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Flavonoids Are Inhibitors of Human Organic Anion Transporter 1 (OAT1)–Mediated Transport

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ABSTRACT

Organic anion transporter 1 (OAT1) has been reported to be involved in the nephrotoxicity of many anionic xenobiotics. As current clinically used OAT1 inhibitors are often associated with safety issues, identifying potent OAT1 inhibitors with little toxicity is of great value in reducing OAT1-mediated drug nephrotoxicity. Flavonoids are a class of polyphenolic compounds with exceptional safety records. Our objective was to evaluate the effects of 18 naturally occurring flavonoids, and some of their glycosides, on the uptake of *para*-aminohippuric acid (PAH) in both OAT1-expressing and OAT1-negative LLC-PK1 cells. Most flavonoid aglycones produced substantial decreases in PAH uptake in OAT1-expressing cells. Among the flavonoids screened, fisetin, luteolin, morin, and quercetin exhibited the strongest effect and produced complete inhibition of OAT1-mediated PAH uptake at a concentration of 50 μM. Further concentration-dependent studies revealed that both morin and luteolin are potent OAT1 inhibitors, with IC_{50} values of <0.3 and 0.47 μM, respectively. In contrast to the tested flavonoid aglycones, all flavonoid glycosides had negligible or small effects on OAT1. In addition, the role of OAT1 in the uptake of fisetin, luteolin, morin, and quercetin was investigated and fisetin was found to be a substrate of OAT1. Taken together, our results indicate that flavonoids are a novel class of OAT1 modulators. Considering the high consumption of flavonoids in the diet and in herbal products, OAT1-mediated flavonoid-drug interactions may be clinically relevant. Further investigation is warranted to evaluate the nephroprotective role of flavonoids in relation to drug-induced nephrotoxicity mediated by the OAT1 pathway.

Introduction

The kidneys play a central role in homeostasis and detoxification through eliminating diverse hydrophilic xenobiotics. In addition to glomerular filtration, the kidneys excrete charged hydrophilic xenobiotics via carrier-mediated transport pathways in proximal tubular cells. Excreting organic ions through proximal tubular cells is a unidirectional process involving two distinct transport steps, including the uptake of charged hydrophilic xenobiotics into the cells from the blood across the basolateral membrane, followed by extrusion across the apical membrane into the urine for elimination (Pritchard and Miller, 1993). During the last decade, several families of organic ion transporters have been identified, among which the organic anion transporter (OAT) family represents one of the most important members due to its critical role in handling an enormous variety of structurally diverse organic anions (You, 2002; Burckhardt, 2012; Wang and Sweet, 2013b). To date, 10 members of the OAT family have been cloned, among which OAT1 was identified in 1996 and represents the first member of the OAT family identified (Lopez-Nieto et al., 1997). OAT1 is expressed predominantly in the kidneys and has been found to be localized exclusively at the basolateral membrane of proximal tubular cells (Hosoyamada et al., 1999). OAT1 demonstrates remarkably broad substrate specificity and has been reported to be actively involved in the tubular uptake of various small, negatively charged molecules, such as endogenous metabolites, toxicants, and numerous clinically used therapeutics (Burckhardt, 2012).

Toxins and cytotoxic drugs that are taken up by OAT1 may exert toxic effects on the proximal tubule cells, resulting in renal injury. Indeed, OAT1 has been reported to be involved, at least partly, in the emergence of the nephrotoxicity of several anionic xenobiotics, including β-lactam antibiotics (e.g., cephaloridine, cephaloglycin, and imipenem), antiviral agents (e.g., cidofovir and adefovir), as well as toxins (e.g., aristolochic acid and mercury) (Hagos and Wolff, 2010; Xue et al., 2011). For example, cephaloridine, a first-generation cephalosporin antibiotic, is rapidly transported into the proximal tubular cell by OATs, including OAT1, but undergoes minimal subsequent movement into the luminal fluid. As a result, the cephaloridine molecules are trapped in the proximal tubular cells, resulting in extremely high and prolonged cellular concentrations of cephaloridine, which subsequently cause severe nephrotoxicity (Tune, 1997). Similarly, the uptake of the antiviral agent cidofovir across the basolateral tubular membrane is mainly mediated by OAT1, and this tubular uptake step is more efficient than the subsequent step of tubular secretion into luminal fluid, leading to the accumulation of cidofovir in renal tubules and cidofovir-induced nephrotoxicity (Ho et al., 2000). Considering the important role of OAT1 in the drug-induced nephrotoxicity,

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ABBREVIATIONS: DMSO, dimethylsulfoxide; EGC, epigallocatechin; EGCG, epigallocatechin gallate; hOAT1, human organic anion transporter 1; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MRP2, multidrug resistance–associated protein 2; NSAID, nonsteroidal anti-inflammatory drugs; OAT, organic anion transporter; PAH, *para*-aminohippuric acid; PBS, phosphate-buffered saline.

coadministration of nephrotoxic drugs with OAT1 inhibitors may represent an effective strategy in reducing OAT1-mediated drug nephrotoxicity. Probenecid, a potent OAT1 inhibitor, has demonstrated in vivo nephroprotective effects and greatly reduced the nephrotoxicity of cephaloridine (Tune et al., 1977) and antiviral drugs (Lacy et al., 1998). Nonsteroidal anti-inflammatory drugs (NSAIDs), including ketoprofen, ibuprofen, and naproxen, exhibited protective effects against human OAT1 (hOAT1)–mediated cytotoxicity of adefovir (Mulato et al., 2000). However, although these OAT1 inhibitors demonstrated nephroprotective effects, they usually need to be administered at a relatively high dose, which is often associated with safety issues, such as gastrointestinal intolerance, renal insufficiency, and hepatotoxicity (Stillman, 1989; Bjorkman, 1998; Mulato et al., 2000). Therefore, identifying potent OAT1 inhibitors with little toxicity is of great value in reducing OAT1-mediated drug nephrotoxicity.

In line with this concept, in the past several years there have been substantial efforts to identify OAT inhibitors from natural sources. Recently, several dietary phenolic acids, including ellagic acid, caffeic acid, ferulic acid, gallic acid, sinapinic acid, and vanillic acid, have been reported to have a potent inhibitory effect on OATs, including OAT1 (Whitley et al., 2005; Uwai et al., 2011; Wang and Sweet, 2012b). In addition, the active components of a few medicinal herbs, including *Salvia miltiorrhiza* (Danshen) and *Rheum* sp., have also been found to potently interfere with OAT-mediated renal elimination (Wang and Sweet, 2012a; Wang et al., 2013). The interactions between OAT1 and flavonoids, a class of polyphenolic compounds with exceptional safety records, have also been evaluated, and a few flavonoids have been found to be potent OAT1 inhibitors in vitro (Hong et al., 2007; Wang and Sweet, 2013a). At this time, only a limited number of flavonoids have been examined. In the present study, we expanded the candidate list and examined the effect of 18 flavonoids, covering six different chemical subclasses of flavonoids (flavones, flavonols, flavanols, isoflavones, chalcones, and flavonolignans), on the uptake of *para*-aminohippuric acid (PAH), a well known OAT1 substrate, in both OAT1-expressing and OAT1-negative cells.

Materials and Methods

Flavonoids and their glycosides investigated in this study [biochanin A, chrysin, fisetin, galangin, luteolin, morin, myricetin, silymarin, diosmetin, diosmin, epigallocatechin (EGC), epigallocatechin gallate (EGCG), genistein, genistin, quercetin, rutin, phloretin, and phloridzin] were obtained from Sigma-Aldrich (St. Louis, MO) and Indofine Chemical Company (Hillsborough, NJ). [^{14}C]PAH (40–60 mCi/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Probenecid, ascorbic acid, and formic acid were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium, Hanks' balanced salt solution, fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). All the chemicals or solvents used for liquid chromatography–tandem mass spectrometry (LC-MS/MS) were commercially available and of high-performance liquid chromatography grade.

Cell Culture

LLC-PK1 cells were used in the PAH uptake studies, flavonoid concentration-dependent inhibition studies, and flavonoid cellular uptake studies. The LLC-PK1 and its subclone that was transfected with hOAT1 were provided by Dr. Guofeng You (Rutgers University, Piscataway, NJ). LLC-PK1 parental cells and LLC-PK1 cells expressing hOAT1 were cultured in 75-cm^2 flasks with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2/95% air atmosphere. For parental LLC-PK1 cells, the culture medium contained 100 units/ml penicillin and 100 μg/ml streptomycin. For LLC-PK1 cells stably transfected with hOAT1, in addition to penicillin/streptomycin, the culture medium also contained 0.8 mg/ml G418. A solution of 0.25% trypsin-EDTA was used to detach the cells from the 75-cm^2 flasks.

[^{14}C]PAH Cellular Uptake Studies

Uptake of [^{14}C]PAH at Various Time Periods. For uptake studies, the cells were seeded at a density of 5×10^5 cells per 35-mm-diameter Petri dish, 2 to 3 days prior to the uptake experiment. The uptake of [^{14}C]PAH (0.5 μM) was performed by incubating cells with 1 ml uptake buffer (PBS, pH 7.4, with 0.1 mM Ca^{2+} and 1 mM Mg^{2+}) at 37°C for various time periods (1, 5, 10, 15, and 30 minutes). The uptake reaction was stopped by aspiration, followed by washing with 3 ml ice-cold buffer (137 mM NaCl and 14 mM Tris base, pH 7.2) three times. Cells were then solubilized in 1 ml of 0.3 N NaOH for 2 hours, and 400 μl was aliquoted for liquid scintillation counting (1900 CA, Tri-Carb liquid scintillation analyzer; PerkinElmer Life and Analytical Sciences).

Uptake of [^{14}C]PAH with and without Flavonoids. The uptake of [^{14}C]PAH (0.5 μM) was performed by incubating cells with 1 ml uptake buffer (PBS, pH 7.4, with 0.1 mM Ca^{2+} and 1 mM Mg^{2+}) together with 50 μM flavonoids or the vehicle [0.3% dimethylsulfoxide (DMSO)] at 37°C for 5 minutes. Eighteen flavonoids, namely, biochanin A, chrysin, fisetin, galangin, luteolin, morin, myricetin, silymarin, diosmetin, diosmin, EGC, EGCG, genistein, genistin, quercetin, rutin, phloretin, and phloridzin, were evaluated in terms of their modulatory effect on OAT1-mediated PAH uptake. Probenecid (200 μM) was used as a positive control. The uptake reaction was stopped by aspiration, followed by washing with 3 ml ice-cold buffer (137 mM NaCl and 14 mM Tris base, pH 7.2) three times. Cells were then solubilized in 1 ml of 0.3 N NaOH for 2 hours, and 400 μl was aliquoted for liquid scintillation counting.

Flavonoid Concentration-Dependent Inhibition Studies

Concentration-dependent studies were performed following the same procedures as described above except that the effects of flavonoids were tested at varying concentrations (0.3−100 μM). Concentration-dependent effects were determined for three flavonoids (luteolin, morin, and quercetin). Specific uptake was obtained by subtracting [^{14}C]PAH uptake into hOAT1-negative cells from the uptake into hOAT1-expressing cells. The specific uptake of [^{14}C]PAH was expressed as percentage of the control (in the presence of 0.3% DMSO). The IC_{50} value, the concentration of flavonoid required to inhibit 50% of specific [^{14}C]PAH uptake, was obtained by fitting data with the following equation using WinNonlin Professional 2.1 (Pharsight, Mountain View, CA):

$$F = 100 \times \left(1 - \frac{I_{max} \cdot C^{\gamma}}{IC_{50}{}^{\gamma} + C^{\gamma}}\right)$$

where C is the concentration of flavonoid, F is the percentage of the specific uptake of [^{14}C]PAH, I_{max} is the maximum percentage of inhibition, and γ is the Hill coefficient. For each flavonoid, the IC_{50} value (expressed as mean ± S.D.) was determined from three separate experiments and each experiment had triplicate measurements.

Flavonoid Cellular Uptake Studies

The intracellular uptake of fisetin, luteolin, morin, and quercetin was examined with or without OAT1 inhibitor (probenecid, 200 μM) in OAT1-overexpressing LLC-PK1/hOAT1 cells.

Briefly, cells were seeded into six-well plates at a density of 5×10^5 cells per well, and uptake studies were started when the cells reached ~80–90% confluence. Stock solution of flavonoids was prepared in DMSO at a concentration of 10 mM. On the day of the experiment, the cells were first washed by PBS twice. The medium containing specific concentrations of flavonoids (fisetin, luteolin, morin, or quercetin), with or without probenecid, was then added to each well. The final DMSO concentration in the transport buffer was 0.1%. After a 1-hour incubation at 37°C, the medium was aspirated and the cells were washed three times with ice-cold PBS. To lyse the attached cells, 1 ml of 0.5% Triton X-100 was added into each well. The concentrations of investigated flavonoids in the cell lysates were analyzed by LC-MS/MS and normalized with cellular protein content. Protein concentration in the cell lysates was determined by the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL).

Sample Preparation and LC-MS/MS Analysis

A 100-μl aliquot of flavonoid samples in cell lysates was deproteinized using an equal volume of methanol. After vortexing, the mixture was centrifuged at

14,000 rpm for 10 minutes. One hundred microliters of the supernatant was transferred into a 200-μl vial insert for LC-MS/MS analysis.

LC-MS/MS was performed using a PE Sciex API-3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA) linked to a TurboIonspray interface and a Shimadzu (Kyoto, Japan) LC10 liquid chromatograph. Analyst 1.4.2 software Applied Biosystems was used for data acquisition and processing. An XTerra MS C18 column (2.1 $\times$ 150 mm i.d., 3.5 μm; Waters Corporation, Milford, MA) was used, and flavonoids were eluted with a mobile phase of acetonitrile/water containing 0.1% formic acid [50:50 (v/v)] at a flow rate of 200 μl/min. To minimize the ion suppression caused by the high concentration of salts present in the transport buffer (Hanks' balanced salt solution), the flow from the LC column was diverted to waste for the first 2 minutes using an API 2000 diverter valve to prevent the early-eluting salts from entering the LC-MS interface. Conditions for MS analysis of tested flavonoids included an ion spray voltage of -4500 V, a nebulizing pressure of 33 psi, and a temperature of 350°C. Nebulizer and curtain gas flow were 10 and 8 ml/min, respectively. The fragment was induced with collision energy of -30 eV. The optimized declustering potential, focusing potential, and collision cell exit potential were -60, -175, and -30V, respectively. The MS was performed in a negative ion mode under multiple reaction monitoring. The mass-to-charge ratios (*m/z*) of molecular ion and product ion of fisetin were 285.0 and 135.1, respectively. The *m/z* values of molecular ion and product ion of luteolin were 285.2 and 132.8, respectively. The *m/z* values of molecular ion and product ion of morin were 301.3 and 150.8, respectively. The *m/z* values of parent ion and product ion of quercetin were 301.1 and 151.1, respectively. The lower limit of quantification of these four flavonoids was 1 ng/ml. The calibration curve was linear over the concentration range of ~1–500 ng/ml for all compounds.

Statistical Analysis

A commercially available package (SPSS 11.0; SPSS Inc., Chicago, IL) was used for all statistics. The differences between the mean values were analyzed for significance using a Student's *t*-test or one-way analysis of variance, followed by Dunnett's test. Differences were considered statistically significant when the P values were <0.05.

Results

Time-Dependent Uptake of [^{14}C]PAH in LLC-PK1 Cells. As shown in Fig. 1, the uptake of [^{14}C]PAH in OAT1-expressing cells was linear over a 5-minute time period. Therefore, we chose 5 minutes as an appropriate time for the following [^{14}C]PAH uptake studies. It should be noted that after 5 minutes, the uptake of PAH decreased with the increase in the incubation time. Ueo et al. (2005) evaluated the time course of PAH in a different cell line (HEK-hOAT1), and they also reported the time-dependent decrease of PAH. The reason for the time-dependent decrease of PAH uptake is unclear.

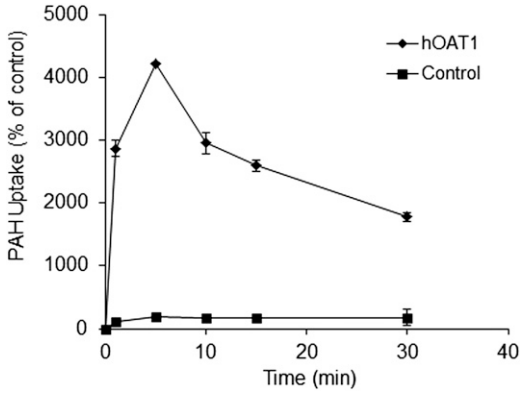


Fig. 1. Time-dependent uptake of [^{14}C]PAH in hOAT1-transfected LLC-PK1 cells and corresponding hOAT1-negative control cells.

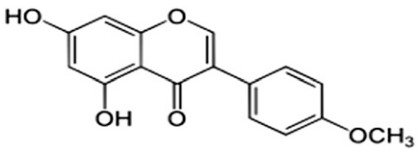
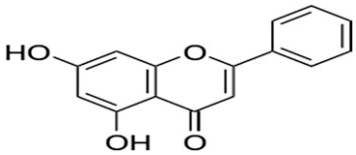
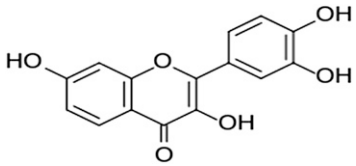
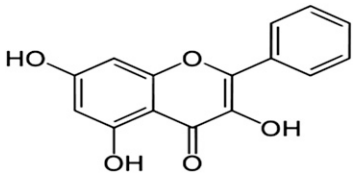
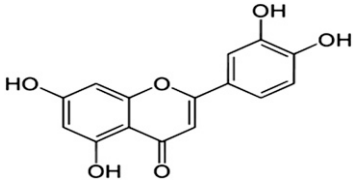
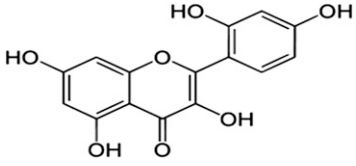
Effects of Flavonoids on OAT1-Mediated [^{14}C]PAH Uptake. To determine whether flavonoids have modulatory effects on hOAT1-mediated transport, uptake studies were conducted with PAH, a well known OAT1 substrate, in OAT1-expressing and OAT1-negative LLC-PK1 cells in the presence or absence of flavonoids (50 μM). As shown in Table 1, when [^{14}C]PAH was added alone, the uptake of PAH in OAT1-expressing cells was substantially higher than that in OAT1-negative control cells (2714% $\pm$ 77.2% versus 100% $\pm$ 2.3% of control; $P < 0.001$). Probenecid (200 μM) markedly decreased [^{14}C]PAH uptake in OAT1-expressing cells ($P < 0.001$; percentage change in mean value, -95.4%), while no significant effect of probenecid was observed on [^{14}C]PAH uptake in OAT1-negative cells ($P > 0.05$; percentage change in mean value, +9.4%). It should be noted that in the presence of probenecid, the intracellular concentration of PAH in OAT1-expressing cells decreased to a level that is very close to that observed in OAT1-negative cells, indicating that OAT1 activity was completely inhibited with 200 μM probenecid. As shown in Fig. 2, among eight flavonoids (namely, biochanin A, chrysin, fisetin, galangin, luteolin, morin, myricetin, and silymarin) that were evaluated, all produced a large and significant decrease in [^{14}C]PAH uptake in OAT1-expressing cells ($P < 0.001$; percentage changes in mean value ranging from -53.7 to -94.8%). In contrast, [^{14}C]PAH uptake in OAT1-negative cells was slightly increased in the presence of these flavonoids (Table 1). The results indicated that these flavonoids are OAT1 inhibitors. Among these eight flavonoids, fisetin, luteolin, and morin produced the greatest inhibitory effect on OAT1, resulting in substantial decreases in [^{14}C]PAH uptake ($P < 0.001$; percentage decreases in mean value of 93.6, 94.8, and 92.3%, respectively), which is comparable to that caused by probenecid in OAT1-expressing cells. In addition, in the presence of fisetin, luteolin, and morin, the intracellular concentration of PAH in OAT1-expressing cells decreased to a level that is comparable to that observed in OAT1-negative cells (Table 1), indicating that OAT1-mediated PAH transport was almost completely blocked with 50 μM of each of these three flavonoids.

Effects of Flavonoids and Their Glycosides on OAT1-Mediated [^{14}C]PAH Uptake. Many flavonoids are known to exist in both aglycone and glycone forms, and different biochemical and pharmacological activities between these two forms have been observed for a number of flavonoids (Kwon et al., 2004; Lin et al., 2005). To compare the modulatory effect of flavonoids and their glycosides on OAT1-mediated [^{14}C]PAH uptake, five pairs of flavonoids and corresponding glycosides, namely, diosmetin and diosmin, EGC and EGCG, genistein and genistin, quercetin and rutin, and phloretin and phloridzin, were characterized in both OAT1-expressing and OAT1-negative LLC-PK1 cells (Fig. 3). As shown in Table 2, in OAT1-expressing cells, both genistein and quercetin greatly inhibited OAT1-mediated [^{14}C]PAH uptake ($P < 0.001$; percentage decreases in mean value of 52.2 and 86.2%, respectively), whereas no significant effect was observed for their glycosides genistin and rutin ($P > 0.05$; percentage changes in mean value of +3.0 and -13.6%, respectively). On the other hand, EGC and its glycoside EGCG and phloretin and its glycoside phloridzin had only marginal effects on OAT1-mediated [^{14}C]PAH uptake in OAT1-expressing cells (Fig. 3). Interestingly, diosmetin and its glycoside diosmin displayed opposite effects on [^{14}C]PAH uptake in OAT1-expressing cells—the intracellular concentration of PAH was substantially decreased in the presence of diosmetin and was significantly increased in the presence of diosmin ($P < 0.001$; percentage changes in mean value of -72.3 and +76.4%, respectively; Table 2), indicating the inhibitory effect of diosmetin and stimulatory effect of diosmin on OAT1. In addition, among diosmetin, genistein, and quercetin, all of which exhibited inhibitory effects on OAT1-mediated

TABLE 1

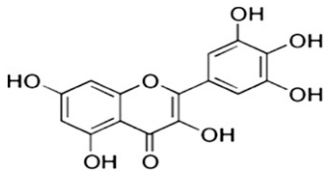
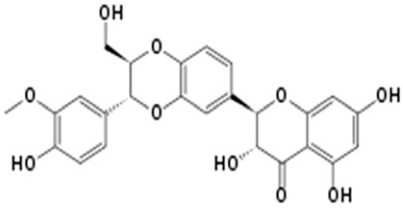
Effects of flavonoids on the uptake of [14C]PAH in both OAT1-expressing and OAT1-negative LLC-PK1 cells

The uptake of [^{14}C]PAH (0.5 μM) in both OAT1-expressing and OAT1-negative LLC-PK1 cells in the absence or presence of flavonoids (50 μM) was performed as described in Materials and Methods. Probenecid (200 μM) was used as a positive control. The uptake of [^{14}C]PAH is expressed as the percentage of control (uptake in OAT1-negative cells). The data are expressed as mean ± S.D.; $n = 4$. The values in the "Change in Mean" columns represent the percentage of decrease or increase in means relative to the control value for either OAT1-expressing or OAT1-negative cells.

	Flavonoids		Chemical Structure	
	OAT1-Expressing Cells		OAT1-Negative Cells	
	Mean ± S.D.	Change in Mean	Mean ± S.D.	Change in Mean
	Percentage			
Control	2714 ± 77.2		100 ± 2.3	
Probenecid	124 ± 4.42***	−95.4	109 ± 5.23	+9.4
Biochanin A 	894 ± 19.6***	−67.1	125 ± 10.9***	+24.9
Chrysin 	480 ± 22.4***	−82.3	121 ± 7.33***	+20.5
Fisetin 	172 ± 7.02***	−93.6	125 ± 7.63***	+24.9
Galangin 	1258 ± 109***	−53.7	106 ± 14.6	+6.4
Luteolin 	142 ± 5.01***	−94.8	112 ± 4.32	+11.8
Morin 	210 ± 7.69***	−92.3	127 ± 10.4***	+27.3

(*continued*)

TABLE 1—*Continued*

		Flavonoids		Chemical Structure	
		OAT1-Expressing Cells		OAT1-Negative Cells	
		Mean ± S.D.	Change in Mean	Mean ± S.D.	Change in Mean
Myricetin		351 ± 8.91***	−87.1	113 ± 9.78	+13.3
Silymarin		525 ± 39.7***	−80.7	115 ± 11.4	+14.8

***$P < 0.001$ versus the control of either OAT1-expressing or OAT1-negative cells.

[^{14}C]PAH transport, quercetin produced the greatest inhibitory effects, with the decrease in [^{14}C]PAH uptake comparable to that caused by probenecid in OAT1-expressing cells. In OAT1-negative control cells, all flavonoids and their glycosides demonstrated negligible effects on [^{14}C]PAH uptake.

It should be noted that in the negative control group in OAT1-expressing cells, the uptake of PAH reported in Table 2 is only half of that in Table 1. The results in Tables 1 and 2 were obtained from two experiments that followed the same protocol, but were conducted on different days using cells with different passage numbers. One possible explanation for the different control values in OAT1-expressing cells is that the OAT1 expression in these cells is different. The OAT1-expressing cells used in experiment 1 (Table 1) appeared to have higher expression of OAT1 than those in experiment 2 (Table 2), resulting in the higher uptake of PAH. In addition, when the uptake of PAH in OAT1-expressing and OAT1-negative cells was compared, the basic assumption is that the expression of endogenous transporters, especially those that are involved in the transport of PAH, remains the same in wild-type and transfected cells. To verify this assumption, further investigation is needed to compare the expression level of endogenous transporters in these two cell lines.

Based on the results presented in this section and the section above, we combined the data and compared the modulatory effects of all 18 flavonoids (13 flavonoid aglycones and 5 flavonoid glycosides) on OAT1-mediated [^{14}C]PAH uptake (Fig. 4). These 18 flavonoids are from six different chemical subclasses, namely flavone, flavonol, flavonolignan, flavanol, isoflavone, and chalcone. As shown in Fig. 4, all of the tested flavonoids from the flavone subclass demonstrated

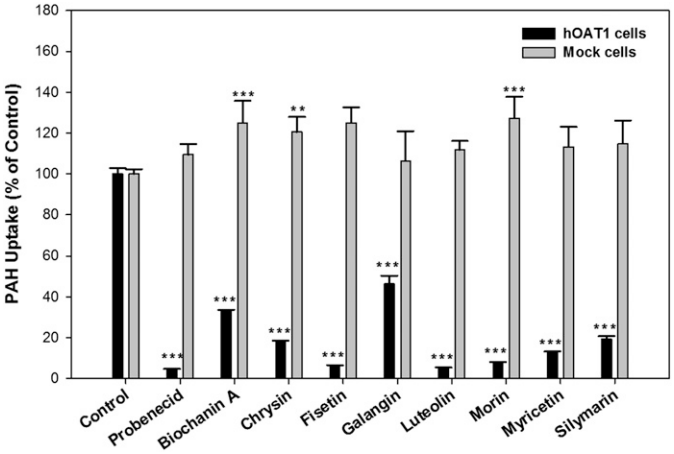


Fig. 2. Effects of flavonoids on [^{14}C]PAH uptake in hOAT1-transfected LLC-PK1 cells and corresponding hOAT1-negative control (Mock) cells. The uptake of [^{14}C]PAH (0.5 μM) in both OAT1-expressing and OAT1-negative LLC-PK1 cells in the absence or presence of flavonoids (50 μM) was performed as described in *Materials and Methods*. Probenecid (200 μM) was used as a positive control. Data are expressed as mean ± S.D. **$P < 0.01$, ***$P < 0.001$, vs. their own negative control in hOAT1 or Mock cells.

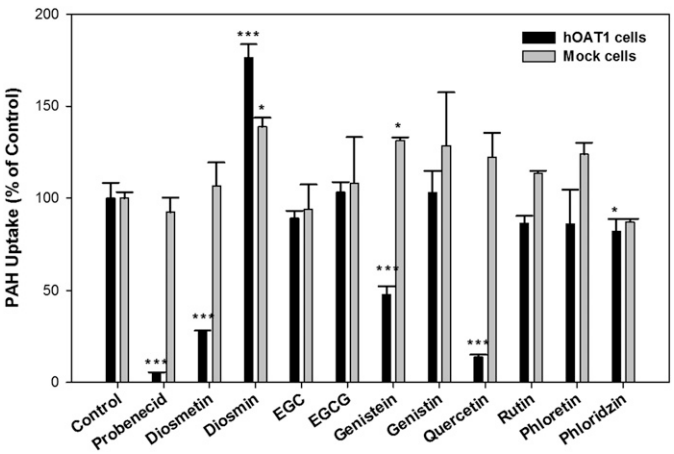


Fig. 3. Effects of flavonoids and their glycosides on [^{14}C]PAH uptake in hOAT1-transfected LLC-PK1 cells and corresponding hOAT1-negative control (Mock) cells. The uptake of [^{14}C]PAH (0.5 μM) in both OAT1-expressing and OAT1-negative LLC-PK1 cells in the absence or presence of flavonoids (50 μM) was performed as described in *Materials and Methods*. Probenecid (200 μM) was used as a positive control. Data are expressed as mean ± S.D. *$P < 0.05$, ***$P < 0.001$, vs. their own negative control in hOAT1 or Mock cells.

TABLE 2

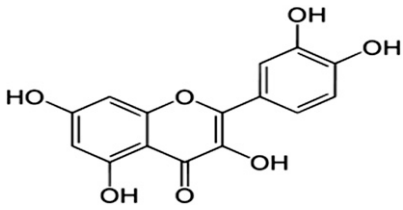
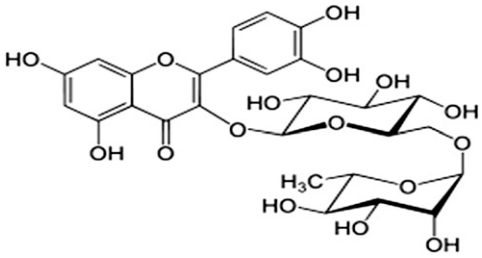
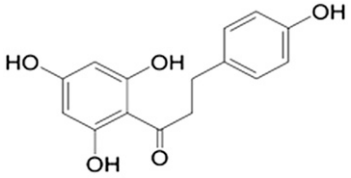
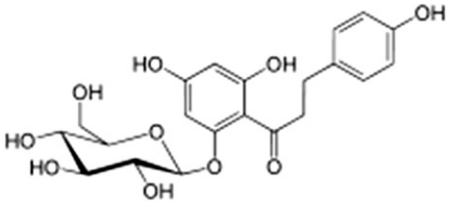
Effects of flavonoids and their glycosides on the uptake of [14C]PAH in both OAT1-expressing and OAT1-negative LLC-PK1 cells

The uptake of [^{14}C]PAH (0.5 μM) in both OAT1-expressing and OAT1-negative LLC-PK1 cells in the absence or presence of flavonoids (50 μM) was performed as described in Materials and Methods. Probenecid (200 μM) was used as a positive control. The uptake of [^{14}C]PAH is expressed as the percentage of control (uptake in OAT1-negative cells). The data are expressed as mean ± S.D. The values in the "Change in Mean" columns represent the percentage of decrease or increase in means relative to the control value for either OAT1-expressing or OAT1-negative cells.

	Flavonoids		Chemical Structure	
	OAT1-Expressing Cells		OAT1-Negative Cells	
	Mean ± S.D.	Change in Mean	Mean ± S.D.	Change in Mean
	Percentage			
Control	1432 ± 119		100 ± 3.31	
Probenecid	75.5 ± 4.29***	−94.7%	92.3 ± 7.96	−7.72%
Diosmetin	396 ± 7.69***	−72.3%	107 ± 12.7	+6.64%
Diosmin	2525 ± 104***	+76.4%	139 ± 4.92*	+38.8%
EGC	1276 ± 57.3	−10.9%	94.1 ± 13.3	−5.95%
EGCG	1478 ± 77.9	+3.28%	108 ± 25.1	+8.16%
Genistein	684 ± 64.1***	−52.2%	131 ± 1.70*	+31.4%
Genistin	1474 ± 169	+3.00%	129 ± 29.0	+28.5%

(*continued*)

TABLE 2—*Continued*

		Flavonoids		Chemical Structure	
		OAT1-Expressing Cells		OAT1-Negative Cells	
		Mean ± S.D.	Change in Mean	Mean ± S.D.	Change in Mean
Quercetin		198 ± 17.6***	−86.2%	124 ± 13.2	+22.3%
Rutin		1237 ± 57.5	−13.6%	87.0 ± 1.25	−13.7%
Phloretin		1232 ± 264	−13.9%	122 ± 6.26	+23.9%
Phloridzin		1172 ± 94.4*	−18.1%	114 ± 1.7	+13.0%

*$P < 0.05$; ***$P < 0.001$ versus the control of either OAT1-expressing or OAT1-negative cells.

potent OAT1 inhibition. In contrast, the tested flavonoids from the flavanol and chalcone subclasses appear to have minimal modulatory effects on OAT1. Regarding the flavonoids from the flavonol and isoflavone subclasses, all flavonoid aglycones demonstrated significant inhibition of OAT1, whereas the flavonoid glycosides showed no inhibitory effect on OAT1 (Fig. 4).

Concentration-Dependent Effects of Flavonoids on OAT1-Mediated [^{14}C]PAH Uptake. Among the flavonoids screened in the PAH uptake studies, concentration-dependent relationships for inhibition of [^{14}C]PAH uptake in OAT1-expressing cells was investigated for three flavonoids, namely, luteolin, morin, and quercetin, all of which demonstrated high OAT1 inhibition activities when they were tested at a concentration of 50 μM. As shown in Fig. 5, luteolin, morin, and quercetin significantly inhibited hOAT1-mediated uptake of [^{14}C]PAH in a concentration-dependent manner. Among these three flavonoids tested, morin and luteolin demonstrated potent inhibitory effects on [^{14}C]PAH uptake in OAT1-expressing cells, with IC_{50} values of <0.3 and 0.47 μM, respectively, indicating that both morin and luteolin are potent OAT1 inhibitors (Fig. 5, A and B). In addition, at concentrations of ≥ 10 μM, quercetin displayed significant inhibitory effect on [^{14}C]PAH uptake in OAT1-expressing cells ($P < 0.05$). In the current study we only evaluated the concentration-dependent effects of morin, luteolin, and quercetin. For fisetin, which demonstrated potent OAT1 inhibition as a competitive OAT1 substrate, further concentration-dependent studies are warranted.

Intracellular Uptake of Flavonoids by OAT1. To further characterize the interaction between flavonoids and OAT1, the uptake of flavonoids, in the absence and presence of the well known OAT1 inhibitor probenecid (200 μM), was examined in hOAT1-transfected LLC-PK1 cells. Among the various flavonoids that were screened in the PAH uptake study, four flavonoids that demonstrated a strong inhibitory effect on OAT1 at a concentration of 50 μM, namely, fisetin, luteolin, morin, and quercetin, were selected for flavonoid uptake studies, and the role of OAT1 in the uptake of these four flavonoids was evaluated. As shown in Fig. 6A, in the presence of probenecid, the intracellular uptake of fisetin was substantially decreased in OAT1-expressing cells ($P < 0.01$), indicating that fisetin is a substrate of OAT1. In contrast, the intracellular concentrations of luteolin, morin, and quercetin did not change significantly ($P > 0.05$) with probenecid coincubation, suggesting that OAT1 does not play

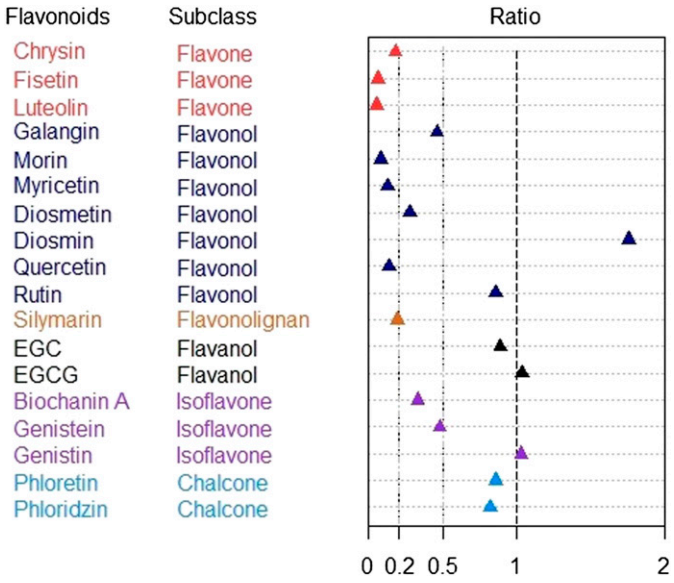


Fig. 4. Forest plot of the percentage changes in OAT1-mediated uptake in the presence of flavonoids from different chemical subclasses.

a major role in the uptake of these three flavonoids. The representative chromatograms of fisetin sample (5 μM) in the presence or absence of probenecid in OAT1-expressing cells are shown in Fig. 6B. The peak area of fisetin decreased more than 5-fold when 5 μM fisetin was coincubated with 200 μM probenecid. No significant changes in peak areas of luteolin, morin, and quercetin were observed when they were coincubated with probenecid (data not shown).

Discussion

OATs belong to the solute carrier 22 transport protein superfamily, and they transport small, amphiphilic organic anions of diverse chemical structures. OAT1, the first OAT to be cloned, is strongly expressed in human kidney and localized at the basolateral membrane of the proximal tubule (Hosoyamada et al., 1999). OAT1 is known to play a central role in the renal uptake of a wide range of anionic xenobiotics, including endogenous metabolic waste products, environmental toxins, and numerous clinically important drugs (e.g., antibiotics, antivirals, anti-inflammatory drugs, diuretics, and anticancer agents) (Rizwan and Burckhardt, 2007; Burckhardt, 2012; Wang and Sweet, 2013b). In addition, OAT1 is involved in the development of nephrotoxicity of many anionic xenobiotics (Hagos and Wolff, 2010). Consequently, preloading of OAT1 inhibitors, including probenecid, betamiprone, and NSAIDs, has been reported to reduce OAT1-mediated drug nephrotoxicity (Tune et al., 1977; Hirouchi et al., 1994; Lacy et al., 1998; Mulato et al., 2000). Probenecid is currently used as a nephroprotectant during clinical therapy with intravenous cidofovir in the treatment of cytomegalovirus retinitis in AIDS patients. As most OAT1 inhibitors evaluated in vivo demonstrate safety issues when they are given at high doses, the identification of OAT1 inhibitors with better safety profiles is highly valuable in reducing the OAT1-mediated drug nephrotoxicity.

Flavonoids are a class of polyphenolic compounds with exceptional safety records. Flavonoids are widely present in fruits, vegetables, and beverages and are the main components of a large number of herbal supplements. Flavonoids have been reported to have many beneficial pharmacological effects, including antioxidative, anti-inflammatory, and anticancer properties. In addition, flavonoids have been found to have modulatory effects on several efflux transporters (Morris and Zhang, 2006) and a few uptake transporters, such as OATP1B1 (Wang et al., 2005) and MCT1 (Wang and Morris, 2007). Regarding the interaction between flavonoids and OAT1, there is only limited information available in the literature (Hong et al., 2007; Wang and Sweet, 2013a).

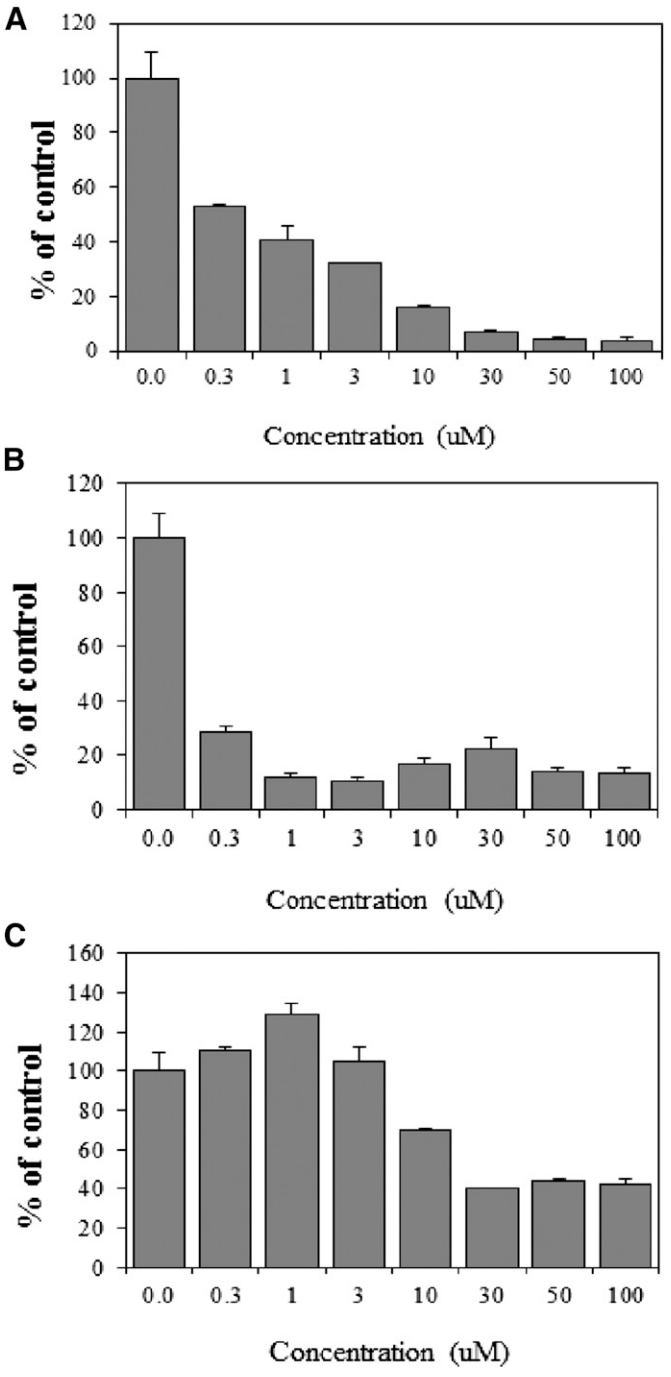


Fig. 5. Concentration-dependent effects of flavonoids on [^{14}C]PAH uptake in hOAT1-transfected LLC-PK1 cells. Concentration-dependent effects were determined for three flavonoids—luteolin (A), morin (B), and quercetin (C)—in the presence of varying concentrations (0.3–100 μM) of flavonoids or vehicle (0.3% DMSO). The IC_{50} values of morin and luteolin were estimated to be <0.3 and 0.47 μM, respectively. The IC_{50} value of quercetin could not be estimated.

In the present study, we examined the modulatory effects of 18 flavonoids (13 flavonoid aglycones and 5 flavonoid glycosides) from six different chemical subclasses (Fig. 4) on OAT1-mediated PAH transport. Our results revealed that in OAT1-expressing cells, 11 flavonoid aglycones (the exceptions being EGC and phloretin) produced

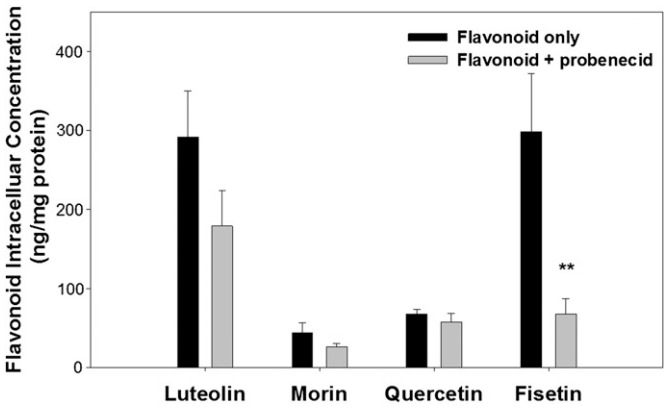


Fig. 6. (The uptake of the flavonoids fisetin, luteolin, morin, and quercetin (5 μM each) in the presence or absence of 200 μM probenecid in hOAT1-transfected LLC-PK1 cells. The concentrations of fisetin, luteolin, morin, and quercetin were determined by LC-MS/MS. Data are presented as mean ± S.D.; $n = 3$. $^{**}P < 0.01$, vs flavonoid only group.

substantial decreases in PAH uptake, whereas no decrease in PAH uptake was observed in OAT1-negative control cells, indicating the inhibitory effect of flavonoids on OAT1-mediated PAH uptake. It should be noted that in OAT1-negative control cells, the general trend was that the uptake of PAH was enhanced in the presence of flavonoids, with the percentage of increase ranging from 6.4 to 31.4%. This phenomenon may be explained by the interaction of these flavonoids with an endogenous efflux transporter(s) expressed in LLC-PK1 cells that mediates PAH transport. Endogenous multidrug resistance–associated protein 2 (MRP2), which has been detected in LLC-PK1 cells (Goh et al., 2002), may be responsible for the small changes in PAH uptake observed, since flavonoids have been reported to interact with MRP2 and PAH is known to be an MRP2 substrate (Van Aubel et al., 2000; Morris and Zhang, 2006). Among these 11 flavonoids that demonstrated OAT1-inhibitory effects, fisetin, luteolin, morin, and quercetin exhibited the largest effects and produced almost complete inhibition of OAT1-mediated PAH uptake at a concentration of 50 μM. Further concentration-dependent effects of luteolin, morin, and quercetin were evaluated, and both morin and luteolin were found to be potent OAT1 inhibitors, with IC_{50} values of <0.3 and 0.47 μM, respectively. It has been reported previously that morin is a potent OAT1 inhibitor with an IC_{50} value of 0.46 μM (Hong et al., 2007), which is very close to our estimate (i.e., <0.3 μM). Morin can be isolated from Osage orange, old fustic, and leaves of common guava. Following the oral administration of 25 mg/kg of morin in rats, the C_{max} of morin was ~3.1 μM (Hou et al., 2003). Luteolin is abundant in the flower of *Chrysanthemum morifolium*, and it accounts for ~7.6% of *C. morifolium* extract (Chen et al., 2007). Dietary sources of luteolin include celery, broccoli, green pepper, and parsley. After a single dose of *C. morifolium* extract (200 mg/kg), the C_{max} of luteolin reached 4 μg/ml (i.e., ~14 μM) (Chen et al., 2007). Because the in vivo C_{max} values of morin and luteolin are much higher than their IC_{50} values for OAT1 inhibition, the potent inhibitory effect of morin and luteolin on OAT1-mediated drug transport observed in vitro might also occur in vivo. Therefore, both morin and luteolin may represent promising nephroprotectants to reduce the drug-induced nephrotoxicity mediated by the OAT1 pathway.

In contrast to the tested flavonoid aglycones, none of the tested flavonoid glycosides showed any inhibition of OAT1. Interestingly, in the presence of diosmin, the intracellular uptake of PAH in OAT1-expressing cells was significantly increased, indicating a possible stimulatory effect of diosmin on OAT1. These results indicate that attachment of a sugar moiety may differentially modulate OAT1 activity. The exact reason for the stimulatory effect of diosmin is currently unknown. A similar phenomenon has been observed when the modulatory effects of flavonoid glycosides were evaluated for OATP1B1 (Wang et al., 2005). Further studies are warranted to clarify the underlying mechanism(s). To further characterize the interaction between flavonoids and OAT1, the uptake of fisetin, luteolin, morin, and quercetin, in the absence and presence of probenecid, was examined in OAT1-expressing cells. The results revealed that fisetin is a substrate of OAT1, while the other three flavonoids are not transported by OAT1. The fact that fisetin is not only an OAT1 inhibitor but also an OAT1 substrate suggests that fisetin may inhibit OAT1-mediated drug uptake through competitive inhibition.

It should be noted that while potent OAT1 inhibitors may serve as nephroprotective agents, with drugs exhibiting nephrotoxicity via the OAT1 pathway, OAT1-mediated drug-drug interactions may not always be beneficial. For those OAT1 substrates that have a narrow therapeutic window and are mainly eliminated through the kidney, coadministration with potent OAT1 inhibitors may increase the incidence of drug toxicity. For example, methotrexate, a drug that is commonly used for the treatment of malignancies and rheumatoid arthritis, is eliminated almost entirely in unchanged form in urine, and OATs, including OAT1, play important roles in its renal elimination. White and colleagues reported that with probenecid coadministration, the mean concentration of methotrexate at 24 hours was 4 times higher than in patients not given probenecid (Aherne et al., 1978). Methotrexate has also been reported to induce severe adverse effects through an increase in blood methotrexate levels when NSAIDs were simultaneously administered (Thyss et al., 1986). In addition to increasing drug toxicity, coadministration with potent OAT1 inhibitors may also lead to reduced drug efficacy, which may be particularly true for those drugs whose pharmacological effect relies on tubular excretion. For example, OAT1 knockout mice manifest impaired diuretic responsiveness to furosemide due to the loss of OAT1-mediated renal secretion (Eraly et al., 2006). Therefore, while flavonoids may represent promising nephroprotectants to reduce drug-induced nephrotoxicity, caution is needed when they are coadministered with OAT1 substrates that have narrow therapeutic windows or whose pharmacological effect is dependent on renal tubular excretion.

In the current study, we only evaluated flavonoids and their glycosides. It is well known that flavonoids usually undergo extensive phase II metabolism to form glucuronide and sulfate conjugates that are excreted in the urine. These conjugated metabolites may contribute to inhibition as well. For example, Wong et al. (2011) reported recently that several quercetin-conjugated metabolites, including quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide, and quercetin-3′-*O*-glucuronide, interact with OATs and attenuate the cytotoxicity of adefovir mediated by OAT1. For the flavonoids investigated in our study, the effects of conjugated metabolites are unknown due to the lack of metabolism by LLC-PK1 cells. However, this may be an important consideration in vivo. In addition, in the current study we only evaluated the modulatory effect of flavonoids on OAT1. It should be noted that, in addition to OAT1, OAT3 is also highly expressed in kidney and actively involved in the tubular uptake of various anionic xenobiotics (Burckhardt, 2012; Wang and Sweet, 2013b). In addition, OAT3 has also been found to play an important role in drug-induced nephrotoxicity (Hagos and Wolff, 2010). As OAT1 and OAT3 have substantial overlap in substrates and modulators (Rizwan and Burckhardt, 2007; Wang and Sweet, 2013b), and considering the potent inhibitory effect of flavonoids on OAT1, we anticipate that these flavonoids may also inhibit OAT3. Therefore, for the flavonoids tested in the current study, further investigations are warranted to evaluate their modulatory effect on OAT3.

In conclusion, many naturally occurring flavonoids can inhibit OAT1-mediated PAH uptake, indicating that they are a novel class of OAT1 modulators. Considering the high consumption of flavonoids in the diet and in herbal products, OAT1-mediated flavonoid-drug interactions may be clinically relevant. Flavonoids may represent promising nephroprotectants to reduce the drug-induced nephrotoxicity mediated by the OAT1 pathway. Further in vivo investigations are warranted to evaluate the nephroprotective role of flavonoids in relation to drug-induced nephrotoxicity mediated by the OAT1 pathway.

Authorship Contributions

Participated in research design: An, Wang, Morris.

Conducted experiments: An, Wang.

Performed data analysis: An, Wang.

Wrote or contributed to the writing of the manuscript: An, Wang, Morris.

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