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Flavonoid conjugates interact with organic anion transporters (OATs) and attenuate cytotoxicity of adefovir mediated by organic anion transporter 1 (OAT1/SLC22A6)

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ABSTRACT

Flavonoids are conjugated by phase II enzymes in humans to form glucuronidated and sulfated metabolites that are excreted in urine via the kidney. In this study, we examined the interaction between metabolites of quercetin and isoflavonoids found *in vivo* with human organic anion transporters 1 (OAT1) and 3 (OAT3) and their potential in attenuating OAT-induced cytotoxicity of adefovir. Accumulation of flavonoid conjugates was studied in human embryonic kidney 293H cells overexpressing OAT1 or OAT3. OAT1-overexpressing cells exhibited an increased uptake of the sulfated conjugates, genistein-4′-*O*-sulfate and quercetin-3′-*O*-sulfate. OAT3-overexpressing cells demonstrated enhanced uptake of glucuronide conjugates, such as daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide and quercetin-3′-*O*-glucuronide. Position of conjugation was important since quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide were poorly transported. Kinetic analysis revealed high affinity uptake of quercetin-3′-*O*-sulfate by OAT1 (K_m = 1.73 μM; V_{max} = 105 pmol/min/mg). OAT3 transported isoflavone glucuronides with lower affinity (K_m = 7.9–19.1 μM) but with higher V_{max} (171–420 pmol/min/mg). Quercetin-3′-*O*-sulfate strongly inhibited OAT1-mediated *p*-aminohippuric acid uptake with an IC_{50} of 1.22 μM. Transport of 5-carboxyfluorescein by OAT3 was potently inhibited by quercetin-3-*O*-glucuronide, quercetin-3′-*O*-glucuronide and quercetin-3′-*O*-sulfate (IC_{50} = 0.43–1.31 μM). In addition, quercetin-3′-*O*-sulfate was shown to effectively reduce OAT1-mediated cytotoxicity of adefovir, an antiviral drug, in a dose-dependent manner. These data suggest that OAT1 and OAT3 are responsible for basolateral uptake of flavonoid conjugates in kidney, and flavonoid conjugates inhibit OAT1 and OAT3 activity at physiologically relevant concentrations. Interaction with OATs limits systemic availability of flavonoids and may be a mechanism of food–drug interaction via inhibition of renal uptake.

1. Introduction

Quercetin is a chemopreventive and anti-inflammatory flavonoid that interacts with nuclear factor E2-related factor 2 (Nrf2) and activates antioxidant response elements (ARE) [1,2], contributing to enhanced cellular protection against carcinogens and oxidative stress [3]. Isoflavones are phytoestrogens that bind to the estrogen receptor [4] and exert preventive effects on hormone-dependent cancer [5], cardiovascular diseases [6], and post-menopausal osteoporosis [7]. Any biological activities of flavonoids, however, are influenced by their metabolism. Dietary flavonoids are metabolized extensively in the intestine and liver, and major forms found *in vivo* are glucuronide, sulfate and/or methyl-conjugates of the parent aglycone [8]. Quercetin is metabolized into quercetin-3-*O*-glucuronide, 3′-methylquercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate following consumption of onions [9]. After soy ingestion, soy isoflavones such as daidzein and genistein are present in the circulation predominantly as their 7-*O*-glucuronide and 4′-*O*-glucuronide, and sulfates were minor metabolites [10]. Concentrations of these metabolites in human plasma are typically low after intake of 50 mg aglycone equivalent and rarely exceed 10 μM even at much higher ingested doses [11].

Interaction with uptake and efflux transporters is considered to be one of the factors that limit the bioavailability of flavonoids, by facilitating the elimination of hydrophilic conjugates into bile

Abbreviations: BCRP, breast cancer resistance protein; MRP, multidrug resistance protein; OAT, organic anion transporter.

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and/or urine [12]. Urinary excretion of flavonoid conjugates has been well documented and varies according to the individual flavonoid aglycones. For the isoflavones daidzein and glycitein, about half of the intake could be recovered in the urine as conjugated metabolites [13]. A high urinary recovery of conjugates was also observed with catechins (~25%) [14]; while for quercetin the urinary excretion is much less than 10% of the intake [15]. Many drugs and their conjugated metabolites are eliminated in the urine via tubular secretion [16]. Vectorial transport of hydrophilic substances across the renal proximal tubules involves the interplay of uptake transporters on the basolateral membrane and the efflux transporters on the apical membrane. Active efflux is carried out by multidrug resistance proteins (MRP), which transport glucuronide and sulfate conjugates of flavonoids such as quercetin, baicalein and genistein [17–19]. The molecular mechanism underlying the possible uptake of flavonoid conjugates into renal tubules via basolateral transporters is not known.

OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*) are highly expressed on the basolateral membrane of proximal tubules [20]. They play an important role in the sodium-dependent renal uptake of structurally diverse organic anions from the blood into proximal tubule cells. OAT1 and OAT3 are capable of mediating the uptake of these organic anions against their electrochemical gradients by coupling to efflux of intracellular α-ketoglutarate. OAT1 interacts with endogenous metabolites, drugs including antivirals, antibiotics, nonsteroidal anti-inflammatory agents and statins, as well as toxicants such as mercury and ochratoxin [21]. OAT3 recognizes many OAT1 substrates; in addition, it has the ability to transport corticosterone, estradiol-17β-glucuronide and taurocholate, that are not substrates of OAT1 [22]. As toxins and cytotoxic drugs are taken up by OAT1 and OAT3, these toxic substances may exert toxic effects on the proximal tubule cells and cause renal injury. *In vitro* studies with OAT1-transfected cells showed that OAT1 over-expression sensitizes the cytotoxicity caused by antiviral drugs, adefovir and cidofovir [23]. Co-administration of the inhibitors of OATs may reduce the extent of OAT-induced nephrotoxicity [24]. On the other hand, inhibition of OAT1 and OAT3 may also alter the pharmacokinetics of a variety of drugs that are OAT substrates. OAT1 inhibitors, such as probenecid, could be administered with other drugs to prolong plasma half lives and improve therapeutic effects [25].

To date, only limited information is available concerning the interaction between OATs and flavonoids. Ellagic acid is a substrate and a potent inhibitor of OAT1 [26], while several flavonoids (naringenin, morin, silybin and quercetin) were also found to inhibit OAT1 and OAT3 [27]. However, the flavonoid aglycones are mostly absent in human plasma. The aim of this work is to investigate the transport of glucuronidated and sulfated metabolites of quercetin and isoflavones, major metabolites *in vivo*, in OAT1- and OAT3-transfected human embryonic kidney 293H cells. We also examined the inhibition of OATs by flavonoid conjugates and the potential of quercetin-3′-*O*-sulfate to attenuate OAT1-induced cytotoxicity of adefovir.

2. Methods and materials

2.1. Chemicals

Genistein and quercetin were obtained from Extrasynthese (Genay, France). Quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucuronide and quercetin-3′-*O*-glucuronide were synthesized from quercetin using human liver S9 (Sigma–Aldrich, St. Louis, MO) and purified by HPLC [28]. Quercetin-3′-*O*-sulfate was chemically synthesized as described and purified by gel filtration [9]. The identity of quercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate was further confirmed by comparing the retention time and the absorption spectra of the authentic standards, kind gifts from Dr. Paul Kroon, Institute of Food Research, Norwich, UK. Purities were checked by HPLC to be over 95%. Daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide, genistein-4′-*O*-sulfate and daidzein-7,4′-*O*-disulfate were synthesized as previously described [10,29]. Adefovir was obtained by the hydrolysis of adefovir dipivoxil (Sigma–Aldrich, St. Louis, MO) using human intestinal S9 (Xenotech LLC, Kansas City, KS) and purified by gel filtration. Human embryonic kidney cells 293H were purchased from Invitrogen (Carlsbad, CA). OAT1 (SLC22A6 transcript variant 2, Genebank Accession Number: NM_153276) and OAT3 (SLC22A8 transcript variant 1, Genebank Accession Number: NM_004254) expression plasmids were obtained from Origene (Rockville, MD). Fugene HD was purchased from Roche (Basel, Switzerland). All other chemicals, unless otherwise stated, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. HEK293 cell culture

293H cells were routinely cultured in 75 cm^2 cell culture flasks (Corning Costar Corp., Cambridge, MA) at 37 °C under a humidified 5% CO_2/O_2 atmosphere. Culture media consisted of Dulbecco's modified minimum essential medium (DMEM) high-glucose media supplemented with 10% fetal bovine serum, 1% nonessential amino acids and 50 U/mL penicillin–streptomycin (all from Sigma–Aldrich). Cells were split in a 1:4 ratio every 48 h. Antibiotics were not added in transfection experiments. All experiments were performed with 293H cells between passages 1 and 20.

2.3. Transfection and uptake transport assay

The cells were seeded into poly-L-lysine coated 24-well plates at a density of 1.2×10^5 cells/well the day prior to transient transfection. OAT1 or OAT3 plasmids, or the empty pCMV-XL6 vector, were transfected into 293H cells with the Fugene HD reagent according to the manufacturer's directions, using an optimized ratio of 3:2 (μL reagent:μg DNA). The transfection complexes were formed in Opti-MEM (Invitrogen, Carlsbad, CA) and then added to cells after incubation for 18 min. Uptake assays were performed 22–24 h after transfection. The over-expression of the OATs was confirmed by quantitative RT-PCR (Taqman assays IDs: Hs00537914_m1 (SLC22A6) and Hs01056647_m1 (SLC22A8)) (Applied Biosystems, Foster City, CA), and by the uptake of model substrates *p*-aminohippuric acid (OAT1), and estrone-3-sulfate (OAT3). Uptake experiments were carried out using HBSS buffer containing 1.8 mM $CaCl_2$ and adjusted to pH 7.4 with 1 M HCl. The media were removed and the monolayers were washed twice with 0.25 mL transport buffer and incubated for 10 min. After that, the buffer was aspirated and 0.25 mL transport buffer containing the test compounds was added. Flavonoid and isoflavone conjugates were dissolved in DMSO (final concentration $< 0.2\%$), except quercetin-3′-*O*-sulfate which was dissolved in water. After the specified incubation time, the uptake was stopped by adding 1 mL ice-cold transport buffer containing 0.2% bovine serum albumin (BSA). This was quickly aspirated and further washed twice with 0.5 mL ice-cold transport buffer with 0.2% BSA. Finally, the cells were rinsed with 1 mL ice-cold transport buffer without BSA. Cells were collected with 0.4 mL lysis solution (50% methanol) and stored at −20 °C. Extraction was performed by sonication for 5 min followed by the addition of 1 mL of ice-cold acetone. The samples were placed in a −20 °C freezer for 1 h and centrifuged at 17,000 × *g* for 5 min. The supernatant was collected and evaporated to dryness *in vaccuo* at 30 °C and stored at −20 °C until analysis. The protein pellet was re-dissolved in 0.1 N NaOH and the protein contents were determined by the Bradford assay. All the uptake values were standardized against protein content.

2.4. Kinetics of uptake of flavonoid and isoflavonoid conjugates

Uptake assays were performed on the 293H cells 20–24 h after transfection. Uptake assays were performed for 10 min, during which uptake is linear with time. Kinetic analyses were performed for genistein-4′-*O*-sulfate and quercetin-3′-*O*-sulfate in OAT1-expressing cells, with a substrate concentration range of 1–50 μM. The uptake of glucuronides by OAT3 was linear over 30 min. The kinetics of uptake of daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide and quercetin-3′-*O*-glucuronide were measured in OAT3-expressing cells for 10 min, with substrate concentrations of 2.5–100 μM. The apparent uptake of OATs was obtained by subtracting the uptake of OAT1 or OAT3 with uptake of control cells at each concentration.

2.5. HPLC analysis of flavonoid and isoflavonoid conjugates

HPLC analyses were carried out on an Agilent 1200 series liquid chromatography system equipped with a diode array detector. The analyses were performed with a Zorbax XDB-C18 column (4.6 mm × 50 mm, 1.8 μm) with 20 mM ammonium formate, pH 4.5 (A) and methanol (B) as the mobile phase. For the analysis of quercetin and conjugates, elution was performed at 0.75 mL/min and gradient started at 30% (B) and increased linearly to 50% in 12 min, and then equilibrated at 30% for 3 min. Quantification of quercetin metabolites was based on peak area of quercetin at 370 nm, assuming similar response factor for quercetin conjugates [30,31]. For the analysis of isoflavones and their conjugates, elution started at 20% (B), increasing to 30% in 5 min and to 70% in 8 min, after which it returned to 20% for 3 min at a flow rate of 0.75 mL/min. The quantification of isoflavone metabolites was based on the peak area of the respective standard curves measured at 254 nm. Typically, 50 μL was injected for the analyses.

2.6. Inhibition of uptake of standard substrates

p-Aminohippuric acid and 5-carboxyfluorescein were used as the model substrates for OAT1 and OAT3, respectively. Uptake of *p*-aminohippuric acid (25 μM) and 5-carboxyfluorescein (100 μM) was measured for 10 min in the presence or absence of 2 or 10 μM test compounds. The intracellular *p*-aminohippuric acid was extracted as in Section 2.3 and was analyzed by HPLC. The analyses were performed with a Zorbax XDB-C18 column (4.6 mm × 50 mm, 1.8 μm) with 20 mM ammonium formate, pH 4.5 as the mobile phase. It was run at a flow rate of 0.75 mL for 3 min. To quantify 5-carboxyfluorescein, the cells were extracted twice with 1 mL methanol in the dark, each for 30 min, and the supernatants were combined and analyzed by HPLC. The analyses were performed with Zorbax XDB-C18 column (4.6 mm × 50 mm, 1.8 μm) and eluted with 20 mM Tris–HCl (pH 8) and 20% methanol, at 0.25 mL/min. 5-Carboxyfluorescein was detected using a fluorescence detector (Agilent) with excitation and emission of 492 and 517 nm, respectively.

2.7. Drug cytotoxicity assays

293H cells were plated onto 35-mm culture plates with a density of 5×10^5 cells/plate. After overnight incubation, the cells were transfected with OAT1, OAT3 or empty plasmids with fugene. Twenty-four hours after transfection, the cells were subcultivated into 96 well plates at 20,000 cells/well and cultured in DMEM complete media. After overnight incubation, various concentrations of adefovir and/or quercetin-3′-*O*-sulfate were added (five replicates), after which the cells were further incubated for another 96 h. At the end of the incubation, cell viability was determined by a modified colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [32]. Briefly, the culture medium was removed and replaced with 100 μL phenol red-free complete media containing 0.5 mg/mL MTT. Following 4 h incubation at 37 °C, 100 μL of a solution containing 10% Triton X-100 and 0.1 N HCl in isopropanol was added. The plate was incubated and gently mixed until MTT formazan crystals were dissolved. Absorbance at 570 nm was measured on a microplate reader and IC_{50} calculated after subtraction of blank values.

2.8. Data analysis

Data are shown as mean ± S.D. (as mean ± S.E. for enzyme kinetics data). Raw data from the enzyme kinetics studies were analyzed using GraphPad Prism 5. K_m and V_{max} were derived from a nonlinear regression fit of the Michaelis–Menten model. Statistical differences were determined using analysis of variance using the Student's *t*-test. Differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Uptake of flavonoid and isoflavone conjugates by OAT1

To test whether flavonoid and isoflavone conjugates are transported by OAT1, 293H cells transfected with empty or OAT1 plasmid were incubated with 25 μM of the test compounds for 30 min. The results are shown in Figs. 1 and 2. Sulfated metabolites quercetin-3′-*O*-sulfate and genistein-4′-*O*-sulfate appeared to be the best substrates of OAT1 (>10-fold over control), especially the former (Figs. 1 and 2). However, daidzein-7,4′-*O*-disulfate was not significantly transported. The glucuronide conjugates were rather poor substrates of OAT1: daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide and quercetin-3′-*O*-glucuronide were not transported; while uptake of quercetin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide was only 2- and 5-fold greater than the control, respectively. Subsequently, we further examined the uptake of the two sulfate conjugates. Uptake of both sulfates was effectively inhibited by 1 mM probenecid, an OAT inhibitor (Fig. 3). It was found that OAT1 mediated a high-affinity transport of quercetin-3′-*O*-sulfate, with

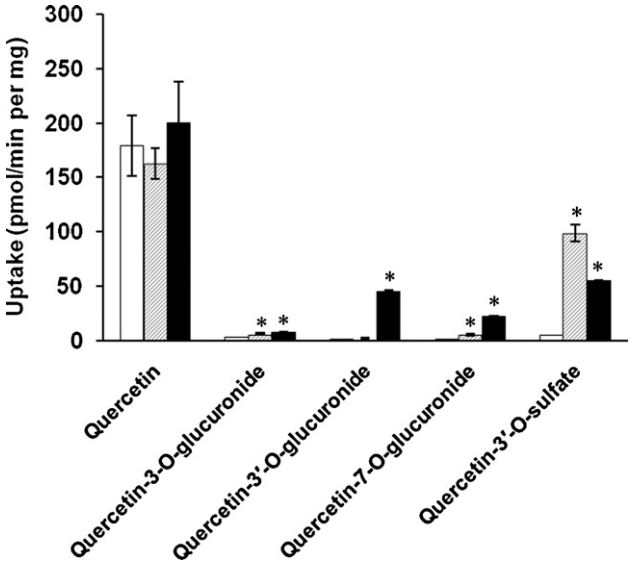

Fig. 1. Uptake of quercetin and its conjugates by OAT1 and OAT3. 293H-control (white bar), 293H-OAT1 (hatched bar) and 293H-OAT3 (black bar) cells were incubated with 25 μM substrate for 30 min. $^*p < 0.05$ compared to control.

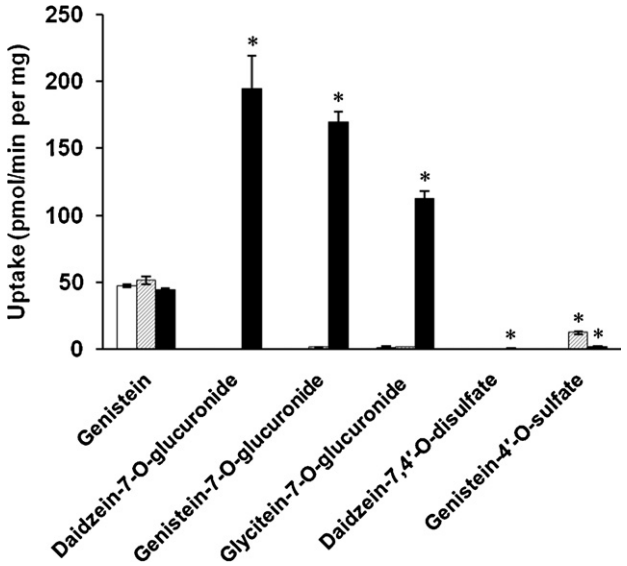

Fig. 2. Uptake of genistein and isoflavone conjugates by OAT1 and OAT3. 293H-control (white bar), 293H-OAT1 (hatched bar) and 293H-OAT3 (black bar) cells were incubated with 25 μM substrate for 30 min. $^{*}p < 0.05$ compared to control.

K_m of 1.73 ± 0.38 μM (Table 1). Uptake of genistein-4′-*O*-sulfate was less active compared to quercetin-3′-*O*-sulfate with a higher K_m and lower V_{max}. Quercetin and genistein, the aglycones with no attached sulfate, were not actively transported by OAT1.

3.2. Uptake of flavonoid and isoflavone conjugates by OAT3

The uptake of conjugates by OAT3 has a markedly different profile compared to OAT1 (Figs. 1 and 2). OAT3 possessed a broader specificity since all the conjugates tested showed a significant increase in their uptake compared to the control transfected cells. Glucuronide conjugates, in particular, were highly transported by OAT3. Uptake of daidzein-7-*O*-glucuronide and genistein-7-*O*-glucuronide in OAT3-expressing cells were in excess of 300-fold over control cells; and for glycitein-7-*O*-glucuronide the transport into cells was 100-fold greater. Uptake of the glucuronide conjugates was completely inhibited by co-incubation with 1 mM probenecid (Fig. 3). Among quercetin conjugates, OAT3-mediated transport of quercetin-3′-*O*-glucuronide and quercetin-3′-*O*-sulfate was most active. Uptake of quercetin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide were comparatively lower. Genistein-4′-*O*-sulfate was also transported by OAT3. Interestingly, daidzein-7,4′-*O*-disulfate, not a substrate for OAT1, was significantly transported by OAT3. However, the rate of transport was low for the tested isoflavone sulfates compared to glucuronide conjugates. We then determined the uptake kinetics of several glucuronide conjugates (Table 1). OAT3 possessed a higher affinity (lower K_m) towards quercetin-3′-*O*-glucuronide and genistein-7-*O*-glucuronide, with a lower affinity (higher K_m) towards daidzein-7-*O*-glucuronide and glycitein-7-*O*-glucuronide. On the other hand, the maximal rate of transport was highest for daidzein-7-*O*-glucuronide, followed by genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide and quercetin-3′-*O*-glucuronide. As seen for OAT1, the aglycones did not appear to be actively transported by OAT3.

3.3. Inhibition of OAT1-mediated uptake of p-aminohippuric acid by flavonoid and isoflavones conjugates

The flavonoid/isoflavone aglycones and conjugates were tested for their ability to inhibit OAT-mediated uptake of the model substrate for OAT1, *p*-aminohippuric acid. Uptake of *p*-aminohippuric acid in OAT1-expressing cells was sensitive to probenecid, an OAT1 inhibitor, with a complete inhibition at 1 mM. We tested the inhibitory effects of the aglycones and conjugates at 2 and 10 μM. At 10 μM, a concentration that is generally considered to be supraphysiological, all flavonoids conjugates tested showed a significant inhibition on OAT1-mediated uptake of *p*-aminohippuric acid (Fig. 4). Quercetin-3′-*O*-sulfate is the most potent inhibitor and almost completely inhibited the uptake of *p*-aminohippuric acid. Quercetin-7-*O*-glucuronide, quercetin-3′-*O*-glucuronide, genistein-4′-*O*-sulfate and the aglycones also resulted in over 50% inhibition of uptake. Isoflavone glucuronides are comparatively weaker inhibitors of OAT1. At a physiologically achievable concentration (2 μM), however, most of the conjugates tested show little inhibition of OAT1. Only the sulfated conjugates daidzein-7,4′-*O*-disulfate, genistein-4′-*O*-sulfate and quercetin-3′-*O*-sulfate demonstrated a statistically significant inhibition of *p*-aminohippuric acid uptake. In particular, quercetin-3′-sulfate demonstrated a strong inhibition (>75%) even at 2 μM. The OAT1-inhibitory activity of quercetin-3′-*O*-sulfate was examined

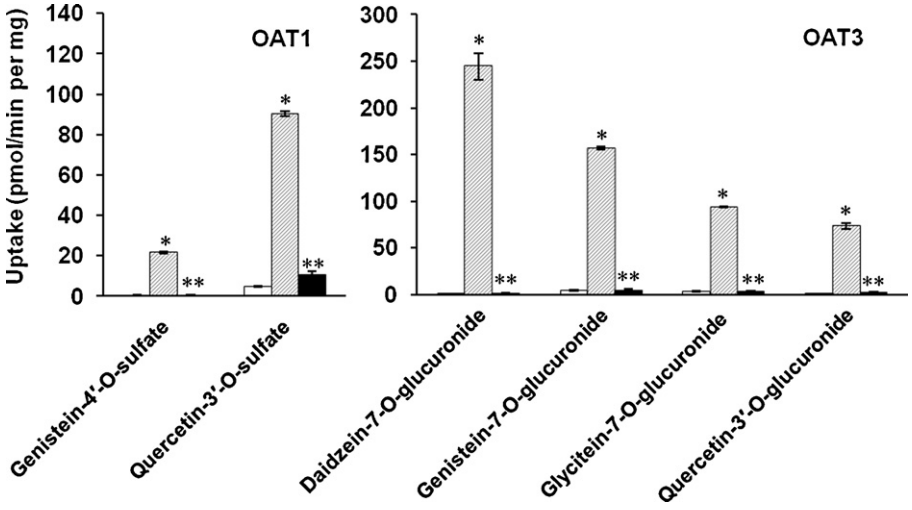

Fig. 3. Effect of probenecid on OAT1- or OAT3-mediated uptake of flavonoid conjugates. 293H-OAT1 (left panel) or 293H-OAT3 (right panel) cells were incubated with 25 μM of flavonoid conjugates without (hatched bar) or with 1 mM probenecid (black bar) for 10 min. $^{*}p < 0.05$ compared to vector control (white bar); $^{**}p < 0.05$ compared to uptake without probenecid.

Table 1
Kinetics of OAT1 and OAT3-mediated uptake of selected flavonoid conjugates.

Substrate	OAT1			OAT3		
	K_m (μM)	V_{max} (pmol/min/mg)	CL (μL/min/mg)	K_m (μM)	V_{max} (pmol/min/mg)	CL (μL/min/mg)
Daidzein-7-*O*-glucuronide				19.1 ± 1.9	420 ± 15	22.0
Genistein-4′-*O*-sulfate	5.07 ± 1.81	28.3 ± 2.9	5.58			
Genistein-7-*O*-glucuronide				7.94 ± 1.42	210 ± 10	26.4
Glycitein-7-*O*-glucuronide				17.6 ± 3.9	171 ± 13	9.72
Quercetin-3′-*O*-sulfate	1.73 ± 0.38	105 ± 7	60.7			
Quercetin-3′-*O*-glucuronide				5.25 ± 0.86	73.0 ± 3.5	13.9

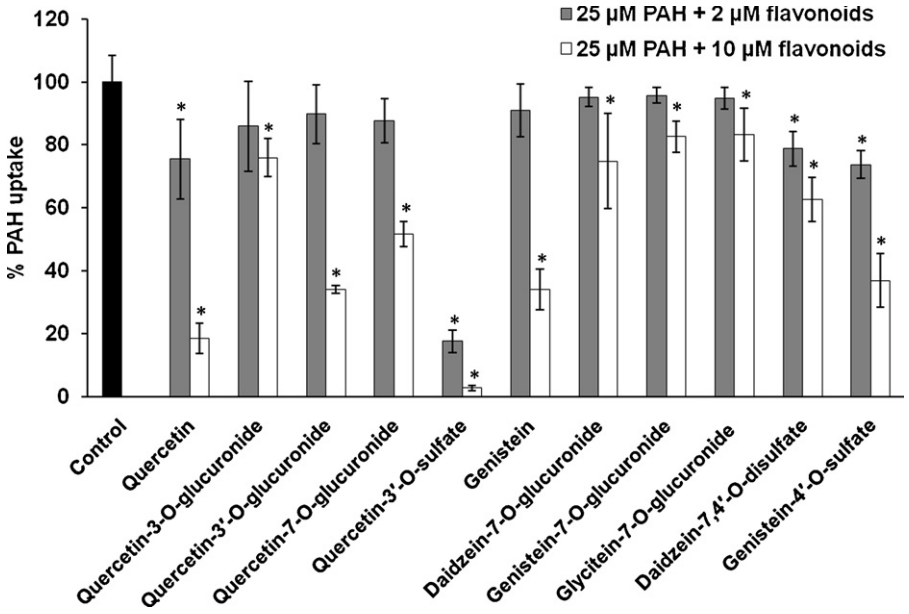

Fig. 4. Inhibition of OAT1-mediated uptake of *p*-aminohippuric acid (PAH) by flavonoid conjugates. OAT1-expressing cells were incubated for 10 min with 25 μM PAH in the presence of 2 (grey bar) and 10 μM (white bar) of flavonoid conjugates. $^*p < 0.05$ compared to control (black bar).

over a range of concentrations (0.1–10 μM). IC_{50} of the inhibition was 1.22 μM, indicating that it is a potent inhibitor of OAT1 (Fig. 5).

3.4. Inhibition of OAT3-mediated transport of 5-carboxyfluorescein by flavonoid and isoflavones conjugates

5-Carboxyfluorescein, a fluorescent marker, is a good model substrate of OAT3 [33]. Flavonoid/isoflavone conjugates were tested for the inhibitory activity on OAT3-mediated uptake of 5-carboxyfluorescein, at 2 and 10 μM concentrations (Fig. 6). We observed that the inhibition of OAT3 by the flavonoid conjugates tested was generally stronger compared to OAT1. All the flavonoids tested showed a considerable (40–90%) inhibition of OAT3-mediated uptake at 10 μM. Quercetin metabolites showed a stronger inhibition (>60%) of OAT3 compared to the isoflavone metabolites. Quercetin and genistein, non-substrates of OAT3, also showed a high inhibition of this transporter. In contrast to OAT1, many flavonoid metabolites tested showed considerable inhibition of OAT3 even at 2 μM. Quercetin metabolites, quercetin-3-*O*-glucuronide, quercetin-3′-*O*-glucuronide and quercetin-3′-*O*-sulfate, showed >60% inhibition of OAT3. Isoflavone glucuronides are poor inhibitors, despite being good substrates of OAT3. The IC_{50} values of inhibition of OAT3 for quercetin-3-*O*-glucuronide, quercetin-3′-*O*-glucuronide and quercetin-3′-*O*-sulfate were determined to be at 0.43 μM, 1.31 μM and 0.75 μM, respectively (Fig. 7). These data imply that physiologically relevant concentrations of quercetin metabolites have a significant impact on OAT3 activity.

3.5. Quercetin-3′-O-sulfate reduces OAT1-induced cytotoxicity of adefovir

Experiments with 293H control and OAT1-expressing cells demonstrated a marked enhancement of adefovir-induced cytotoxicity with OAT1 over-expression. In OAT1-expressing 293H cells, adefovir was highly cytotoxic with an IC_{50} value of 1.5 μM; while in control 293H cells, the IC_{50} value was 124 μM (Fig. 8). Quercetin-3′-*O*-sulfate, a good OAT1 substrate, may thus be protective against cytotoxicity of adefovir. To test this hypothesis,

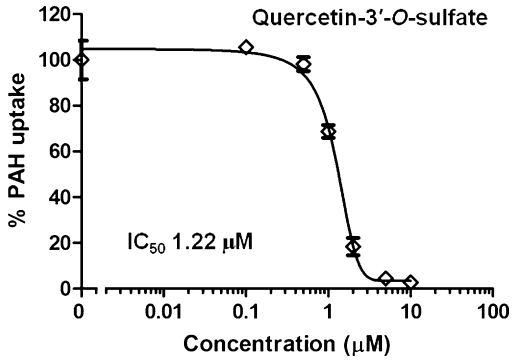

Fig. 5. Concentration-dependent inhibition of OAT1-mediated uptake of *p*-aminohippuric acid (PAH) by quercetin-3′-*O*-sulfate. OAT1-expressing cells were incubated for 10 min with 25 μM PAH in the presence of 0.1–10 μM of quercetin-3′-*O*-sulfate.

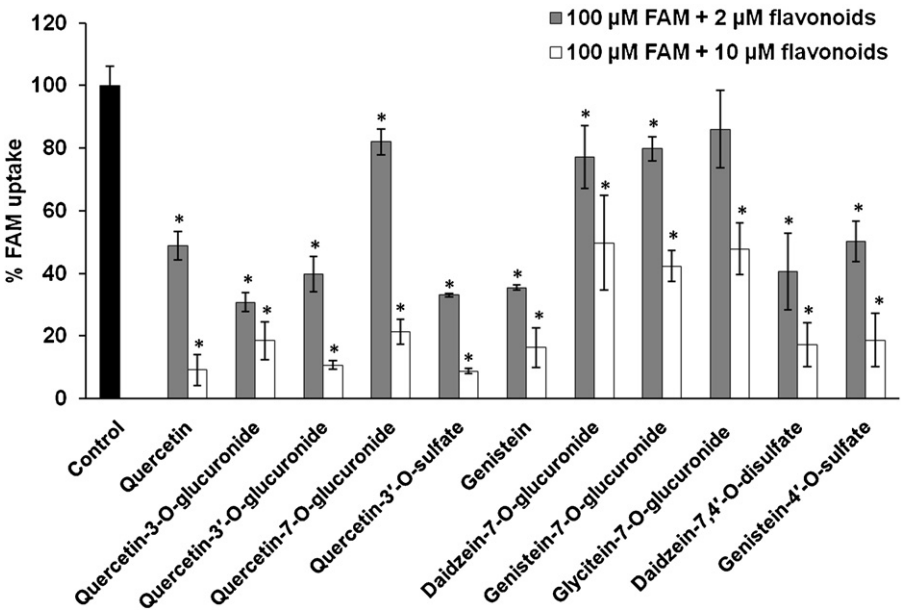

Fig. 6. Inhibition of OAT3-mediated uptake of 5-carboxyfluorescein (FAM) by flavonoid conjugates. OAT3-expressing cells were incubated for 10 min with 25 μM FAM in the presence of 2 (grey bar) and 10 μM (white bar) of flavonoid conjugates. $^{*}p < 0.05$ compared to control (black bar).

we evaluated the effect of co-incubation of adefovir (3 and 10 μM) with different concentrations of quercetin-3′-*O*-sulfate (2–50 μM) (Fig. 8). The results showed that quercetin-3′-*O*-sulfate significantly reduced the cytotoxicity of adefovir dose-dependent at concentrations above 10 μM. Maximal protection was observed at 50 μM, with cell viability increased from 37.3% to 77.4% and 16.2% to 60.1% for 3 and 10 μM adefovir, respectively. Treatment of quercetin-3′-*O*-sulfate alone at concentrations tested (2–50 μM) had no effect on cell viability in OAT1-transfected cells.

4. Discussion

Flavonoids are extensively metabolized in humans by the phase II enzyme families of sulfotransferases and UDP-glucuronosyltransferases following oral administration. Conjugates of glucuronide and/or sulfate are the predominant forms in human plasma, while the parent aglycones are usually absent or present in minute amounts [11]. In the present study, we examined transport of nine quercetin and isoflavone metabolites found *in vivo* in OAT1- or OAT3-overexpressing human embryonic kidney 293H cells. The sulfated conjugates, quercetin-3′-*O*-sulfate and genistein-4′-*O*-sulfate, were efficiently transported by OAT1. On the other hand, glucuronide conjugates including daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide and quercetin-3′-*O*-glucuronide appeared to be preferential substrates for OAT3. Quercetin-3′-*O*-sulfate was also transported by OAT3, but the transport activity was much lower compared with OAT1. Although the aglycones quercetin and genistein were taken up into OAT1- and OAT3-expressing cells, uptake was not significantly different from the control cells. Due to the relative hydrophobicity of the aglycones, their uptake may be attributed to passive diffusion [34]. OAT1 and OAT3 are highly expressed on the basolateral membranes of renal proximal tubules where they mediate the rate-limiting uptake of xenobiotics and their metabolites. The relative specificity and activity of OATs may influence the extent to which flavonoid conjugates are eliminated via the urine. In humans, urinary excretion (20–60% of an ingested dose) is a major pathway of elimination for the isoflavones [11]. Since isoflavone glucuronides are the main metabolites in humans [10], OAT3 may contribute to the urinary excretion of isoflavones by mediating the uptake of glucuronide conjugates from blood into proximal tubules. Hence, OAT3-mediated transport could be one factor limiting the systemic availability of isoflavones. In contrast, a relatively small proportion (1–7%) of ingested quercetin is excreted in urine. Although quercetin-3′-*O*-sulfate and quercetin-3′-*O*-glucuronide are good substrates of OAT1 and OAT3, respectively, quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide, the other major quercetin metabolites *in vivo*, were very poor substrates of OAT1 and OAT3. These data suggest that OAT1- and OAT3-mediated uptake into kidney may play an important role in determining disposition of flavonoid conjugates *in vivo*.

For the flavonoid metabolites, it was observed that OAT1 and OAT3 favored the transport of sulfate and glucuronide conjugates, respectively. OATs have been shown to transport various xenobiotics and their conjugated metabolites. In agreement with our data, OAT1 was reported to mediate the transport of sulfated conjugates such as indoxyl sulfate [35], edaravone sulfate [16] and 5-sulfooxymethylfurfural [36]. Transport of more bulky glucuronidated metabolites by OAT1 was not observed [16,37]. Nonetheless, this study has also identified quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide as potential unique glucuronidated substrates of OAT1. OAT3, on the other hand, has been shown to transport the sulfate or glucuronide conjugates, such as estrone-3-sulfate, edaravone sulfate, edaravone glucuronide [16], and 7-*O*-mycophenolic acid glucuronide [37]. Indeed, all the flavonoid conjugates tested herein showed significantly enhanced uptake in the OAT3-overexpressing cells, suggesting that OAT3 possesses a broader specificity for the flavonoid conjugates compared to OAT1.

Vectorial transport of hydrophilic substances across the renal proximal tubules is achieved through co-operative transport of uptake transporters on the basolateral membrane and efflux transporters on the brush border membrane [38]. In the human kidney, multidrug resistance protein 2 (MRP2/ABCC2) and 4 (MRP4/ABCC4), *p*-glycoprotein (MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2) and OAT4 are expressed on the brush border membrane [39]. Glucuronidated and sulfated conjugates of flavonoids are substrates of apical efflux transporters. Quercetin glucuronides are transported by MRP2 [17] and BCRP [40]; quercetin-3′-*O*-sulfate is transported by MRP2 [41].

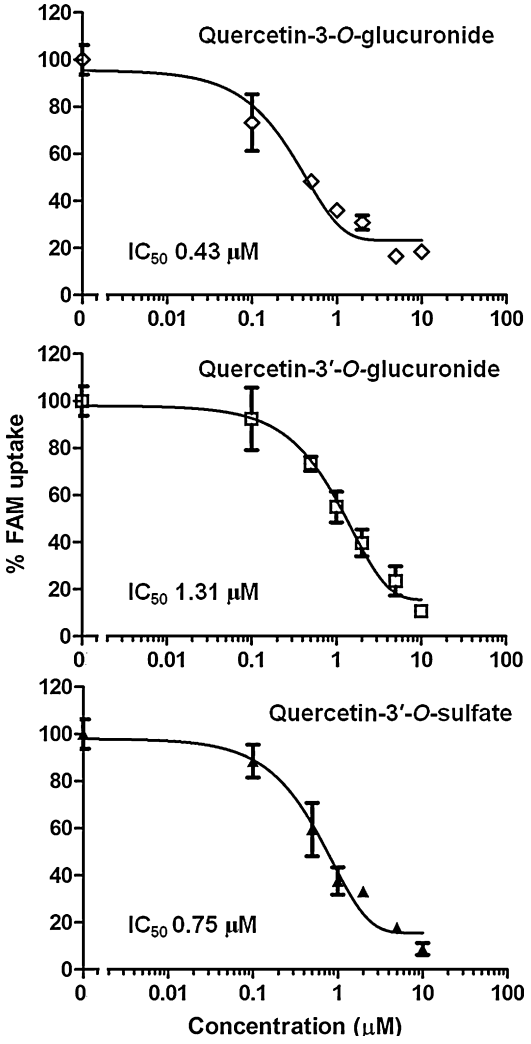

Fig. 7. Concentration-dependent inhibition of OAT3-mediated uptake of 5-carboxyfluorescein (FAM) by quercetin-3-*O*-glucuronide, quercetin-3′-*O*-glucuronide and quercetin-3′-*O*-sulfate. OAT3-expressing cells were incubated for 10 min with 25 μM PAH in the presence of 0.1–10 μM of the quercetin conjugates.

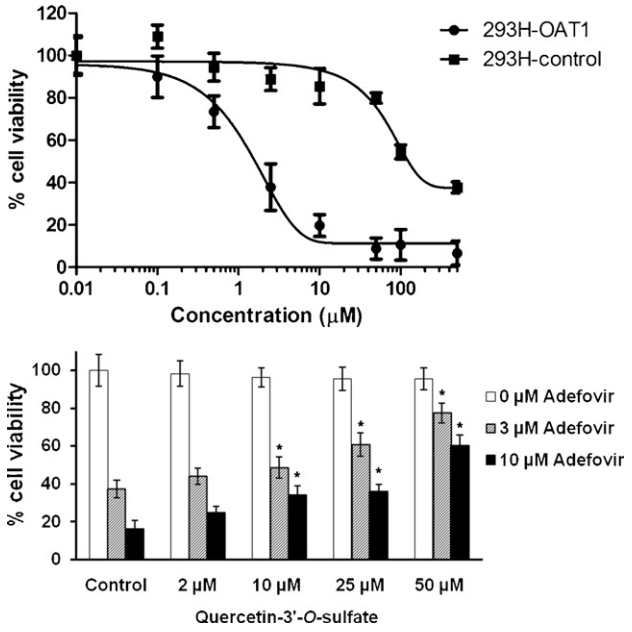

Fig. 8. Quercetin-3′-*O*-sulfate attenuated OAT1-induced cytotoxicity of adefovir. (A) The effect of OAT1 expression on the cytotoxicity of adefovir. 293H-control and 293H-OAT1 cells were incubated with various concentrations of adefovir for 96 h; (B) the effect of quercetin-3′-*O*-sulfate (2–50 μM) on the cytotoxicity of 3 (white bar) and 10 μM (grey bar) adefovir. $^{*}p < 0.05$ compared to control.

BCRP, and to a lesser extent MRP2, were responsible for the apical efflux of isoflavone glucuronides and sulfates in the intestine [18]. In the human kidney, BCRP and MRP2 may play a similar role in the apical efflux of flavonoid conjugates from proximal tubule cells into urine. The coupled action of influx (OAT1 and OAT3) and efflux transporters (BCRP and MRP2) may result in effective elimination of flavonoid metabolites from the circulation, and contribute to the rapid elimination of flavonoids in humans.

Apart from being transported, the present study also shows that flavonoid conjugates inhibited the transport of model substrates by OAT1 and OAT3. Previous investigations showed that ellagic acid and morin are potent inhibitors of OAT1, with IC_{50} values of 0.21 and 0.46 μM, respectively [26,27]. However, the bioavailability of parent aglycones is often minimal due to extensive phase II metabolism. Herein, we demonstrated that some of the metabolites of flavonoids found *in vivo* retained the capability to inhibit OATs. Among the flavonoid conjugates tested, quercetin-3′-*O*-sulfate displayed the most effective inhibition of OAT1 (IC_{50}: 1.7 μM). Indeed, the sulfation of this flavonoid was found to enhance its inhibitory activity on OAT1. Potent inhibition of OAT3-mediated uptake of 5-carboxyfluorescein was also found for quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide and quercetin-3′-*O*-sulfate with IC_{50} values of 0.43–1.31 μM. Isoflavone conjugates are generally weaker inhibitors of OATs. The low IC_{50} of inhibition suggests that physiologically relevant concentrations of quercetin conjugates may have an impact on the OAT-mediated uptake of drugs. Such a flavonoid–drug interaction may be utilized to offer therapeutic benefits, for example, to improve the systemic availability of drugs by limiting the OAT1- or OAT3-mediated renal clearance.

Inhibitors of OATs are potential nephroprotective agents against drug-induced renal injury. The nephrotoxicity of some drugs is caused by the highly concentrative uptake of OATs, leading to excessive accumulation in the renal tubules and development of renal injury. OAT1-mediated transport has been implicated in the nephrotoxicity caused by adefovir, cidofovir and cephalosporin antibiotics [42,43]. It has been suggested that probenecid and non-steroidal anti-inflammatory drugs (NSAIDS), at clinically relevant concentrations, are effective in reducing OAT1-induced nephrotoxicity of drugs [24,42]. Probenecid and NSAIDS, however, are not without adverse effects. Flavonoids may thus be useful alternatives in the management of OAT1-induced nephrotoxicity of drugs. Adefovir, a substrate of OAT1, was used to investigate the potential of quercetin-3′-*O*-sulfate to reduce OAT1-mediated toxicity of drugs. OAT1-transfected cells were shown to be more sensitive to the cytotoxicity of adefovir compared to control cells [24,42]. On the other hand, we showed that quercetin-3′-*O*-sulfate ($\geq$10 μM) effectively reduced the cytotoxicity of adefovir in OAT1-over-expressing cells. This suggests that flavonoids have the potential to limit the severity of drug-induced renal injury via the inhibition of OAT1. Lim et al. [44] also reported that the flavonoid morin protected against imipenem-induced nephrotoxicity, via the inhibition of OAT3-mediated accumulation of this antibiotic. Alternatively, flavonoids may attenuate drug-induced renal damage via free radical scavenging [45,46] or anti-apoptotic mechanisms [47]. Nevertheless, OAT1 and OAT3 may play an active role in these protective effects by mediating the entry of flavonoids and their metabolites into the proximal tubule cells.

In conclusion, our results indicate that both OAT1 and OAT3 are likely to be responsible for uptake of flavonoid sulfates and

glucuronides across the basolateral membrane of the human kidney, respectively. OAT-mediated transport may be one factor limiting the systemic availability of flavonoids *in vivo*. Our study also showed that quercetin conjugates are able to inhibit OAT1 and OAT3 at physiologically achievable concentrations, and quercetin-3′-*O*-sulfate reduces OAT1-mediated cytotoxicity of adefovir, thereby indicating a potential for flavonoid–drug interactions.

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