) into 90 μl of uptake buffer (150 mM mannitol, 2 mM MgSO4, and 50 mM potassium phosphate, pH 6.0), containing 38 μl mCi [14C]urate with or without the putative inhibitor. After exactly 12 s, 4 ml of prechilled uptake buffer was added to stop the uptake process. The sample was immediately filtered through a 0.45-μm mixed cellulose membrane kept under suction. The BBMVs held on the filter were washed twice with the uptake buffer. The radioactivity on the filter was counted on a liquid scintillation counter (Beckman LS2900TR; Beckman Coulter, Fullerton, CA) after adding 4 ml of the scintillant. In most experiments, urate uptake was also measured in the absence of an OH− gradient in which the pH value of the uptake buffer was 7.5 instead of 6.0. Urate uptake through the urate-anion transporter was calculated by subtracting the uptake value in the absence of an outwardly directed OH− gradient (PHin = PHout = 7.5) from that in the presence of the gradient (PHin = 7.5 and PHout = 6.0). The amount of [14C]urate bound on the filter in the absence of BBMV was also measured to serve as the blank for the uptake assessment. The putative uptake inhibitor was originally dissolved in dimethyl sulfoxide and diluted with the appropriate uptake buffer before the assay. The final concentration of dimethyl sulfoxide in the assay was 1%. This concentration of dimethyl sulfoxide was determined in initial experiments to exert minimal effects on the uptake assay. Probenecid was used as a positive control for the uptake inhibition.

In Vivo Action of Morin on Plasma and Urine Urate Levels in Oxonate-Induced Hyperuricemic Rats

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body weight each) were housed individually in metabolic cages in an air-conditioned room (25°C) and had free access to food and water during the period of the experiment. After being stabilized in the experimental set-up for 1 day, rats were orally administered with the vehicle or the designated amount of morin, followed immediately by an i.p. injection of vehicle/potassium oxonate (200 mg/kg). Morin and oxonate were administered via two different routes to avoid any possible interaction between the two agents. Morin was prepared in 0.3% Tween 20, whereas oxonate was suspended in 0.8% sodium carboxymethylcellulose. Before the injection, the suspensions were sonicated at 4°C for 20 min and vortexed vigorously. After the treatments, urine was collected for 5 h. At the end of the 5-h period, rats were anesthetized by i.p. injection of pentobarbital, and blood was sampled by cardiopuncture. Blood was centrifuged at 3000 g for 10 min at 4°C for plasma separation. The plasma and urine samples were stored at −20°C before analyses of uric acid and creatinine concentrations.

Determination of Uric Acid and Creatinine Contents. Uric acid level was determined by an HPLC method using a reverse phase C18 column (Kock et al., 1995) on a Beckman Coulter Gold HPLC system with a manual injector. The plasma and urine samples were pretreated by filtering through 30,000 mol. wt. cut-off centrifugal filters. After suitable dilution with HPLC-grade water, 20 μl of the sample was injected into the column and eluted with the mobile phase containing 27.5 mM NaH2PO4, pH 4.2, 2.5 mM octanesulfonic acid, and 10% acetonitrile at a flow rate of 1 ml/min. The eluate was monitored for absorbance at 292 nm. On the other hand, the plasma and urine creatinine levels were determined spectrophotometrically using the Stanbio Creatinine Kit. The fractional excretion of urate was then calculated as follows to assess the uricosuric effect of the test compound (Dan et al., 1994): Fractional excretion of urate = (urate creatinine)/(urate urate)/(plasma creatinine/plasma urate).

Statistical Analysis
Values are expressed as mean values ± S.E.M. IC50 values were determined from the concentration dependence curves. Data from the in vivo experiments were analyzed using one-way analysis of variance followed by the Dunnett’s multiple comparison test to determine the level of significance. P < 0.05 was considered statistically significant.

Results
The BBMV fraction that we obtained exhibited a 12.9-± 0.4-fold increase in the specific activity of alkaline phosphatase when compared with the homogenate, indicating that it was highly enriched in the brush-border membrane component, in line with published results in the literature (Dan and Koga, 1990; Edwards et al., 1996; Yamada et al., 2000). In the presence of the pH gradient (7.5/6.0), urate uptake proceeded rapidly in a linear manner within 20 s and then gradually leveled off (Fig. 2), again consistent with reports in the literature (Dan and Koga, 1990; Edwards et al., 1996; Yamada et al., 2000), indicating the robustness of our system. Thus, in subsequent experiments, urate uptake was determined at 12 s to obtain the initial velocity. In the absence of the pH gradient, urate uptake was about 50% of that obtained in the presence of the gradient.

The inhibitory effect of morin on the OH−-dependent urate uptake was demonstrated using the validated BBMV system, using probenecid as the control inhibitor. At a concentration of 25 μM, morin inhibited OH−-dependent urate uptake by 58.8%, whereas at the same concentration, probenecid had no inhibitory activity at all (Fig. 3). On the other hand, morin did not show any significant effect on urate uptake in the absence of the pH gradient (data not shown). Figure 3 shows the dose-dependent inhibition of morin on the OH−-dep-
dent urate uptake. IC\textsubscript{50} was observed at a concentration of 18.0 ± 2.5 μM. Subsequent Lineweaver-Burk transformation of the uptake kinetics data revealed that morin inhibited the OH\textsuperscript{-}-dependent urate uptake in a competitive manner (Fig. 4), with a \( K_i \) value of 17.4 ± 3.5 μM.

Morin also inhibited the activity of xanthine oxidase in a concentration-dependent manner, with an IC\textsubscript{50} value of 44 μM (Fig. 5). The inhibition of morin on xanthine oxidase was shown to be reversible because the enzyme activity could be recovered after removal of the inhibitor by dilution. Lineweaver-Burk analysis of the enzyme kinetics data showed that the inhibition of xanthine oxidase by morin was of a mixed type. In mixed inhibition, the inhibitor can bind to the free enzyme as well as to the enzyme-substrate complex (Palmer, 1995). Thus, two inhibitor constants can be defined, with \( K_i \) (inhibitor constant on the enzyme) and \( K_{ies} \) (inhibitor constant on the enzyme-substrate complex) values determined to be 7.9 and 35.1 μM, respectively (Fig. 6).

Figure 7 summarizes the in vivo actions of morin on the oxonate-induced hyperuricemic rats. Oxonate treatment resulted in 57.5% increase in the plasma uric acid level 5 h after treatment, whereas coadministration of morin could effectively reduce such increase in a dose-dependent manner (Fig. 7A). At a morin dose of 80 mg/kg body weight, the plasma uric acid level returned to that of the normal rats. Similarly, the urine urate level was significantly increased (by 3.2-fold) in the oxonate-induced hyperuricemic control rats. Coadministration of morin (5 and 20 mg/kg) further increased the urine urate level. However, at a high morin dose of 80 mg/kg, a decrease in the urine urate level was recorded (Fig. 7B). On the other hand, all the other parameters measured including urine and plasma creatinine levels and urine volume remained essentially the same for the five groups of rats (Fig. 7, C–E). Thus, morin, at a concentration of 20 mg/kg, could significantly increase (by 2-fold) the fractional excretion of urate in the hyperuricemic rats (Fig. 8).

**Discussion**

Antihyperuricemic agents are commonly employed for the treatment of chronic gouty arthritis (Rott and Agudelo, 2003). Current antihyperuricemic agents in use include uric-
cosuric agents, xanthine oxidase inhibitors, and the enzyme urate oxidase (Schlesinger, 2004). Uricosuric agents such as probenecid, sulfinpyrazone, and benzobromarone act on the urate anion transport pathway and inhibit renal proximal tubular urate reabsorption. Xanthine oxidase inhibitors such as allopurinol interfere with the conversion of hypoxanthine to xanthine and then to uric acid. Urate oxidase oxidizes urate to the more soluble allantoin, which is more readily excreted through the kidney.

Although a number of antihyperuricemic agents are available, their utilization is sometimes limited by the associated undesirable side effects. In general, allopurinol is the drug of choice. However, about 5% of patients are unable to tolerate its adverse side effects, which include gastrointestinal irritation, bone marrow suppression, and hypersensitivity syndromes ranging from simple skin rash to life-threatening conditions in which the patients develop toxic epidermal necrolysis, fever, hepatitis, eosinophilia, and worsening renal function (Yale et al., 1996; Umpiérrez et al., 1998; Bomalaski and Clark, 2004). Uricosuric agents are used in patients with
allopurinol-allergic syndromes as well as in underexcreters with normal renal function and no history of urolithiasis. These uricosuric agents are also not without problems. For example, benzbromarone can cause fulminant hepatotoxicity, and this in fact had led to its withdrawal from the French market in 2001 (Bieber and Terkeltaub, 2004), whereas probenecid and sulfinpyrazone have been reported to be nephrotoxic when used to treat hyperuricemia associated with moderate chronic renal insufficiency (Terkeltaub, 2003). Enzyme therapy using urate oxidase is not widely used and is still undergoing clinical trials in the United States. Thus, the search for better antihyperuricemic agents is highly warranted. The present study represents such an effort. From our studies on natural compounds, morin emerged as a potent urate-lowering agent possessing dual mechanisms of action.

In the general population, 80 to 90% of gout patients are underexcreters (Rott and Agudelo, 2003), indicating the importance of the kidney as a therapeutic target. Renal handling of urate is complicated. Not only does urate transport vary among species, but there also exists a bidirectional transport across renal tubule cells. Normally, less than 5% of circulating urate is bound to plasma proteins; thus, most of the urate can be freely filtered through the renal glomerulus (Rafey et al., 2003). More than 90% of the filtered urate is reabsorbed in mature humans (Maesaka and Fishbane, 1998). Recently, Enomoto et al. (2002) identified the long-hypothesized URAT1 in human kidney. Genetic defects of URAT1 cause idiopathic renal hypouricemia (Enomoto et al., 2002). Therefore, it is believed that this urate-anion exchanger is involved in the regulation of blood urate level by mediating urate reabsorption. Immunohistochemical analysis revealed the location of URAT1 protein in the epithelial brush-border membrane of the proximal tubules in the human renal cortex (Enomoto et al., 2002). In rats, urate excretion is similar to that in humans. Urate excretion in both species undergoes bidirectional tubular transport with the final result being net reabsorption (Edwards et al., 1996).

Therefore, the rat renal BBMV is a useful system for studying potential uricosuric agents.

The purity of our BBMV preparation was assessed by the enrichment in alkaline phosphatase activity. The specific enzyme activity enrichment obtained was 12.9 ± 0.4-fold, similar to that reported in the literature (Dan and Koga, 1990; Edwards et al., 1996). Morin could significantly inhibit the OH–-dependent urate uptake in BBMV. Its inhibition was much stronger than that of probenecid. The IC50 value for morin was 18 µM, whereas that for probenecid was 200 µM, which was similar to the previously reported value of 174 µM (Dan and Koga, 1990).

The in vivo experiments demonstrated that morin could indeed exert hypouricemic and uricosuric actions in the oxonate-induced hyperuricemic rats. We have used a dose of 200 mg of oxonate/kg body weight since a higher dose of the drug would result in a much higher increase in urine urate level overshadowing the uricosuric effects being investigated (Sugino and Shimada, 1995). Potassium oxonate is an inhibitor of uricase. An i.p. injection of oxonate could partially block the conversion of uric acid to allantoin and hence artificially elevate the plasma uric acid level in rats to provide a hyperuricemic animal model (Johnson et al., 1969; Osada et al., 1993). Morin, at a concentration of 80 mg/kg, restored the plasma uric acid level of the hyperuricemic rats to that of the normal group. However, at this concentration of morin, the decrease in plasma uric acid level could not be explained simply by its uricosuric action because the urate excreted was actually less for the morin-treated hyperuricemic rats when compared with the hyperuricemic control rats (Fig. 7B). Instead, the hypouricemic action of morin at 80 mg/kg was probably due to the inhibitory effect of morin on xanthine oxidase. The inhibition of morin on xanthine oxidase (IC50 = 44 µM) is moderate when compared with allopurinol (IC50 = 5.43 µM) (Fernandes et al., 2002). At a high dose of morin, however, xanthine oxidase would be significantly inhibited. Thus, although further metabolism of uric acid was inhibited by oxonate, the plasma uric acid level was still low because its synthesis was inhibited. This was probably the reason for the observed low level of urate excretion at this high dose of morin.

Morin is a natural compound found in fig and other moraceae (Kang et al., 2004). Incidentally, this compound occurs abundantly in the twigs of Morus alba L., which is documented in traditional Chinese medicine as a herb used in the treatment of conditions akin to gouty arthritis in modern medicine. Therefore, our results provide a scientific basis for the clinical application of this herb. In addition, the favorable safety profile of this natural compound (Wu et al., 1994) makes it a potential candidate worthy of further investigations.

Elevated uric acid levels have been associated with a number of pathological conditions in humans. On the other hand, uric acid also possesses antioxidant activities. The loss of the uricase gene and hence a high level of plasma urate in humans has been speculated to be an evolutionary mechanism that allows a longer lifespan. In this regard, it is interesting to point out that morin also possesses antioxidant activities (Wu et al., 1994, 1995; Kok et al., 2000). This antioxidant property of morin is actually an added advantage for this compound as a hypouricemic agent because the attenuated antioxidant capacity due to the lowering of uric acid level...
could be compensated by this natural compound. Further studies to delineate its clinical efficacy and to determine the appropriate dosage are highly warranted.

References

**Address correspondence to:** Dr. Christopher H. K. Cheng, Department of Biochemistry, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China. E-mail: chckcheng@uuhk.edu.hk

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