RESEARCH ARTICLE

Antihyperuricemic and nephroprotective effects of resveratrol and its analogues in hyperuricemic mice

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Scope: Stilbenes, of which, resveratrol is a representative compound in foods and plants, possess a variety of bioactivities including antioxidation, anti-inflammation, chemoprevention, and cardioprotection. This study was conducted to evaluate the antihyperuricemic and nephroprotective effects of resveratrol and its analogues and explore the possible mechanisms. The structure–activity relationships were analyzed.

Methods and results: Potassium oxonate-induced hyperuricemic mice were dosed by gavage with eight stilbenes. Uric acid, creatinine, and blood urea nitrogen (BUN) levels in serum and urine, clearance rate of creatinine and BUN, 24-h urate excretion, and fractional excretion of uric acid, uromodulin levels in urine and kidney were determined to evaluate renal urate handling and function. Renal protein levels of organic ion transporters were detected to elucidate the possible mechanisms. Resveratrol, *trans*-4-hydroxystilbene, pterostilbene, polydatin, and mulberroside A were found to have antihyperuricemic activities. These compounds together with *trans*-2-hydroxystilbene provided nephroprotection. *Trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 had no effects.

Conclusion: The uricosuric and nephroprotective actions of resveratrol and its analogues were mediated by regulating renal organic ion transporters in hyperuricemic mice, supporting their beneficial effects for the prevention of hyperuricemia. The number and position, methoxylation and glycosylation of hydroxyl groups in these *trans*-stilbenes were required for their effects.

Keywords:
Gout / Hypouricemic effect / Nephroprotection / Stilbenes / Structure–activity relationship

1 Introduction

Hyperuricemia as a metabolic disorder is usually associated with gout, kidney disease, hypertension, cardiovascular disease, inflammation, diabetes, and metabolic syndrome [1–3]. The reabsorption and secretion of uric acid are controlled by specific organic anion transport proteins in renal apical and basolateral membranes. Urate transporter 1 (URAT1, *SLC22A12*) and glucose transporter 9 (GLUT9, *SLC2A9*) mediate urate reabsorption from kidney tubule lumen to blood and maintain blood urate homeostasis [4, 5]. Human ATP-binding cassette, subfamily G, 2 (*ABCG2*) is located in the brush border membranes of renal proximal tubules to control urate secretion, and its gene mutation in Xenopus oocytes produces the reduction in urate transport rate [6]. *ABCG2* is associated with hyperuricemia and gout in Caucasian, Han Chinese, Japanese, and African–American subjects [7, 8]. Organic anion transporter 1 (OAT1, *SLC22A6*) in the basolateral membranes of renal proximal tubules is responsible for urate secretion from blood to epithelial cells [5]. Uricosuric agents perform their urate-lowering actions by regulating renal URAT1, GLUT9, and OAT1 [4, 8]. Therefore, these renal urate transport-related proteins constitute important targets for the effective agents to prevent and treat hyperuricemia and gout [9]. Renal organic cation/carnitine transporters (OCTs and OCTNs) are involved in the excretion of organic cations including organic drugs and their metabolites. Expression changes of renal OCTs and OCTNs impair kidney organic

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Abbreviations: ABCG2, ATP-binding cassette, subfamily G, 2; **BUN**, blood urea nitrogen; **FEUA**, fractional excretion of uric acid; **GLUT9**, glucose transporter 9; **OAT1**, organic anion transporter 1; **OCTs**, organic cation transporters; **OCTNs**, organic cation/carnitine transporters; **URAT1**, urate transporter 1

cation balance and induce renal solute toxicity [10, 11]. Our previous study demonstrated the downregulation of renal mOCT1, mOCT2, mOCTN1, and mOCTN2 in hyperuricemic mice with renal injury [12]. Uromodulin (UMOD), the most abundant protein in normal urine, is associated with hyperuricemia and kidney diseases [13]. UMOD-deficient mice have reduced creatinine clearance and upregulated expression of the major distal electrolyte transporters [14]. UMOD is a useful marker for renal dysfunction in hyperuricemia with the abnormality of renal organic ion transporters [14]. The effective therapy for hyperuricemia consists of the recommendation for a diet low in purines, alkalinization of urine, and the utilization of antihyperuricemic agents [15]. Allopurinol and probenecid are currently available, but the incidence of side effects such as pruritus, rash, hypersensitivity syndrome, and central nervous toxicity to these agents is still a major clinical problem [16]. Therefore, the search for the effective agents aiming at renal urate elimination targets is necessary.

Stilbene-derived natural compounds with a variety of biological activities have become of great interest to several research groups worldwide [17, 18]. Dietary supply of these stilbenes in grapes, red wine, peanuts, purple grape juice, and berries may have beneficial effects on human health [17–23]. Resveratrol (*trans*-3,4′,5-trihydroxystilbene), one of the most relevant and extensively studied natural stilbenes (Fig. 1), possesses health-promoting properties such as antioxidation, anti-inflammation, cardioprotection, antidiabetes, anticancer, chemoprevention, and neuroprotection [21–25]. As a methylated derivative of resveratrol, pterostilbene has similar bioactivities such as antioxidation [26] and anti-inflammation [27]. Other resveratrol analogues *trans*-4-hydroxystilbene and polydatin are confirmed to have antioxidant activity [25, 28]. Interestingly, resveratrol is able to decrease serum uric acid levels and protects against diabetic nephropathy in diabetic rodents [29]. However, it has a short half-life and limited bioavailability owing to the susceptibility of its phase II metabolism (glucuronidation and sulfation of hydroxyl groups) [20]. Mulberroside A is found abundantly in *Morus alba* L., which is used traditionally in Chinese medicine for the treatment of hyperuricemia and gout. We have recently shown that mulberroside A had uricosuric effects in hyperuricemic mice [12]. Additionally, *Smilax china* L. in which stilbenes are the main active components, is demonstrated to reduce serum urate levels and improve renal function in hyperuricemic mice [30]. These observations suggest that resveratrol analogues may exert urate-lowering effects in hyperuricemia. In this regard, it is necessary to explore the efficacy of resveratrol and its analogues in hyperuricemia and kidney dysfunction.

Therefore, the present study determined the effects of resveratrol and its analogues *trans*-4-hydroxystilbene, *trans*-2-hydroxystilbene, pterostilbene, *trans*-3,4′,5-trimethoxystilbene, *cis*-combretastatin A-4, polydatin, and mulberroside A (Fig. 1), which are structurally similar, on serum and urine biochemical indexes of hyperuricemia and kidney dysfunction such as uric acid, creatinine, and blood urea nitrogen (BUN) levels, together with the clearance of creatinine and BUN, 24-h uric acid excretion, and fractional excretion of uric acid (FEUA) in potassium oxonate-induced hyperuricemic mice. To explore the possible mechanisms underlying the antihyperuricemic action and renal function improvement of resveratrol and its analogues, we detected renal protein levels of mURAT1, mGLUT9, mABCG2, and mOAT1, as well as mOCT1, mOCT2, mOCTN1, and mOCTN2 by Western blot analysis. UMOD levels were simultaneously examined in hyperuricemic mice treated with resveratrol and its analogues in order to improve our understanding of the role of UMOD in mediating their actions. In addition, the structure–activity relationships of resveratrol and its analogues were briefly analyzed in this study.

2 Materials and methods

2.1 Materials and apparatus

Uric acid (>99%), allopurinol (>98%), and potassium oxonate (>97%) were purchased from Sigma (St. Louis, MO, USA). Probenecid (>98.5%) was purchased from San-Jiang Pharmaceutical Chemical Co. Ltd. (Shandong, China). Resveratrol and polydatin (>98%) were purchased from Xi'an Feida Biotech Co. Ltd. (Xian, China). *Trans*-4-hydroxystilbene and *trans*-2-hydroxystilbene (>98%) were purchased from Meryer Chemical Technology Shanghai Co. Ltd. (Shanghai, China). Pterostilbene (>98%) was purchased from Shenyang Dakang Pharmaceutical Technology Co. Ltd. (Shenyang, China). *Trans*-3,4′,5-trimethoxystilbene (>98%) was purchased from Hongkong Advanced Technology and Industrial Co., Ltd. (Shenzhen, China). *Cis*-combretastatin A-4 (>98%) was purchased from Goldwills Pharmaceutical and Chemical Co. Ltd. (Hanzhong, China). Mulberroside A (>98%) was purchased from Shanghai Sunny Biotech Co., Ltd. (Shanghai, China). Assay kits of creatinine and BUN were purchased from Jiancheng Biotech. (Nanjing, China). ELISA assay kit of UMOD was purchased from R&D (Minneapolis, USA). Antibodies used in Western blot analysis were summarized in Table 1. Western blot analysis system was purchased from Bio-Rad Laboratories (Shanghai) Co., Ltd. (Shanghai, China).

2.2 Animals

Male Kun-Ming strain mice (20 ± 2 g) were purchased from the Animal Centre of Qing-Long Shan (Nanjing, China). They were allowed at least 1 week to adapt to the environment before starting experiments. Animals were housed 5 per cage (320 × 180 × 160 cm) under a normal 12-h/12-h light/dark schedule with the light on at 07:00 a.m. (room temperature: 22 ± 2°C; relative humidity: 55 ± 5%), and given a standard

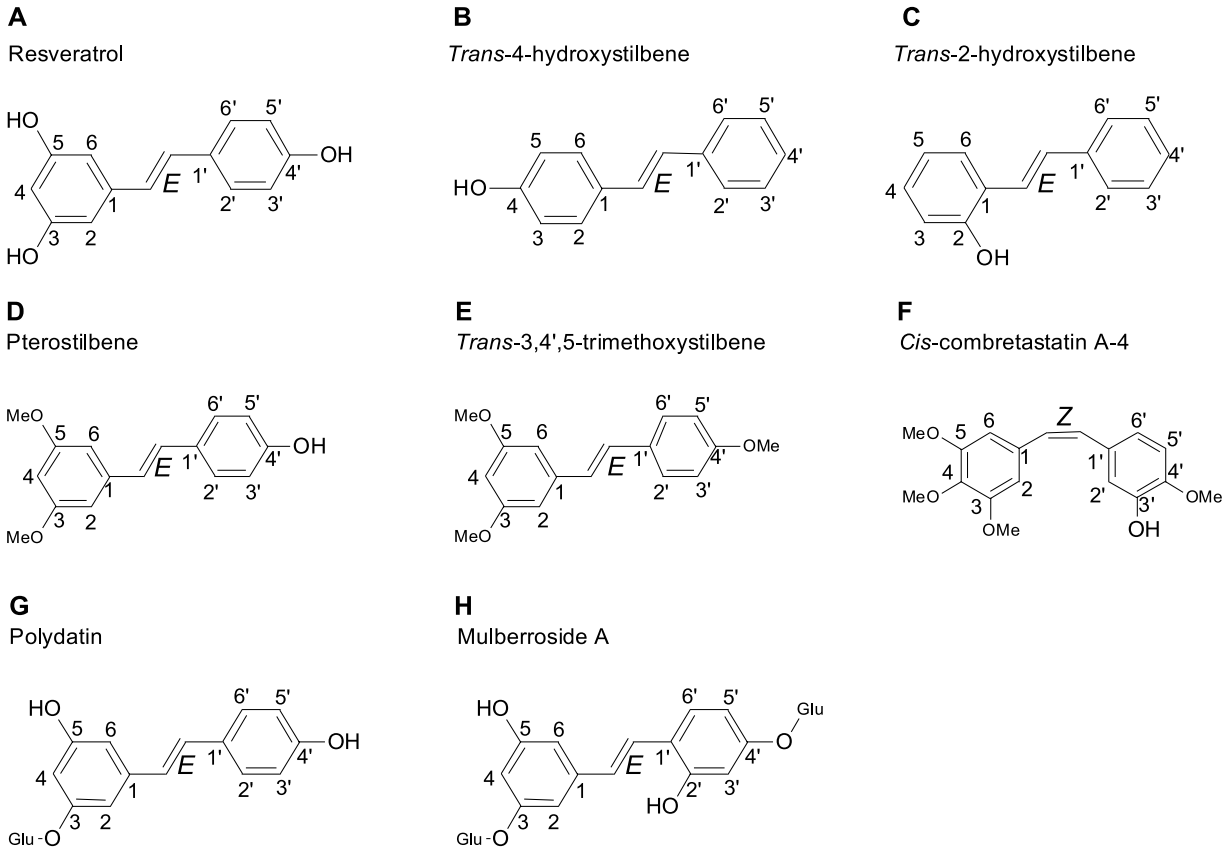

Figure 1. Structures of resveratrol and its analogues.

chow and water ad libitum for the duration of the study. All experiments were carried out in accordance with the Institutional Animal Care Committee at the Nanjing University and the China Council on Animal Care at Nanjing University [SYXK (SU) 2009 – 0017].

An experimental animal model of hyperuricemia induced by uricase inhibitor potassium oxonate has been used to evaluate the effects of drugs or possible therapeutic agents on serum urate levels [12]. Mice were randomly divided into a normal group receiving water, and hyperuricemic groups receiving water, 20 and 40 mg/kg resveratrol, *trans*-4-hydroxystilbene, *trans*-2-hydroxystilbene, pterostilbene, *trans*-3,4′,5-trimethoxystilbene, *cis*-combretastatin A-4, polydatin, and mulberroside A, 5 mg/kg allopurinol, and 100 mg/kg probenecid (positive control drugs), respectively. Each group consisted of eight mice.

Oxonate and all tested samples were freshly dispersed in distilled water, respectively. Food, but not water, was withdrawn from the animals 1 h prior to the administration. Mice were administered in a volume of 15 mL/kg by gavage once daily with oxonate (250 mg/kg) or water (vehicle) at 8:00 a.m. for 7 days. Resveratrol and its analogues, allopurinol and probenecid were orally initiated at 9:00 a.m. on the day when oxonate was given.

2.3 Urine, blood, and kidney tissue sample collection

To check biochemical profiles associated with serum urate level and kidney function, 24-h urine as well as blood samples were collected and assayed. From 24 h before final administration on seventh day, urine sample for each mouse in 24 h was collected in a metabolic cage, and centrifuged at 2000 × *g* for 10 min to remove the particulate contaminants. The supernatant was used for the determination of uric acid, creatinine, BUN, and UMOD levels. Animals were sacrificed by decapitation 1 h after the last treatment and blood samples were collected to get serum samples for uric acid, creatinine, and BUN assays, respectively. Serum and urine samples were stored at −20°C until biochemical assays were performed. Simultaneously, kidney cortex tissues were rapidly separated on ice plate and stored at −70°C until Western blot analysis.

2.4 Determination of uric acid, creatinine, and BUN levels in serum and urine

Uric acid concentrations in serum and 24-h urine were determined by the phosphotungstic acid method [12]. Excretion

Table 1. Summary of antibodies used in Western blot analysis

Company	Description	Catalog number
Saichi Biotech (Beijing, China)	Rabbit mURAT1 antibody	001046-R
	Rabbit mGLUT9 antibody	001052-R
	Rabbit mOAT1 antibody	001019-R
	Rabbit mOCT1 antibody	001017-R
	Rabbit mOCT2 antibody	001018-R
Alpha Diagnostic International Inc. (San Antonio, USA)	Rabbit mOCTN1 antibody	OCTN11-A
	Rabbit mOCTN2 antibody	OCTN21-A
Kangchen Biotech (Shanghai, China)	Mouse mGAPDH monoclonal antibody	KC-5G5
Cell Signaling Technology, Inc. (Boston, MA, USA)	Rabbit mNa^+-K^+-ATPase antibody	#3010S
	Rabbit mABCG2 antibody	#4477S
Jingmei Biotech (Shanghai, China)	Goat antirabbit-immunoglobulin G-horseradish peroxidase	SB-200

of urate in 24 h was calculated using the formula: volume of urine in 24 h × uric acid concentration in 24-h urine. Creatinine levels in serum and 24-h urine as well as BUN levels were determined spectrophotometrically using standard diagnostic kits, respectively. Clearance rate of creatinine was calculated by creatinine concentration in 24-h urine × volume of urine in 24 h / creatinine concentration in serum. Clearance rate of BUN was calculated by BUN concentration in 24-h urine × volume of urine in 24 h / BUN concentration in serum. FEUA was calculated using the formula: FEUA = (uric acid concentration in 24-h urine × creatinine concentration in serum) / (uric acid concentration in serum × creatinine concentration in 24-h urine) × 100, expressed as a percentage [12].

2.5 Determination of urinary and renal UMOD levels

The kidney cortex tissue was homogenized in 100 volume/weight of sodium chloride solution and centrifuged at 5000 × *g* for 10 min at 4°C. UMOD levels in 24-h urine and kidney homogenate supernatant were determined using ELISA kit.

2.6 Western blot analysis

To investigate the possible molecular mechanisms, expression levels of renal urate transport-related proteins were analyzed by Western blot method. Protein levels of mGLUT9, mOAT1, mOCT1, and mOCT2 in renal cortex, and mURAT1, mABCG2, mOCTN1, and mOCTN2 in renal brush border membranes were determined as described in our previous study [12]. Relative quantitation for Western blot analysis was calculated after the normalization to the amount of mouse glyceraldehyde-3-phosphate dehydrogenase (mGAPDH) or mNa^+-K^+-ATPase protein levels.

2.7 Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed using a one-way analysis of variance followed by Dunnett's multiple comparison tests to determine the level of significance. A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Effects of resveratrol and its analogues on serum and urine biochemical indexes of hyperuricemia and kidney dysfunction in oxonate-treated mice

To investigate the antihyperuricemic effect and kidney function improvement of resveratrol and its analogues, serum uric acid levels, the representative biomarker of hyperuricemia, as well as renal function parameters were assayed in oxonate-treated mice. As shown in Fig. 2 , oxonate was further demonstrated to induce hyperuricemia with renal dysfunction in mice [12]. A total of 20 and 40 mg/kg of resveratrol, pterostilbene, polydatin, and mulberroside A and 40 mg/kg of *trans*-4-hydroxystilbene significantly decreased serum uric acid levels near to or below the normal value. Furthermore, resveratrol significantly decreased serum creatinine and BUN levels to the normal value (20 and 40 mg/kg) and increased urine creatinine (20 mg/kg) and BUN (40 mg/kg) levels in hyperuricemic mice. A total of 20 and 40 mg/kg of *trans*-4-hydroxystilbene, polydatin, and mulberroside A, and 40 mg/kg of *trans*-2-hydroxystilbene and pterostilbene significantly decreased serum creatinine and BUN levels to the normal value, simultaneously, they increased urine creatinine and BUN levels in hyperuricemic mice (Fig. 2). Additionally, 40 mg/kg of resveratrol and *trans*-4-hydroxystilbene along with 20 and 40 mg/kg of *trans*-2-hydroxystilbene, pterostilbene, polydatin, and mulberroside A increased clearance rate of creatinine in this model. These compounds at 40 mg/kg and mulberroside A at 20 and 40 mg/kg also increased clearance rate of BUN to the normal value in hyperuricemic mice (Fig. 2).

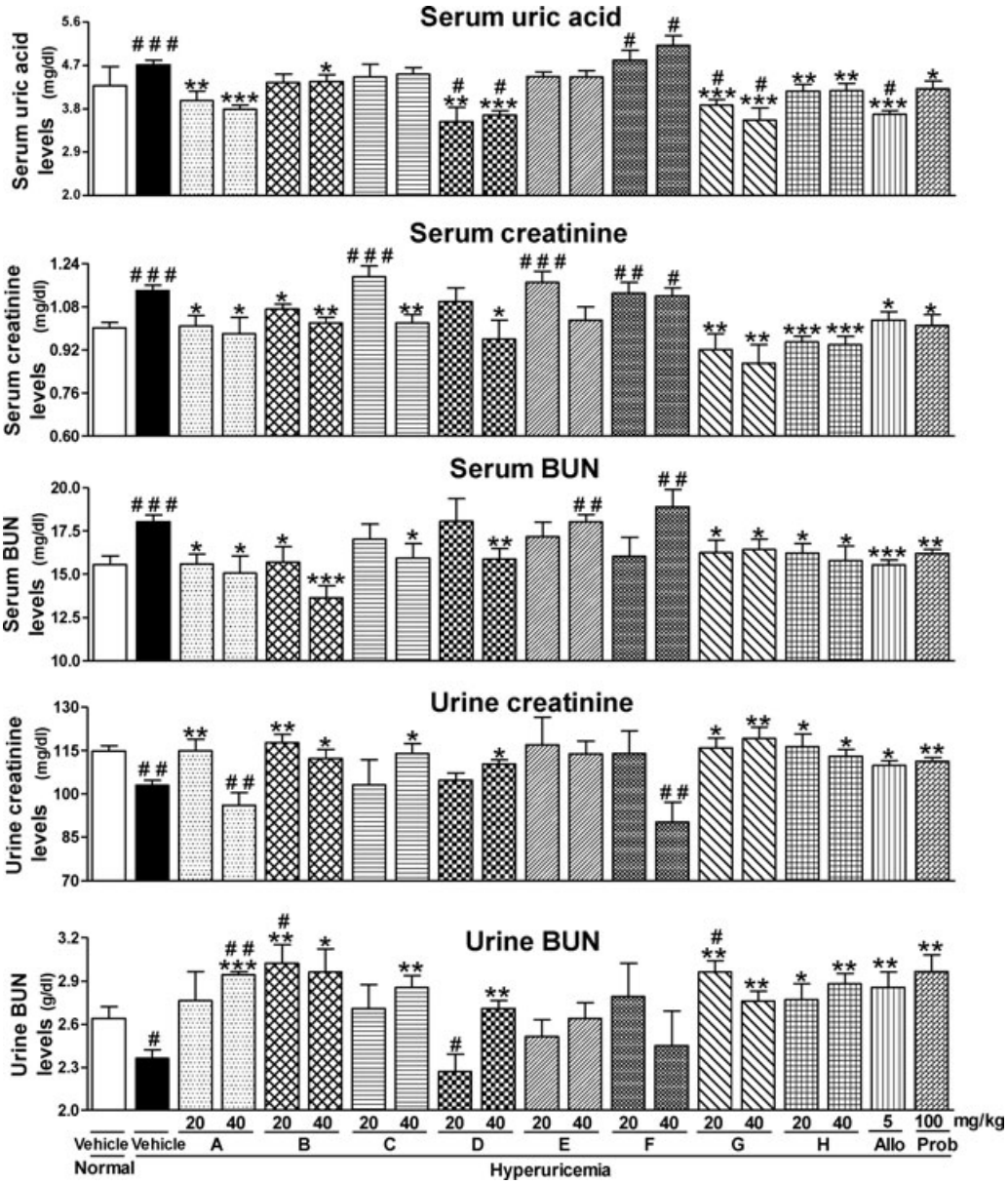

Figure 2. Effects of resveratrol and its analogues on serum and urine uric acid, creatinine and blood urea nitrogen (BUN), clearance rate of creatinine and BUN, 24-h uric acid excretion, and FEUA in hyperuricemic mice. Experiments were performed as described in Section 2. Data were expressed as the mean ± SEM ($n = 8$). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. normal animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. hyperuricemia-vehicle animals. (A) Resveratrol, (B) *trans*-4-hydroxystilbene, (C) *trans*-2-hydroxystilbene, (D) pterostilbene, (E) *trans*-3,4′,5-trimethoxystilbene, (F) *cis*-combretastatin A-4, (G) polydatin, (H) mulberroside A, Allo: allopurinol, Prob: probenecid.

To elucidate the possibility that the reduction of serum urate levels in hyperuricemic mice treated with resveratrol and its analogues was due to the enhancement of renal urate excretion, their effects on urine uric acid concentrations, 24-h urinary urate levels, and FEUA were detected (Fig. 2). Reductions of these indexes were observed in hyperuricemic mice, which were restored by the treatment of 20 and 40 mg/kg of resveratrol, *trans*-4-hydroxystilbene, polydatin, and mulberroside A, and 40 mg/kg of pterostilbene.

These data suggested that resveratrol, *trans*-4-hydroxystilbene, pterostilbene, polydatin, and mulberroside A exhibited antihyperuricemic and nephroprotective effects and *trans*-2-hydroxystilbene had nephroprotection in hyperuricemic animals. However, *trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 did not change these biochemical indexes in this model. As expected [12], allopurinol and probenecid significantly attenuated oxonate-induced these biochemical abnormalities in mice (Fig. 2).

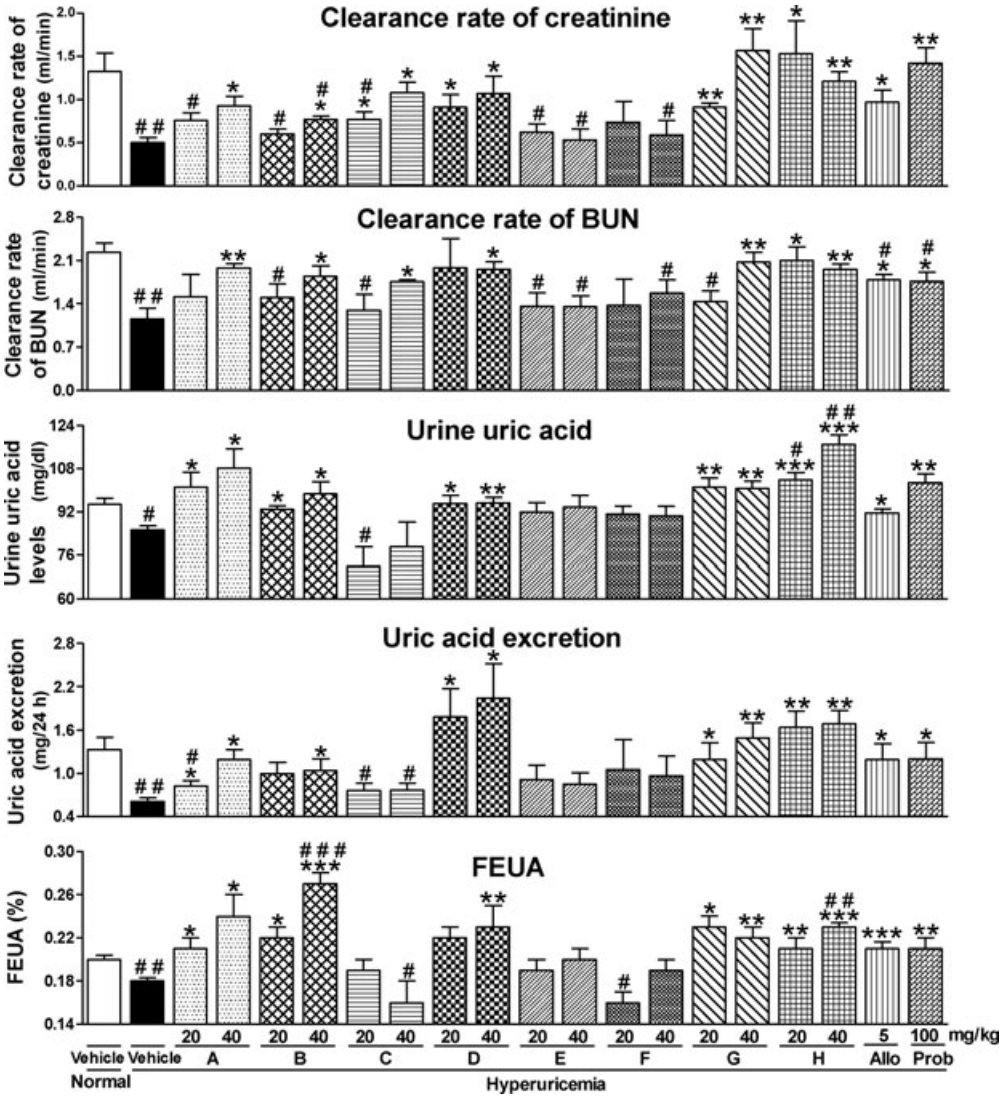

Figure 2. Continued.

3.2 Effects of resveratrol and its analogues on renal expression levels of urate transport-related proteins in oxonate-treated mice

It is well suggested that maintenance of urate homeostasis is critically dependent on urate excretion mediated by renal organic anion transporters. To investigate the possible antihyperuricemic mechanisms, we examined the effects of resveratrol and its analogues on the expression levels of renal mURAT1, mGLUT9, mABCG2, and mOAT1 using Western blot analysis.

As demonstrated in Fig. 3, 20 and 40 mg/kg of resveratrol failed to affect mURAT1 expression, but significantly decreased mGLUT9 protein levels in the kidney of hyperuricemic mice. *Trans*-4-hydroxystilbene and pterostilbene at 40 mg/kg remarkably decreased renal mURAT1 protein levels, but did not change renal mGLUT9 expression. Polydatin and mulberroside A at 20 and 40 mg/kg dramatically reduced renal protein levels of mURAT1 and mGLUT9 in hyperuricemic mice. Furthermore, the increased mABCG2 and decreased mOAT1 at protein levels were observed in the kidney of hyperuricemic animals (Fig. 4). Resveratrol significantly downregulated renal mABCG2 (40 mg/kg) and upregulated renal mOAT1 (20 and 40 mg/kg) in hyperuricemic mice. *Trans*-4-hydroxystilbene at 40 mg/kg and pterostilbene at 20 and 40 mg/kg decreased renal mABCG2 protein levels, without renal mOAT1 change in this model. Polydatin and mulberroside A at 20 and 40 mg/kg remarkably reversed oxonate-induced expression abnormality of renal mABCG2 and mOAT1 in mice. In contrast, *trans*-2-hydroxystilbene, *trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 failed to alter renal mURAT1, mGLUT9, mABCG2, and mOAT1 protein levels in hyperuricemic mice. Allopurinol and probenecid restored

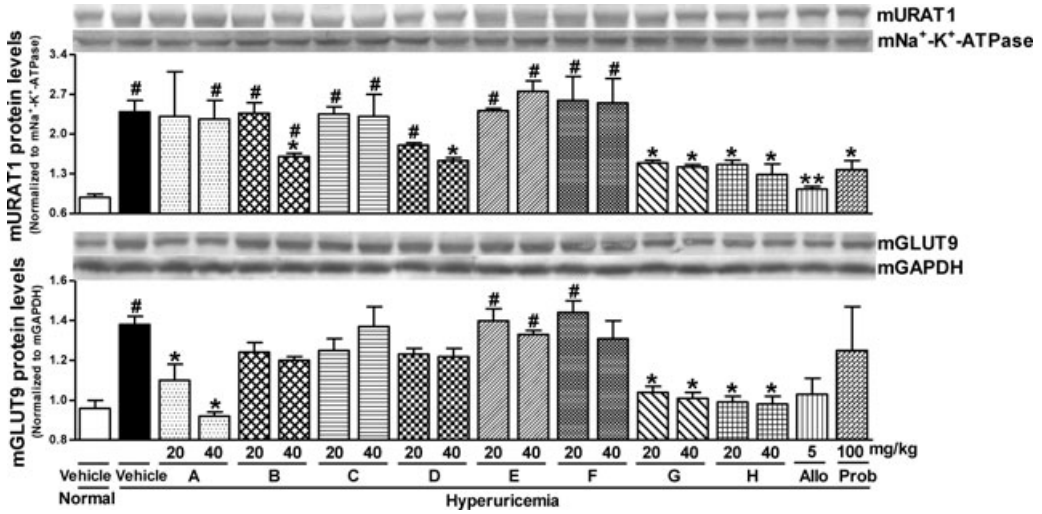

Figure 3. Effects of resveratrol and its analogues on renal mURAT1 and mGLUT9 protein levels in oxonate-induced hyperuricemic mice. Experiments were performed as described in Section 2. Data were expressed as the mean ± SEM ($n = 4$). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. normal animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. hyperuricemia-vehicle animals. (A) Resveratrol, (B) *trans*-4-hydroxystilbene, (C) *trans*-2-hydroxystilbene, (D) pterostilbene, (E) *trans*-3,4′,5-trimethoxystilbene, (F) *cis*-combretastatin A-4, (G) polydatin, (H) mulberroside A, Allo: allopurinol, Prob: probenecid.

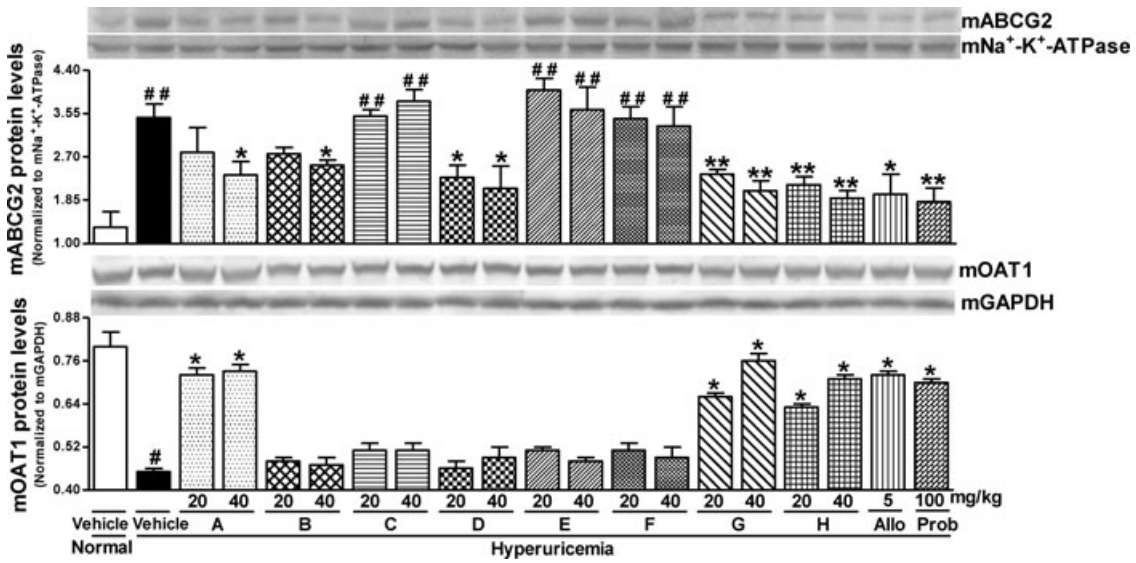

Figure 4. Effects of resveratrol and its analogues on renal mABCG2 and mOAT1 protein levels in oxonate-induced hyperuricemic mice. Experiments were performed as described in Section 2. Data were expressed as the mean ± SEM ($n = 4$). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. normal animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. hyperuricemia-vehicle animals. (A) Resveratrol, (B) *trans*-4-hydroxystilbene, (C) *trans*-2-hydroxystilbene, (D) pterostilbene, (E) *trans*-3,4′,5-trimethoxystilbene, (F) *cis*-combretastatin A-4, (G) polydatin, (H) mulberroside A, Allo: allopurinol, Prob: probenecid.

oxonate-induced expression abnormality of renal mURAT1, mABCG2, and mOAT1 but not mGLUT9 in this model.

3.3 Effects of resveratrol and its analogues on renal protein levels of mOCT1, mOCT2, mOCTN1, and mOCTN2 in oxonate-treated mice

Abnormality of urate transport can develop metabolism disequilibration of renal organic solutes, causing renal dysfunction combined with hyperuricemia [9]. In the present study, we tested the effects of resveratrol and its analogues on protein levels of renal organic cation/carnitine transporters in oxonate-treated mice. As shown in Fig. 5, 20 and 40 mg/kg of resveratrol and mulberroside A, and 40 mg/kg of polydatin significantly upregulated renal mOCT1 protein levels to the normal value, but failed to alter renal mOCT2 expression in hyperuricemic mice. *Trans*-4-hydroxystilbene, *trans*-2-hydroxystilbene, pterostilbene,

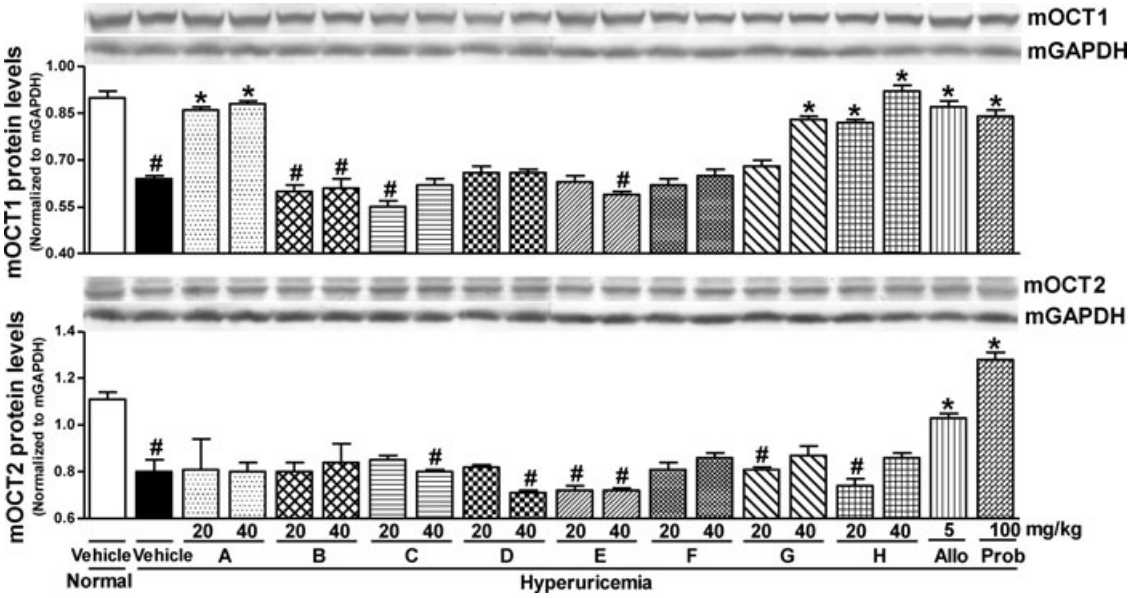

Figure 5. Effects of resveratrol and its analogues on renal mOCT1 and mOCT2 protein levels in oxonate-induced hyperuricemic mice. Experiments were performed as described in Section 2. Data were expressed as the mean ± SEM ($n = 4$). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. normal animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. hyperuricemia-vehicle animals. (A) Resveratrol, (B) *trans*-4-hydroxystilbene, (C) *trans*-2-hydroxystilbene, (D) pterostilbene, (E) *trans*-3,4′,5-trimethoxystilbene, (F) *cis*-combretastatin A-4, (G) polydatin, (H) mulberroside A, Allo: allopurinol, Prob: probenecid.

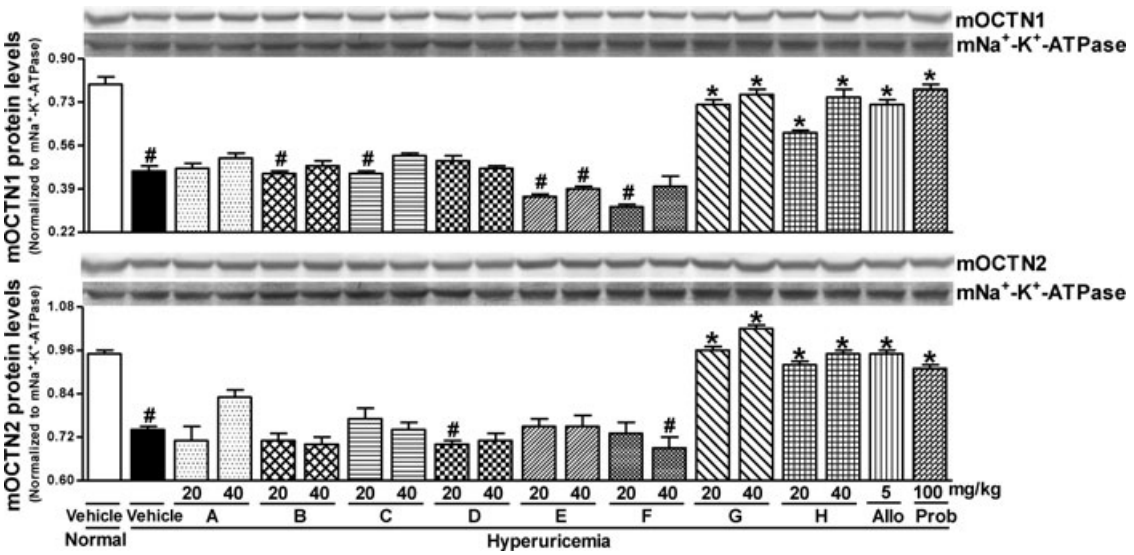

Figure 6. Effects of resveratrol and its analogues on renal mOCTN1 and mOCTN2 protein levels in oxonate-induced hyperuricemic mice. Experiments were performed as described in Section 2. Data were expressed as the mean ± SEM ($n = 4$). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. normal animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. hyperuricemia-vehicle animals. (A) Resveratrol, (B) *trans*-4-hydroxystilbene, (C) *trans*-2-hydroxystilbene, (D) pterostilbene, (E) *trans*-3,4′,5-trimethoxystilbene, (F) *cis*-combretastatin A-4, (G) polydatin, (H) mulberroside A, Allo: allopurinol, Prob: probenecid.

trans-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 did not affect renal mOCT1 and mOCT2 in this model. Furthermore, 20 and 40 mg/kg of polydatin and mulberroside A significantly upregulated renal mOCTN1 and mOCTN2 protein levels in hyperuricemic mice (Fig. 6). However, resveratrol, *trans*-4-hydroxystilbene, *trans*-2-hydroxystilbene, pterostilbene, *trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 failed to alter renal mOCTN1 and mOCTN2 in this model. Allopurinol and probenecid significantly increased renal mOCT1, mOCT2, mOCTN1, and mOCTN2 protein levels in hyperuricemic mice (Figs. 5 and 6).

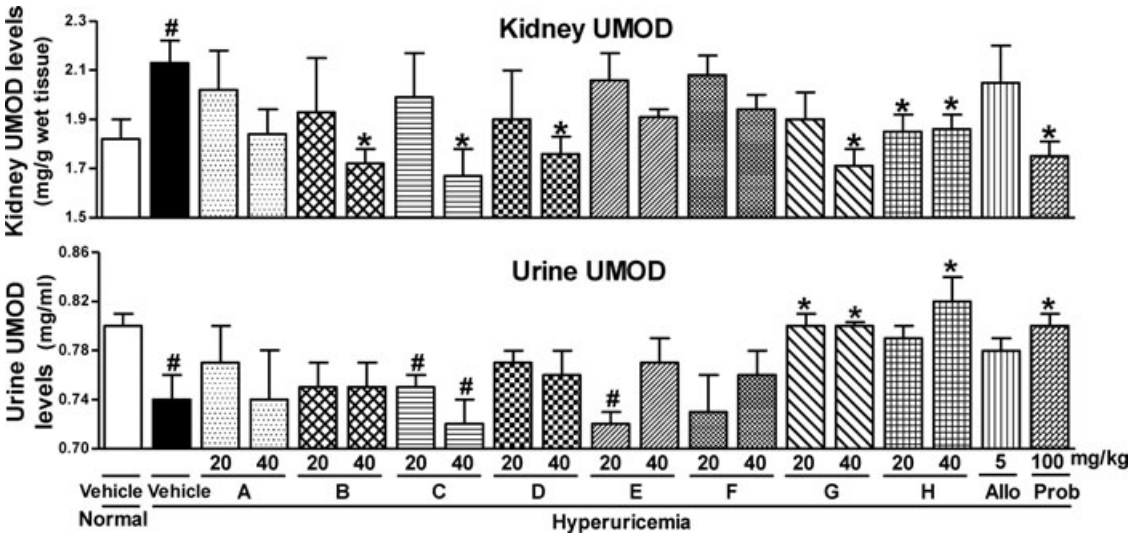

Figure 7. Effects of resveratrol and its analogues on renal and urinary UMOD levels in oxonate-induced hyperuricemic mice. Experiments were performed as described in Section 2. Data were expressed as the mean ± SEM ($n = 4$). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ vs. normal animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. hyperuricemia-vehicle animals. (A) Resveratrol, (B) *trans*-4-hydroxystilbene, (C) *trans*-2-hydroxystilbene, (D) pterostilbene, (E) *trans*-3,4′,5-trimethoxystilbene, (F) *cis*-combretastatin A-4, (G) polydatin, (H) mulberroside A, Allo: allopurinol, Prob: probenecid.

3.4 Effects of resveratrol and its analogues on renal and urine UMOD levels in oxonate-treated mice

UMOD mutation is suggested to be associated with hyperuricemia and renal failure [13]. As shown in Fig. 7, UMOD levels of kidney were increased and of urine were reduced in hyperuricemic mice. A total of 40 mg/kg of *trans*-4-hydroxystilbene, *trans*-2-hydroxystilbene, pterostilbene and polydatin, as well as 20 and 40 mg/kg of mulberroside A significantly decreased renal UMOD levels to the normal value in hyperuricemic mice. However, resveratrol, *trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 failed to alter kidney UMOD levels in this model. Among resveratrol and its analogues, polydatin at 20 and 40 mg/kg and mulberroside A at 40 mg/kg dramatically elevated urinary UMOD levels in hyperuricemic mice. Additionally, probenecid but not allopurinol restored oxonate-induced UMOD alteration of kidney and urine in mice.

4 Discussion

The present study demonstrated that resveratrol and its analogues *trans*-4-hydroxystilbene, pterostilbene, polydatin, and mulberroside A reduced serum urate levels and enhanced urate excretion in oxonate-induced hyperuricemic mice. Their antihyperuricemic effects were related to the regulation of renal mURAT1, mGLUT9, mABCG2, and mOAT1 in hyperuricemic mice. Moreover, the improvement of renal function as well as upregulation of renal mOCT1, mOCT2, mOCTN1, and mOCTN2 protein levels contributed to the nephroprotective effects of resveratrol, *trans*-4-hydroxystilbene, *trans*-2-hydroxystilbene, pterostilbene, polydatin, and mulberroside A. However, *trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 did not have these effects in hyperuricemic mice. Additionally, the number and position, methoxylation and glycosylation of hydroxyl groups may be important requirements of these stilbenes for their antihyperuricemic effects.

Serum urate level is most often consequent to renal urate excretion. Renal urate transport becomes increasingly relevant in blood urate homeostasis. The present study revealed that resveratrol reduced serum urate levels by downregulating mGLUT9 expression to inhibit urate reabsorption, and downregulating mABCG2 with upregulation of mOAT1 expression to increase urate secretion in the kidney of hyperuricemic mice. *Trans*-4-hydroxystilbene and pterostilbene may reduce urate reabsorption by downregulating renal mURAT1 expression and increase urate secretion by downregulating renal mABCG2 in hyperuricemic mice. Compared with the above three stilbenes, polydatin and mulberroside A showed antihyperuricemic actions simultaneously by regulating renal mURAT1 and mGLUT9 expression to decrease urate reabsorption, and renal mABCG2 and mOAT1 expression to elevate urate secretion. However, *trans*-2-hydroxystilbene, *trans*-3,4′,5-trimethoxystilbene, and *cis*-combretastatin A-4 were unable to effectively change renal mURAT1, mGLUT9, mABCG2, and mOAT1 in hyperuricemic mice, which were consistent with the lack of the antihyperuricemic effects observed in Fig. 2. These results suggested that resveratrol, trans-4-hydroxystilbene, pterostilbene, polydatin, and mulberroside A exhibited antihyperuricemic effects through the regulation of different renal urate transport-related proteins to enhance renal urate excretion in hyperuricemic mice. Comparison of

positive control drug, antihyperuricemic efficacies of pterostilbene and polydatin seemed to be similar to allopurinol, and more potent than probenecid in this study. It must be pointed out that this effective size comparison in hyperuricemia may be nearly irrelevant because no correlation was found between serum urate levels in animal studies and in clinical studies. Further studies will be required.

Hyperuricemia is one of well-described risk factors for kidney function disorders. Creatinine as a substrate of OCT1 and OCT2 in renal proximal tubules is also a biomarker of renal dysfunction [11]. Consistent with the amelioration of kidney dysfunction, the nephroprotective effects of resveratrol may be mediated by increasing renal mOCT1 expression, and of *trans*-4-hydroxystilbene, *trans*-2-hydroxystilbene, and pterostilbene at high dose by decreasing renal mUMOD levels in hyperuricemic mice. Compared with the above four stilbenes, polydatin and mulberroside A may similarly exert their potent effects by upregulating renal mOCT1, mOCTN1, mOCTN2, and urine UMOD levels, and simultaneously by reducing renal UMOD levels in hyperuricemic mice. However, *trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 failed to show nephroprotection in this model.

Clinical trials assessing the safety show that resveratrol is safe at 1.0 g daily for 29 days in humans [31]. A total of 3 g/kg pterostilbene and 100 mg/kg polydatin are confirmed to be safer in preclinical efficacy studies of rodent models [32, 33]. However, resveratrol at 5 g/day dosage can develop cast nephropathy in some multiple myeloma patients [34]. Nephropathy and renal toxicity are also observed in rats orally treated with 3 g/kg/day resveratrol for 28 days [35]. These observations suggest that high doses of these stilbenes raise the risks of kidney diseases. Generally, hyperuricemic patients have renal dysfunction. Careful attention must be given in the treatment trials to patients with kidney diseases. Using the body surface area to scale dose between humans and mice [36, 37], the doses of 20 and 40 mg/kg in mice of the present study are converted into 1.6 and 3.2 mg/kg in humans, or 96 and 192 mg/day for a 60 kg individual, respectively. In other animal studies, resveratrol does not cause any toxic effects except at doses above 1 g/kg/day, indicating that daily dose of 450 mg is safe for a 60 kg person [35, 38]. These observations together with the results of the present study indicate that low doses of these stilbenes with novel active targets may be safe and effective in the prevention of hyperuricemia and its associated diseases. Additionally, drug delivery methods to these stilbenes may be provided for enhancing absorption or bioavailability and eventually achieving adequate plasma levels by lower dosages. More important, these methods may prevent or minimize any possible side effects of these stilbenes. Cautious evaluation of the safety and toxicology of these stilbenes as well as delivery methods is necessary to simultaneously investigate their effects on serum urate levels and kidney function in patients with hyperuricemia.

Furthermore, it was worth to understand relationship of main structural features of resveratrol analogues and their antihyperuricemic properties. The tested compounds with hydroxyl group at C-4 position (*para*), such as resveratrol, *trans*-4-hydroxystilbene, pterostilbene, and polydatin significantly showed antihyperuricemic effects. Besides C-4 hydroxyl group, resveratrol has C-3 and C-5 hydroxyl groups. Compared with *trans*-4-hydroxystilbene, the number of hydroxyl groups is greater in resveratrol, the action improved, indicating that C-3 and C-5 hydroxyl groups may act as important groups in its antihyperuricemic action. The increased number of hydroxyl group in stilbene molecules may result in high hydrophilicity in vivo circumstance, which may be convenient to interact with hydrophilic groups or functional domains of renal urate transport-related proteins. On the other hand, resveratrol analogues in which hydroxyl groups are substituted by methoxyl groups exhibited different antihyperuricemic effects in comparison to the parent compound. Pterostilbene is derived from 3- and 5-hydroxyl groups of resveratrol substituted by methoxyl. Compared with resveratrol, its antihyperuricemic effects are greater. These results may be explained such that the methoxylation makes pterostilbene lipophilicity increase and then enter cells more easily, showing better pharmacokinetic properties than resveratrol. However, the entire methoxylation of three hydroxyl groups in resveratrol molecule, such as *trans*-3,4′,5-trimethoxystilbene makes the compound difficult to interact with renal urate transport-related proteins by hydrogen bonds, possibly showing disappearance of antihyperuricemic action in hyperuricemic mice. Furthermore, polydatin with glycosylation of hydroxyl groups remarkably produced potent antihyperuricemic effects in comparison to the parent compound resveratrol in hyperuricemic mice. Resveratrol is metabolized and circulated as glucuronides or sulfates occurring on hydroxyl groups [39]. Polydatin with 3-glycosylation and C-4 hydroxyl group is hydrolyzed in the body as resveratrol [40]. Similar to polydatin, mulberroside A in which hydroxyl groups at C-3 and C-4-positions are glycosides is metabolized into oxyresveratrol-2-O-β-D-glucopyranoside, oxyresveratrol-3′-O-β-D-glucopyranoside, and oxyresveratrol in rats [41]. The glycosylation may change the metabolism of related compounds in vivo, and be more bioavailable than resveratrol. Thus, the glycosylation of hydroxyl groups at the active sites (C-3, C-4, and C-5 positions) is suggested to strengthen their actions on the reduction of serum urate levels in hyperuricemic mice. Conversely, cisoid stilbene, *cis*-combretastatin A-4 with hydroxyl group at C-3′ position and four methoxy groups at C-3, C-4, C-5, and C-4′ positions did not show any effects in hyperuricemic mice, indicating that transoid configuration may be a key character for antihyperuricemic compounds resveratrol, *trans*-4-hydroxystilbene, pterostilbene, polydatin, and mulberroside A. These analyses could help in the elucidation of the important functional information about the number and position, methoxylation and glycosylation of hydroxyl groups, and double bond configuration in these natural resveratrol analogue molecules targeting renal organic ion transporters. Furthermore, the present study should be beneficial in future rational drug design

for hyperuricemia to develop drug candidates of structurally modified resveratrol analogues with the favorable bioavailability, safety, and efficacy.

Stilbenes are abundant in many foods and beverages, such as resveratrol and polydatin contained in mulberries, grapes, red wine, as well as *Polygonum cuspidatum*, *Cassia quinquangulata*, and *S. china*, resveratrol and pterostilbene in *Vaccinium Berries*, mulberroside A in *M. alba*, and pterostilbene in peanuts [42–45]. Thus, foods and herbs containing these beneficial nutrient stilbenes may be natural remedies to prevent hyperuricemia and its associated diseases. It is of interest to detect their contents in foods and herbs, as which may generate testable predictions and provide a dietary supplement for lowering serum urate in patients with hyperuricemia. Moreover, the combination of these stilbenes may be suggested to be best for preventing hyperuricemia and its associated diseases. Therapeutic action and underlying mechanisms of the combination of these stilbenes need to be further investigated.

In summary, the present study demonstrated the antihyperuricemic and nephroprotective activities of resveratrol, *trans*-4-hydroxystilbene, pterostilbene, polydatin, and mulberroside A in oxonate-induced hyperuricemic mice. These effects were mediated by regulating renal expression levels of mGLUT9, mABCG2, mOAT1, mOCT1 for resveratrol, of mURAT1, mABCG2, and mUMOD for *trans*-4-hydroxystilbene, of mURAT1, mAGCG2, and mUMOD for pterostilbene, of mURAT1, mGLUT9, mAGCG2, mOAT1, mOCT1, mOCTN1, mOCTN2, and mUMOD for polydatin and mulberroside A in hyperuricemic mice. *Trans*-2-hydroxystilbene showed renal protective activity by decreasing renal UMOD levels in hyperuricemic mice. However, *trans*-3,4′,5-trimethoxystilbene and *cis*-combretastatin A-4 were unable to show any effects in this model. The number and position, methylation and glycosylation of hydroxyl groups in natural *trans*-stilbenes may be required for their actions. These experimental results in animals provide the evidence for the possible preventive efficacy on hyperuricemia with renal dysfunction by resveratrol analogues. Foods and plants with these abundant stilbenes, as a daily dietary supplement of adults, may be natural remedies to safely and effectively prevent hyperuricemia with kidney dysfunction.

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