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Interactions of human organic anion transporters with diuretics

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human organic anion transporter, hOAT

rat organic anion transporter, rOAT

the first, second and third segments of proximal tubule, S_1, S_2, S_3

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estrone sulfate, ES

tetraethylammonium, TEA

organic anion-transporting peptide, oatp

multidrug resistance-associated protein, MRP

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Abstract

The tubular secretion of diuretics in the proximal tubule has been shown to be critical for the action of drugs. In order to elucidate the molecular mechanisms for the tubular excretion of diuretics, we have elucidated the interactions of human organic anion transporters (hOATs) with diuretics using cells stably expressing hOATs. Diuretics tested were thiazides including chlorothiazide, cyclothiazide, hydrochlorothiazide and trichlormethiazide; loop diuretics including bumetanide, ethacrynic acid and furosemide; and carbonic anhydrase inhibitors including acetazolamide and methazolamide. These diuretics inhibited organic anion uptake mediated by hOAT1, hOAT2, hOAT3 and hOAT4 in a competitive manner. HOAT1 exhibited the highest affinity interactions for thiazides, whereas hOAT3 did those for loop diuretics. HOAT1, hOAT3 and hOAT4 but not hOAT2, mediated the uptake of bumetanide. HOAT3 and hOAT4 but not hOAT1 mediated the efflux of bumetanide. HOAT1 and hOAT3, but not hOAT2 and hOAT4 mediated the uptake of furosemide. In conclusion, it was suggested that hOAT1 may play an important role in the basolateral uptake of thiazides, and hOAT3 in the uptake of loop diuretics. In addition, it was also suggested that bumetanide taken up by hOAT3 and/or hOAT1 is excreted into the urine by hOAT4.

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Diuretics cause natriuresis, and are therefore used to treat patients with volume overload including hypertension, liver cirrhosis, nephrotic syndrome and congestive heart failure (Ives, 2001). Thiazides and loop diuretics exhibit their diuretic effects from the luminal side by inhibiting the Na^+-Cl^- cotransporter of the distal tubule and the Na^+-K^+-$2Cl^-$ cotransporter of the loop of Henle, respectively (Ives, 2001). In addition, since the binding of diuretics to plasma proteins is generally high (more than 90%), tubular secretion is the main route of urinary excretion of the diuretics. Thus, tubular secretion has been thought to be critical for the action of loop and thiazide diuretics. Renal tubular secretion of diuretics has been demonstrated in studies including the secretion of bumetanide and furosemide in the isolated perfused rat kidney (Berkersky and Popick, 1986; Lee et al., 1986) and renal tubular secretion of chlorothiazide and hydrochlorothiazide in the avian kidney (Odlind and Lonnerholm, 1982). As shown in Table 1, thiazide and loop diuretics, which carry as a common chemical characteristic a sulfamoyl group (sulfonamide diuretics), are weak organic acids. Consistent with this, the involvement of the organic anion transport system in the tubular secretion of diuretics has been suggested in studies including bumetanide inhibition of para-aminohippuric acid (PAH) transport in rat renal slices (Gemba et al., 1981) and PAH inhibition of furosemide excretion in the rabbit (Bidiville and Roch-Ramel, 1986).

Carbonic anhydrase plays an important role in bicarbonate reabsorption from the proximal tubule (Ives, 2001). Acetazolamide, a major carbonic anhydrase inhibitor, blocks the reabsorption of sodium bicarbonate, resulting in an alkaline diuresis with loss of sodium and bicarbonate in the urine. Tubular secretion of acetazolamide was demonstrated to be important for the elimination of this drug in the study using the isolated rat perfused kidney (Taft and Sweeney,

1995).

The secretion of numerous organic anions, including endogenous metabolites, drugs and xenobiotics, is an important physiological function 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. Recently, cDNAs encoding the human organic anion transporter (hOAT) family have been successively cloned including hOAT1 (Reid et al., 1998; Hosoyamada et al., 1999), hOAT2 (Enomoto et al., 2002), hOAT3 (Cha et al., 2001) and hOAT4 (Cha et al., 2000). As shown Fig. 1, hOAT1, hOAT2 and hOAT3 have been shown to be localized to the basolateral side of the proximal tubule (Hosoyamada et al., 1999; Cha et al., 2001; Motohashi et al., 2002; Enomoto et al., 2002), whereas hOAT4 is localized to the apical side of the proximal tubule (Cha et al., 2000).

Although Uwai et al. (2000) have already demonstrated the interactions of rat OAT1 (rOAT1) with diuretics using an oocyte expression system, the molecular mechanisms underlying the renal handling of diuretics have been poorly clarified. Thus, the purpose of this study was to elucidate the interactions of hOATs with diuretics using the second segment of the proximal tubule (S_2) cells stably expressing hOAT1, hOAT2, hOAT3 and hOAT4 (S_2 hOAT1, S_2 hOAT2, S_2 hOAT3 and S_2 hOAT4). Diuretics tested in the current study are listed in Table 1.

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Materials and Methods

Materials

[^{14}C] PAH (1.86 GBq/mmol), [^{3}H]prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) (6808 GBq/mmol), [^{3}H]estrone sulfate (ES) (1961 GBq/mmol) and [^{14}C]tetraethylammonium (TEA) (2.035 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). [^{3}H]bumetanide (185.6 GBq/mmol) was purchased from Muromachi Chemicals (Tokyo, Japan). Diuretics were obtained from Sigma Chemical Co. (St. Louis, MO). Other materials used included fetal bovine serum, trypsin and geneticin from Invitrogen (Carlsbad, CA), recombinant epidermal growth factor from Wakunaga (Hiroshima, Japan), insulin from Shimizu (Shizuoka, Japan), RITC 80-7 culture medium from Iwaki Co. (Tokyo, Japan) and TfX-50 from Promega (Madison, WI).

Cell culture

S$_2$ cells were established by culturing the microdissected S$_2$ segment derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen gene. The establishment and characterization of S$_2$ hOAT1, S$_2$ hOAT2, S$_2$ hOAT3 and S$_2$ hOAT4 were reported previously (Takeda et al., 2002a, b). Briefly, the full-length cDNAs of hOAT1, hOAT2, hOAT3 and hOAT4 were subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA), a mammalian expression vector. S$_2$ hOAT1, S$_2$ hOAT2, S$_2$ hOAT3 and S$_2$ hOAT4 were obtained by transfecting S$_2$ cells with pcDNA3.1-hOAT1, pcDNA3.1-hOAT2, pcDNA3.1-hOAT3 and pcDNA3.1-hOAT4, using TfX-50 according to the manufacturer's instructions. S$_2$ cells transfected with pcDNA3.1 lacking an insert were designated as S$_2$ pcDNA 3.1 (mock), and used as control. These cells were grown in a humidified incubator at 33^{o}C and under 5% CO$_2$ using

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RITC 80-7 medium containing 5% fetal bovine serum, 10 mg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor and 400 mg/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 25~35 passages. Clonal cells were isolated using a cloning cylinder and screened by determining the optimal substrate for each transporter, i.e., [^{14}C]PAH for hOAT1 (Hosoyamada et al., 1999), [^{3}H]$PGF_{2\alpha}$ for hOAT2 (Enomoto et al., 2002) and [^{3}H]ES for hOAT3 and hOAT4 (Cha et al., 2000; Cha et al., 2001)

Uptake experiments

Uptake experiments were performed as previously described (Takeda et al., 2002a, b). The S_2 cells were seeded in 24-well tissue culture plates at a density of 1 x 10^5 cells/well. After the cells were cultured for two days, the cells were washed three times with Dulbecco's modified phosphate-buffered saline solution (containing in mM: 137 NaCl, 3 KCl, 8 $NaHPO_4$, 1 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$; pH 7.4), and then preincubated in the same solution in a water bath at 37^{o}C for 10 min. The cells were then incubated in a solution containing various substrates at 37^{o}C for 30 min. 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).

Inhibition study

To evaluate the inhibitory effects of diuretics on organic anion uptake mediated by hOAT1, hOAT2, hOAT3 and hOAT4, the cells were incubated in a

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solution containing either [^{14}C]PAH for 2 min (hOAT1), [^{3}H]PGF$_{2\alpha}$ for 20 sec (hOAT2) or [^{3}H]ES for 2 min (hOAT3 and hOAT4) in the absence or presence of various concentrations of diuretics at 37^{o}C. Each hOAT mediates the transport of each substrate with a high affinity, which is consistent with the original reports (Hosoyamada et al., 1999; Enomoto et al., 2002; Cha et al., 2000, 2001). The K_m value of hOAT1 for PAH is 20. 1 μM, that of hOAT2 for PGF$_{2\alpha}$ is 425 nM, that of hOAT3 for ES is 2.2 μM and that of hOAT4 for ES is 9.9 μM. Diuretics were dissolved in dimethylsulfoxide and diluted with the incubation medium. The final concentration of dimethylsulfoxide in the incubation medium was adjusted to less than 0.2%. We have chosen the incubation time for the inhibition experiment based on the following reasons. One is that the uptake of the standard substrate by each hOAT increased linearly. Another is that the uptake of the standard substrate by each hOAT was much higher than that by mock, as described in previous reports (Takeda et al., 2002a; Enomoto et al., 2002). The uptake by hOAT1, hOAT2, hOAT3 and hOAT4 were approximately 16-fold, 26-fold, 37-fold and 31-fold higher than that by mock, respectively. Thus, even if diuretics exerted any inhibitory effects on organic anion uptake by mock, it would be negligible. Based on these results, we have not performed the inhibition experiments on mock.

Kinetic analysis of inhibition

After preincubation as described above, S$_2$ hOAT1, S$_2$ hOAT2, S$_2$ hOAT3 and S$_2$ hOAT4 were incubated in a solution containing [^{14}C]PAH (hOAT1), [^{3}H]PGF$_{2\alpha}$ (hOAT2) and [^{3}H]ES (hOAT3 and hOAT4) at various concentrations in the absence or presence of diuretics at 37^{o}C either for 2 min (hOAT1, hOAT3 and hOAT4) or 20 sec (hOAT2). The concentrations of diuretics used were as follows; 100 μM hydrochrolothiazide, 20 μM bumetanide, 50 μM

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furosemide and 150 μM acetazolamide for hOAT1; 2000 μM hydrochlorothiazide, 300 μM bumetanide and 1000 μM furosemide for hOAT2; 2000 μM hydrochrolothiazide, 5 μM bumetanide, 25 μM furosemide and 1500 μM acetazolamide for hOAT3; 750 μM bumetanide, 100 μM furosemide and 1000 μM acetazolamide for hOAT4. Analyses of Lineweaver-Burk plots were performed as previously described by us (Babu et al., 2002b).

High performance liquid chromatography (HPLC) analysis of furosemide

After preincubation as described above, S2 hOAT1, S2 hOAT2, S2 hOAT3, S2 hOAT4 and mock were incubated in a solution containing 500 μM furosemide at 37°C for 2 min. After washing the cells with D-PBS, the cells were extracted with 0.1 N NaOH, centrifugated at 14,000 rpm for 10 min, and the supernatants were neutralized with 8N HCl. The intracellular concentrations of furosemide were determined by HPLC as follows. High-performance liquid chromatograph was equipped with a Model 880-PU pump (JASCO Co., Tokyo, Japan), a Model 880-5 degasser (JASCO Co.), a Model 875-UV variance wavelength dtector (JASCO Co.) and a Model chromatocorder 12 (SIC Co., Tokyo, Japan) under following conditions: a reverse phase column (Develosil ODS-UG-5, 250 mm×4.6 mm I.D., Nomura Chemicals. Co., Aichi, Japan) with a guard column (Develosil ODS-UG-5, 10 mm × 4.0 mm I.D., Nomura Chemicals. Co.); mobile phase, acetonitrile/water/acetic acid=70/30/0.1 v/v; flow rate, 1.0 ml/min; wavelength, 270 nm; and temperature, ambient temperature.

Bumetanide efflux

The efflux study was performed as previously described (Takeda et al., 2002a; Babu et al., 2002a, b). The S2 hOAT1, S2 hOAT3, S2 hOAT4 and mock were seeded in 24-well tissue culture plates at a cell density of 1 x 10^5cells/well.

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After the cells were cultured for two days, the cells were washed three times with D-PBS and then preincubated in the same solution for 10 min in a water bath at 37°C. S2 hOAT1 exhibited the highest uptake activity compared with mock for 5 min incubation (data not shown). Thus, the monolayers were incubated at 37°C in a solution containing 300 nM [^{3}H] bumetanide for 5 min for hOAT1 and 30 min for hOAT3 and hOAT4, washed immediately with D-PBS, and incubated at 37°C in 500 μl of D-PBS. After incubation for the indicated periods, 50 ul of supernatant was collected. After the incubation, the medium was aspirated immediately and the cell monolayers were washed three times with the medium and solubilized in 0.5 ml of 0.1 N sodium hydroxide. The amount of bumetanide in the supernatant and in the cell lysate was measured. The rate of efflux at each time point was calculated using the following formula; (effluxed bumetanide by S2 hOATs − effluxed bumetanide by mock) ／ (bumetanide accumulated by S2 hOATs at time 0− bumetanide accumulated by mock at time 0).

Statistical Analysis

Data are expressed as means ± S.E. Statistical differences were determined using one-way ANOVA with Dunnett's post-hoc test for the inhibition experiments and Student's *t*-test for the uptake experiments. Differences were considered significant at $P<0.05$.

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Results

Effects of diuretics on organic anion uptake mediated by hOATs

We examined the inhibitory effects of various concentrations of diuretics on the organic anion uptake mediated by hOAT1, hOAT2 and hOAT3 and hOAT4. Fig. 2 shows the effects of various concentrations of bumetanide on the organic anion uptake mediated by hOAT1, hOAT2, hOAT3 and hOAT4. Bumetanide inhibited the organic anion uptake mediated by hOAT1 (A), hOAT2 (B), hOAT3 (C) and hOAT4 (D) in a dose-dependent manner (N=8, *P<0.001, **P<0.01 and ***P<0.05 vs. control). Similarly, other diuretics tested also inhibited the organic anion uptake mediated by hOAT1, hOAT2, hOAT3 and hOAT4 in a dose-dependent manner, whereas acetazolamide and methazolamide did not inhibit that by hOAT2, and cyclothiazide, hydrochlorothiazide and metazolamide did not inhibit that by hOAT4. The IC_{50} values are listed in Table 2.

In order to further elucidate the inhibitory effects of diuretics on the organic anion uptake mediated by hOATs, we performed the kinetic analyses of inhibitory effects. As shown in Fig. 3, bumetanide competitively inhibited the organic anion uptake mediated by hOAT1 (A), hOAT2 (B), hOAT3 (C) and hOAT4 (D). Competitive inhibition was also seen for the inhibitory effects of hydrochlorothiazide, furosemide and acetazolamide for hOAT1, hydrochlorothiazide and furosemide for hOAT2, hydrochlorothiazide, furosemide and acetazolamide for hOAT3, and furosemide and acetazolamide for hOAT4 (data not shown).

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Bumetanide uptake mediated by hOATs

In order to determine whether hOATs mediate the uptake of diuretics, we evaluated the uptake activities of [^{3}H]bumetanide by hOATs. As shown in Figs. 4A, B and C, the uptake rates of [^{3}H]bumetanide by hOAT1, hOAT3 and hOAT4, but not by hOAT2, were higher than those by mock (N=8, *P<0.001, **P<0.01 and ***P<0.05 vs. control). In order to further elucidate the transport property of bumetanide mediated by hOAT3 and hOAT4, we performed kinetic analysis of bumetanide uptake in S2 hOAT3 and S2 hOAT4. As shown in Figs. 5, hOAT3 (A) and hOAT4 (C) mediated the dose-dependent uptake of bumetanide and analysis of the Eadie-Hofstee revealed that the K_m values for hOAT3- (B) and hOAT4- (D) mediated bumetanide uptake was 1586±69.4 nM (N=6) and 306 ±63.2 nM (N=6), respectively.

Bumetanide efflux by hOATs

In order to determine whether hOAT1, hOAT3 and hOAT4 mediate the efflux of bumetanide, we measured the efflux of bumetanide from S2 hOAT1, S2 hOAT3, S2 hOAT4 and mock preincubated in a solution containing bumetanide. As shown in Fig. 6, when the efflux from mock was subtracted, S2 hOAT3 (A) and S2 hOAT4 (B) mediated the efflux of bumatnide, whereas S2 hOAT1 did not (data not shown).

HPLC analysis of furosemide

In order to elucidate whether hOATs mediate the uptake of furosemide, we have measured intracellular furosemide content using HPLC. As shown in Fig. 7, the uptake rates of furosemide by hOAT1 and hOAT3, but not hOAT2 and hOAT4, were higher than those by mock (N=6, *P<0.05 and **P<0.01 vs. mock).

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Discussion

HOAT1 and hOAT3 have been shown to mediate the transport of nonsteroidal anti-inflammatory drugs, anti-tumor drugs, histamine H_2-receptor antagonist, prostaglandins, anti-virals, angiotensin-converting enzyme inhibitors and beta-lactam antibiotics (Hosoyamada et al., 1999; Cha et al., 2001; Takeda et al., 2002a,b; Khamdang et al., 2002). In addition, it is reasonable to think that both hOAT1 and hOAT3 exhibit transport properties as an exchanger (Sekine et al., 1997; Hosoyamada et al., 1999; Cha et al., 2001; Sweet et al., 2003). However, some differences in characteristics exist between hOAT1 and hOAT3, such as substrate specificity and localization: hOAT1 is localized at S_2 segment of the proximal tubule (Hosoyamada et al., 1999), whereas hOAT3 is localized at the first, second and third segments (S_1, S_2 and S_3) of the proximal tubule (Cha et al., 2001). HOAT2 also mediates the basolateral uptake of organic anions including salicylate and $PGF_{2\alpha}$ (Enomoto et al., 2002). On the other hand, hOAT4 also mediates the apical transport of various anionic drugs in the proximal tubule (Babu et al., 2002b); however, this transporter exhibits relatively narrow substrate recognition compared to hOAT1 and hOAT3 (Cha et al., 2000). Using the cells stably expressing hOATs, we have elucidated the interactions of hOATs with diuretics.

Comparing the IC_{50} values of diuretics among hOATs, hOAT1 exhibited the highest affinity for chlorothiazide, hydrochlorothiazide, trichlorothiazide and acetazolamide; and hOAT3 exhibited that for bumetanide, ethacrynic acid, furosemide and methazolamide. In contrast, hOAT2 exhibited the lowest

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affinity for ethacrynic acid, furosemide and acetazolamide; hOAT4 exhibited that for cyclothiazide, hydrochlorothiazide and bumetanide; and hOAT2 and hOAT4 exhibited that for chlorothiazide, trichlorothiazide and methazolamide. Thus, hOAT1 and hOAT3 generally exhibited higher affinity interactions with diuretics than hOAT2 and hOAT4. In addition, generally, hOAT1 appears to exhibit the highest affinity interactions with thiazides except cyclothiazide, and hOAT3 appears to do that with loop diuretics. On the other hand, it was suggested that hOAT4 exhibits higher affinity with loop diuretics than with thiazides. Furthermore, the interactions of hOATs with carbonic anhydrase inhibitors were the weakest among the diuretics tested.

HOAT3 mediated the uptake as well as efflux of bumetanide, and hOAT1 mediated the uptake of bumetanide. It is possible that the reason why hOAT1 exhibited no efflux activity of bumetanide is because it did not show as much bumetanide uptake activity as hOAT3 and hOAT4, as shown in Fig. 4. The magnitude of bumetanide uptake by hOAT3 was approximately 20-fold larger than that by hOAT1. The IC_{50} values of bumetanide for hOAT1 and hOAT3 in our system were similar to that of bumetanide for the basolateral PAH transport system in the isolated S_2 segments of rabbit kidney proximal tubules, 3 μM (Bartel et al., 1993). Based on these findings, it was suggested that hOAT3 mediates the uptake as well as efflux of bumetanide, and hOAT1 mediates the uptake of bumetanide in the basolateral side of the proximal tubule.

HOAT1 and hOAT3 exhibited high affinity interactions with furosemide. In addition, hOAT1 and hOAT3 mediated the uptake of furosemide. It was reported that furosemide inhibited the basolateral PAH transport system in the isolated S_2 segments of rabbit kidney proximal tubules, with an IC_{50} value of 40 μM (Bartel et al., 1993). The value was similar to those of furosemide for

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hOAT1 and hOAT3. Bidiville and Roch-Ramel (1986) demonstrated that probenecid inhibited the renal secretion of furosemide by 95%, whereas PAH depressed the renal secretion of furosemide by only 44 to 66% in rabbit kidneys. Based on this, Uwai et al. (2000) hypothesized the existence of a transporter besides OAT1 that mediates the renal excretion of furosemide. Considering these lines of evidence, it was suggested that hOAT1 and hOAT3 mediate the transport of furosemide in the basolateral side of the proximal tubule.

Furosemide uptake activity by hOAT1 and the IC_{50} value of furosemide for hOAT1 were consistent with those of rOAT1 (Uwai et al., 2000). However, the IC_{50} values of acetazolamide, bumetanide and hydrochlorothiazide for hOAT1 were different between humans and rats (more than threefold difference; Zhang et al., 1998). The reason for this may be due to the interspecies difference in the interactions of OAT1 with these drugs between humans and rats or the difference concerning the expression system, i.e., mammalian expression system for hOAT1 versus *Xenopus oocyte* expression system for rOAT1.

In addition to bumetanide and furosemide, since hOAT1 and hOAT3 exhibited high affinity interactions with ethacrynic acid and thiazides, it is speculated that these transporters play important roles in the basolateral uptake of these diuretics in the proximal tubule. The IC_{50} value of hydrochrolothiazide for hOAT1 (67.3 μM) was comparable to that of hydrochlorothiazide for cellular PAH uptake across the basolateral membrane of isolated S_2 segments in rabbits, i.e., 140 μM (Bartel et al., 1993). In contrast, the interactions of hOAT1 and hOAT3 with carbonic anhydrase inhibitors were the weakest among the diuretics tested. However, since hOAT1 and hOAT3

appear to be a major transporters mediating the basolateral uptake of various anionic drugs (Hosoyamada et al., 1999; Cha et al., 2001; Takeda et al., 2002a,b; Khamdang et al., 2002), it is possible that these two transporters are involved in the transport of carbonic anhydrase inhibitors. The involvement of transporters other than hOAT1 and hOAT3 in the basolateral transport of carbonic anhydrase inhibitors in the proximal tubule should also be considered. In contrast, comparing the affinities of hOATs with diuretics, hOAT2 may not significantly contribute to the transport of all the diuretics tested except cyclothiazide.

HOAT4 mediated the uptake and the efflux of bumetanide, and exhibited higher affinity interactions with loop diuretics than with other diuretics. Thus, it is suggested that hOAT4 mediates the reabsorption or the efflux of loop diuretics in the apical side of the proximal tubule. In addition, considering the above-mentioned evidence that hOAT1 and hOAT3 mediate the basolateral uptake of bumetanide, it is possible that bumetanide taken up by hOAT1 and/or hOAT3 is effluxed into the urine by hOAT4, which represents the mechanism of the unidirectional transport of bumetanide. Other than the hOAT family, the interactions of other human transporters and human homologues of rodent transporters mediating organic anion transport in the apical side of the proximal tubule with diuretics should be investigated, including OAT-K1 (Saito et al., 1996), OAT-K2 (Masuda et al., 1999), organic anion-transporting peptide 1 (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), MRP4 (van Aubel et al., 2002) and human-type I sodium-dependent inorganic phosphate transporter (Uchino et al., 2000). In this regard, the interaction of OAT-K1 with furosemide, as well as that of OAT-K2 with furosemide and

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spironolactone, was reported (Saito et al., 1996; Masuda et al., 1999). In addition, furosemide was shown to inhibit N-ethylmalemide glutathione uptake mediated by MRP2 (Bakos et al., 2000).

The therapeutically relevant plasma concentration of a drug is considered to be within fivefold of the maximum steady-state plasma concentration of a drug (Zhang et al.,2000). Considering this as well as the plasma concentrations of diuretics and their unbound fractions (Hardman and Limbird, 2001), the therapeutically relevant concentrations of unbound hydrochlorothiazide, trichloromethiazide, bumetanide, furosemide and acetazolamide are considered to be 2.62, 0.173, 0.22, 0.41 and 33.8 μM, respectively. Thus, the IC_{50} values of hOAT1 for hydrochlorothiazide, trichlormethiazide and acetazolamide are approximately 25, 100 and 2 times higher than the therapeutically relevant concentrations of unbound drugs, and those of hOAT3 for bumetanide and furosemide are approximately 3 and 18 times higher than the therapeutically relevant concentrations of unbound drugs. Thus, it is predicted that hOATs could transport these diuretics *in vivo*, whereas the rates of transport vary from drug to drug.

In conclusion, it was suggested that hOAT1 plays an important role in the basolateral uptake of thiazides, and hOAT3 in the uptake of loop diuretics in the proximal tubule. In addition, it was also suggested that bumetanide taken up by hOAT1 and/or hOAT3 is excreted into the urine by hOAT4, which may be the mechanism underlying the tubular secretion of bumetanide.

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Footnote

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References

Abe T, Kakyo M, Sakagami H, Tokui T, Nishio T, Tanemoto M, Nomura H, Hebert SC, Matsuno S, Kondo H and Yawo H (1998) Molecular characterization and tissue distribution of a new organic anion transporter subtype (oatp3) that transports thyroid hormones and taurocholate and comparison with oatp2. *J Biol Chem* **273**:22395-22401.

Babu E, Takeda M, Narikawa S, Kobayashi Y, Yamamoto T, Cha SH, Sekine T, Sakthisekaran D and Endou H (2002a) Human organic anion transporters mediate the transport of tetracycline. *Jpn J Pharmacol* **88**: 69-76.

Babu E, Takeda M, Narikawa S, Kobayashi Y, Tojo A, Cha SH, Sekine T, Sakthisekaran D and Endou H (2002b) Characterization of ochratoxin A transport by human organic anion transporter 4. *Biochim Biophys Acta* **1540**:64-75.

Bakos E, Evers R, Sinko E, Varadi A, Borst P and Sarkadi B (2000) Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. *Mol Pharmacol* **57**:760-768.

Bartel C, Wirtz C, Brandle E and Greven J (1993) Interaction of thiazide and loop diuretics with the basolateral para-aminohippurate transport system in isolated S_2 segments of rabbit kidney proximal tubules. *J Pharmacol Exp Ther* **266**:972-927.

Bekersky I and Popick A (1986) Disposition of bumetanide in the isolated

perfused rat kidney: effects of probenecid and dose response. *Am J Cardiol* **57**: 33A-37A.

Bidiville J and Roch-Ramel F (1986) Competition of organic anions for furosemide and p-aminohippurate secretion in the rabbit. *J Pharmacol Exp Ther* **237**:636-643.

Cha SH, Sekine T, Kusuhara H, Yu E, Kim YJ, Kim DK, Sugiyama Y, Kanai Y and Endou H (2000) Molecular cloning and characterization of multispecific organic anion transporter 4 expressed in the placenta. *J Biol Chem* **275**:4507-4512.

Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T and Endou H (2001) Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59**:1277-1286.

Enomoto A, Takeda M, Shimoda M, Narikawa S, Kobayashi Y, Kobayashi Y, Yamamoto T, Sekine T, Cha SH, Niwa T and Endou H (2002) Interaction of human organic anion transporters 2 and 4 with organic anion transport inhibitors. *J Pharmacol Exp Ther* **301**:797-802.

Gemba M, Taniguchi M and Matsushima Y (1981) Effect of bumetanide on p-aminohippurate transport in renal cortical slices. *J Pharmacobiodyn* 4:162-166.

Hardman, J.G., Limbird, L.E., 2001. In The pharmacological basis of therapeutics (tenth edition), McGraw-Hills, New York.

Hosoyamada M, Sekine T, Kanai Y and Endou H (1999) Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. *Am J Physiol* **276**:F122-F128.

Ives HE (2001) Diuretic agents. *Basic & Clinical Pharmacology* (eight edition), pp 245-265, edited by Katzung BG, McGraw-Hill, New York.

Jacquemin E, Hagenbuch B, Stieger B, Wolkoff AW and Meier PJ (1994) Expression cloning of a rat liver Na^+-independent organic anion transporter. *Proc Natl Acad Sci USA* **91**:133-137.

Khamdang S, Takeda M, Narikawa S, Enomoto A, Anzai N, Piyachaturawat P and Endou H (2002) Interactions of human organic anion transporters and human organic cation transporters with nonsteroidal anti-inflammatory drugs. *J Pharmacol Exp Ther* **303**: 534-539.

Lee LJ, Cook JA and Smith DE (1986) Renal transport kinetics of furosemide in the isolated perfused rat kidney. *J Pharamacokinet Biopharm* **14**: 157-174.

Leier I, Hummel-Eisenbeiss J, Cui Y and Keppler D (2000) ATP-dependent para-aminohippurate transport by apical multidrug resistance protein MRP2. *Kidney Int* **57**: 1636-1642.

Masuda S, Ibaramoto K, Takeuchi A, Takeuchi A, Saito H, Hashimoto Y and Inui KI (1999) Cloning and functional characterization of a new multispecific organic anion transporter, OAT-K2, in rat kidney. *Mol Pharmacol* **55**: 743-753.

Motohashi H, Sakurai Y, Saito H, Masuda S, Urakami Y, Goto M, Fukatsu A, Ogawa O and Inui K (2002) Gene expression levels and immunolocalization of organic ion transporters in the human kidney. *J Am Soc Nephrol* **13**:866-874.

Noe B, Hagenbuch B, Stieger B and Meier PJ (1997) Isolation of a multispecific organic anion and cardiac glycoside transporter from rat brain. *Proc Natl Acad Sci USA* **94**:10346-10350.

Odlind B and Lonnerholm G (1982) Renal tubular secretion and effects of chlorothiazide, hydrochlorothiazide and clopamide: a study in the avian kidney. *Acta Pharmacol Toxicol* **51**:187-197.

Reid G, Wolff NA, Dautzenberg FM and Burckhardt G (1998) Cloning of a human renal p-aminohippurate transporter, hROAT1. *Kidney Blood Press Res*

121:233-237.

Saito H, Masuda S and Inui KI (1996) Cloning and functional characterization of a novel rat organic anion transporter mediating basolateral uptake of methotrexate in the kidney. *J Biol Chem* **271**: 20719-20725.

Sekine T, Watanabe N, Hosoyamada M, Kanai Y and Endou H (1997) Expression cloning and characterization of a novel multispecific organic anion transporter. *J Biol Chem* **272**: 18526-18529.

Sweet DH, Chan LM, Walden R, Yang XP and Miller DS (2003) Organic anion transporter 3 (Slc22a8) is a dicarboxylate exchanger indirectly coupled to the Na^+ gradient. Am J Physiol Renal Physiol **284**:F763-769.

Taft DR and Sweeney KR (1995) The influence of protein binding on the elimination of acetazolamide by the isolated perfused rat kidney: evidence of albumin-mediated tubular secretion. *J Pharmacol Exp Ther* **274**:752-760.

Takeda M, Babu E, Narikawa S and Endou H (2002a) Interaction of human organic anion transporters with various cephalosporin antibiotics. *Eur J Pharmacol* **438**:137-142.

Takeda M, Khamdang S, Narikawa S, Kobayashi Y, Yamamoto T, Kimura H, Cha SH, Sekine T and Endou H (2002b) Human organic anion transporters and organic cation transporters mediate renal antiviral transport. *J Pharmacol Exp Ther* **300**: 918-924.

Uchino H, Tamai I, Yamashita K, Minemoto Y, Sai Y, Yabuuchi H, Miyamoto KI, Takeda E and Tsuji A (2000) *p*-aminohippuric acid transport at renal apical membrane mediated by human inorganic phosphate transporter NPT1. *Biochem Biophys Res Commun* **270**: 254-259.

Uwai Y, Saito H, Hashimoto Y and Inui KI (2000) Interaction and transport of thiazide diuretics, loop diuretics, and acetazolamide via rat renal organic anion

transporter rOAT1. *J Pharmacol Exp Ther* **295**:261-265.

van Aubel RA, Smeets PH, Peters JG, Bindels RJ and Russel FG (2002) The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. *J Am Soc Nephrol* **13**:595-603.

Zhang L, Schaner ME and Giacomini KM (1998) Functional characterization of an organic cation transporter (hOCT1) in a transiently transfected human cell line (HeLa). *J Pharmacol Exp Ther* **286**: 354-361.

Zhang L, Gorset W, Washington CB, Blaschke TF, Kroetz DL and Giacomini KM (2000) Interactions of HIV protease inhibitors with a human organic cation transporter in a mammalian expression system. *Drug Metab Disp* **28**: 329-334.

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Legends for figures

Fig. 1 Diagram of proximal tubular cells and membrane localization of hOATs.

Fig. 2 Effects of various concentrations of bumetanide on the organic anion uptake mediated by hOATs. S2 hOAT1 (A), S2 hOAT2 (B), S2 hOAT3 (C) and S2 hOAT4 (D) were incubated in solution containing 5 μM [^{14}C]PAH (hOAT1), 50 nM [^{3}H]PGF$_{2\alpha}$ (hOAT2) or 50 nM [^{3}H]ES (hOAT3 and hOAT4) in the presence of various concentrations of bumetanide at 37^{o}C for 2 min (hOAT1, hOAT3 and hOAT4) or 20 sec (hOAT2). Each value represents the mean ± S.E. of eight monolayers from two separate experiments. *P<0.001, **P<0.01 and ***P<0.05 vs. control.

Fig. 3 Kinetic analyses of the effects of bumetanide on organic anion uptake mediated by hOATs. S2 hOAT1, S2 hOAT2, S2 hOAT3 and S2 hOAT4 were incubated in solution containing either [^{14}C]PAH (hOAT1), [^{3}H]PGF$_{2\alpha}$ (hOAT2) or [^{3}H]ES (hOAT3 and hOAT4) at various concentrations in the absence or presence of bumetanide either at 20 μM (hOAT1), 300 μM (hOAT2), 5 μM (hOAT3) or 750 μM (hOAT4) at 37^{o}C for 2 min (hOAT1, hOAT3 and hOAT4) or 20 sec (hOAT2). Analyses of Lineweaver-Burk plots were performed. (A): hOAT1; (B): hOAT2; (C): hOAT3; (D): hOAT4. Each value represents the mean ± S.E. of four monolayers from one typical experiment of two separate

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experiments.

Fig. 4 Bumetanide uptake by hOATs. (A) S2 hOAT1, S2 hOAT2, S2 hOAT3, S2 hOAT4 and mock were incubated in solution containing 100 nM [^{3}H]bumetanide at 37^{o}C for 5 min. S2 hOAT3 (B), S2 hOAT4 (C) and mock were incubated in solution containing 100 nM [^{3}H]bumetanide at 37^{o}C for incubation times of 30 min (hOAT3) and 15 min (hOAT4). Each value represents the mean ± S.E. of eight monolayers from two separate experiments. *P<0.001, **P<0.01 and ***P<0.05 vs. mock.

Fig. 5 Dose-dependent uptake of bumetanide by hOAT3 and hOAT4, and its kinetic analysis. S2 hOAT3 (A), S2 hOAT4 (C) and mock were incubated in solution containing various concentrations of [^{3}H]bumetanide at 37^{o}C for 1 min. Eadie-Hofstee plot analysis of bumetanide uptake by hOAT3 (B) and hOAT4 (D) was performed. Each value represents the mean ±S.E. of four monolayers from one typical experiment of two separate experiments.

Fig. 6 Efflux of bumetanide by hOAT3 and hOAT4. After a 30-min incubation in solution containing 300 nM bumetanide at 37°C, S2 hOAT3 (A), S2 hOAT4 (B) and mock were incubated at 37°C for 30 min. The amount of bumetanide in the supernatant and in the cell lysate was determined. The rate of efflux at each time point was calculated with the following formula; (effluxed bumetanide by S2 hOAT3 or S2 hOAT4 − effluxed bumetanide by mock) ／ (bumetanide accumulated by S2 hOAT3 or S2 hOAT4 at time 0 − bumetanide accumulated by mock at time 0). Each value represents the mean ± S.E. of four determinations from one typical experiment of two separate experiments.

Fig. 7 Furosemide uptake by hOATs. S2 hOAT1, S2 hOAT2, S2 hOAT3, S2 hOAT4 and mock were incubated in solution containing 500 μM furosemide at 37^{o}C for 2 min. Each value represents the mean±S.E. of six monolayers from

two separate experiments. After washing the cells with a solution, the intracellular contents were determined using HPLC. *P<0.05 and **P<0.01 vs. mock.

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Table 1 Diuretic drugs tested in this study

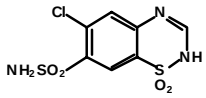
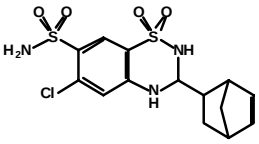
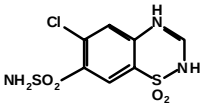
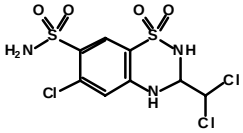
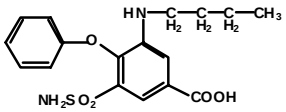
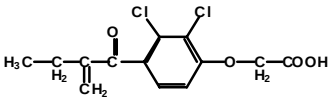
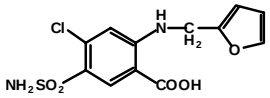
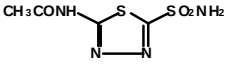
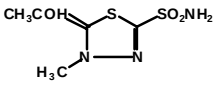
Name	Chemical structure
Thiazide	
Chlorothiazide	
Cyclothiazide	
Hydrochlorothiazide	
Trichlormethiazide	
Loop diuretic	
Bumetanide	
Ethacrynic acid	
Furosemide	
Carbonic anhydrase inhibitor	
Acetazolamide	
Methazolamide	

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Table 2 IC_{50} values of various diuretics for organic anion uptake mediated by hOAT1, hOAT2, hOAT3 and hOAT4

Diuretics	hOAT1	hOAT2	hOAT3	hOAT4
				(μM)
Thiazide				
Chlorothiazide	3.78±0.41	2205±143	65.3±4.83	2632±132
Cyclothiazide	84.3±7.32	39.2±4.10	27.9±2.12	5000>
Hydrochlorothiazide	67.3±5.32	1023±53.2	942±76.5	5000>
Trichlormethiazide	19.2±1.12	1220±101	71.2±4.32	1505±98.3
Loop diuretic				
Bumetanide	7.60±0.58	77.5±5.38	0.75±0.07	348±23.2
Ethacrynic acid	29.6±1.63	121±9.83	0.58±0.08	8.76±0.91
Furosemide	18.0±1.10	603±46.3	7.31±0.81	44.5±2.53
Carbonic anhydrase inhibitor				
Acetazolamide	75.0±6.52	5000>	816±67.3	425±39.3
Methazolamide	438±32.1	5000>	97.5±9.84	5000>

S_2 hOAT1, S_2 hOAT2, S_2 hOAT3 and S_2 hOAT4 were incubated in a solution containing either 5 μM [^{14}C]PAH (hOAT1), 50 nM [^{3}H] $PGF_{2\alpha}$ (hOAT2) or 50 nM [^{3}H]ES (hOAT3 and hOAT4) in the absence or presence of various concentrations of diuretics for 2 min (hOAT1, hOAT3 and hOAT4) or 20 sec (hOAT2). Each value represents the mean ± S.E. of eight monolayers from two separate experiments.

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Table 3 IC_{50} values of various diuretics for organic anion uptake mediated by hOAT1 and rOAT1

Diuretic	hOAT1	rOAT1*
		(μM)
Acetazolamide	75.0±6.52	1100
Bumetanide	7.60±0.58	1.9
Furosemide	18.0±1.10	7.4
Hydrochlorothiazide	67.3±5.32	265

S_2 hOAT1 was incubated in a solution containing either 5 μM [^{14}C]PAH in the absence or presence of various concentrations of diuretics for 2 min at 37^{o}C. Each value represents the mean ± S.E. of eight monolayers from two separate experiments. *From Uwai et al. (2000) J Pharmacol Exp Ther.

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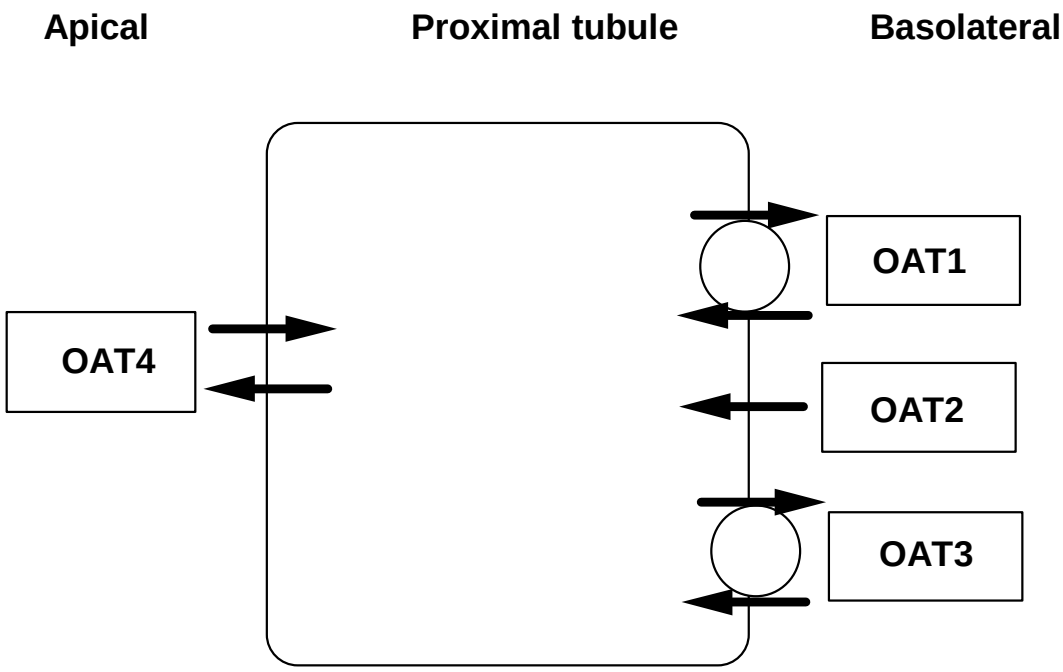

Fig. 1 Hasannejad et al.

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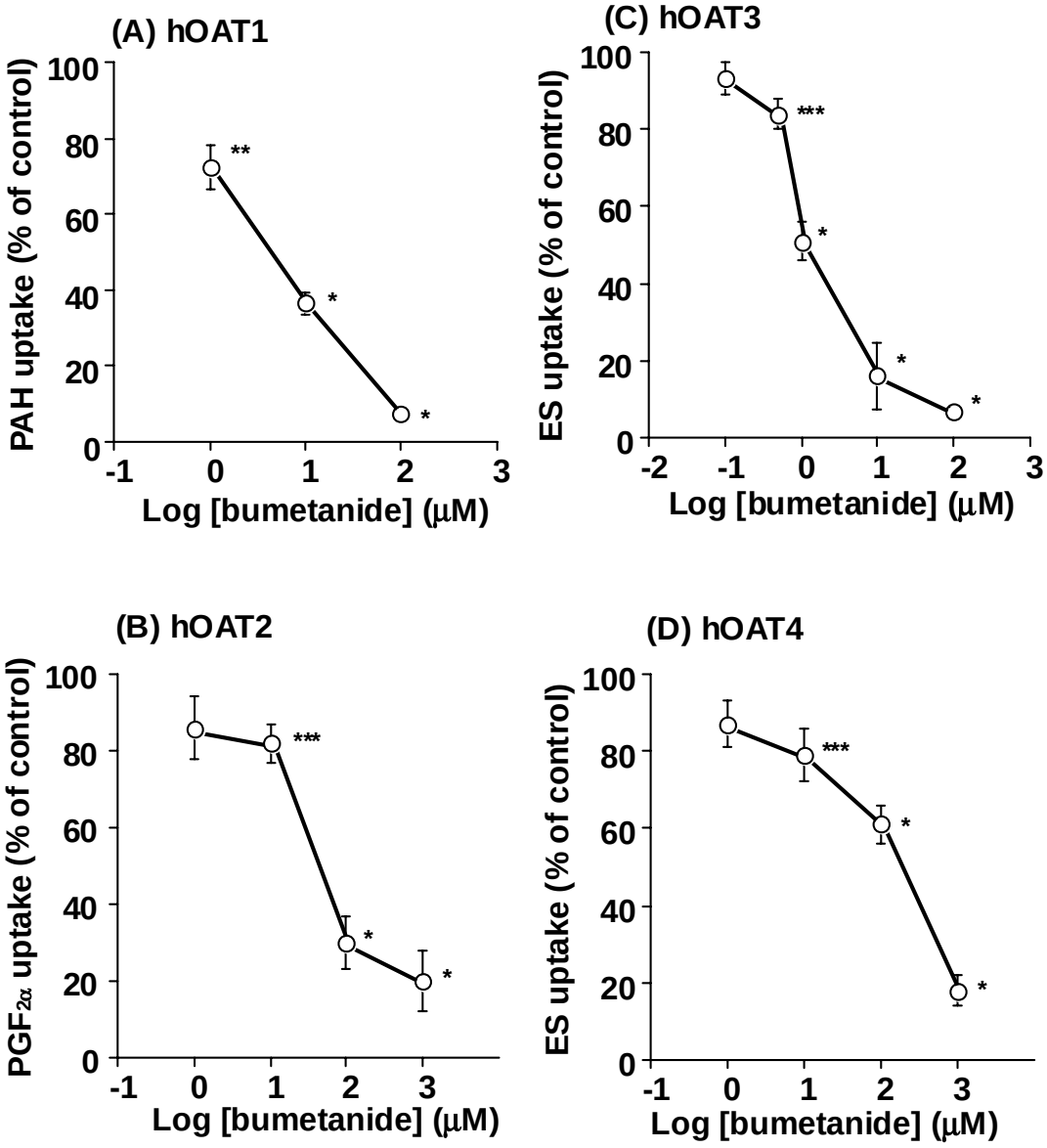

Fig. 2 Hasannejad et al.

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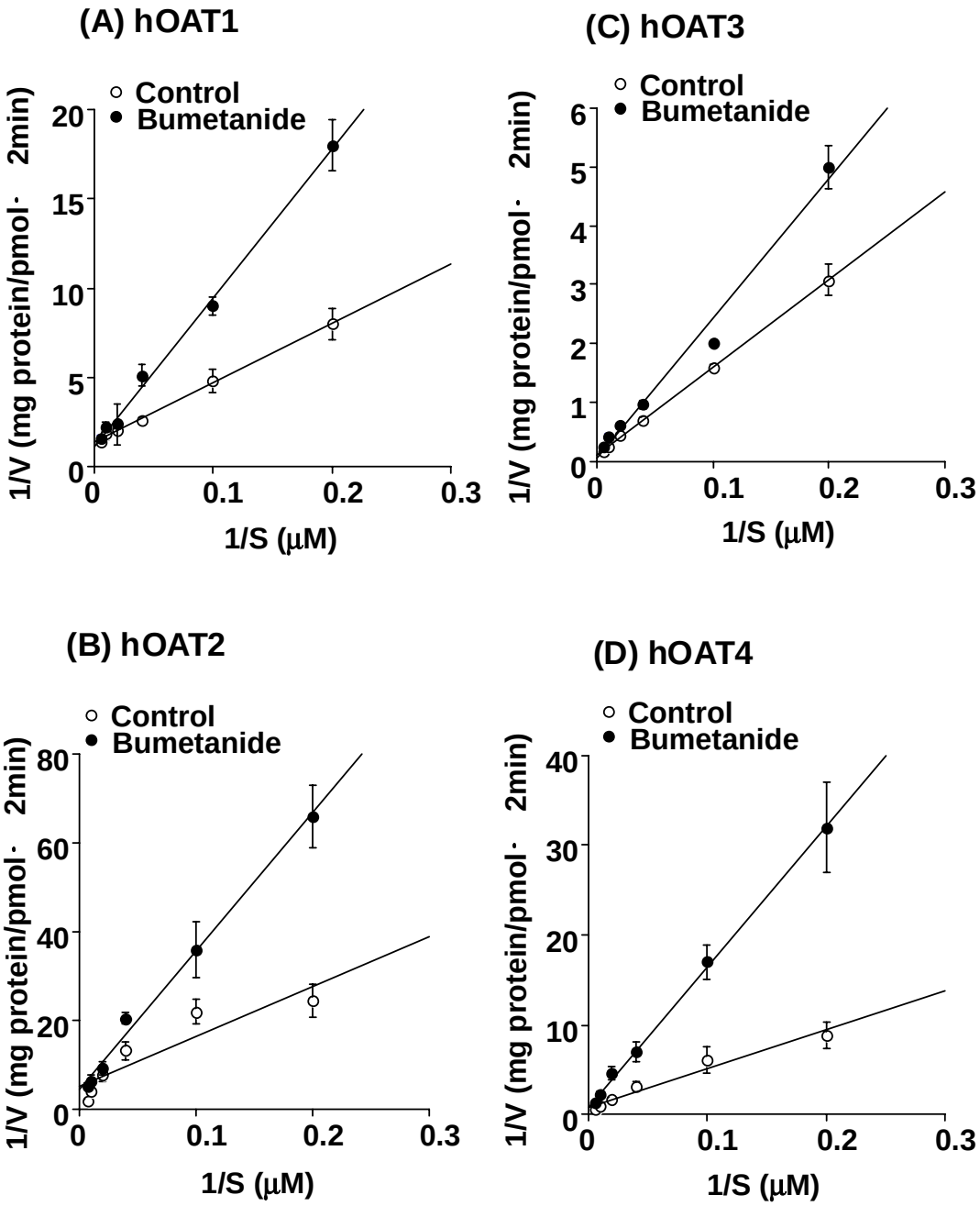

Fig. 3 Hasannejad et al.

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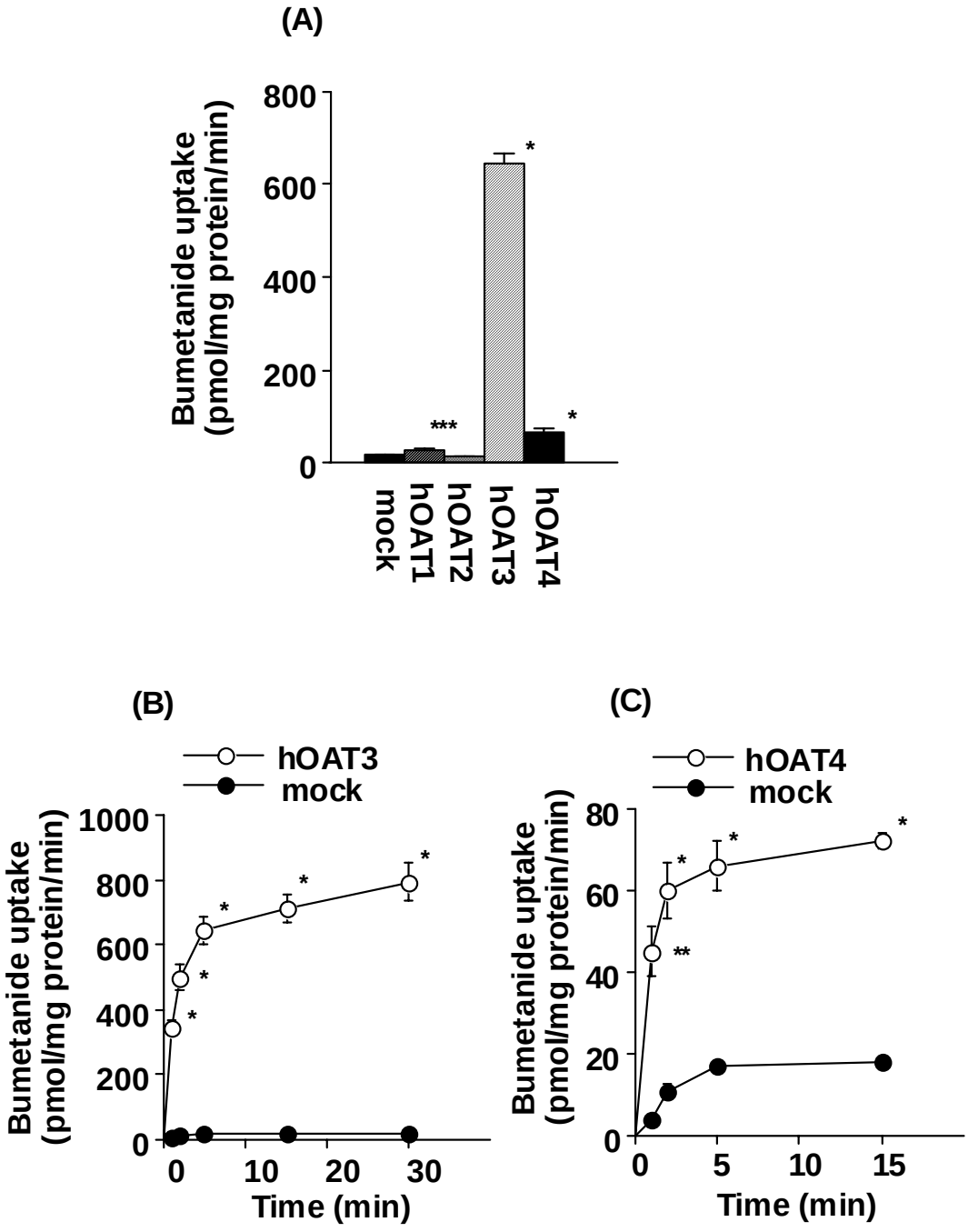

Fig. 4 Hasannejad et al.

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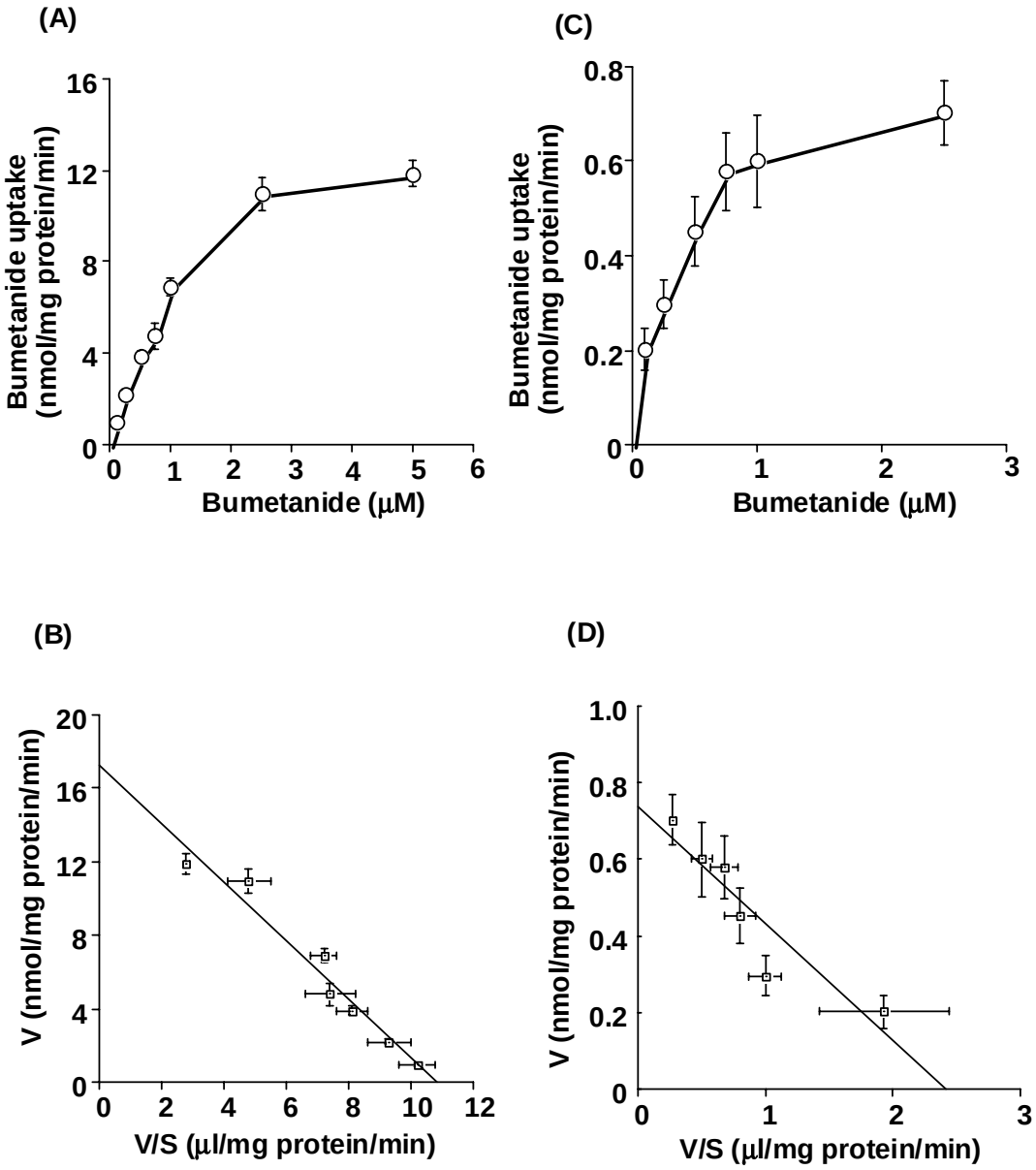

Fig. 5 Hasannejad et al.

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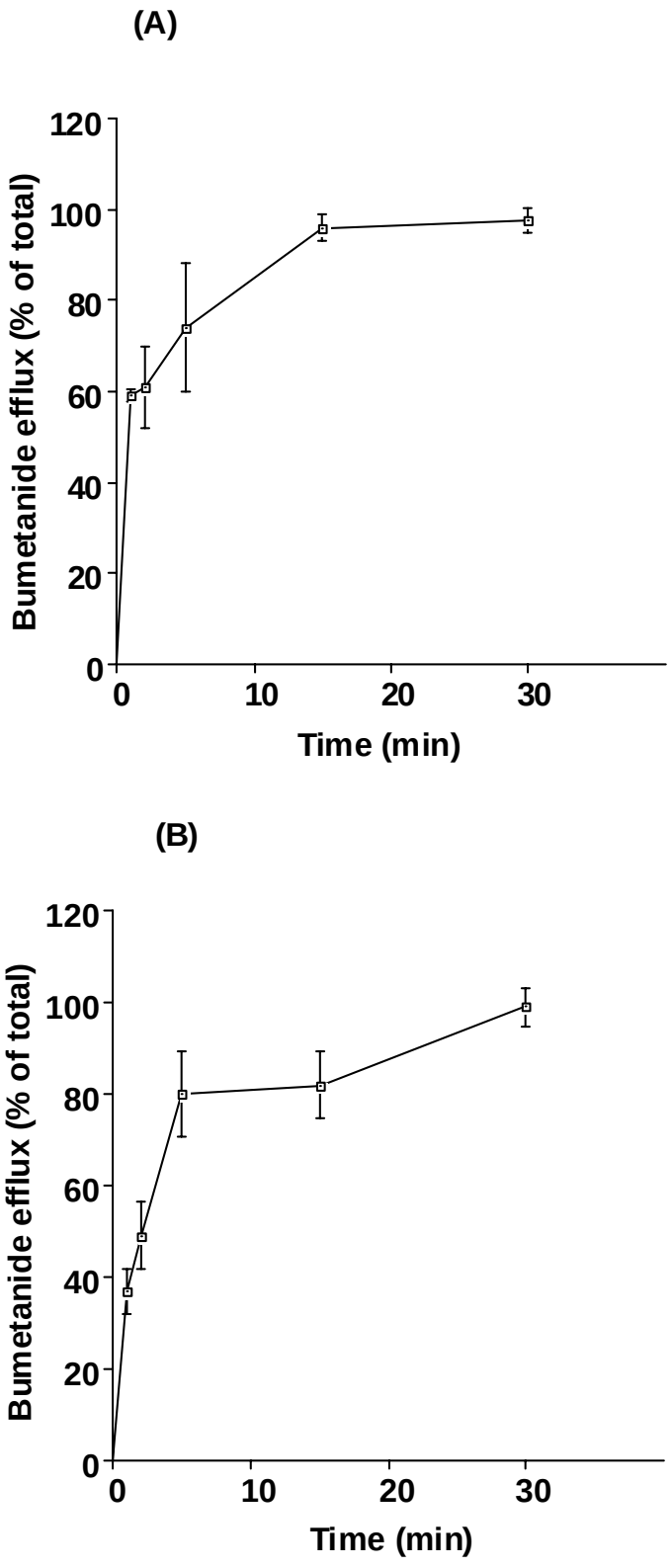

Fig. 6 Hasannejad et al.

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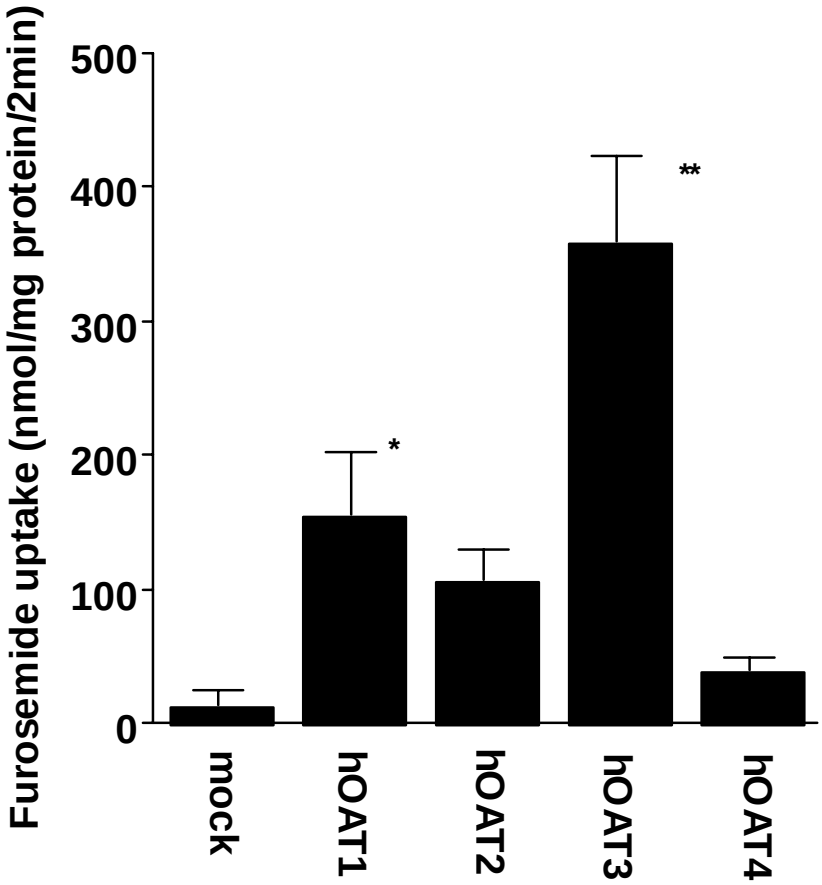

Fig. 7 Hasannejad et al.