JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 1499–1505

Received October 28, 2005
Accepted August 7, 2006
DOI 10.1211/jpp.58.11.0011
ISSN 0022-3573

Inhibitory effects of angiotensin II receptor antagonists and leukotriene receptor antagonists on the transport of human organic anion transporter 4

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Abstract

Human organic anion transporter 4 (OAT4) is the only member of the OAT family that is expressed in the placenta and also expressed in kidney. Although OAT4 has been shown to transport certain organic anions as well as other members of the OAT family, fewer numbers of substrates have been identified for OAT4 compared with OAT1 and OAT3, suggesting that the substrate specificity of OAT4 is greater than other OAT members. However, the substrate specificity of OAT4 remains to be investigated in detail. The aim of this study was to examine the effects of various drugs on the OAT4-mediated transport of estrone-3-sulfate, a typical substrate of OAT4, by using human embryonic kidney cells stably transfected with OAT4 (HEK-OAT4). HEK-OAT4 cells exhibited concentration-dependent uptake of estrone-3-sulfate, with a K_m value of 20.9 ± 3.53 μM. Dehydroepiandrosterone sulfate and probenecid potently inhibited estrone-3-sulfate uptake. We also searched for the potential inhibitors of OAT4 and identified candesartan, candesartan cilexetil, losartan, losartan carboxyl (EXP3174) and valsartan as inhibitors of OAT4, with K_i values of 88.9, 135.2, 24.8, 13.8 and 19.6 μM, respectively. The above angiotensin II receptor antagonists and leukotriene receptor antagonists share a common structural feature, that is the tetrazole group. Although pranlukast is devoid of anionic motifs other than the tetrazole group, it potently inhibited the OAT4-mediated uptake of estrone-3-sulfate, indicating that a tetrazole group may be one important structural feature in substrate recognition by OAT4.

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Funding: This study was supported in part by a Grant-in-Aid from the Ministry of Education, Sports, Science and Technology.

Introduction

Human organic anion transporter 4 (OAT4) was cloned from human kidney and shown to be predominantly expressed in the kidney and placenta (Cha et al 2000). It is the sole member of the OAT family expressed on the apical membrane of the proximal tubules in the kidney and considered to be involved in the absorption and elimination of endogenous metabolites and xenobiotics, including drugs and environmental toxins, from the intestine and into the urine, respectively (Babu et al 2002a). In the placenta, OAT4 is expressed on the basal membranes of trophoblast cells, and is considered to be involved in the transfer of various compounds across the blood–placental barrier.

Functional characterization studies have revealed that OAT4 transports various compounds with diverse structures. OAT4 expressed in *Xenopus laevis* oocytes transports estrone-3-sulfate and dehydroepiandrosterone sulfate (DHEAS), a major circulating steroid secreted from the adrenal cortex (Cha et al 2000). In addition, uptake studies using cultured cells have shown that OAT4 transports anti-inflammatory drugs, anti-tumour drugs, antiviral drugs, prostaglandins, environmental toxins, antibiotics and diuretics (Babu et al 2002a, b; Khamdang et al 2002; Kimura et al 2002; Takeda et al 2002a, b; Hasannejad et al 2004).

Although OAT4 transports various compounds as well as other members of the OAT family, its substrate specificity is different from that of other members. A study on the transport of salicylate, ibuprofen, indometacin and ketoprofen in stable expression

systems indicated that OAT1 and OAT3 transport all of them, while OAT2 transports only salicylate, and OAT4 transports salicylate and ketoprofen (Khamdang et al 2002). In the same system, OAT1 and OAT3 transported both bumetanide and furosemide, while OAT4 transported only bumetanide, and OAT2 did not transport either (Hasannejad et al 2004). These results suggest that the substrate selectivity of OAT4 is relatively greater than other isoforms. However, the substrate recognition of OAT4 has not been well characterized.

Most substrates and inhibitors of OAT4 have anionic motifs. For example, the uptake of estrone-3-sulfate into *Xenopus* oocytes expressing OAT4 was inhibited by 5 μM β-estradiol sulfate but not inhibited by 5 μM β-estradiol or β-estradiol-3-D-glucuronide (Cha et al 2000). They have also shown that the OAT4-mediated uptake was decreased by other anions that are structurally unrelated to β-estradiol, ibuprofen and bumetanide (Cha et al 2000). These results strongly imply that substrate recognition by OAT4 is dependent on the small anionic motif and not the hydrophobic part such as a steroidal structure or bulky anion such as glucuronide. However, some compounds without anionic motifs, such as piroxicam, has been shown to inhibit the OAT4-mediated uptake (Babu et al 2002a; Takeda et al 2002b).

In the present study, we aimed to investigate the inhibitory profiles of various known OAT inhibitors and other drugs in order to characterize the substrate recognition pattern of OAT4, which is the only OAT family member expressed at the apical membrane of proximal tubular cells and at the basal membrane of trophoblast cells.

Materials and Methods

Materials

[^{3}H]Estrone-3-sulfate ammonium salt (43.1 Ci mmol^{-1}) was purchased from NEN Life Science Products, Inc. (Boston, MA, USA). [^{3}H]Valproate (55 Ci mmol^{-1}) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). Cholate, cimetidine, ciclosporin A, DHEAS, enoxacin, erythromycin, estrone-3-sulfate, geneticin, glutathione, α-ketoglutarate, ketoprofen, phenobarbital, probenecid, progesterone, valproate, taurochenodeoxycholate and mitoxantrone were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). *d*-Chlorpheniramine, ibuprofen, *p*-aminohippurate (PAH), salicylate, streptomycin, taurocholate, tetracycline, theophylline and verapamil were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Benzylpenicillin, carbamazepine and valinomycin were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Carvedilol and levofloxacin were kind gifts from Daiichi Pharmaceutical Co., Ltd (Tokyo, Japan). Candesartan, candesartan cilexetil and manidipine were kind gifts from Takeda Chemical Industries, Ltd (Osaka, Japan). Ramosetron was a kind gift from Astellas Pharma, Inc. (Tokyo, Japan). Valsartan was a kind gift from Novartis Pharma KK (Tokyo, Japan). Losartan and losartan carboxyl (EXP3174) were kind gifts from Banyu Pharmaceutical Co., Ltd (Tokyo, Japan). Clarithromycin was a kind gift from Taisho Pharmaceutical Co., Ltd (Tokyo, Japan). Montelukast was a kind gift from Kyorin Pharmaceutical Co., Ltd (Tokyo, Japan). Pranlukast was a kind gift from Ono Pharmaceutical Co., Ltd (Osaka, Japan). Zafirlukast was a kind gift from AstraZeneca PLC (London, UK). All other chemicals and reagents used were of analytical grade and were obtained from commercial sources.

Establishment and culture of HEK-OAT4

Full-length OAT4 coding sequences corresponding to published sequences (GenBank accession number AB026116) were amplified by polymerase chain reaction from human kidney cDNA (BD Biosciences Clontech, CA, USA). The OAT4 cDNA fragments, amplified using the forward primer 5′-ATGAGAATGCGGCCGCGCAGTTAGGTCAGCAGTC-3′ and the reverse primer 5′-CCGGAATTCGAGGCAACTC TTTCCAG-3′, were subcloned into pIRESneo vector (BD Biosciences Clontech). A clone (pIRES-OAT4) containing the complete open reading frame of OAT4 was isolated.

pIRES-OAT4 and pIRESneo vector were transfected into human embryonic kidney cells (HEK293) using LipofectAMINE 2000 Reagent (Invitrogen Corporation, Carlsbad, CA, USA). These cells were grown in a humidified incubator under 5% CO_2 at 37°C, using Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) containing 10% FBS, 70 μg mL^{-1} benzylpenicillin, 100 μg mL^{-1} streptomycin and 500 μg mL^{-1} geneticin (Sigma-Aldrich Co.) for 2 weeks and then single colonies were isolated. Clones transfected with pIRES-OAT4 and with pIRESneo were established and designated as HEK-OAT4 and HEK-Mock, respectively.

Western blot analysis

HEK-OAT4 and HEK-Mock cells were suspended in lysis buffer (100 mL of Tris HCl (pH 7.6), 150 mM NaCl, 1mM $CaCl_2$, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), 0.1% Np-40, 1 mM phenylmethyl sulfonyl fluoride, 0.01 mg mL leupeptin, 0.01 mg mL^{-1} aprotinin and 1 mM sodium vanadate), and the supernatant was used for Western blot analysis. The concentration of membrane protein was determined by the Lowry method with bovine serum albumin as a standard (Lowry et al 1951). The membrane was blocked overnight with phosphate-buffered saline (PBS) containing 5% non-fat powdered milk at 4°C, washed with PBS, and then incubated for 1 h at room temperature in PBS containing 1% non-fat milk and 1000-fold diluted anti-OAT4 rabbit serum (Transgenic Inc., Kumamoto, Japan). Horseradish peroxidase-conjugated rabbit anti-mouse IgG (ICN Biochemicals Inc., OH, USA) was used as the secondary antibody. Detection was carried out with ECL reagents according to the manufacturer's instructions.

Uptake experiments

HEK-OAT4 or HEK-Mock cells were seeded in 96-well tissue culture plates at a density of 8×10^4 cells/well and cultivated for 2 days. For uptake studies, cells were washed three times and pre-incubated for 10 min with 0.8 mL of uptake buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.4). Uptake

was initiated by replacing the uptake buffer with buffer supplemented with [^{3}H]estrone-3-sulfate at 37°C. At the end of the experiments, the cells were washed three times with 0.5 mL of ice-cold buffer and solubilized in 200 μL of 1 M NaOH, followed by neutralization with 100 μL of 2 M HCl. Aliquots of 200 μL were transferred to scintillation vials, and the radioactivity associated with the cells and that in the medium were determined with a liquid scintillation counter (LS6500, Becton Instrument, Inc., CA, USA). The remaining 50-μL aliquots of the cell lysate were used for protein assay by the Lowry method with bovine serum albumin as a standard. HEK-Mock cells were used to obtain the background activity. The uptake of [^{3}H]estrone-3-sulfate into the cells is presented as the cell-to-medium ratio, determined as the amount of [^{3}H]estrone-3-sulfate associated with the cells divided by the concentration of [^{3}H]estrone-3-sulfate in the uptake buffer.

Inhibition study

HEK-OAT4 and HEK-Mock cells were incubated in the uptake buffer containing 1 μM estrone-3-sulfate in the absence and presence of various compounds at 37°C for 20 s, according to the method described above. Candesartan, candesartan cilexetil, carbamazepine, clarithromycin, ciclosporin A, digoxin, ibuprofen, levofloxacin, losartan, losartan carboxyl (EXP3174), manidipine, montelukast, phenobarbital, pranlukast, progesterone, valinomycin, valsartan, verapamil and zafirlukast were each dissolved in dimethyl sulfoxide, and diluted with the uptake buffer. The final concentration of dimethyl sulfoxide in the uptake buffer was adjusted to less than 0.5%.

Kinetic analysis

OAT4-mediated uptake of estrone-3-sulfate was determined as the difference between the uptake by HEK-OAT4 and the uptake by HEK-Mock cells. The kinetic parameters were estimated from the following equation:

$$CL = V_{max}/(K_m + C)$$

where CL is the uptake clearance (μL (mg protein)$^{-1}$/20 s), V_{max} is the maximum uptake rate (pmol (mg protein)$^{-1}$/20 s), K_m is the Michaelis constant (μM), and C is the concentration of estrone-3-sulfate in the uptake buffer (μM).

Inhibitory constants (K_i values) of a series of compounds were obtained by examining the concentration dependency of their inhibition on OAT4-mediated uptake of estrone-3-sulfate. Equation 2 was fitted to the observed CL′/CL values by use of the non-linear least-squares regression analysis program MULTI (Yamaoka et al 1981) to obtain the kinetic parameters:

$$CL'/CL = 1/(1 + I^n/K_i^n)$$

where CL′ is uptake clearance of estrone-3-sulfate in the presence of inhibitor (μL (mg protein)$^{-1}$/20 s), CL is that in the absence of inhibitor (μL (mg protein)$^{-1}$/20 s), I is concentration of inhibitor (μM), and n is the Hill coefficient. The calculated kinetic parameters were expressed as the estimate ± s.d.

Statistical analysis

All the data are expressed as the mean ± s.e.m. The significance of differences between the means was determined by using the Student's *t*-test or one-way analysis of variance followed by Dunnett's post-hoc test. A *P* value of less than 0.05 was considered statistically significant.

Results and Discussion

Transport characteristics of OAT4

The expression of OAT4 in HEK-OAT4 and HEK-Mock cells was determined by Western blot analysis (Figure 1 inset). An immunoreactive band with a molecular mass of approximately 70 kDa was observed in HEK-OAT4 cells but not in HEK-Mock cells. The uptake of estrone-3-sulfate, a typical endogenous substrate of OAT4, in HEK-OAT4 cells was investigated. The uptake of estrone-3-sulfate in HEK-OAT4 cells at 37°C was significantly greater than that in HEK-Mock cells (data not shown). Since the uptake increased linearly for 30 s, the uptake at 20 s was used as the

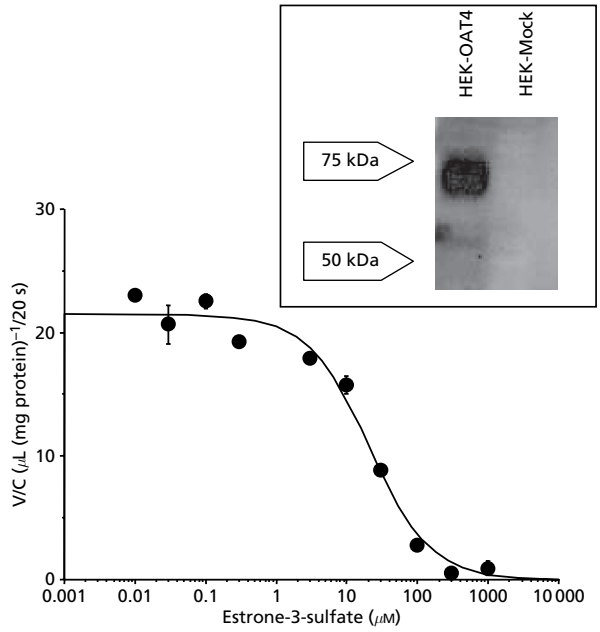


Figure 1 Concentration dependence of OAT4-mediated uptake of estrone-3-sulfate. The uptake rate of estrone-3-sulfate by HEK-OAT4 or HEK-Mock cells for 20 s was measured at various concentrations. OAT4-mediated transport was determined by subtracting the uptake velocity in HEK-Mock cells from that in HEK-OAT4 cells. Each point represents observed data and the fitted line is shown. The K_m and V_{max} values were 20.9 μM and 449.0 pmol (mg protein)$^{-1}$/20 s, respectively. The uptake of 0.01 μM estrone-3-sulfate by HEK-OAT4 and HEK-Mock was 27.3 ± 1.8 and 5.9 ± 1.8 μL (mg protein)$^{-1}$/20 s, respectively. Each point represents the mean ± s.e.m., n = 4. Inset: Western blot analysis of OAT4. Crude membrane proteins of HEK-OAT4 100 μg (lane 1) and HEK-Mock 100 μg (lane 2) were resolved by 10% polyacrylamide gel.

initial uptake. The initial uptake was saturable, with K_m and V_{max} values of 20.9 ± 3.5 μM and 449.0 ± 71.1 pmol (mg protein)$^{-1}$/20 s, respectively (Figure 1). The K_m value is relatively higher than that in previous reports; the K_m values obtained by using *Xenopus laevis* oocyte and mouse proximal tubule cells stably expressing OAT4 were 1 μM and 9.9 μM, respectively (Cha et al 2000; Hasannejad et al 2004).

Inhibitory effects of typical inhibitors on the uptake of estrone-3-sulfate in HEK-OAT4 cells

The inhibitory effects of typical OAT family inhibitors on the uptake of estrone-3-sulfate in HEK-OAT4 cells were investigated (Figure 2). Estrone-3-sulfate itself, DHEAS and probenecid potently inhibited the uptake of estrone-3-sulfate in a concentration-dependent manner. On the other hand, benzylpenicillin, α-ketoglutarate, PAH, valproate, tetracycline and taurocholate showed weaker inhibition, although they are statistically significant because of their small standard deviations. Their inhibitory potency was weak. Cimetidine did not inhibit the uptake of estrone-3-sulfate. These results are consistent with previous reports (Cha et al 2000; Babu et al 2002a, b). The inhibitory effects of some drugs such as PAH and α-ketoglutarate seem to be dose-independent (Figure 2). However, their inhibitory effects were relatively weak and so much higher concentrations may be needed to determine for sure if their effects are dose-dependent or not. It has been reported that valproate inhibits the uptake of PAH by rat OAT1 (Sekine et al 1997). The present study demonstrated that valproate inhibits the uptake of estrone-3-suflate in HEK-OAT4 cells (Figure 2). We also investigated the uptake of 7.4 nM [^{3}H]valproate in HEK-OAT4 and HEK-Mock cells. However, the OAT4-mediated uptake of valproate was not observed, that is the uptake of [^{3}H]valproate into HEK-OAT4 and HEK-Mock cells was 1.6 ± 0.1 and 4.0 ± 1.4 μL (mg protein)$^{-1}$/5 min, respectively (n = 3–4, not statistically significant).

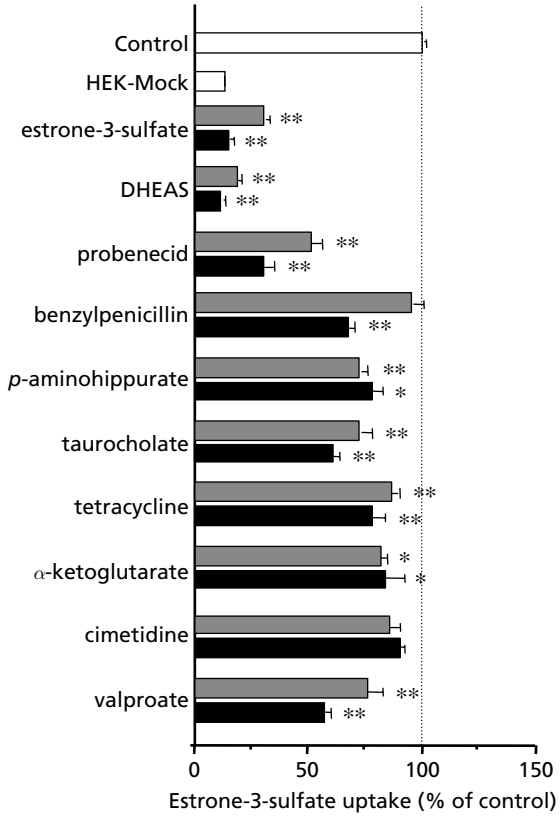


Figure 2 Effects of typical inhibitors on the uptake of estrone-3-sulfate into HEK-OAT4 cells. The uptake rates of estrone-3-sulfate (1 μM) by HEK-OAT4 and HEK-Mock cells were determined in the absence (open column) or presence of 100 μM (grey column) or 500 μM (closed column) inhibitor at 37°C for 20 s. The values were expressed as the percentage of [^{3}H]estrone-3-sulfate uptake by HEK-OAT4 cells in the absence of inhibitor. The mean values of the uptake of 1 μM estrone-3-sulfate by HEK-OAT4 and HEK-Mock cells were 20.0 ± 0.4 and 1.3 ± 1.8 μL (mg protein)$^{-1}$/20 s, respectively. Each column represents the mean ± s.e.m., n = 4. *$P < 0.05$ and **$P < 0.01$, significantly different compared with the control (analysis of variance followed by Dunnet's test).

Inhibitory effects of various drugs on the uptake of estrone-3-sulfate in HEK-OAT4 cells

To investigate the substrate selectivity of OAT4, the effects of various compounds on the uptake of estrone-3-sulfate by HEK-OAT4 cells were investigated (Figure 3). The uptake of estrone-3-sulfate into HEK-OAT4 cells was potently inhibited by candesartan, candesartan cilexetil, cholate, glutathione, ketoprofen, losartan, losartan carboxyl (EXP3174), valsartan, montelukast, pranlukast and zafirlukast. Glutathione also inhibited the function of OAT4 (Figure 3). It may be possible that reduced GSH may have exerted a remodelling and a reduction of OAT4 protein. The mechanism of the inhibitory effects of glutathione on OAT4 remains to be elucidated. We further investigated the inhibitory properties of the angiotensin II receptor antagonists, candesartan, candesartan cilexetil, losartan, losartan carboxyl and valsartan on the function of OAT4. The K_i values of the inhibitory effects of these angiotensin II receptor antagonists were 88.9 ± 17.6, 135.2 ± 49.3, 24.8 ± 6.2, 13.8 ± 3.3 and 19.6 ± 2.4 μM, respectively (Table 1). On the other hand, therapeutic unbound plasma concentrations of candesartan, losartan and valsartan in humans are 1.3, 9.4 and 338 nM, respectively (Nakashima et al 1995; Suzuki & Yano 1996; Cyong & Uebaba 1998), and far lower than the above K_i values. Therefore, the inhibition of OAT4 by these drugs is not feasible in-vivo in humans under therapeutic conditions. We also investigated the inhibitory properties of the leukotriene receptor antagonists. With regard to three leukotriene receptor antagonists, the uptake study to determine the K_i values were experimentally not feasible owing to their low solubility.

Although MRP1, a member of the ABC transporters, has also been demonstrated to transport estrone-3-sulfate in an ATP-dependent manner, the function of OAT4 was not inhibited by ciclosporin A, an inhibitor of MRP2 and P-gp, progesterone, an inhibitor of P-gp, or mitoxantrone, an inhibitor of breast cancer resistance protein (BCRP) (Figure 3). Therefore, the substrate specificity of OAT4 is considered to be

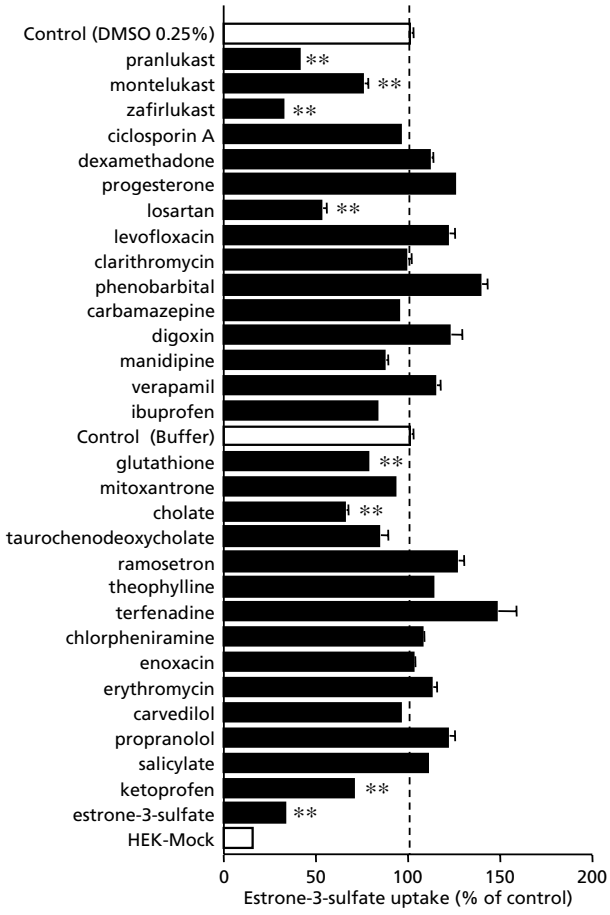


Figure 3 Effects of various drugs on the uptake of estrone-3-sulfate by HEK-OAT4 cells. The uptake rates of estrone-3-sulfate (1 μM) by HEK-OAT4 and HEK-Mock cells were determined in the absence (open column) or presence of 100 μM (closed columns) inhibitors, except for ciclosporin A (40 μM), gluthathione (20 mM) and progesterone (500 μM) at 37°C for 20 s. The values were expressed as the percentage of [^{3}H]estrone-3-sulfate uptake by HEK-OAT4 cells in the absence of the inhibitor. The mean values of the uptake of estrone-3-sulfate by HEK-OAT4 and HEK-Mock cells were 20.6 ± 1.6 and 1.8 ± 0.2 μL (mg protein)$^{-1}$/20 s, respectively. Each value represents the mean ± s.e.m., n = 4. *$P < 0.05$ and **$P < 0.01$, significantly different compared with the control (analysis of variance followed by Dunnet's test).

distinct from that of BCRP or MRP1, although there may be overlap in substrate recognition to some extent between OAT4 and BCRP or MRP1.

Khamdang et al (2002) reported that OAT4 transported salicylate and ketoprofen. This results seems to be inconsistent with the fact that 100 μM salicylate did not inhibit OAT4 in the present study (Figure 3). However, Cha et al (2000) demonstrated that the uptake of estrone-3-sulfate in the oocyte expressing OAT4 was not inhibited by salicylate at less than 50 μM. These inconsistencies may be owing to the difference in the concentration of salicylate used in the study.

Essential structure for substrate recognition by OAT4

Although cholate, taurocholate and taurochenodeoxycholate share common structural features, only taurochenodeoxycholate, which has a large anionic motif, did not inhibit OAT4. While it seems difficult to find common structural features other than the anionic motif, the native form of the cyclopentanoperhydrophenanthrene nucleus may be one of the preferred motifs for OAT4 inhibition. This result is consistent with the finding of Cha et al (2000) that the small anionic motif rather than the hydrophobic part is important for substrate recognition. Indeed, other OAT4 inhibitors investigated in this study (i.e. valproate, α-ketoglutarate, *p*-aminohippuric acid, benzylpenicillin and ketoprofen) are structurally quite unrelated and share only the carboxyl moiety.

The angiotensin II receptor antagonists and leukotriene receptor antagonists used in the present study contain a tetrazole group in their structures, except for montelukast and zafirlukast (Figure 4). However, another common structure was not found among the inhibitors. Cefamandole and cefoperazone, and cephem antibiotics also contain a tetrazole group and have been shown to inhibit the uptake of estrone-3-sulfate by S2-OAT4 (Takeda et al 2002b). Although some OAT4 inhibitors such as cefamandole, cefoperazone, candesartan and losartan carboxyl contain both a tetrazole group and a carboxyl group, other OAT4 inhibitors such as cancesartan, losartan and pranlukast contain a tetrazole group but not carboxyl groups. Taken together, these results suggest that OAT4 may recognize not only the carboxylate group but also

Table 1 Inhibitory constants of various drugs on the initial human organic anion transporter 4 (OAT4)-mediated uptake of estrone-3-sulfate

Inhibitor	K_i (μM)	n	Plasma concentration (μM)[a]	Unbound fraction (%)	Unbound plasma concentration (μM)[b]
Candesartan	88.9 ± 17.6	0.57 ± 0.06	0.13	1	0.0013
Candesartan cilexetil	135.2 ± 49.3	0.52 ± 0.12	–	–	–
Losartan	24.8 ± 6.2	0.98 ± 0.13	0.47	2	0.0094
Losartan carboxyl	13.8 ± 3.3	1.45 ± 0.19	1.05	–	–
Valsartan	19.6 ± 2.4	0.71 ± 0.04	6.5	5.2	0.338

HEK-OAT4 cells were incubated with uptake buffer containing 1 μM estrone-3-sulfate for 20 s at 37°C in the absence or presence of various concentrations of candesartan, candesartan cilexetil, losartan, losartan carboxyl and valsartan. The uptake in mock cells was subtracted from that in HEK-OAT4 cells. Each value represents the estimate ± s. d., n = 4. [a]The plasma concentrations were taken from the product information of the listed drugs. [b]The unbound plasma concentrations were calculated from total plasma concentrations and unbound fractions of drugs.

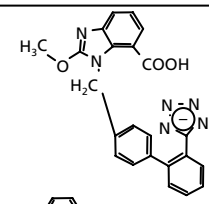
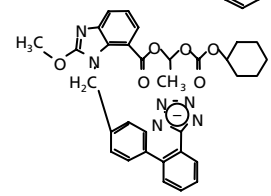
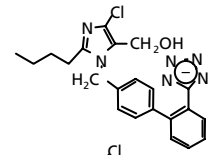
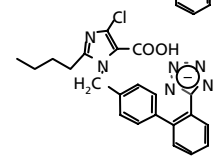
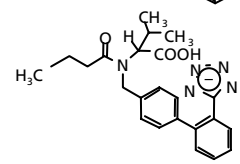
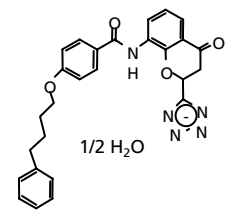
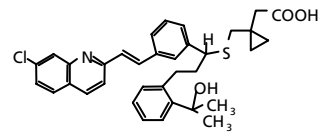
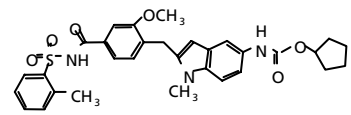
Name	Chemical structure
Angiotensin II receptor antagonists	
Candesartan	
Candesartan cilexetil	
Losartan	
Losartan carboxyl (EXP3174)	
Valsartan	
Leukotriene receptor antagonists	
Pranlukast	
Montelukast	
Zafirlukast	

Figure 4 Chemical structures of the angiotensin receptor antagonists and leukotriene receptor antagonists.

the tetrazole group as an anionic motif. The possibility therefore exists that cephem antibiotics, anti-allergic agents and other drugs containing a tetrazole group may interact with OAT4.

In conclusion, an anionic motif that is recognized by OAT4 is not limited to a carboxyl group and a sulfate group, but contains a tetrazole group.

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