RESEARCH ARTICLE

Interaction of hydroxycinnamic acids and their conjugates with organic anion transporters and ATP-binding cassette transporters

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Received: December 21, 2010
Revised: February 7, 2011
Accepted: February 23, 2011

Scope: Hydroxycinnamic acids are abundant antioxidants in our diet. In humans, hydroxycinnamic acids are metabolized to form sulfates and glucuronides, with the majority recovered in urine.
Methods and results: We assessed the potential roles of organic anion transporters (OATs) and ATP-binding cassette (ABC) transporters in the renal uptake and efflux of hydroxycinnamic acid conjugates. Uptake studies using OAT1 (SLC22A6)-, OAT2 (SLC22A7)-, and OAT3 (SLC22A8)-expressing 293H embryonic kidney cells showed that OAT1 and OAT3, but not OAT2, accepted hydroxycinnamic acid conjugates as substrates. OAT1 and OAT3 mediated the basolateral uptake of hydroxycinnamic acid sulfates and glucuronide conjugates, respectively. Hydroxycinnamic acid sulfates are substrates of OAT4 and were capable of *trans*-stimulating 5-carboxyfluorescein uptake mediated by OAT4. On the other hand, hydroxycinnamic acid conjugates are not substrates for the ABC transporters, multidrug resistance protein 2 (MRP2/ABCC2) or breast cancer resistance protein (BCRP/ABCG2), demonstrated by the inability to alter ATPase activity. *Cis*-inhibition studies with OATs and MRPs revealed that hydroxycinnamic acid conjugates have limited impact on the transport of model substrates significantly at physiological concentrations.
Conclusion: Concerted action of OAT1, OAT3, and OAT4 is involved in the elimination of hydroxycinnamic acid conjugates into urine, whereas MRP2 and breast cancer resistance protein are not involved in the disposition of these conjugates.

Keywords:
Bioavailability / Hydroxycinnamic acids / Multidrug resistance transporters / Organic anion transporters

1 Introduction

Hydroxycinnamic acids are natural antioxidants ubiquitously found in fruits, vegetables, and coffee [1]. Intakes of hydroxycinnamic acids can be very high (up to 1000 mg/day), and studies have suggested that the consumption of hydroxycinnamic acid-rich foods may be beneficial to health [2–4]. In humans, hydroxycinnamic acids are extensively metabolized by phase II enzymes, in particular sulfotransferases and to a lesser extent UDP-glucuronosyltransferases, to give sulfated and/or glucuronidated conjugates as the predominant forms in plasma [5, 6]. When administered as free acids, hydroxycinnamic acids are well absorbed and 40–60% of the ingested dose can be recovered in urine [7, 8]. After consumption of coffee (200 mL serving), a rich source of chlorogenic acids (quinic acid esters), as much as 30% of the ingested dose was recovered in the urine as hydroxycinnamic acid conjugates

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Abbreviations: ABC transporter, ATP-binding cassette transporter; **BCRP,** breast cancer resistance protein; **MRP,** multidrug resistance protein; **OAT,** organic anion transporter

(Fig. 1) [6]. Therefore, urinary excretion may limit the systemic availability of hydroxycinnamic acids, resulting in low concentrations of hydroxycinnamic acids and their conjugates in the plasma ($\leq 2\,\mu M$) after consumption of hydroxycinnamic acid-rich foods [9–11].

Tubular secretion in the kidney is an important mechanism for the excretion of endogenous metabolites and xenobiotics. Numerous endogenous phenols, such as the neurotransmitter metabolites homovanillic acid and dihydroxyphenylacetic acid, are avidly excreted into the urine via active secretion [12, 13]. Sulfated conjugates, including indoxyl sulfate and estrone sulfate, are also eliminated via tubular secretion [14, 15]. Given the structural similarity of dietary hydroxycinnamic acids to aforementioned neurotransmitter metabolites, and their propensity to be sulfated, it is likely that they may be actively secreted, at least in part, via the proximal tubules in kidney. Tubular excretion involves vectorial transport of hydrophilic substances across polarized cell monolayers, accomplished by basolaterally located uptake transporters acting in concert with apically located efflux transporters. In the human kidney, basolateral uptake involves members of the organic anion transporter (OAT/SLC22A) family, whereas efflux is mainly carried out by several ATP-binding cassette (ABC) transporters and/or OAT/SLC22A transporters that are expressed on the brush-border membrane.

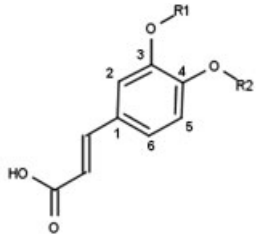
OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*) are highly expressed in proximal tubules where they have been localized on the basolateral membrane [16]. OAT1 and OAT3 mediate the sodium-dependent uptake of diverse organic anions, including endogenous metabolites, toxicants, and numerous drugs [15, 17, 18]. OAT2 (*SLC22A7*) is also found on the basolateral membrane of proximal tubules [19], but unlike OAT1 and OAT3, it is ubiquitously expressed in many tissues with particularly high expression in the liver [20]. OAT2 mediates facilitative uptake of organic anions such as dicarboxylates, cAMP, and some drugs. On the brush-border membrane, efflux is mediated by ABC transporters such as the multidrug resistance protein 2 (ABCC2/MRP2) and 4 (ABCC4/MRP4), breast cancer resistance protein (BCRP/ABCG2), as well as OAT4 (OAT4/SLC22A11). MRP2 and BCRP are broad specificity transporters that mediate the active efflux of endogenous compounds, drugs, toxicants, and their sulfated and glucuronidated metabolites [21, 22]. OAT4 is an SLC22A member unique to humans; it is involved in the reabsorption and/or efflux of sulfate conjugates of steroid hormones, urate, and anionic drugs [23]. The co-operative transport of the basolateral influx and apical efflux transporters is responsible for the effective elimination of unwanted metabolites and xenobiotics from the blood into the urine.

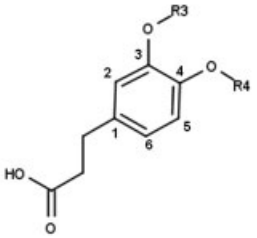
Given that urinary excretion is a primary route for the elimination of hydroxycinnamic acids and their conjugated metabolites, they may be substrates of influx and efflux transporters expressed in proximal tubules. Moreover, due to the high dietary load of hydroxycinnamic acids in some populations, the possibility of interaction with other compounds for renal transporters warrants investigation. To our knowledge, no previous reports have examined the potential interaction of hydroxycinnamic acids and their conjugates with OATs and ABC transporters. In this study, transport of hydroxycinnamic acids and their conjugated metabolites by OAT1, OAT2, OAT3, OAT4, MRP2, and BCRP was assessed. In addition, the interaction of these dietary components with model substrates of these transporters was also investigated.

2 Materials and methods

2.1 Chemicals

Caffeic acid, ferulic acid, and isoferulic acid were purchased from Sigma-Aldrich (St. Louis, MO). Dihydrocaffeic acid and dihydroferulic acid were obtained from Alfa Aesar (Lancashire, UK). Caffeic-3-*O*-glucuronide, caffeic acid-4-*O*-glucuronide, caffeic acid-3-*O*-sulfate, caffeic acid-4-*O*-sulfate, dihydrocaffeic acid-3-*O*-glucuronide, dihydrocaffeic acid-





1. R1 = H, R2 = H: Caffeic acid
2. R1 = CH_3, R2 = H: Ferulic acid
3. R1 = H, R2 = CH_3: Isoferulic acid
4. R1 = GluA, R2 = H: Caffeic acid-3-*O*-glucuronide
5. R1 = H, R2 = GluA: Caffeic acid-4-*O*-glucuronide
6. R1 = SO_3^-, R2 = H: Caffeic acid-3-*O*-sulfate
7. R1 = H, R2 = SO_3^-: Caffeic acid-4-*O*-sulfate
8. R1 = CH_3, R2 = GluA: Ferulic acid-4-*O*-glucuronide
9. R1 = CH_3, R2 = SO_3^-: Ferulic acid-4-*O*-sulfate
10. R1 = GluA, R2 = CH_3: Isoferulic acid-3-*O*-glucuronide
11. R1 = SO_3^-, R2 = CH_3: Isoferulic acid-3-*O*-sulfate
12. R3 = H, R4 = H: Dihydrocaffeic acid
13. R3 = CH_3, R4 = H: Dihydroferulic acid
14. R3 = GluA, R4 = H: Dihydrocaffeic acid-3-*O*-glucuronide
15. R3 = H, R4 = GluA: Dihydrocaffeic acid-4-*O*-glucuronide
16. R3 = SO_3^-, R4 = H: Dihydrocaffeic acid-3-*O*-sulfate
17. R3 = H, R4 = SO_3^-: Dihydrocaffeic acid-4-*O*-sulfate
18. R3 = CH_3, R4 = GluA: Dihydroferulic acid-4-*O*-glucuronide
19. R3 = CH_3, R4 = SO_3^-: Dihydroferulic acid-4-*O*-sulfate

Figure 1. Structures of hydroxycinnamic acids, sulfates, and glucuronides conjugates. GluA, glucuronic acid.

4-*O*-glucuronide, dihydrocaffeic acid-3-*O*-sulfate, dihydrocaffeic-4-*O*-sulfate, dihydroferulic acid-4-*O*-glucuronide, dihydroferulic acid-4-*O*-sulfate, ferulic acid-4-*O*-glucuronide, ferulic acid-4-*O*-sulfate, isoferulic acid-3-*O*-glucuronide, and isoferulic acid-3-*O*-sulfate were synthesized as described previously [24]. Human embryonic kidney 293H (293H) cells were purchased from Invitrogen (Carlsbad, CA). OAT1 (SLC22A6 transcript variant 2, Genebank accession number: NM_153276), OAT2 (SLC22A7 transcript variant 1, Genebank accession number: NM_006672.2), OAT3 (SLC22A8 transcript variant 1, Genebank accession number: NM_004254), and OAT4 (Genebank accession number: NM_018484.2) expression plasmids were obtained from Origene (Rockville, MD). Fugene HD was purchased from Roche (Nutley, NJ). All other chemicals, unless otherwise stated, were purchased from Sigma-Aldrich.

2.2 293H cell culture and transfection

293H cells were cultured in Dulbecco's modified Minimum Essential Medium (DMEM) high-glucose medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 50 U/mL penicillin–streptomycin, in 5% CO_2 at 37°C. Antibiotics were not included in transfection experiments. Experiments were performed with cells between passages 1 and 20. The day before transfection, cells were seeded into poly-L-lysine coated 24-well plates at a density of 1.2×10^5 cells/per well. OAT1, OAT2, OAT3, or OAT4 plasmids (2 μg), or empty pCMV-XL6 vector was mixed with 3 μL Fugene HD reagent, according to the manufacturer's manual, in 100 μL Opti-MEM (Invitrogen). After incubation for 18 min at room temperature, 25 μL of transfection complex was added to each well. Uptake assays were performed 22–24 h after transfection. Overexpression of OATs was confirmed by real-time RT-PCR using Taqman assays (Hs00537914_m1 [SLC22A6], Hs00198527_m1 [SLC22A7], Hs01056647_m1, [SLC22A8], Hs00218486_m1 [SLC22A11]) (Applied Biosystems), which showed very high overexpression of OAT1 (1.19×10^5-fold), OAT2 (1.28×10^5-fold), OAT3 (1.10×10^5-fold), and OAT4 (7.62×10^4-fold). The transporter activity was further confirmed by the uptake of model substrates *p*-aminohippuric acid (OAT1 and OAT2), estrone-3-sulfate (OAT3), and carboxyfluorescein (OAT4).

2.3 Uptake assays

Uptake experiments were carried out in Hank's balanced salt solution (HBSS) containing 1.8 mM $CaCl_2$ and 1.8 mM $MgCl_2$ at pH 7.4. Medium was removed and the monolayer was washed twice with 0.25 mL transport buffer. After 10 min, buffer was replaced with transport buffer containing the test compounds. Hydroxycinnamic acids were dissolved in DMSO (final concentration, $<0.2\%$); all the other hydroxycinnamic acid conjugates were dissolved in water. After the incubation period, uptake was stopped by adding 1 mL ice-cold transport buffer containing 0.2% bovine serum albumin (BSA). This was quickly aspirated and washed twice with 0.5 mL ice-cold transport buffer with 0.2% BSA. The final wash was performed with 1 mL ice-cold transport buffer without BSA. Cells were lysed in 0.4 mL 50% methanol, sonicated for 5 min, followed by the addition of 1 mL of ice-cold acetone. Samples were kept in a −20°C freezer for 1 h and centrifuged at $17\,000 \times g$ for 5 min. The supernatant was evaporated to dryness in vacuo and stored at −20°C until analysis. The amount of hydroxycinnamic acids and conjugates taken up into the cells was measured by HPLC (Section 2.5), and the protein pellet was redissolved in 0.1 N NaOH and protein content was determined by the Bradford assay. All the uptake values were standardized against protein content.

2.4 Kinetics of uptake of hydroxycinnamic acid and conjugates

Initial time-course studies showed that the uptake of caffeic acid and dihydrocaffeic acid was linear for at least 10 min; for the sulfated conjugates, uptake was linear for at least 5 min. Kinetic analysis were performed for hydroxycinnamic acid and conjugates in control or OAT1-expressing cells at a substrate concentration range of 2–100 μM. The amount of hydroxycinnamic acids and conjugates taken up into the cells was measured by HPLC, and the protein content was determined by the Bradford assay. Apparent uptake of OATs was obtained by subtracting the uptake values of control cells at each individual concentration from the uptake values in transporter-expressing 293H-OAT1 cells.

2.5 HPLC analysis of hydroxycinnamic acid and 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 × 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 hydroxycinnamic acids, elution started at 15% (B), increasing to 40% in 8 min and to 90% for 2 min, after which it returned to 15% for 4 min at a flow rate of 0.75 mL/min. For the analysis of hydroxycinnamic acid conjugates, elution was performed at 0.75 mL/min and the gradient started at 5% (B), increased linearly to 10% in 5 min, increased to 35% in 4 min, to 90% for 2 min, and returned to 5% for 4 min. Quantification of cinnamic acids and dihydrocinnamic acids was based on the peak area at 310 and 280 nm, respectively. The on-column limit of quantification of this HPLC method for the quantification of

cinnamic acids was 0.5 pmol, and that for the quantification of dihydrocinnamic acids was 2.5 pmol.

2.6 Inhibition or stimulation of uptake of model substrates

p-Aminohippuric acid was used as the model substrate for OAT1, whereas 5-carboxyfluorescein was used as the substrate for OAT3 and OAT4, respectively. Uptake of *p*-aminohippuric acid (25 μM) was measured for 10 min in the presence or absence of 10 μM test compounds. The uptake *p*-aminohippuric acid was measured by HPLC. The analyses were performed with a Zorbax XDB-C18 column (4.6 × 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. For OAT3 and OAT4, uptake of 5-carboxyfluorescein (100 μM) was measured for 10 min in the presence or absence of 10 μM test compound. For *trans*-stimulation experiments, 293H-OAT4 cells were preloaded for 4 h with 1 mM test substances before uptake. To analyze 5-carboxyfluorescein, the cell monolayers were extracted twice with 1 mL methanol in the dark, each for 30 min, and the supernatants were analyzed using HPLC with isocratic elution with 20 mM Tris-HCl buffer (pH 8) and 20% methanol as the mobile phase 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 Interaction of hydroxycinnamic acid conjugates with MRPs

Isolated membranes from Sf9 insect cells expressing high levels of human ABCC2 or ABCG2 (SOLVO Biotechnology, Budapest, Hungary) were used to measure vanadate-sensitive ATPase activity during incubation with test compounds as described previously [25, 26]. The compounds were dissolved in water. The efficiency of hydroxycinnamic acid conjugates (0.41–300 μM) to stimulate ATPase is expressed on a 0–100% scale, where 100% is defined as the activation observed in the presence of sulfasalazine (10 μM for BCRP; 100 μM for MRP2). ATPase inhibition efficacy is defined as the maximal inhibitory effect obtained as a percentage of baseline activity. Inhibitory effects of hydroxycinnamic acid conjugates (0.41–300 μM) on the vesicular transport of ^{3}H-estradiol-17β-glucuronide in membrane vesicles obtained from Sf9 insect cells overexpressing the human MRP2 transporter, and on the transport of ^{3}H-estrone-3-sulfate in vesicles from Sf9 insect cells overexpressing human BCRP were investigated as described previously [27, 28]. The ATP-dependent transport was calculated by subtracting the background obtained in the presence of AMP from those in the presence of ATP. The inhibition is expressed on a 0–100% scale, where 0% is defined as transport without any inhibitors.

2.8 Data analysis

Experiments were performed in triplicate. Data are shown as mean ± SD (as mean ± SE for enzyme kinetic data). Raw data from the enzyme kinetic studies were analyzed using GraphPad Prism 5 (GraphPad software, CA). K_m and V_{max} were derived from a nonlinear regression fit of the Michaelis–Menten model. Statistical differences were determined using the analysis of variance using the Student's *t*-test. Differences were considered significant when $p < 0.05$.

3 Results

3.1 Uptake of hydroxycinnamic acids by OATs

Uptake of hydroxycinnamic acids by control, OAT1, OAT2, OAT3, and OAT4 overexpressing 293H cells is shown in Fig. 2. In 293H control cells, there was significant uptake of dihydroferulic acid, ferulic acid, and isoferulic acid, whereas uptake of caffeic acid and dihydrocaffeic acid was not detected. The expression of OAT1, and to a lesser extent OAT3, resulted in a significant uptake of caffeic acid and dihydrocaffeic acid. The uptake of dihydroferulic acid and ferulic acid was also enhanced ~2-fold in the OAT1-expressing cells. On the other hand, uptake of all the hydroxycinnamic acids tested was not enhanced by either OAT2 or OAT4, indicating that hydroxycinnamic acids are

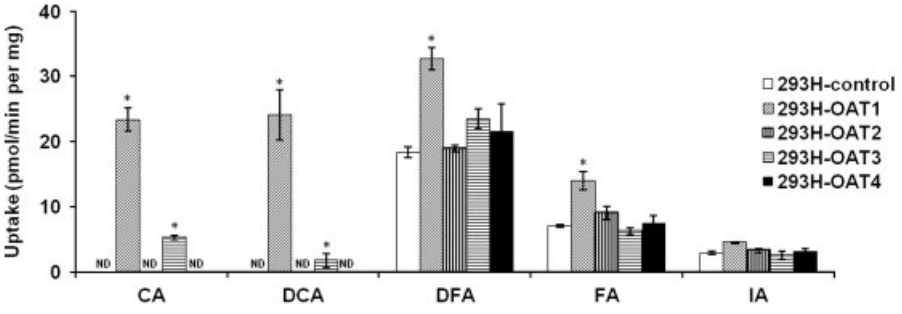


Figure 2. Uptake of hydroxycinnamic acid by 293H-control, 293H-OAT1, 293H-OAT2, 293H-OAT3, and 293H-OAT4 cells. Cells were incubated with 25 μM substrate for 30 min ($n = 3$). CA, caffeic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; FA, ferulic acid; IA, isoferulic acid. *$p < 0.05$ compared with control. ND, not detected.

not substrates for these transporters. OAT1-mediated uptake was completely blocked by 1 mM probenecid, an inhibitor of OATs. The uptake of caffeic acid and dihydrocaffeic acid by OAT1 was further characterized to obtain the kinetic parameters (Table 1–Supporting Information Fig. 1). The uptake of both hydroxycinnamic acids over a range of concentrations (2.5–100 μM) showed a sigmoidal plot, with minimal uptake at concentrations ≤ 5 μM. When modeled with the Hill equation, the Hill coefficient (n) was >1, suggesting the presence of positive cooperativity (Table 1). OAT1 had a higher efficiency (V_{max}/K_m) for the transport of dihydrocaffeic acid compared with caffeic acid.

3.2 Uptake of hydroxycinnamic acid sulfates by OATs

In 293H control cells, the uptake of hydroxycinnamic acid sulfates was not detected. The expression of OAT1, OAT3, or OAT4 resulted in a significantly increased uptake of these sulfated metabolites, whereas OAT2 overexpression had no effect (Fig. 3). OAT1-mediated uptake, in particular, was very high compared with OAT3 and OAT4. Transport of the 3-*O*-sulfates, caffeic acid-3-*O*-sulfate, dihydrocaffeic acid-3-*O*-sulfate, and isoferulic acid-3-*O*-sulfate by OAT1 was higher compared with their corresponding 4-*O*-sulfates. OAT3-mediated transport was 2- to 10-fold lower than OAT1, and there was no obvious preference for 3-*O*-sulfates over 4-*O*-sulfates. For OAT4, the transport activity was very low, with only detectable uptake of caffeic acid-3-*O*-sulfate, caffeic acid-4-*O*-sulfate, ferulic acid-4-*O*-sulfate, and isoferulic acid-3-*O*-sulfate, at levels 30- to 200-fold lower compared with OAT1. The uptake of dihydrocinnamic acid sulfates by OAT4 was not detected. Analysis of the kinetics of OAT1-mediated uptake showed that hydroxycinnamic acid sulfates were taken up more efficiently than their corresponding hydroxycinnamic acids, especially at low substrate concentrations (Supporting Information Fig. 2). The uptake of hydroxycinnamic acid sulfates followed simple hyperbolic kinetics, and was completely inhibited by 1 mM probenecid (data not shown). Cinnamic acids had higher affinity (K_m) but lower V_{max} compared with the corresponding dihydrocinnamic acids. Based on their intrinsic clearance (V_{max}/K_m), isoferulic acid-3-*O*-sulfate was the most actively transported sulfate, followed by caffeic acid-3-*O*-sulfate and dihydrocaffeic acid-3-*O*-sulfate (Table 1).

3.3 Uptake of hydroxycinnamic acid glucuronides by OATs

OATs possess a markedly different specificity toward hydroxycinnamic acid glucuronides compared with aglycones and sulfate conjugates (Fig. 4). Similar to sulfated conjugates, we could not detect the uptake of glucuronide conjugates in 293H control cells. In this case, OAT3 appeared to be the predominant transporter mediating glucuronide uptake. It significantly increased the uptake of all the hydroxycinnamic acid glucuronides tested. The transport activities of the glucuronides by OAT3 were similar or lower compared with their corresponding sulfated conjugates, and much lower than OAT1-mediated uptake of sulfates. OAT3-mediated uptake was the highest for dihydrocaffeic acid-3-*O*-glucuronide and dihydrocaffeic acid-4-*O*-glucuronide, followed by dihydroferulic acid-4-*O*-glucuronide; glucuronides of cinnamic acids were transported less efficiently. OAT3 did not appear to transport either the 3-*O*-glucuronide or the 4-*O*-glucuronide conjugates preferentially. Several glucuronides, including the 3-*O*- and 4-*O*-glucuronides of caffeic acid and dihydrocaffeic acid, were also transported by OAT1 at lower rates. OAT2 and OAT4 did not show any transport activity toward the hydroxycinnamic acid glucuronides tested.

3.4 Inhibition of OAT1-mediated uptake of *p*-aminohippuric acid by hydroxycinnamic acids and conjugates

The inhibitory effect of hydroxycinnamic acids and their conjugates toward OAT1-mediated uptake was evaluated using *p*-aminohippuric acid as the model substrate. The uptake of *p*-aminohippuric acid (25 μM) in OAT1-expressing cells was over 20-fold greater than that of control 293H cells and was sensitive to probenecid with complete inhibition at

Table 1. Kinetics of OAT1 and OAT3-mediated uptake of selected hydroxycinnamic acids and their conjugates

Substrate	OAT1			
	K_m (μM)	V_{max} (pmol/[min × mg])	V_{max}/K_m (μL/[min × mg])	Hill coefficient
Caffeic acid	28.6 ± 3.7	183 ± 14	6.40	1.92 ± 0.33
Caffeic acid-3-*O*-sulfate	38.9 ± 3.3	1190 ± 40	30.6	
Dihydrocaffeic acid	21.4 ± 4.2	218 ± 25	10.2	2.35 ± 0.85
Dihydrocaffeic acid-3-*O*-sulfate	76.7 ± 16.3	2070 ± 240	27.0	
Dihydroferulic acid-4-*O*-sulfate	115 ± 31	1530 ± 260	11.3	
Ferulic acid-4-*O*-sulfate	54.2 ± 3.6	454 ± 15	8.38	
Isoferulic acid-3-*O*-sulfate	32.7 ± 5.3	1290 ± 90	39.4	

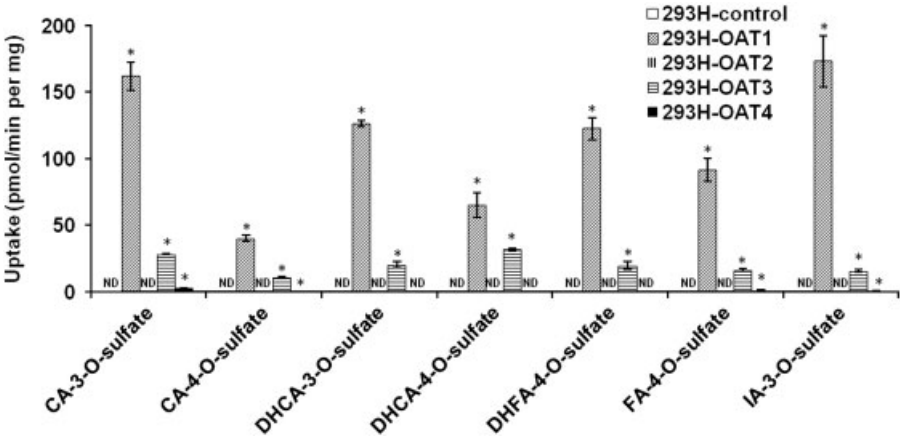


Figure 3. Uptake of hydroxycinnamic acid sulfates by 293H-control, 293H-OAT1, 293H-OAT2, 293H-OAT3, and 293H-OAT4 cells. Cells were incubated with 25 μM substrate for 30 min ($n = 3$). CA, caffeic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; FA, ferulic acid; IA, isoferulic acid. $^{*}p < 0.05$ compared with control. ND, not detected.

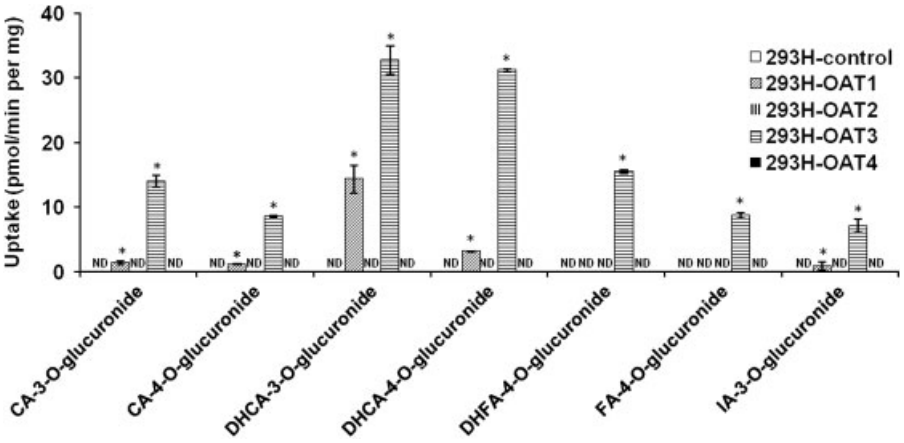


Figure 4. Uptake of hydroxycinnamic acid glucuronides by 293H-control, 293H-OAT1, 293H-OAT2, 293H-OAT3, and 293H-OAT4 cells. Cells were incubated with 25 μM substrate for 30 min ($n = 3$). CA, caffeic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid, FA, ferulic acid; IA, isoferulic acid. $^{*}p < 0.05$ compared with control. ND, not detected.

1 mM (data not shown). The inhibitory effect at maximum physiological concentration (10 μM) is shown in Fig. 5A. All the hydroxycinnamic acid aglycones demonstrated significant inhibition of *p*-aminohippuric acid transport by OAT1, with caffeic acid and ferulic acid being the strongest inhibitors (>50% inhibition). All hydroxycinnamic acid sulfates, except caffeic acid-4-*O*-sulfate, inhibited OAT1 activity. However, the inhibitory activity was moderate (10–25%). None of the hydroxycinnamic acid glucuronides tested was able to inhibit OAT-mediated uptake of *p*-aminohippuric acid at 10 μM. The inhibitory activity of caffeic acid and ferulic acid was further evaluated over a range of concentrations (0.1–1000 μM) (Fig. 5B). IC_{50} values of inhibition for caffeic acid and ferulic acid were 5.22 and 9.01 μM, respectively.

3.5 Inhibition of OAT3- and OAT4-mediated uptake of 5-carboxyfluorescein by hydroxycinnamic acids and conjugates

The ability of hydroxycinnamic acids and their conjugates to inhibit OAT3 or OAT4-mediated uptake of 5-carboxyfluorescein, a fluorescent tracer for activity of OATs [29], was also tested. 5-Carboxyfluorescein was highly transported by both OAT3 and OAT4, and its uptake was sensitive to inhibition by 1 mM probenecid (data not shown). At 10 μM, hydroxycinnamic acid and their conjugates did not appreciably affect OAT3-mediated uptake of 5-carboxyfluorescein (Fig. 6A). Caffeic acid and dihydrocaffeic acid inhibited the activity of OAT3. As shown in Fig. 6B, caffeic acid and dihydrocaffeic acid inhibited OAT3 in a concentration-dependent manner, with IC_{50} values of 30.8 and 14.2 μM, respectively. On the contrary, caffeic acid-3-*O*-sulfate, caffeic acid-4-*O*-sulfate, and dihydrocaffeic acid-3-*O*-sulfate were found to weakly stimulate OAT3-mediated uptake (~30%). On the other hand, hydroxycinnamic acids and conjugates did not demonstrate any effect on OAT4-mediated uptake of 5-carboxyfluorescein. These data suggest that hydroxycinnamic acids and their conjugates have a limited impact on OAT3 and OAT4 activity at physiologically relevant concentrations.

3.6 *Trans*-simulation of OAT4 transport activity by hydroxycinnamic acid conjugates

To examine the potential of OAT4 to act as an efflux transporter for hydroxycinnamic acid conjugates, *trans*-stimulation

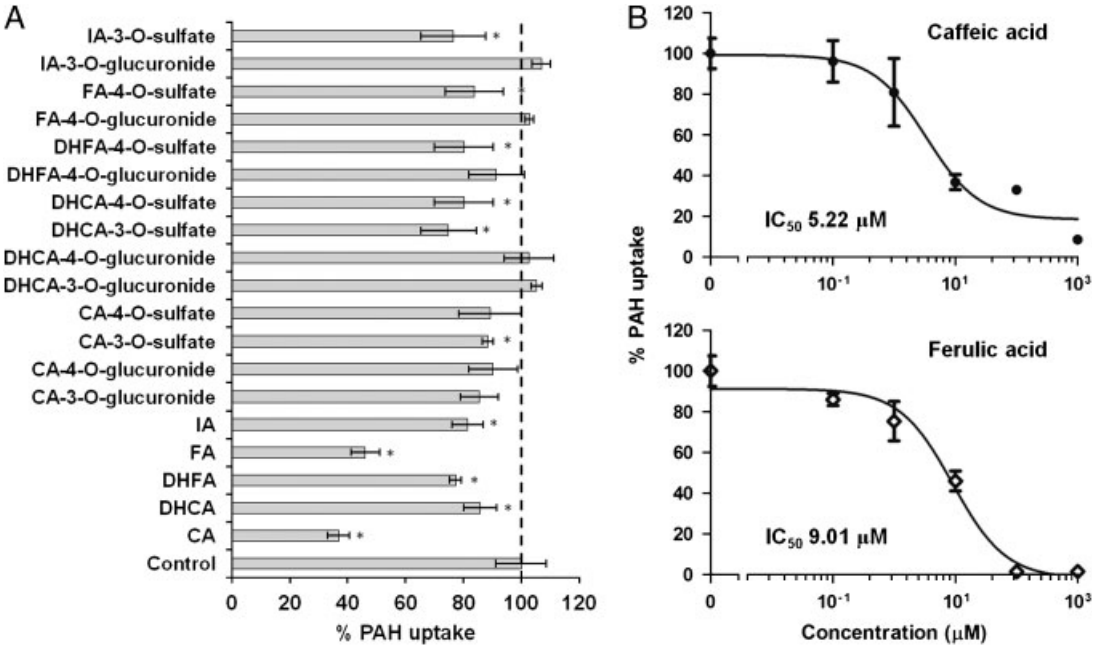


Figure 5. (A) Inhibition of OAT1-mediated uptake of *p*-aminohippuric acid (PAH) by hydroxycinnamic acids and their conjugates. OAT1-expressing cells were incubated for 10 min with 25 μM PAH in the presence of 10 μM of hydroxycinnamic acids and their conjugates. (B) Concentration-dependent inhibition of OAT1-mediated uptake of *p*-aminohippuric acid (PAH) by caffeic acid and ferulic acid. OAT1-expressing cells were incubated for 10 min with 25 μM PAH in the presence of 0.1–1000 μM caffeic acid and ferulic acid ($n = 3$). CA, caffeic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; FA, ferulic acid; IA, isoferulic acid. $^*p < 0.05$ compared with control.

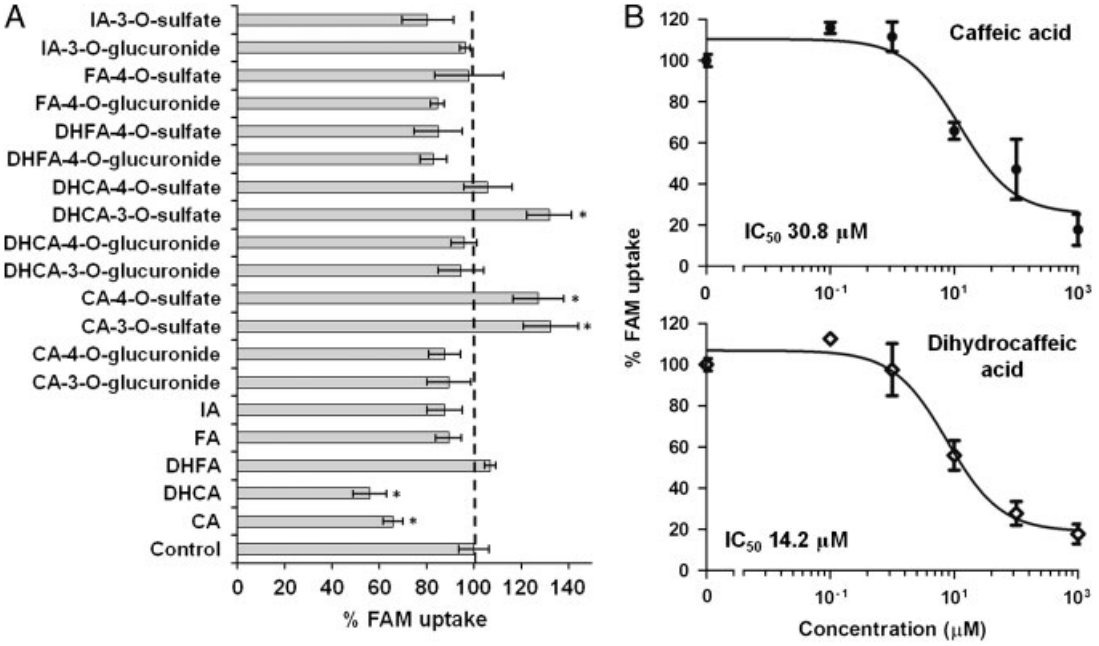


Figure 6. (A) Inhibition of OAT3-mediated uptake of 5-carboxyfluorescein (FAM) by hydroxycinnamic acids and their conjugates. OAT3-expressing cells were incubated for 10 min with 100 μM FAM in the presence of 10 μM of hydroxycinnamic acids and their conjugates (B) Concentration-dependent inhibition of OAT3-mediated uptake of 5-carboxyfluorescein (FAM) by caffeic acid and dihydrocaffeic acid. OAT3-expressing cells were incubated for 10 min with 100 μM FAM in the presence of 0.1–1000 μM of caffeic acid and dihydrocaffeic acid ($n = 3$). CA, caffeic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; FA, ferulic acid; IA, isoferulic acid. $^*p < 0.05$ compared with control.

experiments were performed by preloading the cells for 4 h with 1 mM test substrates. Then, the uptake of 5-carboxyfluorescein was measured. Figure 7 shows that preloading with caffeic acid-3-*O*-sulfate and isoferulic acid-3-*O*-sulfate resulted in twofold increase in the uptake of 5-carboxyfluorescein; with dihydrocaffeic acid-3-*O*-sulfate and ferulic acid-4-*O*-sulfate, the uptake was increased by 50% ($p<0.05$). Dihydrocaffeic acid-4-*O*-sulfate and dihydroferulic acid-4-*O*-sulfate also slightly increased uptake, but the effect was not statistically significant. Preloading with hydroxycinnamic acid glucuronides had no apparent effect on 5-carboxyfluorescein uptake. Thus, hydroxycinnamic acid sulfates, but not glucuronides, *trans*-stimulate OAT4-mediated 5-carboxyfluorescein uptake.

3.7 Interaction of hydroxycinnamic acid conjugates with selected ABC transporters

We examined the interaction of the hydroxycinnamic acid conjugates with ABC transporters expressed in kidney [30], using an in vitro ATPase assay [26]. Substrates activate the ATPase activity, whereas inhibitors may be identified by inhibition of vesicular transport [31]. At physiological concentrations ($\leq 10\,\mu M$), hydroxycinnamic acid conjugates did not have an effect on MRP2 and BCRP activity (data not shown). At supra-physiological concentration (300 μM), hydroxycinnamic acid conjugates only interact weakly with MRP2 and BCRP (Supporting Information Table 1).

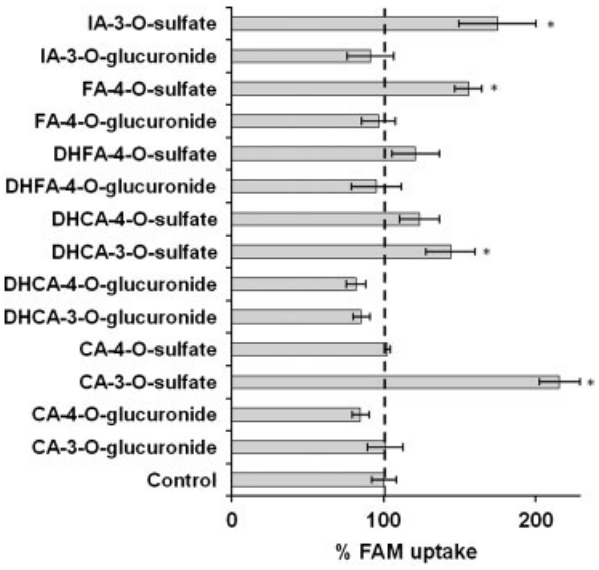


Figure 7. *Trans*-stimulation of the OAT4-mediated uptake of 5-carboxyfluorescein (FAM) by hydroxycinnamic acid conjugates. OAT4-expressing cells were incubated for 4 h with 1 mM hydroxycinnamic acid conjugates. The uptake of 100 μM FAM was then measured over 10 min ($n=3$). CA, caffeic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; FA, ferulic acid; IA, isoferulic acid. *$p<0.05$ compared with control.

4 Discussion

In the present study, we studied the transport of five hydroxycinnamic acids and 14 of their sulfated and glucuronidated metabolites by organic anion transporters (OAT1-4) and ABC transporters (MRP2 and BCRP) (Fig. 1). Basolateral uptake is a rate limiting step in the tubular secretion of drugs and xenobiotics [32]. OAT1 and OAT3 may be involved in the uptake of hydroxycinnamic acids and their conjugates into the proximal tubules. Passive diffusion is not expected to play a major role in the transport of conjugates; there was no detectable uptake of conjugates in 293H control cells even at the highest substrate concentration used (100 μM). Hydroxycinnamic acid sulfates were efficiently transported by OAT1, and to a lesser extent, by OAT3 (Fig. 3). Transport by OAT1 was regioselective, with a preference for 3-*O*-sulfates compared with 4-*O*-sulfates. The estimated K_m values of the selected hydroxycinnamic acid sulfates (28.6–115 μM) were higher than concentrations normally found in human plasma ($\leq 2\,\mu M$). However, OAT1-mediated uptake of sulfates was highly significant at low concentrations (Supporting Information Fig. 2). Hydroxycinnamic acid glucuronides are good substrates of OAT3, but most of them are poor substrates of OAT1 (Fig. 4). Similar to our observations, OAT1 was reported to transport simple, sulfated metabolites (indoxyl sulfate and edaravone sulfate) [33–35], whereas transport of the more bulky sulfate and glucuronide conjugates (estrone-3-sulfate and estradiol-17β-glucuronide) is carried out by OAT3 [15]. In contrast, nonconjugated hydroxycinnamic acids are poor substrates of OATs. Although the uptake of caffeic acid and dihydrocaffeic acid was significantly enhanced by OAT1, extent of uptake was much lower compared with the sulfates (Figs. 2 and 3). Moreover, no apparent transport was observed at concentrations $\leq 5\,\mu M$. At physiologically achievable concentrations, OAT1-mediated uptake of caffeic acid and dihydrocaffeic acid may thus be inactive. Our data are consistent with the minimal urinary excretion of hydroxycinnamic acids in humans, whereas sulfates are the major forms excreted, most likely via tubular secretion [5, 6]. Overall, the hydroxycinnamic acid sulfates, the predominant metabolites formed by phase II metabolism in humans, are best transported by the basolateral OATs. Conjugation of hydroxycinnamic acids by sulfotransferases, SULT1A1, SULT1A3, and SULT1E1 [5], in the liver and the intestine is therefore part of the mechanism for the elimination of these compounds upstream of OAT1 efflux.

Apical efflux of sulfate and glucuronide conjugates in kidney is believed to involve members of the ABC transporter family [36]. However, none of the tested hydroxycinnamic acid conjugates evaluated showed stimulation of ATPase activity of these transporters. Several sulfate and glucuronide conjugates inhibited the vesicular transport of MRP2 and BCRP at high concentrations ($\geq 10\,\mu M$), indicating that they may be weak inhibitors of apically expressed MRP2 or BCRP. Emerging evidence has suggested that the apically expressed OAT4 is an asymmetric transporter that

facilitates the efflux of some organic anions into the lumen [23]. *p*-Aminohippuric acid and glutarate were very poorly taken up by OAT4-expressing cells and did not inhibit OAT4-mediated uptake of estrone-3-sulfate; however, they *trans*-stimulated OAT4-mediated uptake of model substrates carboxyfluorescein and estrone-3-sulfate [23, 37]. Here, we observed that hydroxycinnamic acid sulfates, including caffeic acid-3-*O*-sulfate, dihydrocaffeic acid-3-*O*-sulfate, ferulic acid-4-*O*-sulfate, and isoferulic acid-3-*O*-sulfate, were also able to significantly *trans*-stimulate the uptake of 5-carboxyfluorescein. This indicates that OAT4 may play a role in mediating the efflux of hydroxycinnamic acid sulfates from the proximal tubular cells into the urine. Glucuronide conjugates were not found to inhibit or *trans*-stimulate OAT4-mediated uptake. For glucuronide conjugates, further investigation is needed to establish the molecular basis for their efflux in the kidneys. MRP4 or URAT1 (SLC22A12) that are expressed on brush-border membrane may be possible candidates for the export of these conjugates in humans [38].

Due to the highly hydrophilic nature of hydroxycinnamic acid conjugates, their distribution in the body is highly dependent on the presence of appropriate uptake transporters. OAT1 and OAT3, the most active transporters, are predominantly expressed in kidney [18]. In animal models, dietary supplementation of hydroxycinnamic acids resulted in enhanced renal accumulation, where they exerted antioxidant and anti-inflammatory effects [39, 40]. Hence, OAT-mediated uptake may play a role in the targeting of hydroxycinnamic acids to renal tissues, and hydroxycinnamic acids may confer beneficial effects intracellularly during the transit through proximal tubule cells. The present study also raised the question of whether hydroxycinnamic acid–drug interactions could occur via altering OAT-mediated transport, given the high dietary load in some populations (1 g/day). Although some of the hydroxycinnamic acids were found to inhibit OAT1 and OAT3, the IC_{50} values (~5–30 μM) were above physiological concentrations. Hydroxycinnamic acid glucuronides and sulfates, on the other hand, interact very weakly with OAT1 and OAT3 even at supraphysiological concentration (10 μM). Some conjugates also weakly inhibited MRP2 and BCRP-mediated efflux, albeit at nonphysiological concentrations. These data indicated that normal levels of dietary intake of hydroxycinnamic acids are not likely to result in significant interaction with drugs through the inhibition of OATs, MRP2, or BCRP.

In summary, the present study provides evidence that OAT1 and OAT3 may be involved in renal uptake of hydroxycinnamic acid conjugates, and OAT4 may mediate apical efflux. Interplay between the uptake and the efflux transporters may lead to efficient tubular secretion and limit the systemic availability of hydroxycinnamic acids in humans. Furthermore, the dietary intake of hydroxycinnamic acids is unlikely to result in significant influence on OATs or MRPs-mediated transport of drugs.

The authors thank the Nestlé Research Center, Lausanne, Switzerland for funding.

The authors have declared no conflict of interest.

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