Interaction of Human Organic Anion Transporters 2 and 4 with Organic Anion Transport Inhibitors

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ABSTRACT

The organic anion transport system is involved in the tubular excretion and reabsorption of various drugs and substances. The purpose of this study was to characterize the effects of various organic anion transport inhibitors on renal organic anion transport using proximal tubule cells stably expressing human organic anion transporter 2 (hOAT2) and hOAT4. Immunohistochemical analysis revealed that hOAT2 is localized to the basolateral side of the proximal tubule in the kidney. hOAT2 mediated a time- and concentration-dependent increase in prostaglandin F2α (PGF2α) uptake. The organic anion transport inhibitors used for this study were probenecid, 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902), betamipron, and cilastatin. Probencid, but not KW-3902, betamipron, and cilastatin, significantly inhibited hOAT2-mediated PGF2α uptake. In contrast, probenecid, KW-3902, and betamipron, but not cilastatin, inhibited hOAT4-mediated estrone sulfate (ES) uptake. Kinetic analyses revealed that these inhibitions were competitive. The K_i value of probenecid for hOAT2 was 766 μM, whereas those of probenecid, KW-3902, and betamipron for hOAT4 were 54.9, 20.7, and 502 μM, respectively. These results suggest that probenecid, KW-3902, and betamipron could inhibit hOAT4-mediated ES uptake in vitro, whereas probenecid alone could only inhibit the hOAT2-mediated PGF2α uptake. Comparing the K_i values with the therapeutically relevant concentrations of unbound inhibitors in the plasma, probenecid alone was predicted to inhibit hOAT4-mediated organic anion transport in vivo.

Various organic anion transport inhibitors are used experimentally and clinically. Probencid is a conventional and standard organic anion transport inhibitor experimentally, but it is used as a uricosuric drug clinically. In addition, KW-3902, developed as an adenosine A1 receptor antagonist (Shimada et al., 1991), was also shown to inhibit organic anion transport in the basolateral membrane of opossum kidney cells derived from the American opossum kidney (Nagai et al., 1999). On the other hand, betamipron and cilastatin are administered in combination with carbapenem antibiotics, panipenem, and imipenem, respectively (Birnbaum et al., 1985; Shiba et al., 1991). Betamipron inhibits the uptake of panipenem and imipenem into proximal tubule cells (Hirouchi et al., 1994). On the other hand, imipenem is degraded by human renal dehydropeptidase-I and therefore must be administered in combination with cilastatin, a dehydropeptidase-I inhibitor, to prevent loss of antimicrobial activity in urine and limit potential nephrotoxicity associated with renal metabolism (Craig, 1997).

In our previous study, we elucidated the interaction of human organic anion transporter 1 (hOAT1) and hOAT3 with organic anion transport inhibitors including probenecid, KW-3902, betamipron, and cilastatin (Takeda et al., 2001). Thus, the purpose of this study was to characterize the interaction of hOAT2 and hOAT4 with these organic anion transport inhibitors using cells derived from the second portion of the proximal tubule (S2) from mice stably expressing hOAT2 and hOAT4 (S2 hOAT2 and S2 hOAT4, respectively).

Experimental Procedures

Materials. [3H]prostaglandin F2α (PGF2α) (6808 GBq/mmol) and [3H]estrone sulfate (ES) (1861 GBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Other materials used included fetal bovine serum, trypsin, and gentamicin from Invitrogen.

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ABBREVIATIONS: KW-3902, 8-(noradamantan-3-yl)-1,3-dipropylxanthine; hOAT, human organic anion transporter; S2, the second segment of proximal tubule; PGF2α, prostaglandin F2α; ES, estrone sulfate; oatp, organic anion-transporting peptide; PAH, para-aminohippuric acid.
Cell Culture and Establishment of \( S_2 \) hOAT2 and \( S_2 \) hOAT4.

\( S_2 \) cells, derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen gene, were established as described previously by us (Hosoyamada et al., 1996). The full-length cDNA of hOAT2 was isolated by screening the human kidney cDNA library using rat hOAT2 cDNA (Sekine et al., 1998) as a probe (GenBank accession number: AF210455). The amino acid sequences homology between hOAT2 and rat OAT2 is 77%. The full-length cDNAs of hOAT2 and hOAT4 (Chu et al., 2000) were subcloned into pcDNA3.1 (Invitrogen), a mammalian expression vector. \( S_2 \) hOAT2 and \( S_2 \) hOAT4 were obtained by transfecting \( S_2 \) cells with pcDNA3.1-hOAT2 and pcDNA3.1-hOAT4 coupled with pSV2neo, a neomycin resistance gene, using Tx-50 according to the manufacturer’s instructions. \( S_2 \) cells transfected with pcDNA3.1 lacking an insert and pSV2neo were designated as \( S_2 \) pcDNA 3.1 and used as the control (mock cells). These cells were grown in a humidified incubator at 33°C and under 5% CO\(_2\) using Tx-50 medium containing 5% fetal bovine serum, 10 μg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor, and 400 μg/ml geneticin. The cells were subcultured in a medium containing 0.05% trypsin-EDTA solution (containing 137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4 mM NaHCO\(_3\), 0.5 mM EDTA, and 5 mM HEPES, pH 7.2) and used for 25 to 35 passages. Clonal cells were isolated using a cloning cylinder and screened by determining the optimal substrate for each transporter [i.e., \([\text{14}^C]\)salicylate for OAT2 (Sekine et al., 1998) and \([\text{3}^H]\)ES for hOAT4 (Cha et al., 2000)]. \( S_2 \) hOAT4 exhibited a dose- and time-dependent increase in the uptake of ES. When \( S_2 \) hOAT2 and \( S_2 \) hOAT4 cells were cultured on permeable support (Transwell chambers; Coster, Cambridge, MA) and incubated in a solution containing \([\text{3}^H]\)mannitol on the apical or the basolateral side, the amounts of basal to apical and apical to basal transepithelial transport of \([\text{3}^H]\)mannitol were similar; thus, \( S_2 \) hOAT2 and \( S_2 \) hOAT4 cells were determined to be leaky. In addition, vertical sections of \( S_2 \) hOAT2 and \( S_2 \) hOAT4 stained with polyclonal antibodies against hOAT2 and hOAT4, respectively, showed that the subcellular localization of proteins for hOAT2 and hOAT4 was mainly on the cell membrane (unpublished observation). Both the basolateral and apical portions of the membrane showed positive staining. Therefore, the cells were cultured on a solid support for use in the following experiments.

Immunohistochemical Analysis of hOAT2 Protein in Human Kidney.

Human kidney tissues were collected previously from patients with urethral tract carcinoma after giving informed consent. For the generation of the antibody against hOAT2, rabbits were immunized with keyhole limpet hemocyanin-conjugated synthesized peptides, CSLQEEEMPMEQVQN, corresponding to cysteine and the 14 amino acids of the COOH terminus of hOAT2. The IgG fraction of the polyclonal antibodies for the synthesized peptide was purified from the serum of immunized rabbits using a protein A column.

Light-microscopic analysis of the hOAT2 protein was performed as previously described (Tojo et al., 1999). Briefly, waxed sections of the human kidney cortex (2 μm) were cut and stained by the labeled streptavidin-biotin method. After dewaxing, the sections were incubated with 3% H\(_2\)O\(_2\) for 15 min and then with blocking serum for 15 min. The sections were then incubated with a polyclonal antibody against hOAT2 (1:500 dilution) for 2 h. The sections were rinsed with Tris-buffered saline containing 0.1% Tween-20 and incubated with the biotinylated secondary antibody against rabbit immunoglobulin (Dako, Glostrup, Denmark) for 1 h. After rinsing with Tris-buffered saline containing 0.1% Tween-20, the sections were incubated for 30 min with horseradish peroxidase-conjugated streptavidin solution. Horseradish peroxidase labeling was detected using a peroxidase substrate solution with diaminobenzidine (0.8 mM; Dojindo Laboratories, Kumamoto, Japan). The sections were counterstained with hematoxylin before examination under a light microscope.

Uptake Experiments.

Uptake experiments were performed as previously described (Takeda et al., 1999). The cells were seeded in 24-well tissue culture plates at a cell density of 1 × 10\(^5\) cells/well. After a 2-day culture, they were washed three times with Dulbecco’s modified phosphate-buffered saline solution (containing 137 mM NaCl, 3 mM KCl, 8 mM Na\(_2\)HPO\(_4\), 1 mM KH\(_2\)PO\(_4\), 1 mM CaCl\(_2\), and 0.5 mM MgCl\(_2\), pH 7.4), and then preincubated in the same solution in a water bath at 37°C for 10 min. \( S_2 \) hOAT2 cells were incubated in a solution containing 5 nM \([\text{3}^H]\)PGF\(_2\alpha\) for use in time course experiments and 50 nM \([\text{3}^H]\)PGF\(_2\alpha\) for use in inhibition experiments. \( S_2 \) hOAT4 cells were incubated in a solution containing 50 nM \([\text{3}^H]\)ES for use in the inhibition experiments. The uptake was stopped by the addition of ice-cold Dulbecco’s modified phosphate-buffered saline, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N sodium hydroxide and 2.5 ml of Aquasol-2, and radioactivity was determined using a β-scintillation counter (LSC-3100; Aloka, Tokyo, Japan).

Kinetic Analysis.

After the preincubation as described above, \( S_2 \) hOAT2 and \( S_2 \) hOAT4 cells were incubated in a solution containing \([\text{3}^H]\)PGF\(_2\alpha\) or \([\text{3}^H]\)ES at different concentrations in the absence or presence of various inhibitors at 37°C for 20 s (for hOAT2) or 2 min (for hOAT4). Probenecid and cilastatin were dissolved in H\(_2\)O, whereas KW-3902 and betamipron were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was adjusted to less than 0.1%, which did not affect the hOAT2- and hOAT4-mediated organic anion uptake in our system. Based on the \([\text{3}^H]\)PGF\(_2\alpha\) and \([\text{3}^H]\)ES uptake under each condition, double reciprocal plot analyses were performed as previously described (Apiswasanakul et al., 1999). For accurate analysis, the transformed data points were analyzed using weighted linear regression, with a weighting factor of 1/y or 1/y\(^2\). When the inhibition was competitive, the \( K_i \) values were calculated based on the following equation: 

\[ K_i = \frac{V_{max} \cdot [I]}{V_{max} - V_{max} \cdot [I]} \]

where \( V_{max} \) is the maximum velocity, \([I]\) is the inhibitor concentration, and \( K_i \) is the inhibitor dissociation constant.
Statistical Analysis. Data are expressed as means ± S.D. or means ± S.E. Statistical differences were determined using analysis of variance with Dunnett’s posthoc test. Differences were considered significant at \( P < 0.05 \).

Results

Immunohistochemical Analysis of hOAT2 Protein in Human Kidney. Light-microscopic analysis of 2-μm waxed sections demonstrated that hOAT2 immunoreactivity was detected in the basolateral side of the proximal tubules (Figs. 2, A and B). Arrows indicate stained proximal tubules.

Time- and Concentration-Dependent Uptake of PGF\(_{2α}\) in S\(_2\) hOAT2. We examined the time-dependent uptake of PGF\(_{2α}\) in S\(_2\) hOAT2. As shown in Fig. 3A, S\(_2\) hOAT2 exhibited much higher PGF\(_{2α}\) uptake than mock cells. The kinetics of PGF\(_{2α}\) uptake were examined to evaluate the pharmacological characteristics of hOAT2 upon the uptake of PGF\(_{2α}\). We analyzed the mean data using the Michaelis-Menten equation and determined the \( K_m \) and \( V_{max} \) values. Using these parameters, we made a theoretical curve. As shown in Fig. 3B, S\(_2\) hOAT2 exhibited a concentration-dependent increase in PGF\(_{2α}\) uptake. Eadie-Hofstee plot of the concentration dependence of PGF\(_{2α}\) uptake in S\(_2\) hOAT2 after subtraction of uptake by mock cells revealed that the estimated \( K_m \) value of PGF\(_{2α}\) uptake by hOAT2 was 425 ± 53.0 nM (data not shown). These results suggest that hOAT2 mediates the transport of PGF\(_{2α}\).

Effects of Various Organic Anion Transport Inhibitors on Organic Anion Uptake by hOAT2 and hOAT4. We examined the effects of probenecid, KW-3902, betamipron, and cilastatin at different concentrations on PGF\(_{2α}\) uptake in S\(_2\) hOAT2 and ES uptake in S\(_2\) hOAT4. As shown in Fig. 4, probenecid, but not KW-3902, betamipron, and cilastatin, significantly inhibited PGF\(_{2α}\) uptake by hOAT2 \((n = 4; *P < 0.0001 \text{ versus control})\). In addition, probenecid exhibited a dose-dependent decrease in PGF\(_{2α}\) uptake by hOAT2 over the concentration range of 1 to 2000 μM (data not shown). In contrast, probenecid, KW-3902, and betamipron, but not cilastatin, significantly inhibited ES uptake by hOAT4 in a dose-dependent manner (data not shown).
We analyzed the kinetics of the inhibitory effects of probenecid, KW-3902, betamipron, and cilastatin on PGF$_2\alpha$ uptake by hOAT2. As shown in Fig. 5, analysis of the Lineweaver-Burke plot of the effects of probenecid on PGF$_2\alpha$ uptake by hOAT2 revealed that the mode of the inhibitory effect was competitive. Similarly, as shown in Fig. 6, probenecid (A), KW-3902 (B), and betamipron (C) inhibited hOAT4-mediated ES uptake in a competitive manner. Table 1 shows the $K_i$ values of various organic anion transport inhibitors for hOAT2 and hOAT4.

**Discussion**

The secretion and reabsorption of numerous organic anions, including endogenous metabolites, drugs, and xenobiotics, are important physiological functions of the renal proximal tubule. The process of secreting organic anions through the proximal tubule cells is achieved via unidirectional transcellular transport involving the uptake of organic anions into the cells from the blood across the basolateral membrane, followed by extrusion across the brush-border membrane into the proximal tubule fluid (Pritchard and Miller, 1993). Recently, cDNA-encoding transporters mediating renal organic anion transport have been successively cloned, including OAT1 (Sekine et al., 1997; Reid et al., 1998;
Hosoyamada et al., 1999), OAT2 (Sekine et al., 1998), OAT3 (Kusuhara et al., 1999; Cha et al., 2001), OAT4 (Cha et al., 2000), OAT-K1 (Saito et al., 1996), OAT-K2 (Masuda et al., 1999), organic anion-transporting polypeptide ( oatp1) (Jacquemin et al., 1994), oatp2 (Noe et al., 1997), oatp3 (Abe et al., 1998), multidrug resistance-associated protein 2 (MRP2) (Leier et al., 2000) and human-type I sodium-dependent inorganic phosphate transporter (NPT1) (Uchino et al., 2000). Among them, hOAT1 and hOAT3 are thought to be the major OATs responsible for the basolateral uptake of various organic anions (Hosoyamada et al., 1999; Cha et al., 2001). H0AT4, localized to the apical side of the proximal tubule (Babu et al., 2002), was reported to mediate the transport of various anionic drugs, including para-aminomophipic acid (PAH), ES, methotrexate, and ochratoxin A (Cha et al., 2000).

At first, we characterized the localization and functional properties of hOAT2. Immunohistochemically, hOAT2 was shown to be localized to the basolateral side of the proximal tubule. Although salicylate was found to be the best substrate for rat OAT2 (Sekine et al., 1998), the background uptake by mock cells was high (unpublished observation). In the current study, we found that PGF2α was the better substrate for hOAT2, whereas the background uptake by mock cells was much lower. H0AT2 mediated a time- and dose-dependent increase in PGF2α uptake. Based on these observations, we used PGF2α as a substrate to elucidate the interaction of hOAT2 with various organic anion transport inhibitors. In addition, since hOAT1, hOAT2, and hOAT3 (Hosoyamada et al., 1999; Cha et al., 2001) are localized to the basolateral side of the proximal tubule, the functional difference among these transporters should therefore be elucidated.

Probencid, KW-3902, and betamipron, but not cilastatin, significantly inhibited ES uptake by hOAT4, whereas probencid alone inhibited the hOAT2-mediated PGF2α uptake. These results are in contrast to the previous results that probencid, KW-3902, betamipron, and cilastatin significantly inhibited PAH uptake by hOAT1 and ES uptake by hOAT3 (Takeda et al., 2001). However, the rank order of the inhibitory effects on hOAT4-mediated ES uptake (i.e., KW-3902 > probencid > betamipron) was the same as that for hOAT1-mediated PAH uptake and hOAT3-mediated ES uptake (Takeda et al., 2001).

Probencid has been widely used to analyze organic anion transport systems. In the current study, probencid was shown to inhibit organic anion uptake mediated by hOAT2-mediated PGF2α uptake and hOAT4-mediated ES uptake in vitro. The maximum steady-state plasma concentration and unbound fraction of probencid were reported to be 170 μM (Nierenberg, 1983) and 11.0% (Dayton et al., 1963), respectively. Thus, the maximum steady-state concentration of unbound probencid in the plasma is estimated to be approximately 18.7 μM. Since the therapeutically relevant plasma concentrations of a drug is thought to be within 5-fold of the maximum steady-state plasma concentration of a drug (Zhang et al., 2000), the therapeutically relevant concentration of unbound probencid in the plasma is thought to be 93.5 μM. In addition, since the unbound drug could be filtered through the glomerulus, the concentration of unbound probencid with the tubular fluid in the apical side of the proximal tubule would be within 93.5 μM. Based on these observations, since the Ki value of probencid for hOAT2-mediated PGF2α uptake and hOAT4-mediated ES uptake were 766 and 54.9 μM, respectively (Table 1), it was predicted that probencid could inhibit the reabsorption of organic anions by hOAT4 on the apical side of the proximal tubule in vivo.

KW-3902 is selective and is the most potent adenosine A1 receptor antagonist known to date (Suzuki et al., 1992). In animal studies, this compound was shown to have diuretic activity and renal-protective effect against cephaloridine-induced nephrotoxicity (Mizumoto et al., 1993; Nagashima et al., 1994). However, this compound was excluded from clinical development based on the results of a phase II trial study, which showed that this compound did not exert sufficient diuretic action (K. Hakko and K. Co, unpublished observation). In this study, KW-3902 was shown to inhibit hOAT4-mediated ES uptake but not hOAT2-mediated PGF2α uptake in vitro. Based on the rank order of the Ki values of various inhibitors shown in Table 1, KW-3902 was the most potent inhibitor of hOAT4-mediated ES uptake. Thus, it was suggested that KW-3902 could be a powerful pharmacological agent for analyzing hOAT4-mediated organic anion transport in vitro. However, the Ki values of KW-3902 for hOAT4-mediated PGF2α uptake (20.7 μM) were much higher than the maximum plasma concentration of KW-3902 in a healthy volunteer [i.e., 0.196 μM (KW-3902 product brochure; Kyowa Hakko Kogyo Co.) (more than 3-fold; Zhang et al., 1998)]. Thus, it was predicted that this compound would exhibit no significant inhibitory effects on hOAT4-mediated organic anion uptake in vivo.

Betamipron significantly inhibited ES uptake by hOAT4 but not PGF2α uptake by hOAT2 in vitro. However, the Ki value of betamipron for hOAT4-mediated ES uptake was

**TABLE 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Probencid</th>
<th>KW-3902</th>
<th>Betamipron</th>
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<tbody>
<tr>
<td>hOAT2</td>
<td>766 ± 72.9</td>
<td>20.7 ± 3.11</td>
<td>502 ± 22.8</td>
</tr>
<tr>
<td>Ki value (μM)</td>
<td>1000.0</td>
<td>20.0</td>
<td>500.0</td>
</tr>
<tr>
<td>Inhibitor (μM)</td>
<td>170.0</td>
<td>0.196</td>
<td>94.8</td>
</tr>
<tr>
<td>Total plasma concentration (μM)</td>
<td>11.0</td>
<td>26.9</td>
<td>25.5</td>
</tr>
<tr>
<td>Unbound fraction (%)</td>
<td></td>
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much higher than the therapeutically relevant concentration of unbound betamipron in the plasma (25.5 μM) (Shiba et al., 1991; Zhang et al., 2000). These results suggest that betamipron exerts no significant inhibitory effects on hOAT4-mediated ES transport in vivo.

In the current study, cistatin was found to have no significant inhibitory effects on hOAT2-mediated PGF<sub>2α</sub> uptake and hOAT4-mediated ES uptake. In contrast, cistatin significantly inhibited hOAT1-mediated PAH uptake and hOAT3-mediated ES uptake. In vitro, whereas it was predicted that cistatin exhibits significant inhibitory effects on hOAT1- but not hOAT3-mediated organic anion uptake in vivo (Takeda et al., 2001).

In addition to the hOATs used in the current study, it is important to understand the interaction of apical transporters mediating organic anion transport (i.e., OAT-K1, OAT-K2, oatp1, MRP2, and NPT1) with various organic anion inhibitors (Inui et al., 2000). However, these topics are beyond the scope of this study, and further study should be performed to elucidate them.

In conclusion, the results suggest that in vitro hOAT2 interacts with only probenecid, whereas hOAT4 interacts with probenecid, KW-3902, and betamipron. In addition, by comparing the K<sub>i</sub> values of the inhibitors with their therapeutically relevant concentrations of unbound inhibitors in the plasma, it was predicted that hOAT4-mediated organic anion uptake would be inhibited by probenecid in vivo, whereas none would inhibit hOAT2-mediated organic anion uptake in vivo. The cells used in this study would serve as a good tool to characterize newly developed organic anion transport inhibitors.

References


