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Characterization of organic anion transport inhibitors using cells stably expressing human organic anion transporters

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Abstract

The organic anion transport system is involved in the tubular excretion of various clinically important drugs. The purpose of this study was to characterize the effects of various organic anion transport inhibitors on organic anion transport using proximal tubule cells stably expressing human organic anion transporter 1 (human-OAT1) and human-OAT3, which are localized to the basolateral membrane of the proximal tubule. Organic anion transport inhibitors including betamipron, cilastatin, KW-3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine) and probenecid significantly inhibited human-OAT1- and human-OAT3-mediated organic anion uptake in a dose-dependent manner. Kinetic analyses revealed that these inhibitions were competitive. The K_i values of betamipron, cilastatin, KW-3902 and probencid for human-OAT1 were 23.6, 1470, 7.82 and 12.1 μM, whereas those for human-OAT3 were 48.3, 231, 3.70 and 9.0 μM. These results suggest that betamipron and probenecid could inhibit both human-OAT1- and human-OAT3-mediated organic anion transport in vivo, whereas cilastatin could inhibit only human-OAT3-mediated one. In contrast, KW-3902 did not exert the effects of significance, whereas KW-3902 was the most potent.

Keywords: Organic anion transporter, inhibitor; Proximal tubule; Betamipron; Cilastatin; KW-3902; Probenecid

1. Introduction

There are various organic anion transport inhibitors used clinically. 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 consequently must be administered in combination with the dehydropeptidase-I inhibitor, cilastatin, to prevent low antimicrobial activity in urine and limit potential nephrotoxicity associated with renal metabolism (Craig, 1997). Although betamipron was shown to be an organic anion transport inhibitor, it remains unknown whether cilastatin acts as an organic anion transport inhibitor or not. On the other hand, KW-3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine), developed as an adenosine A_1 receptor antagonist (Mizumoto et al., 1992), was also shown to inhibit organic anion transport in the basolateral membrane of opossum kidney (OK) cells, derived from the American opossum kidney (Nagai et al., 1999). Furthermore, probenecid is a conventional and standard organic anion transport inhibitor experimentally, which is used as an uricosuric drug clinically.

The purpose of this study was to characterize the effects of these organic anion transport inhibitors on organic anion uptake using cells from the second portion of the proximal tubule (S_2) cells, stably expressing human-OAT1 and human-OAT3 (S_2 human-OAT1 and S_2 human-OAT3, respectively).

2. Materials and methods

2.1. Materials

[^{14}C]*para*-aminohippuric acid (53.1 mCi/mmol) and [^{3}H]estrone sulfate (53 Ci/mmol) were purchased from

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New England Nuclear (Boston, MA, USA). Other materials used included fetal bovine serum, trypsin and geneticin from Gibco/BRL (Gaithersburg, MD, USA), recombinant epidermal growth factor from Wakunaga (Hiroshima, Japan), insulin from Shimizu (Shizuoka, Japan), RITC 80-7 culture medium from Iwaki (Tokyo, Japan), probenecid from Sigma (St. Louis, MO, USA) and TfX-50 from Promega (Madison, WI, USA). Betamipron, cilastatin and KW-3902 were kind gifts of Sankyo Pharmaceutical (Tokyo, Japan), Banyu Pharmaceutical (Tokyo, Japan) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively.

2.2. Cell culture and establishment of S_2 human-OAT1 and S_2 human-OAT3

S_2 cells, derived from transgenic mice harboring the simian virus 40 large T-antigen gene, were established as described previously by us (Hosoyamada et al., 1996). S_2 is the segment of the proximal tubule where human-OAT1 and human-OAT3 were shown to be localized (Hosoyamada et al., 1999; Cha et al., in press). The full-length cDNAs of human-OAT1 and human-OAT3 were subcloned into pcDNA 3.1 (In Vitrogen, San Diego, CA, USA), a mammalian expression vector. S_2 human-OAT1 and S_2 human-OAT3 were obtained by transfecting S_2 cells with pcDNA3.1-human-OAT1 and pcDNA3.1-human-OAT3 coupled with pSV_2neo, a neomycin resistance gene, using TfX-50 according to the manufacturer's instructions. S_2 cells transfected with pcDNA3.1 lacking an insert and pSV_2neo were designated as S_2 pcDNA 3.1 and used as a control. These cells were grown in a humidified incubator at 33°C and under 5% CO_2 using RITC 80-7 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 in mM: 137 NaCl, 5.4 KCl, 5.5 glucose, 4 $NaHCO_3$, 0.5 EDTA and 5 HEPES; pH 7.2) and used for 60–100 passages. Clonal cells were isolated using a cloning cylinder and screened by determination of the optimal substrate for each transporter, i.e. [^{14}C]*para*-aminohippuric acid for human-OAT1 (Hosoyamada et al., 1999) and [^{3}H]estrone sulfate for human-OAT3 (Cha et al., in press). The S_2 monolayer was determined to be leaky based on the results of a study in which we estimated paracellular secretion from cells cultured on a permeable support, using D-[^{3}H]mannitol as an indicator. In addition, vertical sections of S_2 human-OAT1 and S_2 human-OAT3 stained with polyclonal antibodies against human-OAT1 and human-OAT3, respectively, showed that the subcellular localization of human-OAT1 and human-OAT3 proteins was mainly on the cell membrane (unpublished observation). In generating these antibodies, rabbits were immunized with keyhole limpet hemocyanin-conjugated synthesized peptides, CMV-PLQASAQEKNGL, corresponding to cysteine and the 14 amino acids of the COOH terminus of human-OAT1, and CRIPLQPHGPGLGSS, corresponding to cysteine and the 14 amino acids of the COOH terminus of human-OAT3. Both the basolateral and apical portions of the membrane showed positive staining. Therefore, the cells were cultured on a solid support for the following experiments.

2.3. Uptake experiments

Uptake experiments were performed as previously described (Takeda et al., 1999). Uptake experiments are commonly performed at 37°C when mammalian cells or tissues are used. On the other hand, since S_2 cells are encoded with the temperature-sensitive simian virus 40 large T-antigen gene (Hosoyamada et al., 1996), the cells grow at a permissive temperature (33°C), but not at a nonpermissive temperature (37°C). In the preliminary studies, we found no difference in the amount of uptake between 33°C and 37°C (data not shown). Thus, uptake experiments were performed at 37°C in the current study, whereas culture was performed at 33°C. These cells had been seeded in 24-well tissue culture plates at a cell density of 1×10^5 cells/well. After these cells had been cultured for 2 days, they were washed three times with Dulbecco's modified phosphate-buffered saline (D-PBS) solution (containing in mM: 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$; pH 7.4), and then preincubated in the same solution for 10 min in a water bath at 37°C. The uptake was stopped by the addition of ice-cold D-PBS, 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 (Aloka, LSC-3100). As human-OAT1 has an exchanger property, extracellular *para*-aminohippuric acid enhances the efflux of [^{14}C]*para*-aminohippuric acid incorporated with the cells (data not shown). However, it was also noted that during 2 min incubation, which was also the incubation time for the inhibition study and the kinetic analysis, no significant enhancement of efflux was observed in cells treated with the vehicle (data not shown). Thus, contribution of efflux to the net uptake results may not be significant.

2.4. Kinetic analysis

After preincubation as described above, S_2 human-OAT1 and S_2 human-OAT3 were incubated in D-PBS containing [^{14}C]*para*-aminohippuric acid or [^{3}H]estrone sulfate at different concentrations in the absence or presence of various inhibitors for 2 min. Cilastatin and probenecid were dissolved in H_2O, whereas betamipron and KW-3902 were dissolved in dimethylsulfoxide. The final concentration of dimethylsulfoxide was adjusted to less than 0.1%, which did not affect the human-OAT1- and human-OAT3-mediated organic anion uptake in our sys-

tem. Dimethylsulfoxide treatment probably does not interfere at the used concentration (less than 0.1%) with organic anion transport. In fact, interference of this product is observed only at higher concentrations (between 0.1% and 5%) (data not shown). Based on the organic anion uptake under each set of conditions, double reciprocal plot analyses were performed as previously described (Apiwattanakul et al., 1999). When the inhibition was competitive, the K_i values were calculated based on the following equation,

$$K_i = \text{concentration of inhibitor} \Big/ \Big[\Big(K_m \text{ of } \textit{para}\text{-aminohippuric acid or estrone sulfate with inhibitor} \Big/ K_m \text{ of } \textit{para}\text{-aminohippuric acid or estrone sulfate without inhibitor} \Big) - 1 \Big].$$

2.5. Statistical analysis

Data are expressed as means ± S.E. Statistical significance of differences was determined using Student's unpaired *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Characterization of organic anion uptake in S_2 human-OAT1 and S_2 human-OAT3

S_2 human-OAT1 and S_2 human-OAT3 exhibited a dose- and time-dependent increase in the uptake of *para*-aminohippuric acid and estrone sulfate, respectively. Eadie–Hofstee analysis showed that the Michaelis constants (K_m) were 20.1 ± 2.3 ($N = 4$) and 2.21 ± 0.27 μM ($N = 4$), which are similar to those reported in experi-

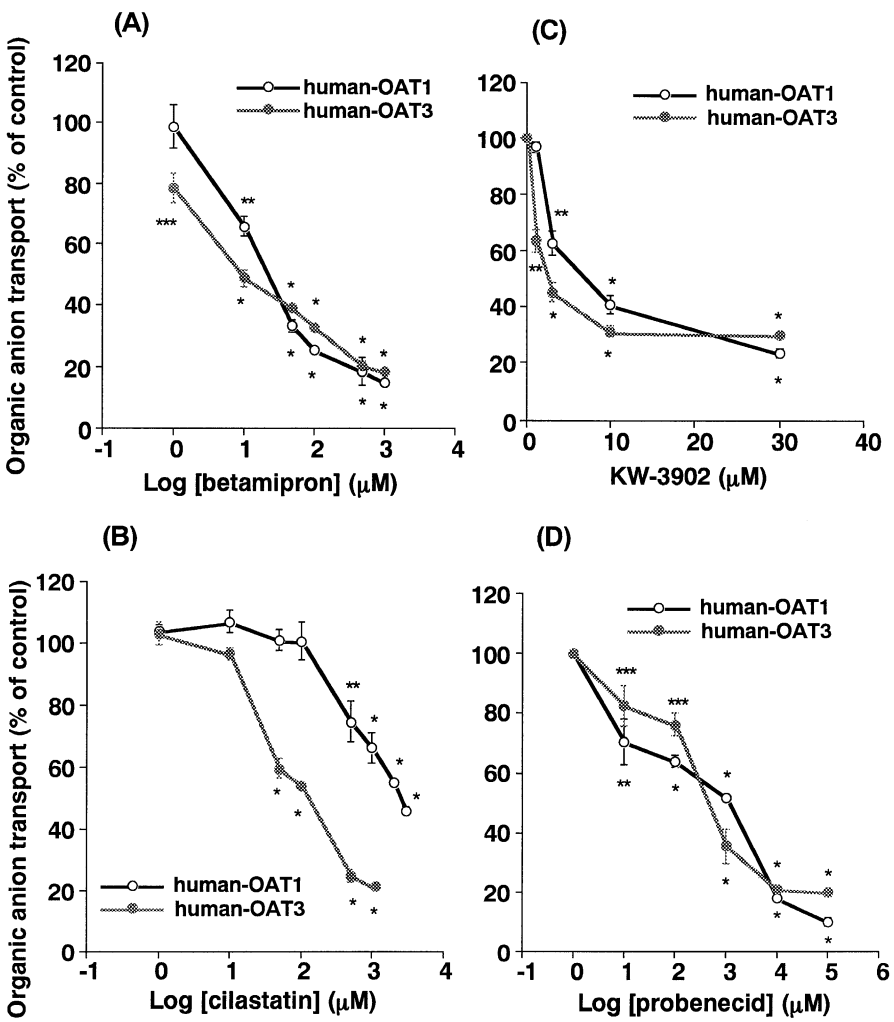

Fig. 1. Effects of various concentrations of betamipron (A), cilastatin (B), KW-3902 (C) and probenecid (D) on organic anion uptake in S_2 human-OAT1 and S_2 human-OAT3. The cells were incubated in a medium containing [^{14}C]*para*-aminohippuric acid or [^{3}H]estrone sulfate for 2 min in the absence or presence of various concentrations of betamipron, cilastatin, KW-3902 and probenecid. Each value represents the mean ± S.E. of four determinations. $^{*}P < 0.001$, $^{**}P < 0.01$ and $^{***}P < 0.05$ vs. control.

ments using *Xenopus* oocytes, 14.3 (Hosoyamada et al., 1999) and 3.1 μM (Cha et al., in press), respectively. *Para*-aminohippuric acid and probenecid, an organic anion transport inhibitor, exhibited significant inhibition of [^{14}C]*para*-aminohippuric acid uptake in S_2 human-OAT1, and estrone sulfate and probenecid did so on [^{3}H]estrone sulfate uptake in S_2 human-OAT3. These results suggest that S_2 human-OAT1 and S_2 human-OAT3 are characterized by the transport of organic anions as previously reported (Hosoyamada et al., 1999; Cha et al., in press).

3.2. Effects of various concentrations of organic anion transport inhibitors on organic anion uptake in S_2 human-OAT1 and S_2 human-OAT3

We described the effects of betamipron, cilastatin, KW-3902 and probenecid at different concentrations on organic anion uptake in S_2 human-OAT1 and S_2 human-OAT3. As shown in Fig. 1, betamipron (A), cilastatin (B), KW-3902 (C) and probenecid (D) significantly inhibited organic anion uptake by human-OAT1 and human-OAT3 in a dose-dependent manner ($N = 4$, $^{*}P < 0.001$, $^{**}P < 0.01$ and $^{***}P < 0.05$ vs. control).

3.3. Kinetic analysis of effects of various organic anion transport inhibitors on organic anion uptake in S_2 human-OAT1 and S_2 human-OAT3

We analyzed the kinetics of the inhibitory effects of betamipron, cilastatin, KW-3902 and probenecid on organic anion uptake in S_2 human-OAT1 and S_2 human-OAT3. As shown in Figs. 2 and 3, analysis of the Lineweaver–Burke plot of the effects of betamipron (A), cilastatin (B), KW-3902 (C) and probenecid (D) on organic anion uptake in S_2 human-OAT1 (Fig. 2) and S_2 human-OAT3 (Fig. 3) showed that these organic anion transport inhibitors inhibited human-OAT1- and human-OAT3-mediated organic anion uptake in a competitive

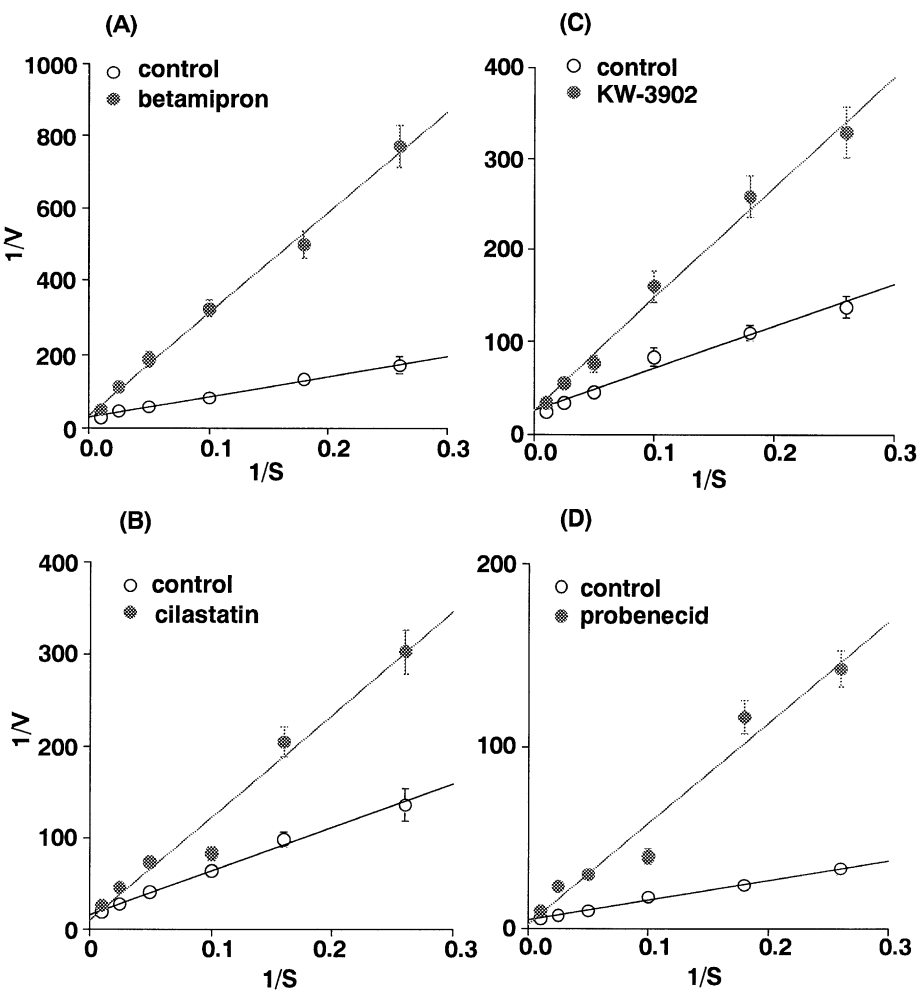

Fig. 2. Kinetic analysis of the inhibitory effects of betamipron, cilastatin, KW-3902 and probenecid on organic anion uptake in S_2 human-OAT1. Organic anion uptake in S_2 human-OAT1 was measured at various concentrations of [^{14}C]*para*-aminohippuric acid in the presence or absence of 100 μM betamipron, 2000 μM cilastatin, 10 μM KW-3902 and 50 μM probenecid for 2 min at 37°C, and Lineweaver–Burke plot analyses were performed. (A) betamipron, (B) cilastatin, (C) KW-3902, and (D) probenecid. Each value represents the mean ± S.E. of four determinations.

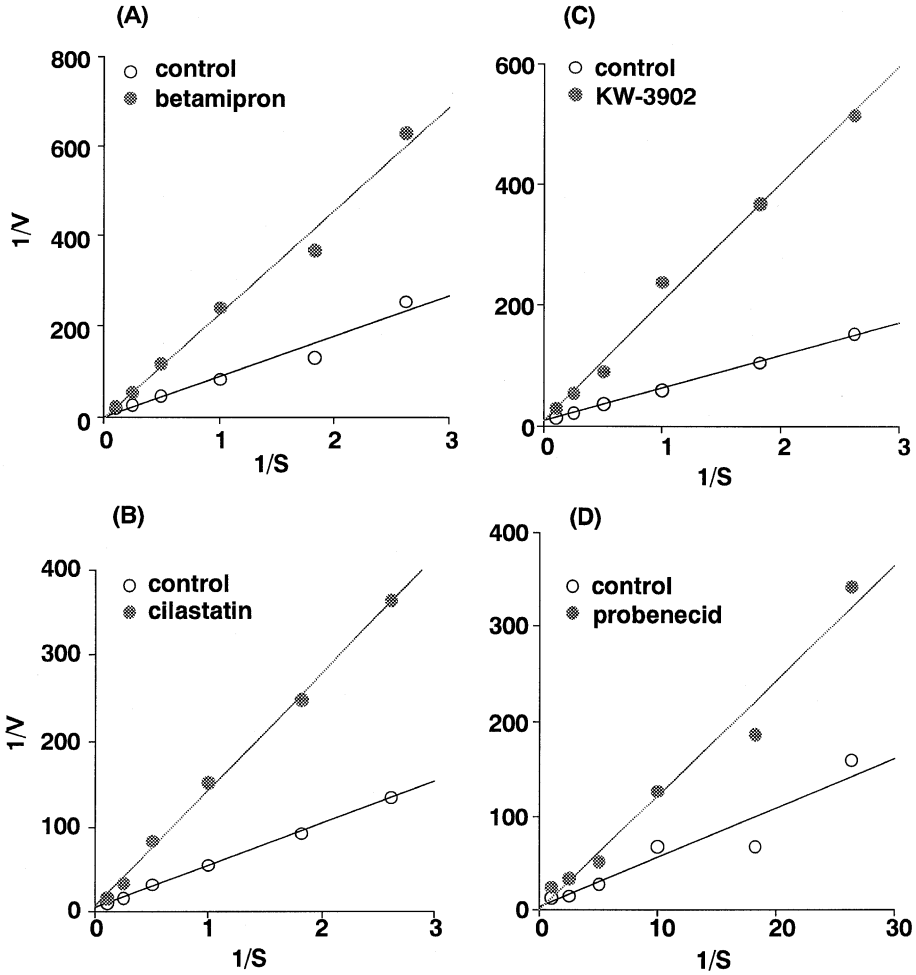

Fig. 3. Kinetic analysis of the inhibitory effects of betamipron, cilastatin, KW-3902 and probenecid on organic anion uptake in S_2 human-OAT3. Organic anion uptake in S_2 human-OAT3 was measured at various concentrations of [^{3}H]estrone sulfate in the presence or absence of 100 μM betamipron, 500 μM cilastatin, 10 μM KW-3902 and 10 μM probenecid for 2 min at 37°C, and Lineweaver–Burke plot analyses were performed. (A) betamipron, (B) cilastatin, (C) KW-3902, (D) probenecid. Each value represents the mean ± S.E. of four determinations.

manner. The table shows the K_i values of various organic anion transport inhibitors for human-OAT1 and human-OAT3.

4. Discussion

The secretion of numerous organic anions, including endogenous metabolites, drugs and xenobiotics, is an important physiological function of renal proximal tubules. 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, cDNAs encoding renal organic anion transporters (OATs) have been successively cloned, including OAT1 (Sekine et al., 1997; Hosoyamada et al., 1999), OAT2 (Sekine et al., 1998), OAT3 (Kusuhara et al., 1999; Cha et al., in press), 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), multiple resistance-associated protein 2 (MRP2) (Leier et al., 2000) and human-type I sodium-dependent inorganic phosphate transporter (NPT1) (Uchino et al., 2000). Among members of the OAT family, human-OAT1 and human-OAT3 were reported to be involved in the active uptake of various drugs and endogenous substances via the basolateral membrane of the proximal tubule, i.e. nonsteroidal anti-inflammatory drugs, antitumor drugs, histamine H_2 receptor antagonist, prostaglandins, diuretics, angiotensin-converting enzyme inhibitors and beta-lactam antibiotics (Hosoyamada et al., 1999; Sekine et al., 2000; Cha et al., in press). Some differences in characteristics exist between human-OAT1 and human-OAT3, such as substrate specificity and localization: human-OAT1 at the basolateral side of the S_2 segment of the proximal tubule

(Hosoyamada et al., 1999) vs. human-OAT3 at the first, second and third segments (S_1, S_2 and S_3) of the proximal tubule (Cha et al., in press). In addition, human-OAT1, but not human-OAT3, exhibits transport properties as an exchanger (Hosoyamada et al., 1999; Cha et al., in press). This study focused on the basolateral OATs, i.e. human-OAT1 and human-OAT3, which determine the tubular excretion of various anionic drugs. The characterization of the interaction between various organic anion inhibitors and apical OATs, i.e. OAT-K1, OAT-K2, oatp1, MRP2, OAT4 and NPT 1 (Inui et al., 2000), is beyond the scope of this study; therefore, further study should be performed to elucidate this. The localization of oatp2 and oatp3 remains unknown.

The inhibitory effects of betamipron on human-OAT1- and human-OAT3-mediated organic anion uptake were consistent with the preventive effect of betamipron on nephrotoxicity and the uptake of panipenem in the rabbit renal cortex (Hirouchi et al., 1994). The K_i values of betamipron for human-OAT1 and human-OAT3 were comparable to its therapeutical plasma concentration (within fivefold the maximum plasma concentration) in patients treated with panipenem/betamipron, the maximum plasma concentration of which was 94.8 μM (Zhang et al., 2000; Shiba et al., 1991). These results suggest that betamipron could inhibit both human-OAT1- and human-OAT3-mediated organic anion transport in vivo. The K_i value of betamipron for human-OAT1 was similar to the IC_{50} value recently reported for the effect of betamipron on the human-OAT1-mediated uptake of adenovir at a concentration of 6.0 μM (Mulato et al., 2000). There was no significant difference in betamipron effects between *para*-aminohippuric acid and adenovir as substrates. The significant difference in K_i value or IC_{50} value was thought to be more than threefold (Zhang et al., 1998). However, we had already found that the K_i values of drugs that affect organic anion transport are significantly variable depending on the substrate used in S_2 human-OAT1 and S_2 human-OAT3, i.e. methotrexate vs. *para*-aminohippuric acid or estrone sulfate (data not shown). In this regard, the current results with a standard substrate for human-OAT1, *para*-aminohippuric acid, could be important information about the pharmacological properties of betamipron.

In the current study, we observed the inhibitory potency of cilastatin against organic anion transport mediated by human-OAT1 and human-OAT3. The K_i value of cilastatin for human-OAT1 was similar to its therapeutic plasma concentration in patients treated with imipenem/cilastatin, the maximum plasma concentration of which was 83.0 μM (Saito et al., 1985). In contrast, the K_i value of cilastatin for human-OAT3 was much higher than its therapeutic plasma concentration (about 18-fold). These results suggest that cilastatin could inhibit organic anion transport mediated by human-OAT1, but not by human-OAT3 in vivo. In addition, the K_i value of cilastatin for human-OAT1-mediated organic anion transport was about sixfold higher than human-OAT3-mediated one, whereas those of betamipron, KW-3902 and probenecid differed by about twofold. In this regard, cilastatin could be utilized as a relatively specific inhibitor for human-OAT3-mediated organic anion transport.

KW-3902 is selective and is the most potent adenosine A_1 receptor antagonist known (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 the phase II trial, in which this compound did not exert sufficient diuretic action (unpublished observation). In the present study, KW-3902 was shown to inhibit both human-OAT1- and human-OAT3-mediated organic anion transport. However, the K_i values for human-OAT1 and human-OAT3 were much higher than therapeutic plasma concentration of KW-3902, the maximum plasma concentration of which in a healthy volunteer was 0.196 μM (KW-3902: product brochure; Kyowa Hakko Kogyo). Thus, the current results suggest that this compound has no clinical impact on the organic anion transport system. However, based on the rank order of the K_i values of various inhibitors shown in Table 1, KW-3902 is the most potent inhibitor of human-OAT1- and human-OAT3-mediated organic anion transport. Thus, this compound could be a powerful pharmacological tool for analyzing an organic anion transport system. KW-3902 was also shown to inhibit organic anion uptake in the basolateral membrane of OK cells (Nagai et al., 1999). In the present study, organic anion transport was estimated using [^{14}C]*para*-aminohippuric acid, suggesting that the results mainly reflect the activity of OAT1. Consistent with this, there was no significant difference between the IC_{50} value obtained with OK cells and the K_i with S_2 human-OAT1, i.e. 10.7 vs. 7.82 μM, but it is possible that there is a significant difference in these values between opossum and human.

Table 1
K_i values of various organic anion transport inhibitors that competitively inhibit the uptake of [^{14}C]*para*-aminohippuric acid in S_2 human-OAT1 and [^{3}H]estrone sulfate in S_2 human-OAT3
S_2 human-OAT1 and S_2 human-OAT3 were incubated with solution containing various concentrations of [^{14}C]*para*-aminohippuric acid or [^{3}H]estrone sulfate, in the absence or presence of various organic anion transport inhibitors. The K_i values were estimated from a Lineweaver–Burke plot.

Drug	Human-OAT1		Human-OAT3	
	K_i value (μM)	Inhibitor (μM)	K_i value (μM)	Inhibitor (μM)
Betamipron	23.6 ± 3.10	100	48.3 ± 5.30	100
Cilastatin	1470 ± 201	2000	231 ± 20.6	500
KW-3902	7.82 ± 0.86	10	3.70 ± 0.26	10
Probenecid	12.1 ± 1.51	50	9.0 ± 0.82	10

Although probenecid has been widely used for analyzing an organic anion transport system, its molecular target, mode of action and relative potency have not been sufficiently identified. In the current study, probenecid was shown to be a potent and competitive inhibitor of organic anion transport driven by human-OAT1 and human-OAT3. The K_i values of probenecid for human-OAT1 and human-OAT3 were much lower than the maximum plasma concentration in patients, 170 μM (Nierenberg, 1983). Probenecid was shown to increase the plasma concentration of various drugs including methotrexate (Nierenberg, 1983; Basin et al., 1991), penicillin G (Nierenberg et al., 1986; Shanson et al., 1984; Shinn and Shrewbury, 1985) and antiviral drugs (Laskins et al., 1982; Mulato et al., 2000) by competing for tubular secretion via OAT. The current results may provide a molecular basis for a broad drug interaction of probenecid. The K_i value of probenecid for human-OAT1 was similar to the IC_{50} value for the effect of probenecid on adenovir uptake in human-OAT1-expressing Chinese hamster ovary cells, 6.0 μM (Mulato et al., 2000).

In conclusion, we showed the molecular target and the kinetic properties of various organic anion transport inhibitors using cells stably expressing human-OAT1 and human-OAT3. In addition, by comparing the K_i values of inhibitors with their therapeutical plasma concentrations, the clinical significance of various inhibitors was estimated. The cells used in this study would provide a good tool for characterizing newly developed organic anion transport inhibitors.

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