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Title:

Inhibition of OAT3-mediated renal uptake as a mechanism for drug-drug interaction between fexofenadine and probenecid

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Running title:

Drug interaction of fexofenadine with probenecid involves OAT3

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Abbreviations: PAH, *p*-aminohippurate; PCG, benzylpenicillin; RND, ranitidine; TEA, tetraethylannmonium; hOATs, human organic anion transporters; hOCTs, human organic cation transporters; OATP8, organic anion transporting peptide 8; AUC, area under the plasma concentration- time curve;

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Abstract

Fexofenadine, a nonsedating antihistamine drug, is effective for the treatment of seasonal allergic rhinitis and chronic urticaria. Simultaneous administration of probenecid increases the plasma concentration of fexofenadine due to an inhibition of its renal elimination in healthy volunteers (Yasui-Furukori et al., *Clin Pharmacol Ther*. 77:17-23, 2005). The purpose of the present study is to investigate the possibility that the drug-drug interaction between fexofenadine and probenecid involves the renal basolateral uptake process. The uptake of fexofenadine was determined in HEK293 cells expressing human organic anion transporter 1 (OAT1/*SLC22A6*), OAT2 (*SLC22A7*), OAT3 (*SLC22A8*) and organic cation transporter 2 (OCT2/*SLC22A2*). Only hOAT3-HEK showed a significantly greater accumulation of fexofenadine than that in vector-HEK, which was saturable with K_m and V_{max} values of 70.2 μM and 120 pmol/min/mg protein, respectively. Inhibition potency of probenecid for the uptake of fexofenadine was compared between hOAT3 and organic anion transporting peptide 1B3 (hOATP1B3), a transporter responsible for the hepatic uptake of fexofenadine (Shimizu et al., *Drug Metab Dispos*. 33:1477-81, 2005). The Ki values were determined to be 1.86 and 282 μM, for hOAT3 and hOATP1B3, respectively, with Hill coefficient of 0.76 and 0.64, respectively. The Ki value of probenecid for hOAT3, but not for hOATP1B3, was significantly lower than the maximum unbound plasma concentration of probenecid at clinical dosages. These results suggest that the renal drug-drug interaction between fexofenadine and probenecid is likely explained by an inhibition of the renal uptake of fexofenadine via hOAT3, at least in part.

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Introduction

The kidney plays important roles in the detoxification of xenobiotics and endogenous wastes as well as maintaining stable levels of electrolytes and nutrients in the body. Urinary excretion consists of glomerular filtration in the glomeruli, tubular secretion across the proximal tubules and reabsorption. Many studies have shown the importance of transporters in the tubular secretion of a large number of organic compounds and a number of studies have described the role of multispecific organic anion and cation transporters (OAT/*SLC22* and OCT/*SLC22*) in the renal uptake of drugs. OCT2 (*SLC22A2*) plays a predominant role in the renal uptake of organic cations in the human kidney, while OCT1 plays a predominat in the hepatic uptake of organic cations in the human liver (Koepsell, 2004; Lee and Kim, 2004; Wright and Dantzler, 2004). Three isoforms of the organic anion transporter family (OAT1/*SLC22A6*, OAT2/SLC22A7 and OAT3/*SLC22A8*) have been identified on the basolateral membrane of the human proximal tubules (Lee and Kim, 2004; Miyazaki et al., 2004; Wright and Dantzler, 2004). It has been suggested that hOAT1 plays an important role in the renal uptake of hydrophilic organic anions with a low molecular weight, while hOAT3 plays an important role in the renal uptake of amphipathic organic anions as well as a basic drug, famotidine (Hasegawa et al., 2003; Tahara et al., 2005a). The mRNA of hOAT2 in the kidney is markedly lower than that of hOAT1 and hOAT3 (Motohashi et al., 2002), and its role in drug transport in the kidney remains unknown. Identification of the basolateral transporters provides a clue to understanding the molecular mechanisms of drug-drug interactions involving tubular secretion. Takeda et al (2002) and Nozaki et al (2004) have shown that rOat3/hOAT3-mediated renal uptake can be a potential drug-drug interaction site with some non-steroidal anti-inflammatory drugs at clinical dosages by comparing their K_i values for rOat3/hOAT3 with the unbound plasma concentration at their clinical dosages. In addition, we have reported that OAT3 could be the site of an interaction between famotidine and probenecid in humans (Tahara et al., 2005a).

Fexofenadine, an active metabolite of terfenadine, is a non-sedating histamine H_1 receptor antagonist that is prescribed for the oral treatment of allergic rhinitis and chronic idiopathic urticaria. After oral administration of [^{14}C]-fexofenadine to healthy volunteers, 92% of the total dose was recovered, 12% in urine and 80% in feces, as the unchanged form (Lippert, 1995). Since the average absolute oral bioavailability of fexofenadine was reported to be 33% (Dresser et al., 2005), about 36% of the bioavailable fexofenadine can be excreted into the urine during a 24-hour period,

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and renal elimination makes a significant contribution to the total body clearance in addition to biliary excretion. Interactions of fexofenadine with drugs and food have been reported. The interactions with rifampicin (Hamman et al., 2001), St John's wort (Wang et al., 2002) and fruit juice (Dresser et al., 2002) caused a reduction in the AUC of fexofenadine after oral administration, and these are hypothesized to include modulation of P-glycoprotein or inhibition of OATP2B1 in the small intestine (Cvetkovic et al., 1999; Nozawa et al., 2004). The interactions with verapamil (Yasui-Furukori et al., 2005) and ketoconazole (Simpson and Jarvis, 2000) increased the AUC of fexofenadine probably because of an increase in the oral absorption produced by inhibition of intestinal P-glycoprotein. Probenecid-treatment caused a significant reduction in the unbound renal clearance of fexofenadine (Yasui-Furukori et al., 2005). Since probenecid is a potent inhibitor of OATs (Tahara et al., 2005a), it is possible that this interaction involves renal transporters, such as OAT1, OAT2 and OAT3.

In the present study, to obtain an insight into the basolateral uptake mechanism of fexofenadine, the uptake was determined in cDNA transfected cells expressing hOAT1, hOAT2, hOAT3 and hOCT2, and the effect of probenecid on the uptake was determined to examine whether it is inhibited by a clinically relevant concentration of probenecid.

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Materials and Methods

Fexofenadine hydrochloride was purchased from Toronto Research Chemicals (North York, ON, Canada). Ranitidine was purchased from Sigma-Aldrich (St Louis, MO). [^{3}H]PAH (151 GBq/mmol) was purchased from Perkin Elmer Life Sciences. [^{3}H]PCG (740 GBq/mmol) was purchased from Amersham Biosciences UK (Little Chalfont, Buckinghamshire, UK). All other chemicals and reagents were obtained from Kanto Kagaku (Tokyo, Japan) or Wako Pure Chemicals (Osaka, Japan) and were of the highest grade available.

The stable transfectants expressing hOAT1-, hOAT2-, hOAT3-, (Tahara et al., 2005a), hOCT2 (Schlatter et al., 2002) and hOATP1B3-HEK (Shimizu et al., 2005) were established as described previously. These cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and G418 sulfate (400 μg/ml) at 37°C with 5% CO_2 and 95% humidity on the bottom of a dish. hOAT1-, hOAT2-, hOAT3-, hOCT2- and hOATP1B3-HEK were seeded in polylysine-coated 12-well plates at a density of 1.2×10^5 cells/well. The transport activity by each cell-line was confirmed by examining the uptake of ranitidine by hOAT1, hOAT2, hOAT3, and hOCT2.

Transport Studies

Transport studies were carried out as described previously (Tahara et al., 2005a). Uptake was initiated by adding medium containing 10 μM of the compounds after the cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. The Krebs-Henseleit buffer consisted of 142 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$ adjusted to pH 7.4. The uptake was terminated at a designed time by adding ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Then, cells were washed twice with 1 ml ice-cold Krebs-Henseleit buffer. For the determination of the uptake of fexofenadine, cells were dissolved in 300 μl 0.2 N NaOH and kept overnight. Aliquots (150 μl) were transferred to vials after adding 30 μl 1 N HCl. Aliquots (100 μl) were used for LC-MS quantification as described below.

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The remaining 10 µl of the aliquots of cell lysate were used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard. Ligand uptake was given as the cell-to-medium concentration ratio determined as the amount of ligand associated with cells divided by the medium concentration.

Quantification of fexofenadine by LC-MS

A sensitive method was developed to determine fexofenadine by HPLC-electrospray ionization mass spectrometry with midazolam as the internal standard (Tahara et al, 2005b). The LC-MS consisted of an Alliance™HT 2795 separation module with an autosampler (Waters, Milford, MA) and micromass ZQ mass spectrometer with an electro ion spray interface (Waters). The optimum operating conditions used were as follows: electrospray probe (capillary) voltage 2.7 kV, sample cone voltage 35 V and source temperature 100°C. The spectrometer was operated at a drying desolvation gas flow-rate of 300 L/h. The mass spectrometer was operated in the selected ion monitoring mode using the respective MH+ ions, *m/z* 502.3 for fexofenadine and *m/z* 326.3 for the internal standard. The mobile phase used for HPLC was: (A) methanol and (B) 0.05% formic acid. Chromatographic separation was achieved on a C_{18} column (Capcell pak C_{18}, MG, 4.6 mm I.D. x 75 mm, particle size 3 µm, Shiseido, Tokyo, Japan) using a linear gradient from 55% A to 70% A over 5 min and returning to 55% A within 2 min. The quantification limit of this method was 5 nmol/L in the cell lysate. Instrument control and data analysis were performed using Mass Lynx application software from Waters.

Kinetic analyses

Kinetic parameters were obtained using the Michaelis-Menten equation:

$$v = V_{max} \times S / (K_m + S)$$

where v is the uptake rate of the substrate (pmol/min/mg protein), S is the substrate concentration in the medium (µM), K_m is the Michaelis-Menten constant (µM) and V_{max} is the maximum uptake rate (pmol/min/mg protein). To obtain the kinetic parameters, the equation was fitted to the uptake velocity using a MULTI program (Yamaoka et al., 1981). The input data were weighted as the reciprocals of the squares of the observed values. Inhibition constants (K_i) of several compounds were calculated assuming competitive inhibition using the following equation.

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$$CL_{+inh} = CL / (1 + (I/K_i)^S)$$

where CL is the uptake clearance, *I* is the concentration of inhibitor (μM) and S is the Hill coefficient. The subscript (+inh) represents the value in the presence of inhibitor. The substrate concentration was low compared with its K_m value in the inhibition study. The two-tailed unpaired t test was used for a statistical analysis and value of P less than 0.05 was considered significant.

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Results

Time-profile of the uptake of fexofenadine by hOAT1-, hOAT2-, hOAT3- and hOCT2-HEK

Figure 1 shows the time-profiles of the uptake of the typical substrates and fexofenadine by hOAT1-, hOAT2-, hOAT3-, hOCT2- and vector-HEK cells. Consistent with previous our report (Tahara et al., 2005a; Tahara at al., 2005c), the uptake of the typical substrates by the cDNA transfectants was significantly greater than that in vector-HEK. The uptake of fexofenadine by hOAT3-HEK was significantly greater than that in vector-HEK at all time points, whereas the uptake by hOAT1-, hOAT2- and hOCT2-HEK was very similar to that of vector-HEK (Figure 1). Since the uptake of fexofenadine by hOAT3-HEK increased linearly up to 5 min of incubation, the uptake of fexofenadine for 5 min was used for further characterization.

Concentration-dependence of the uptake of fexofenadine by hOAT3-HEK, and the effect of probenecid

The concentration-dependence of the uptake of fexofenadine by hOAT3-HEK was examined (Figure 2). The uptake was saturable, and the K_m and V_{max} values, determined by non-linear regression analysis, were 70.2 ± 2.7 μM and 120 ± 3 pmol/min/mg protein, respectively. The inhibitory effect of probenecid on hOAT3-mediated uptake of fexofenadine was examined (Figure 3). The K_i value of probenecid for the uptake of fexofenadine by hOAT3-HEK was determined to be 1.30 ± 0.30 μM with Hill coefficient of 0.76.

Time-profile of the uptake of fexofenadine by hOATP1B3-HEK, and the effect of probenecid

As reported previously by Shimizu et al (2005), the uptake of fexofenadine using the same hOATP1B3-HEK was greater than that by mock cells (7.59 ± 0.26 versus 3.97 ± 0.22 μl/mg protein at 5 min) (Figure 4A). The K_i value of probenecid for the uptake of fexofenadine by hOATP1B3-HEK was determined to be 130 ± 40 μM with Hill coefficient of 0.64 (Figure 4B). Probenecid is a 100-fold more potent inhibitor of hOAT3 than hOATP1B3.

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Discussion

Fexofenadine is an orally-active non-sedative histamine H1 receptor antagonists. Only a small amount of the orally-administered [^{14}C]-fexofenadine was recovered in the urine of healthy volunteers (12%), and urinary excretion has been considered to be a minor elimination pathway. However, the fact that the absolute oral bioavailability of fexofenadine is, on average, 33% means that a considerable amount of fexofenadine is excreted into the urine over a 24-hour period (36% of the amount absorbed into the circulating blood), and suggests that renal elimination makes a significant contribution to the total clearance. The renal clearance of fexofenadine is greater than the glomerular filtration rate, indicating that tubular secretion accounts for the major part of the renal clearance (Table 1). Simultaneously administered probenecid caused about a 50% increase in the AUC of fexofenadine in healthy subjects, and this is largely explained by a 73% inhibition of the renal clearance of fexofenadine (Table 1) (Yasui-Furukori et al., 2005). In the present study, we examined the possible role of renal organic anion and cation transporters in the drug-drug interaction between fexofenadine and probenecid.

In cDNA transfected cells, fexofenadine is efficiently transported only by hOAT3, while specific uptake by hOAT1, hOAT2 and hOCT2 was below the limit of detection, suggesting that hOAT3 plays a major role in the renal uptake of fexofenadine (Figure 1). The transport activity of fexofenadine by hOAT3 was much lower than that of benzylpenicillin (1.71 versus 10.7 μl/min/mg protein). This was in good agreement with clinical data showing that the renal tubular secretion clearance of benzylpenicillin in healthy volunteers was 983 ml/min (Bins and Mattie, 1988), at least 9-fold higher than that of fexofenadine (113 ml/min, Table 1). Probenecid is a potent inhibitor of hOAT3, and the unbound plasma concentration of probenecid at clinical doses (0.5-2.0 g), ranging from 12 to 52 μM (Selen et al., 1982), is greater than its K_i value for hOAT3 (Table 1, Figure 3). Therefore, probenecid will produce almost complete inhibition of hOAT3 in clinical situations, consistent with clinical report, 73% inhibition of the renal clearance of fexofenadine by probenecid (Yasui-Furukori et al., 2005). Therefore, inhibition of basolateral uptake can be one of the sites of interaction between fexofenadine and probenecid. Cimetidine inhibits the renal clearance of fexofenadine by 39% on average in healthy subjects (Table 1). Since the clinical plasma concentration of unbound cimetidine at a dose of 400 mg was reported to be 5.2 μM (van Crugten et al., 1986), far below its K_m and IC_{50} values for hOAT3 (113 μM;Tahara et al. 2005c,

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and 92.4 μM;Khamdang et al. 2004, respectively), it is unlikely that the interaction involves hOAT3. Cimetidine may inhibit efflux process across the brush border membrane of the proximal tubules. Although fexofenadine has been shown to be a substrate of P-gp (Cvetkovic et al., 1999; Tahara et al., 2005b), the steady-state plasma concentration was unchanged in Mdr1a/1b knockout mice (Tahara et al., 2005b), suggesting its limited role in the urinary and biliary excretion, and the transporter responsible for the luminal efflux remains unknown. Further studies are necessary to investigate whether the transporter responsible for the luminal efflux is another site of drug-drug interaction with probenecid and cimetidine.

The non-renal clearance of fexofenadine is explained by biliary excretion. It was found that fexofenadine is a substrate of hOATP1B3, while the specific uptake of fexofenadine by OATP1B1 and OATP2B1 is very low (Shimizu et al., 2005). Based on quantitative prediction using the concept of a relative activity factor, hOATP1B3 has been suggested to play a major role in the hepatic uptake of fexofenadine (Shimizu et al., 2005). Our inhibition study revealed that probenecid is a weak inhibitor of hOATP1B3 with a K_i value greater than the unbound concentration achieved by a clinical dose (1 g) of probenecid (24 μM, Selen et al., 1982). Therefore, probenecid likely exhibits only a minimal inhibitory effect on the hepatic uptake of fexofenadine via hOATP1B3. This is consistent with the kinetic consideration that the drug-drug interaction is largely explained by a 73% inhibition of the renal clearance of fexofenadine.

The effect of probenecid on the total body clearance will be less potent since the contribution of the renal clearance of fexofenadine to the total clearance was smaller in rats (15-20%) (Kamath et al., 2005). There are two possibilities to account for this. One is the species difference in OAT3-mediated transport, *i.e.,* basolateral uptake process since OAT3-mediated transport shows poor correlation between rat and human (Tahara at al., 2005c). The other is reabsorption mediated by Oatp1a1 in rats. Oatp1a1 is localized on the brush border membrane of the kidney (Bergwerk et al., 1996), whereas its human homolog, OATP1A2, exhibits brain-specific distribution (Abe et al., 1999). Oatp1a1 has been suggested to be involved in the reabsorption of organic anions (Gotoh et al., 2002). Since fexofenadine is a substrate of Oatp1a1 (Cvetkovic et al., 1999), it is likely that it undergoes reabsorption from the lumen by Oatp1a1 in the kidney. Oatp1a1 expression exhibits gender difference, leading to the gender difference in the renal clearance of amphipathic organic anions (Gotoh et al., 2002). Female rats may be better animal model to investigate the pharmacokinetics in human.

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The present study highlights the underlying mechanism of the drug-drug interaction with probenecid focusing on OAT3. Probenecid is also a potent inhibitor of OAT1, and its Ki value is smaller than the clinical unbound plasma concentration of probenecid. Therefore, both OAT1 as well as OAT3 can be a site of drug-drug interaction with probenecid. This is why probenecid causes a drug-drug interaction with a number of drugs in terms of renal elimination (Cunningham et al., 1981). Adefovir and cidofovir have been suggested to taken up by the kidney via human OAT1(Ho et al., 2000; Mulato et al., 2000). They are nucleoside phosphonate analogs, a class of novel antivirals structurally related to natural nucleotides, and nephrotoxicity is their main dose-limiting toxic effect. Ho et al. (2000) and Mulato et al. (2000) have demonstrated that hOAT1 is directly involved in the induction of nephrotoxicity since the expression of hOAT1 sensitized a mammary cell line to adefovir and cidofovir, and probencid reduced the cytotoxicity (Ho et al., 2000; Mulato et al., 2000). In such circumstances, combination with probenecid will have a beneficial effect in suppressing the nephrotoxicity as well as prolonging their plasma retention time leading to an increase in the concentration in the liver, the target organ for the treatment of hepatitis B.

In conclusion, hOAT3 shows specific uptake of fexofenadine among basolateral transporters, and accounts for its renal uptake. Probenecid is a potent inhibitor of hOAT3, and inhibition of hOAT3 is a likely mechanism to account for the increase in the AUC of fexofenadine caused by probenecid treatment in healthy subjects.

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Foot notes

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Legends to figures

Figure 1. Time-profile of the uptake of typical substrates and fexofenadine by hOAT1-, hOAT2-, hOAT3-, and hOCT2-HEK

The time-dependent uptake of the typical substrates and fexofenadine (10 μM) by hOAT1-, hOAT2-, hOAT3-, and hOCT2-HEK was examined at 37°C. Closed and open circles represent the uptake by OATs/OCTs-HEK and vector-HEK, respectively. Statistical differences in the uptake of OATs/OCTs-HEK were compared with vector-HEK by a two-tailed unpaired *t*-test with $p<0.05$ as the limit of significance (*$p<0.05$; **$p<0.01$). Each point represents the mean ± S.E. (n=3).

Figure 2. Concentration-dependence of the uptake of fexofenadine by hOAT3-HEK

The time-dependent uptake of fexofenadine (10 μM) by hOAT3-HEK was examined at 37°C. The concentration-dependence of hOAT3-mediated fexofenadine uptake is shown as Eadie-Hofstee plots. The hOAT3-mediated uptake of fexofenadine for 5 min was determined at various concentrations (5 to 100 μmol/L, range of concentrations used). The hOAT3-mediated transport was obtained by subtracting the transport velocity in vector-HEK from that in rOAT3-HEK. Each point represents the mean ± S.E. (n=3). Where bars are not shown, the S.E. is contained within the limits of the symbol.

Figure 3. Inhibitory effect of probenecid on the uptake of fexofenadine by hOAT3- HEK.

The uptake of fexofenadine (10 μM) by hOAT3- and hOATP1B3-HEK for 5 min was determined in the absence or presence of probenecid at the designated concentrations. The values are expressed as a percentage of fexofenadine transport by hOAT3- or hOATP1B3-HEK in the presence of inhibitors *versus* that in the absence of inhibitors. The Hill coefficient value was 0.763 ± 0.047. Each point represents the mean ± SE. (n=3).

Figure 4. Time-profile of the uptake of fexofenadine by hOATP1B3 and the inhibitory effect of probenecid on the

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uptake of fexofenadine by hOATP1B3-HEK.

The time-dependent uptake of fexofenadine (10 μM) by hOATP1B3-HEK was examined at 37°C. Closed and open circles represent the uptake by hOATO1B3-HEK and vector-HEK, respectively (A). The uptake of fexofenadine (10 μM) by hOATP1B3-HEK for 5 min was determined in the absence or presence of probenecid at the designated concentrations (B). The values are expressed as a percentage of fexofenadine transport by hOATP1B3-HEK in the presence of inhibitors *versus* that in the absence of inhibitors. The Hill coefficient value was 0.642 ± 0.079. Statistical differences in the uptake of hOATP1B3-HEK were compared with that by vector-HEK using a two-tailed unpaired *t*-test with $p<0.05$ as the limit of significance (*$p<0.05$; **$p<0.01$). Each point represents the mean ± S.E. (n=3).

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Table 1 Effects of cimetidine, and probenecid treatments on pharmacokinetic parameters of fexofenadine

Parameter	Control	Cimetidine	Probenecid
$AUC_{(0-\infty)}$ (ng·h/mL)[a)]	3637±1199	4124±2019	6150±3972
Ratio to control	1	1.08	1.53
$CL_{renal,u}$ (mL/min)[a)]	230±78	152±70	74±52
Ratio to control	1	0.610	0.270
CL_{sc} (mL/min)[b)]	133	55.0	0 (almost)
Ratio to control	1	0.414	0 (almost)
$I_{u,max}$ [c)]		5.20	24.0
K_i (K_m) for hOAT3		(113)	1.30
R[d)]		0.956	0.0514

a) $CL_{renal,u}$, unbound renal clearance, (Yasui-Furukori et al., 2005).

b) CL_{sc}, tubular secretion clearance ($CL_{renal,u}$-$CL_{creatinine}$), Creatinine clearance value was used for the value of GFR (97 mL/min, van Crugten et al., 1985).

c) $I_{u,max}$, maximum unbound plasma concentration of inhibitor (van Crugten et al., 1985; Selen et al., 1982).

d) R value was calculated according to following equation, $R=1/(1+I_{u,max}/K_i)$.

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Figure 1

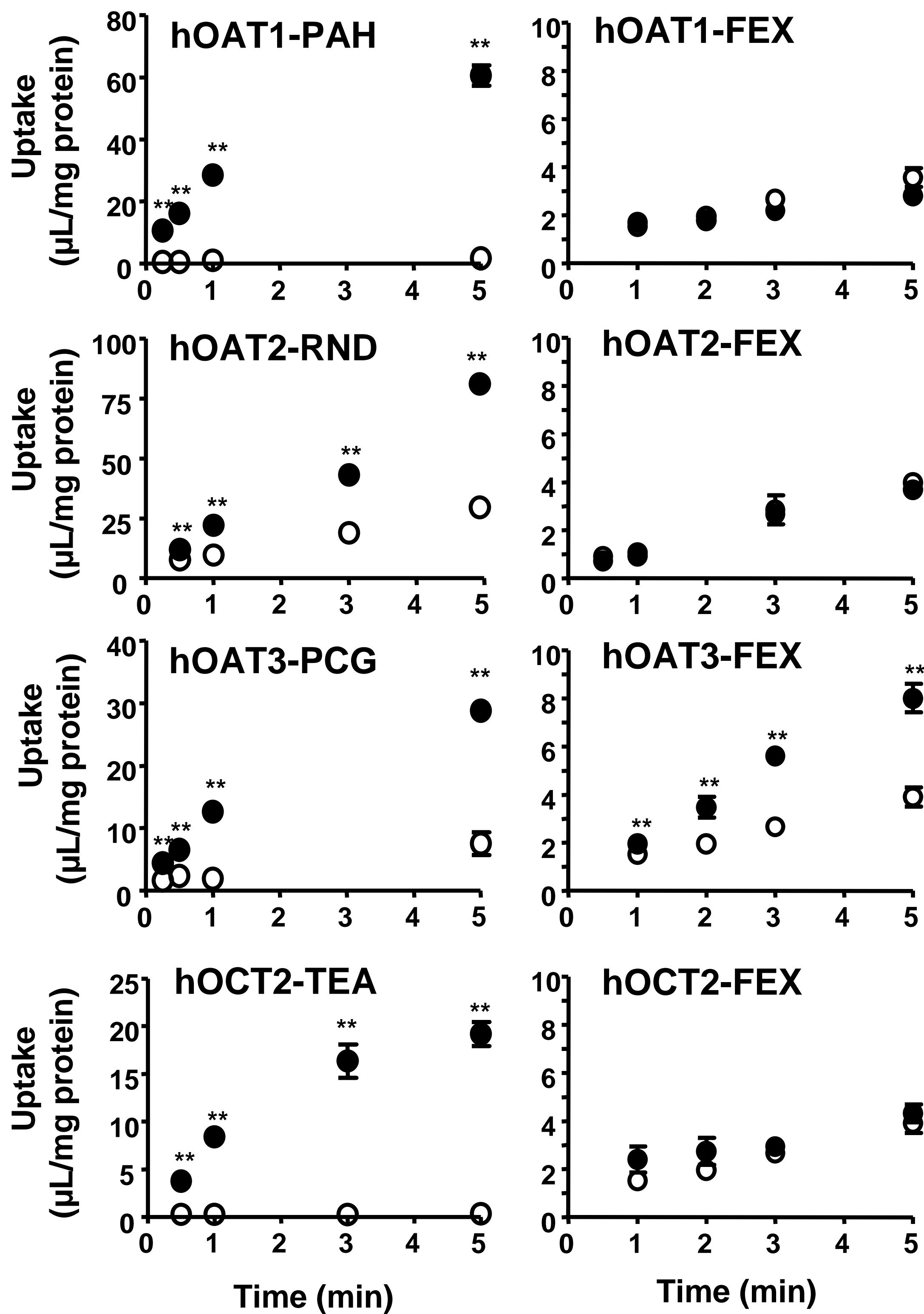

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Figure 2

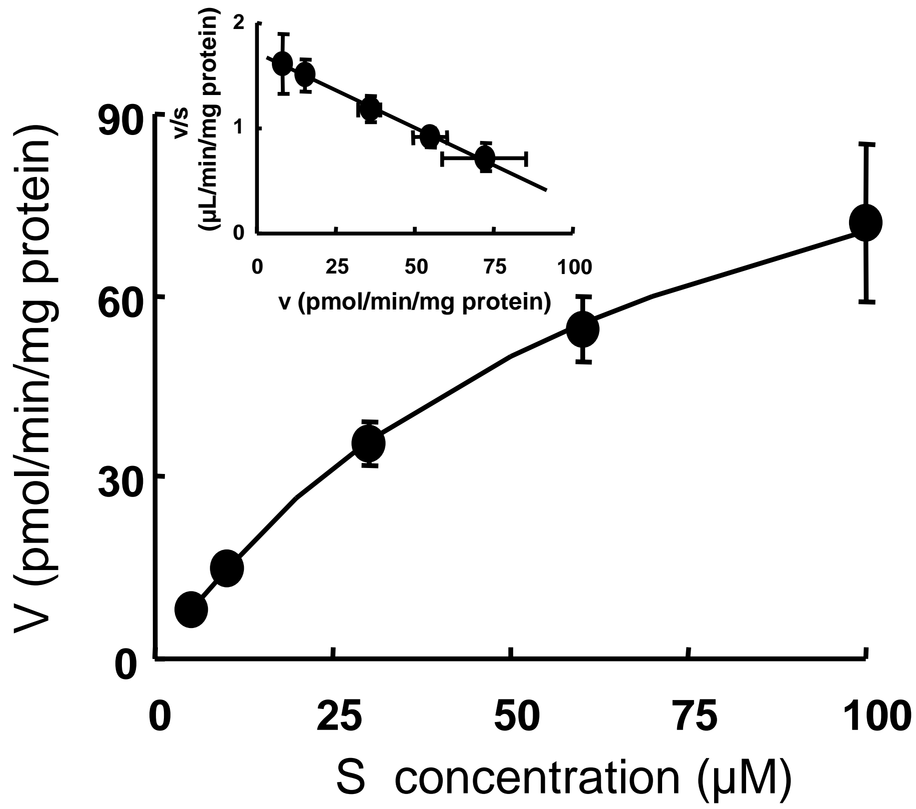

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Figure 3

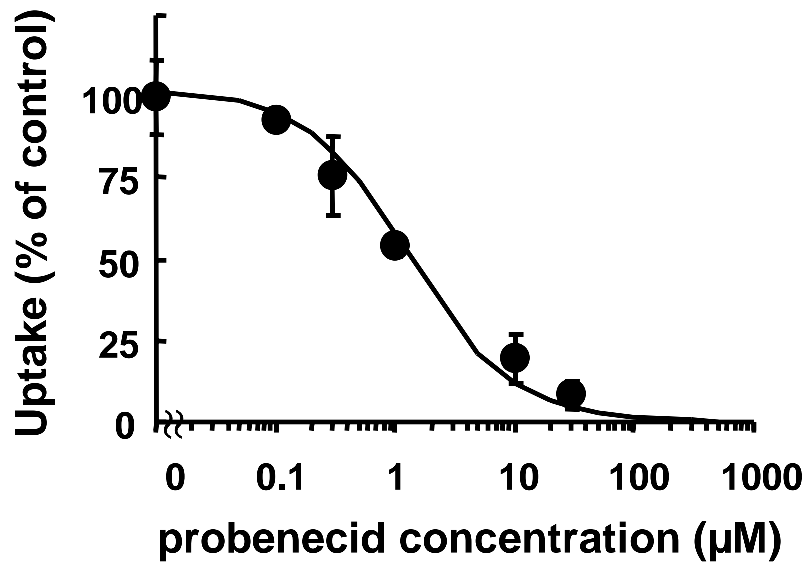

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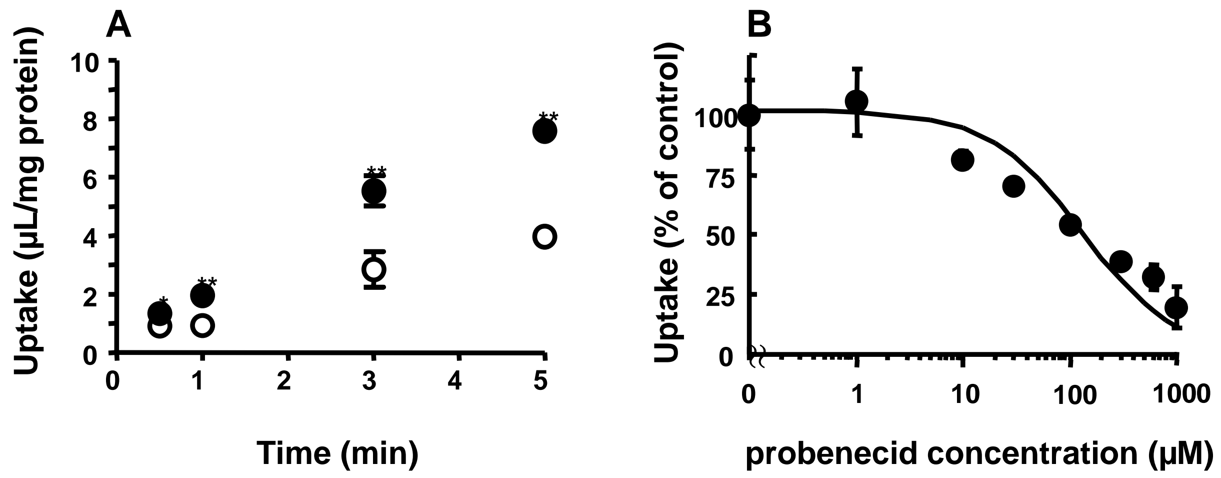
Figure 4



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