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Interactions of Amoxicillin and Cefaclor with Human Renal Organic Anion and Peptide Transporters

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Running title: Transport of amoxicillin and cefaclor by hPepT1 and hPepT2

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Abbreviations: UTI, urinary tract infection; hOAT1, human organic anion transporter 1; hPepT1, human peptide transporter 1; hPepT2, human peptide transporter 2; MDCK, Mardin-Darby canine kidney cells; YFP, yellow fluorescence protein; RFP, red fluorescence protein; ACE, angiotensin-converting enzyme; PAH, *para*-aminohippuric acid; Gly-Sar, Glycyl-Sarcosine; MSD, mass selective detector.

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Abstract

Amoxicillin and cefaclor are two of the widely used β-lactam antibiotics in the treatment of urinary tract infections. Both drugs are eliminated mainly by the kidney and also rely on renal excretion to exert their antibacterial activities in the urinary tract. Previous studies have suggested the involvement of organic anion and oligopeptide transporters in membrane transport of β-lactams. The objective of the current study is to examine the kinetics of amoxicillin and cefaclor interactions with human renal transporters hOAT1, hPepT1 and hPepT2 in detail, both as substrates and as inhibitors. Using fluorescence protein tagging and cell sorting, we established MDCK cell lines stably expressing highly functional hOAT1, hPepT1 and hPepT2. Amoxicillin and cefaclor inhibited hOAT1-mediated ^{3}H-PAH uptake (K_i = 11.0 and 1.15 mM, respectively). However, uptake study revealed that neither drugs were transported by hOAT1. Amoxicillin and cefaclor competitively inhibited hPepT2-mediated ^{3}H-Gly-Sar uptake (K_i = 733 μM and 65 μM, respectively), while much lower affinity for hPepT1 was observed with both antibiotics. Direct uptake studies demonstrated that amoxicillin and cefaclor were transported by hPepT1 and hPepT2. Kinetic analysis showed that hPepT2-mediated uptake of both drugs was saturable with K_m of 1.04 mM for amoxicillin and 70.2 μM for cefaclor. hPepT2, and to a less extent hPepT1, may play an important role in apical transport of amoxicillin and cefaclor in the renal tubule. hOAT1, in contrast, is not involved in basolateral uptake of these antibiotics.

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Introduction

Urinary tract infections (UTIs) are common occurrences and account for about 7 to 8 million physician visits a year in the United States (Bacheller and Bernstein, 1997). β-lactam antibiotics, trimethoprim-sulfamethoxazole and quinolones are the standard medications for treatment of UTIs. Among them, amoxicillin is one of the most widely prescribed β-lactam antibiotics, while cephalosporin antibiotics, such as cefaclor, are particularly useful in patients with recurrent or chronic UTIs.

Despite their low lipophilicity and zwitterionic nature at physiological pH, both amoxicillin and cefaclor (Figure 1) exhibit good oral bioavailability (Zarowny et al., 1974; Meyers, 2000). There is ample evidence in the literature to suggest that carrier-mediated active uptake plays an important role in the intestinal absorption of many β-lactam antibiotics (Bretschneider et al., 1999). In particular, the oligopeptide transporter PepT1 (Liang et al., 1995) expressed on the brush border membrane of the enterocytes is considered the major mechanism for the absorptive transport of β-lactam antibiotics. PepT1 is a proton-coupled oligopeptide transporter, which has been shown to transport di/tri-peptides and peptidomimetics, including β-lactam antibiotics and ACE inhibitors. Specifically, amoxicillin has been reported to inhibit hPepT1 with low affinity, and cefaclor uptake by hPepT1 was also observed (Han et al., 1999).

Renal excretion is the primary elimination route for most β-lactam antibiotics. Excretion into urine is of particular importance in the treatment of UTIs as it is the major route for delivering the antibiotic to its site of action. Both amoxicillin and cefaclor are eliminated mainly by the kidneys and have renal clearance exceeding glomerular filtration rate (GFR), which suggests a significant contribution of tubular secretion in the excretory process. However, the particular transporters involved in the renal tubular secretion of these drugs have not been

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identified. Based on the pK_a values of amoxicillin (2.8, 7.2) and cefaclor (2.43, 7.16), a large fraction of the drugs carry a net negative charge at physiological pH. Early *in vivo* studies showed that probenecid, a classic inhibitor of the renal organic anion transport system, significantly reduced the renal clearance of many β-lactam antibiotics including amoxicillin and cefaclor (Shanson et al., 1984; Brown, 1993; Shitara et al., 2005), which suggested the involvement of organic anion transporters. Recent molecular studies have identified the Organic Anion Transporter 1 (OAT1), expressed on the basolateral membrane of proximal tubule cells, as one of the key players for tubular secretion of small and hydrophilic anionic drugs (Hosoyamada et al., 1999). In addition, several other OAT isoforms (e.g. OAT3 and OAT4) are also expressed in the kidneys, where they may play a role in renal excretion of drugs. Although many β-lactam antibiotics have been shown to inhibit OATs (Takeda et al., 2002), only a few β-lactams have been shown to be transported by OAT transporters cloned from mammalian species (Jariyawat et al., 1999). Definitive data are lacking regarding whether or not these β-lactams are substrates of the human OAT transporters.

Despite their tubular secretion, amoxicillin and cefaclor are likely to be reabsorbed via the oligopeptide transporters located at the apical membrane of the renal tubular cells. In addition to PepT1, which is expressed in the S1 segment of the proximal tubule, hPepT2, a closely related isoform of hPepT1, is also expressed at the S2 and S3 regions of the proximal tubule (Daniel and Kottra, 2004). Aside from the physiological roles of PepT1 and PepT2 in recovering small peptides from the glomerular filtrate, these transporters may also contribute to the retrieval of readily filtered drugs. Although PepT2 shares high amino acid identity and significant functional similarity with PepT1, it has distinct substrate specificity compared with PepT1 and generally exhibits higher affinity (Terada et al., 2000). There are a number of drugs

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including amoxicillin and cefaclor reported to inhibit rat PepT2 (Luckner and Brandsch, 2005), but few of them have been shown as the substrates. Hence, detailed substrate and kinetic information for the human isoform, hPepT2, in transporting β–lactams is needed.

The goal of this study is to provide a detailed examination of the interaction of amoxicillin and cefaclor with human renal transporters hOAT1, hPepT1 and hPepT2, both as substrates and as inhibitors. The information is important for understanding the specific roles of these transporters in renal handling of amoxicillin and cefaclor, and in predicting the potential for drug interactions with these two antibiotics. Our results demonstrate that amoxicillin and cefaclor are weak inhibitors, but not substrates for hOAT1. In contrast, both antibiotics are transported by hPepT1 and hPepT2. These data suggest that amoxicillin and cefaclor are actively secreted into the urine through a transport system distinct from the classic renal organic anion carrier OAT1. Furthermore, in spite of a net secretion, the two β–lactams may be reabsorbed to a significant extent by renal peptide transporters.

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Materials and Methods

Materials. ^{3}H-Glycylsarcosine (Gly-Sar) (0.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). ^{3}H-PAH (4.18 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). All other unlabeled chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest quality available. All cell culture media and reagents were from Invitrogen (Carlsbad, CA).

cDNA Constructs for Expression. The full length cDNA encoding hOAT1 was isolated from human kidney by RT-PCR using the following set of primers: 5'-GCGAATTCCACC-ATGGCCTTTAATGACCTCCTGCAGCAGG-3' (sense) and 5'-GACTCGAGCTGTA-GGACCTTCCCTCCCTTTAGGGTTCTGT-3' (antisense). hPepT1 cDNA was isolated from human intestine using the following primer pair: 5'-TAGTAGCTCGAGCCGCCATGGGAA-TGTCCAAATCAC-3'(sense) and 5'-TGATGAGGATCCCCAATGGAGTGTCCTGCTACCT-G-3' (antisense). The isolated hOAT1 and hPepT1 cDNAs were subcloned into the yellow fluorescence vector pEYFP-C1 (Clontech, Palo Alto, CA). hPepT2 cDNA was amplified by PCR procedure with sense primer (5'-TGATGAAAGCTTCAGCCATGAATCCTTTCCAGAA-3') and antisense primer (5'-TAGTAGGGATCCCAGAATCTAGGGAGTCATCAGAGT-3') from the ATCC clone (IMAGE ID:5288920) and was subcloned into pDsRed2-C1 vector (Red fluorescence vector, Clontech). All three full-length cDNAs were confirmed by direct sequencing. The sequences of the cloned cDNAs were identical to the published sequences of hOAT1 (Hosoyamada et al., 1999), hPepT1 (Liang et al., 1995) and hPepT2 (Liu et al., 1995).

Transfection and Cell Culture. cDNA constructs were transfected into MDCK cells with Lipofectamine™2000 (Invitrogen) as described previously (Engel et al., 2004). Respective empty vectors were also transfected into MDCK cells to serve as controls. Transfected cells

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were selected with 1 mg/ml G418 for two weeks. Fluorescence-positive cells were purified by a FACS Vantage SE sorter (BD Biosciences, San Jose, CA). The cells were maintained in minimum Eagle's medium supplemented with 10% FBS and 500 µg/ml G418 at 37ºC in 95% air, 5% CO_2 with 95% humidity.

Visualization of hOAT1, hPepT1 and hPepT2 Tagged with Fluorescence Proteins. One million cells were grown in four-well Lab-Tek borosilicated cover glass chambers (Nalge Nunc International Corp, Naperville, IL) until reaching differentiation. Images were obtained by a Leica confocal microscope equipped with Argon and DPSS laser as the light sources. Images were captured by excitation at 488 nm and emission at 520-540 nm for YFP or excitation at 561 nm and emission at 580-600 nm for RFP.

Time Course and Kinetics of PAH Uptake by hOAT1, Gly-Sar Uptake by hPepT1 and hPepT2. Transfected MDCK cells were plated into 24-well tissue culture plates. Two days post seeding, cells were preincubated in the uptake buffer for 10 min in a 37ºC water bath, which consists of 20 mM MES (pH 6.0 for hPepT1 and hPepT2) or Tris-HCl (pH 7.4 for hOAT1), 3 mM K_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2$, 130 mM NaCl and 5 mM glucose. hOAT1 uptake time course was measured with 5 µM ^{3}H-PAH in Tris buffer (pH 7.4); time course of hPepT1 and hPepT2 uptake was determined in MES buffer (pH 6.0) containing 20 µM ^{3}H-Gly-Sar or 5 µM ^{3}H-Gly-Sar, respectively. The concentration dependence of hOAT1-mediated PAH uptake was examined by incubating 0.2-100 µM of PAH for 2 min at Tris buffer (pH 7.4), while the K_m values of hPepT1- and hPepT2-mediated Gly-Sar uptake were determined by incubating varying total concentrations of Gly-Sar in pH 6.0 MES buffer for 2 min. At the end of the incubation, uptake was terminated by removal of the medium and washing the monolayer three times with ice-cold DPBS. The cells were solubilized with 0.5 ml of 1 N sodium hydroxide and

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neutralized with 0.5 ml 1 N hydrochloric acid. The radioactivity was quantified by liquid scintillation counting. The protein content of cells was determined using BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

Amoxicillin and Cefaclor Inhibition of hOAT1-, hPepT1- and hPepT2-mediated Uptake. All inhibition experiments were conducted in triplicate two days after cell seeding. Transport experiments were conducted in the uptake buffer containing 0.1 μM ^{3}H-PAH (hOAT1) or 0.2 μM ^{3}H-Gly-Sar (hPepT1 and hPepT2) in the presence of varying concentrations of cold amoxicillin or cefaclor. At the end of the incubation, cells were washed three times with ice-cold buffer and processed as described above. To determine the inhibition mechanism of hPepT2 by amoxicillin and cefaclor, hPepT2 cells were incubated with ^{3}H-Gly-Sar (5-80 μM) either alone or in the presence of amoxicillin (1 mM) or cefaclor (60 μM).

Amoxicillin and Cefaclor Uptake by hOAT1, hPepT1 and hPepT2. Stably transfected cells and vector control cells were seeded in 6-well plates for uptake study. Kinetic study of hPepT2-mediated amoxicillin and cefaclor uptake was done with 12-well plates instead. Two days after seeding, uptake experiments were performed by incubating cells with specified concentrations of amoxicillin and cefaclor in Tris or MES buffer as stated above. Uptake was stopped by removal of the medium and cell monolayers were washed three times with ice-cold DPBS medium. Cefadroxil (internal standard) was added to each well and cells were lysed with 1 ml ice-cold acetonitrile / H_2O (v/v: 7:3), scraped off on ice and collected. The samples were sonicated for 10 min and centrifuged for 5 min at 12,000 g. The supernatant was evaporated to dryness and the residues were reconstituted with 150 μl acetic acid (85 mM). The resulting solution was filtered through a membrane filter (Spin-XLC 0.22 μM, Costar Corp, Cambridge, MA) and analyzed by liquid chromatography/mass spectrometry (LC-MS).

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Drug Assay. The cellular content of amoxicillin and cefaclor was quantified using a modified LC-MS method of Chen et al. (Chen et al., 2003). Briefly, 10 µl of each sample was injected onto Restek Ultra Aqueous C_{18} column (200 mm × 2.1 mm, 5 µ, Restek Corp., PA) connected to an Agilent 1100 LC-MS system. Chromatographic separation was achieved by the use of gradient elution with mobile phase consisting of 85 mM acetic acid and acetonitrile. The flow rate was set at 0.25 ml/min. Mass selective detector (MSD) was operated in the atmospheric pressure ionization electrospray mode with negative polarity. The ions monitored were m/z 364 for amoxicillin, m/z 322 for cefaclor and m/z 362 for cefadroxil. Antibiotics content in samples was determined using a standard curve prepared with known concentrations of the antibiotics.

Kinetic Analysis. Experimental data were fitted by KaleidaGraph (Synergy Software, Reading, PA). The values of apparent Michaelis constant (K_m) and maximal rate of uptake (V_{max}) were determined by fitting the data to the Michaelis-Menten equation using nonlinear regression. IC_{50} values were determined by fitting the data to the equation $V = V_0 / \left[1 + \left(I / IC_{50} \right)^r \right]$, where V_0 and V are the initial uptake rates in the absence and in the presence of the inhibitors respectively, I is the inhibitor concentration, r is the Hill coefficient and IC_{50} is half-maximal inhibitory concentration. Assuming the inhibition is competitive; the inhibition constants (K_i) were calculated by the equation $K_i = IC_{50} / \left(1 + C / K_m \right)$, where C represents the concentration of the model substrate, and K_m is the apparent affinity of the substrate. Data were reported as mean ± S.D. of one representative experiment from 2-4 experiments of similar results. Statistical differences were determined using two-tailed unpaired student's *t*-test. Differences were considered significant at $P < 0.05$.

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Results

Expression and Localization of hOAT1, hPepT1 and hPepT2 in Stably Transfected MDCK Cells. To establish hOAT1, hPepT1, hPepT2 stably transfected cell lines, MDCK cells were chosen for transporter expression because it is a renal epithelial cell line and has relatively low background transport activity. To facilitate the establishment of cell lines stably expressing hOAT1, hPepT1 and hPepT2, we tagged YFP or RFP to the N-termini of these transporters. After G418 selection and cell sorting, the majority of the sorted cells exhibited membrane expression of the corresponding transporters. Both hOAT1 and hPepT2 were primarily expressed on the plasma membrane (Figure 2A and C). In contrast, intracellular hPepT1 expression was observed in addition to its plasma membrane expression (Figure 2B). The membrane localization of the expressed transporters in differentiated MDCK cells was further examined by confocal microscopy. Vertical cross-section images showed that hOAT1 was mainly localized to the basolateral membrane with minor expression at the apical membrane (Figure 2D), whereas hPepT1 and hPepT2 were predominantly expressed on the apical membrane (Figure 2E and F).

Functional Characterization of hOAT1, hPepT1 and hPepT2 in MDCK Cells. To evaluate the functional properties of the tagged transporters, uptake studies were performed with ^{3}H-labeled model substrates (PAH for hOAT1 and Gly-Sar for hPepT1 and hPepT2). Figure 3 shows that Gly-Sar uptake by hPepT1 and hPepT2 was linear for 7 min and 5 min respectively, while linear PAH uptake by hOAT1 was observed up to 5 min. At 5 min, a 27-, 60-, and 30-folds increase in uptake activity was observed in respective cells expressing hOAT1, hPepT1 and hPepT2 relative to cells transfected with empty vectors. Concentration-dependent uptake studies were performed to obtain the apparent affinity (K_m) and maximal velocity (V_{max}) values. All

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transporters exhibited saturable uptake of their prototype substrates. The estimated K_m values were 22.2 $\pm$ 5.0 µM for hOAT1-PAH uptake, 637 $\pm$ 50 and 183 $\pm$ 19 µM for Gly-Sar uptake by hPepT1 and hPepT2, respectively. The $V_{\max}$ values of hOAT1, hPepT1 and hPepT2 were 0.073 $\pm$ 0.006, 12.93 $\pm$ 0.32, and 4.87 $\pm$ 0.22 nmol/mg protein/min, respectively. The K_m values determined in our study are consistent with the previously reported values of the corresponding untagged transporters (Aslamkhan et al., 2003; Terada et al., 2004; Zhang et al., 2004), suggesting minimal kinetic changes associated with YFP or RFP tagging.

Inhibition of Amoxicillin and Cefaclor on hOAT1, hPepT1 and hPepT2. As a first step towards characterizing the interactions of amoxicillin and cefaclor with hOAT1, hPepT1 and hPepT2, we examined the inhibitory effect of amoxicillin and cefaclor on the uptake of respective prototype substrates and determined their inhibition potencies (K_i). Typical inhibition profiles are shown in Figure 4. Both antibiotics exhibited moderate to weak inhibitory effects on hOAT1 activity; K_i values were 1.15 mM for cefaclor and 11.0 mM for amoxicillin (Table 1). Amoxicillin and cefaclor also inhibited hPepT2-mediated Gly-Sar in a dose-dependent manner and the respective K_i values were 733 µM and 65 µM (Table 1). Compared with hPepT2, lower inhibition potencies of both antibiotics were observed for hPepT1 with K_i at 4.52 mM for cefaclor and 66.2 mM for amoxicillin. To examine the mechanism of inhibition of amoxicillin and cefaclor on hPepT2, Gly-Sar uptake by hPepT2 at various concentrations was determined in the absence or presence of the antibiotics. Analysis by Lineweaver-Burk plots demonstrated that these antibiotics inhibited the hPepT2-mediated Gly-Sar uptake in a competitive manner (Figure 5).

Uptake of Amoxicillin and Cefaclor by hOAT1, hPepT1 and hPepT2. The inhibition studies suggested that both amoxicillin and cefaclor interact with hOAT1, hPepT1 and hPepT2. To

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determine whether they are substrates of these transporters, we developed LC/MS methods to directly measure the cellular accumulation of amoxicillin and cefaclor by stably transfected MDCK cells and vector control cells. As shown in Figure 6, there was no significant difference in amoxicillin or cefaclor uptake between hOAT1 expressing cells and the control cells, suggesting that the two compounds were not transported by hOAT1. In contrast, a 7-fold increase in amoxicillin uptake and a 65-fold increase in cefaclor uptake were observed in cells expressing hPepT1 after 30 min incubation. The addition of 10 mM Gly-Sar in the incubation buffer significantly reduced cellular uptake of both compounds. Similarly, there was a 6.5-fold increase in amoxicillin uptake and a 12-fold increase in cefaclor uptake in cells expressing hPepT2, which was also sensitive to Gly-Sar inhibition. These results clearly demonstrated that amoxicillin and cefaclor were transported by hPepT1 and hPepT2. Kinetic analysis of hPepT2-mediated amoxicillin and cefaclor uptake was performed to determine K_m and V_{max} values. Linear uptake was observed up to 10 min for both drugs (data not shown), and initial uptake rates were determined at 10 min accordingly. As shown in Figure 7, hPepT2-mediated uptake of amoxicillin and cefaclor was concentration-dependent. The estimated K_m and V_{max} values were 1.04 $\pm$ 0.04 mM and 144 $\pm$ 10.2 pmol/mg protein/min for amoxicillin, and 70.2 $\pm$ 7.4 µM and 223 $\pm$ 7.5 pmol/mg protein/min for cefaclor. The apparent affinities are in good agreement with the K_i values obtained from the inhibition studies.

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Discussion

β-lactam antibiotics, which include penicillins and cephalosporins, have had a long history of clinical use. However, the identity of transporters involved in their intestinal absorption and renal elimination has only been investigated in recent years following the cloning of many intestinal and renal drug transporters. Amoxicillin and cefaclor are two commonly used β-lactam antibiotics in the treatment of UTIs. Because renal excretion (secretion and/or reabsorption) is an important route of elimination for both antibiotics and their excretion into urine governs their antimicrobial efficacy in UTIs, a better understanding of the underlying mechanism of their renal transport is of clinical relevance. In the present study, we focused on characterizing the interactions of amoxicillin and cefaclor with human renal transporters hOAT1, hPepT1 and hPepT2.

With the aid of fluorescence protein tagging, we were able to establish stable cell lines with high transport activities by flow cytometry based cell sorting. Kinetic analysis with probe substrates indicated that YFP or RFP tagging did not significantly alter the functionality of these transporters. The expression of fluorescence fusion protein also allowed us to directly examine the cellular localization of hOAT1, hPepT1 and hPepT2 in a renal epithelial cell line. Our results clearly indicated predominant plasma membrane localization of these transporters (Figure 2). Interestingly, clustered intracellular expression was also observed in hPepT1-expressing MDCK cells. Although intracellular existence of a low affinity peptide transporter was previously suggested (Bockman et al., 1997; Zhou et al., 2000), no conclusive evidence is available to support hPepT1 expression in intracellular compartments (Sun et al., 2001). The exact intracellular localization of hPepT1 within epithelial cells requires further investigation. Our vertical localization results in differentiated MDCK cells indicated the primary basolateral

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expression of hOAT1 and apical expression of hPepTs. The results are consistent with the physiological function of the transporters in the kidney and are in agreement with previous tissue immunostaining results (Shen et al., 1999; Sun et al., 2001; Motohashi et al., 2002). However, we did occasionally observe minor hOAT1 expression on the apical membrane in a few cells, which might be due to incomplete cellular differentiation of some MDCK cells.

Both amoxicillin and cefaclor are primarily eliminated by the kidney and their net renal clearances are larger than GFR, suggesting that they undergo active tubular secretion. Based on *in vivo* drug interaction and *in vitro* inhibition studies (Brown, 1993; Takeda et al., 2002), it was suggested that OAT1, one of the classic renal PAH transporter, is responsible for concentrative uptake of many β-lactams from blood into the renal tubular cells. Using MDCK cells stably expressing hOAT1, we investigated the interactions of amoxicillin and cefaclor with hOAT1. Our results showed that amoxicillin and cefaclor inhibited hOAT1 with low affinities, at K_i value of 11.0 mM and 1.15 mM respectively (Figure 3). We then determined whether amoxicillin and cefaclor were transported by hOAT1. While the hOAT1-expressing cells exhibited high transport activity for PAH, no significant hOAT1-mediated uptake of amoxicillin or cefaclor was detected (Figure 6A). It is unlikely that the lack of transport activity of amoxicillin and cefaclor by hOAT1-expressing cells was due to restricted substrate access to the basolateral aspect of the MDCK monolayer, because cells used in all uptake studies were unpolarized and high level of PAH uptake was observed. Our finding differs from a previous report where a moderate increase in ^{3}H-labled amoxicillin uptake by CHO cells expressing hOAT1 was observed (Hill et al., 2002). The reason for this discrepancy is unclear and may be due to different experimental conditions used. Nevertheless, the low affinity of hOAT1 in interacting with amoxicillin and cefaclor and its minimal uptake of these antibiotics suggest that hOAT1 does not play a

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significant role in the basolateral uptake of the two antibiotics into the tubular cells. It is possible that hOAT3 or another yet-to-be identified transporter is responsible for the basolateral uptake of amoxicillin and cefaclor. A recent study indicated the involvement of hOAT3 in renal secretion of cefazolin, a cephalosporin antibiotic (Sakurai et al., 2004), whereas another report showed that cefaclor was not a substrate for rat OAT3 (Kuroda et al., 2005). Hence, further investigation is needed to elucidate the molecular mechanism underlying active section of β-lactam antibiotics in the kidney.

Renal clearance is the net result of glomerular filtration, tubular secretion and reabsorption. Although many β-lactams have renal clearance larger than GFR, indicating net renal secretion, there is evidence for active reabsorption. An increase in renal clearance at high doses was observed for cefadroxil both in rats and in humans (Garrigues et al., 1991; Garcia-Carbonell et al., 1993). Non-linearity of renal clearance with increasing dose of amoxicillin in rats was also previously reported (Torres-Molina et al., 1992). Saturation of the renal reabsorption process has been suggested to account for the observations in these studies. The presence of peptide transporters on the apical membrane and the inward-directed proton gradient in the renal tubules indicate that these transporters may be able to mediate tubular reabsorption of β-lactams. In this study, we investigated hPepT1 and hPepT2 as potential reabsorption transporters for amoxicillin and cefaclor. Both antibiotics exhibited relatively high affinity (K_i) towards hPepT2 (733 μM for amoxicilin and 65 μM for cefaclor). Kinetic analysis revealed that the inhibition of both antibiotics on Gly-Sar uptake by hPepT2 was competitive, suggesting that they interacted with the same substrate binding site. Direct evidence for transport was further obtained by measuring amoxicillin and cefaclor cellular uptake. Our results unequivocally demonstrated that both drugs were efficiently transported by hPepT2 (Figures 6) with K_m values

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at 1.04 mM for amoxicillin and 70.2 μM for cefaclor, which were consistent with the estimated K_i from the inhibition study.

If the filtrate concentration of amoxicillin and cefaclor in proximal tubule can be approximated by their plasma concentrations, which are within a low micromolar range, hPepT2-mediated reabsorption may be an important factor determining renal elimination rate of cefaclor. Given that local drug concentration may be higher in the renal tubules due to water reabsorption, the role of hPepT2 in apical reabsorption of cefaclor and amoxicillin may be even more prominent. The recent study in PepT2 knockout mice showed an abolished renal reabsorption of Gly-Sar and a concomitant 2-fold increase in its renal clearance (Ocheltree et al., 2005), highlighting the significant *in vivo* impact of PepT2 in the kidney. If hPepT2-mediated reabsorption is significantly involved in the renal handling of β-lactams, it is possible that the renal clearance of these β-lactams may be altered when hPepT2 is saturated at high dose, or the activity of the transporter is changed by other drugs or by genetic factors.

In addition to hPepT2, hPepT1 is also expressed on the apical membrane of renal tubular cells. Compared with hPepT2, lower inhibition potencies of both antibiotics were observed for hPepT1 with K_i at 4.52 mM for cefaclor and 66.2 mM for amoxicillin (Table 1) which is in accordance with the reported low affinity profile of hPepT1. Our uptake data clearly demonstrated that both amoxicillin and cefaclor are transported by hPepT1 (Figure 6). Given the low affinities, hPepT1 may play a less important role than hPepT2 in the renal reabsportion of both drugs. In contrast, in the intestine, which highly expresses hPepT1 and where the luminal concentration of the antibiotics can be high, hPepT1 may be critical for the intestinal absorption of amoxicillin and cefaclor as the passive permeability of these two drugs is limited by their low lipophilicity.

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In conclusion, using MDCK cells stably expressing hOAT1, hPepT1 and hPepT2, we demonstrated that amoxicillin and cefaclor are transported by hPepT2 and hPepT1, but not by hOAT1. Our data suggest that peptide transporters are a mediator of renal reabsorption and intestinal absorption of the two antibiotics. hOAT1, in contrast, is not involved in renal secretion of these drugs. The present results also underscore the complexity of the renal handling of β-lactam antibiotics in which transporter-mediated active reabsorption may co-exist with active secretion.

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Footnote

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Legends for Figures

Figure 1. Chemical structures of amoxicillin and cefaclor.

Figure 2. Expression and localization of YFP-hOAT1, YFP-hPepT1 and RFP-hPepT2 in MDCK cells. The stably transfected cells were seeded in Lab-Tek chambered coverglass and visualized by a Leica confocal microscope. hOAT1 **(A)**, hPepT1 **(B)** and hPepT2 **(C)** expression was imaged in the *x-y* plane in undifferentiated cells. Vertical localization of the transporters in differentiated MDCK cells was visualized by *x-z* plane scan, YFP-hOAT1 **(D)**, YFP-hPepT1 **(E)** and RFP-hPepT2 **(F)**.

Figure 3. Time course of PAH uptake by hOAT1, Gly-Sar uptake by hPepT1 and hPepT2 expressed in MDCK cells. Transporters (●) and vector-transfected (■) MDCK cells were cultured in 24-well plates for two days. hOAT1-mediated ^{3}H-PAH **(A)** and hPepTs-mediated ^{3}H-Gly-Sar **(B and C)** uptake were performed in Tris buffer at pH 7.4 or MES buffer at pH 6.0 respectively. The data are shown as mean $\pm$ S.D. of triplicate determinations.

Figure 4. Inhibition of hOAT1-, hPepT1- and hPepT2-mediated uptake by amoxicillin and cefaclor. MDCK cells expressing hOAT1, hPepT1, hPepT2 and corresponding vector control cells were incubated in Tris buffer containing 0.1 μM ^{3}H-PAH **(A)** or MES buffer containing 0.2 μM ^{3}H-Gly-Sar **(B and C)** in the presence of varying concentrations of amoxicillin and cefaclor. Transporter-specific uptake was calculated by subtracting the uptake of vector control cells. Data are mean $\pm$ S.D. of the triplicate. All fits were performed by non-linear fitting using Kaleidagraph.

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Figure 5. Kinetic analysis of amoxicillin and cefaclor inhibition on hPepT2 mediated ^{3}H-Gly-Sar uptake. Initial ^{3}H-Gly-Sar uptake rates by hPepT2 were measured by incubating hPepT2 with ^{3}H-Gly-Sar (5−80 μM) for 2 min in the absence (■) or presence (●) of 1 mM amoxicillin **(A)** or 60 μM cefaclor **(B)**. Each point represents mean ± S.D (n=3).

Figure 6. **Amoxicillin and cefaclor uptake by hOAT1, hPepT1 and hPepT2.** Cells were seeded in six-well plates. Uptake of amoxicillin or cefaclor at 500 μM over 30 min interval by hOAT1- **(A)**, hPepT1- **(B)** and hPepT2- **(C)** expressing MDCK cells (solid columns) and vector-transfected cells (open columns) was determined. Bars represent mean ± S.D. (n=3). The values of amoxicillin or cefaclor uptake in the absence or presence of 10 mM Gly-Sar were compared using two-tailed unpaired student's *t*-test. * $P < 0.05$, significantly different from uptake controls without Gly-Sar.

Figure 7. **Concentration dependency of amoxicillin (A) and cefaclor (B) uptake by hPepT2.** MDCK cells expressing hPepT2 were seeded in 12-well plates and cultured for two days. Initial rates of amoxicillin and cefaclor uptake by hPepT2 were determined by measuring 10 min uptake at varying concentrations of the two antibiotics. hPepT2-mediated uptake was calculated as the uptake in hPepT2-expressing cells subtracted by the uptake in vector control cells. *Insets*, Eadie-Hofstee plots.

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Tables

Table 1. Inhibition constants (K_i) of amoxicillin and cefaclor on hOAT1-, hPepT1-, and hPepT2-mediated uptake. ^{3}H-PAH or ^{3}H-Gly-Sar uptake (2 min) by hOAT1 or hPepT1- and hPepT2-expressing MDCK cells were measured in the presence of varying concentrations of amoxicillin or cefaclor. Data represent mean ± S.D. (n=3).

	K_i (mM)	
	Amoxicillin	Cefaclor
hOAT1	11.0 ± 0.78	1.15 ± 0.15
hPepT1	66.2 ± 24.3	4.52 ± 0.28
hPepT2	0.73 ± 0.11	0.065 ± 0.007

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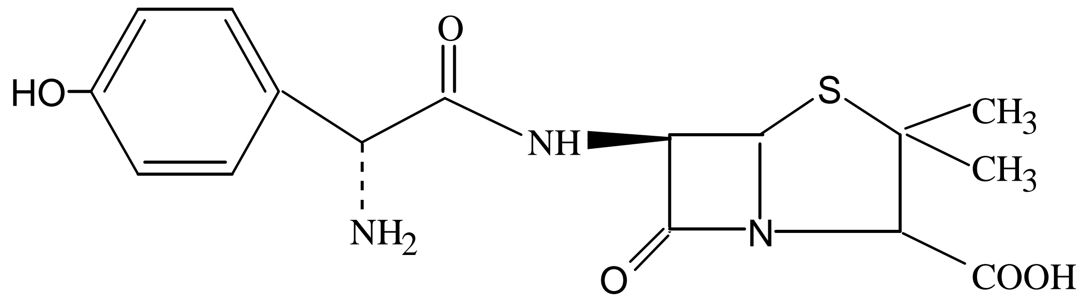
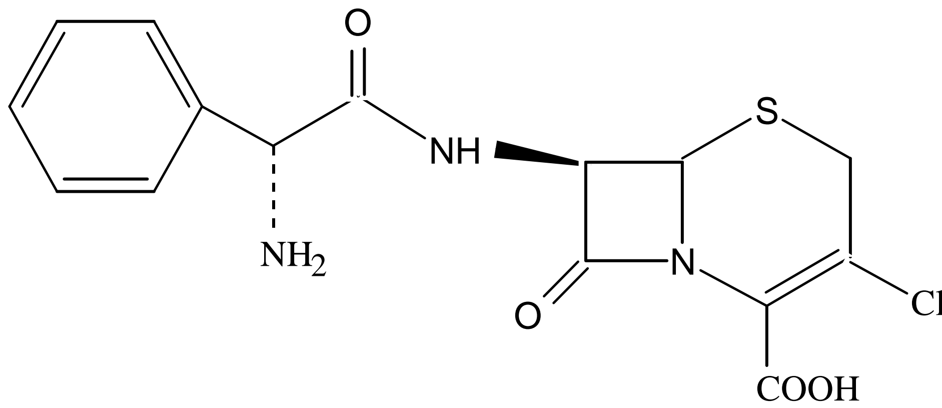
Figure 1



Amoxicillin



Cefaclor

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Figure 2

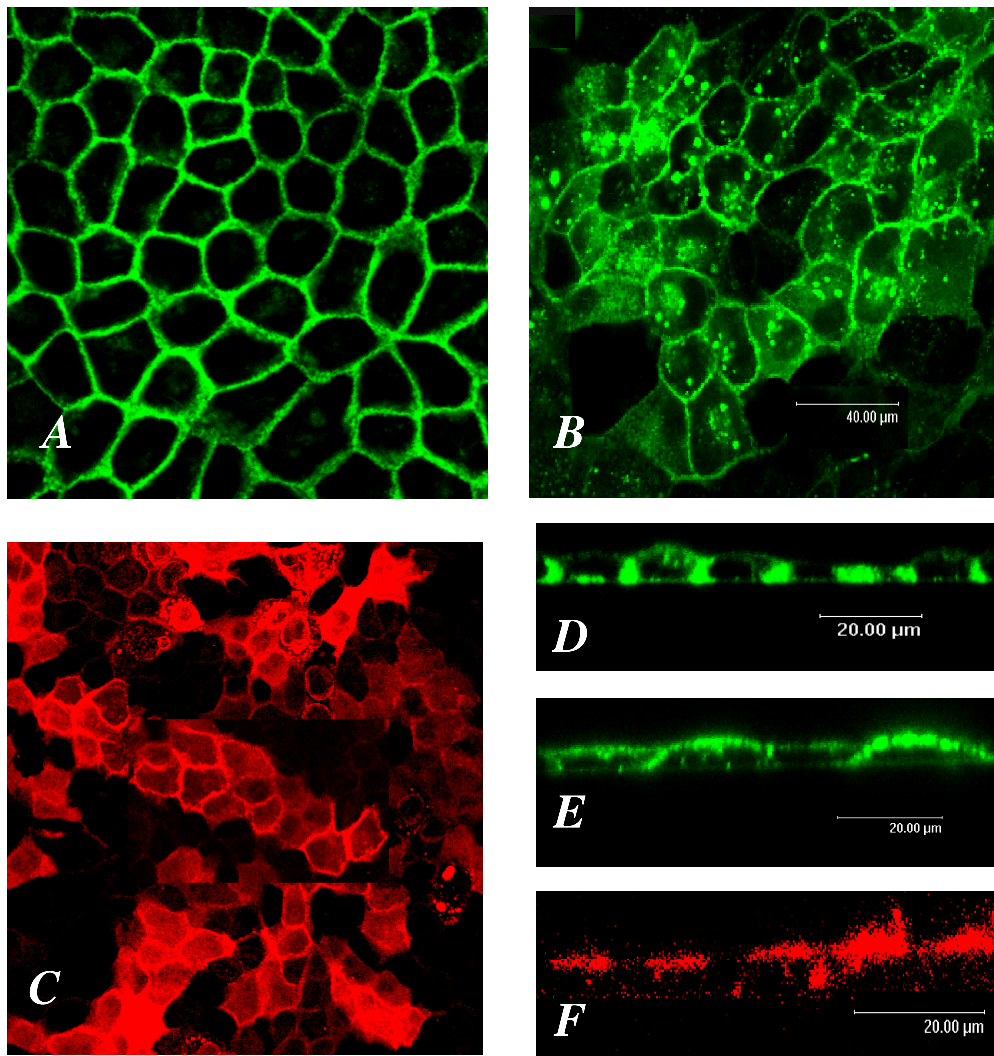

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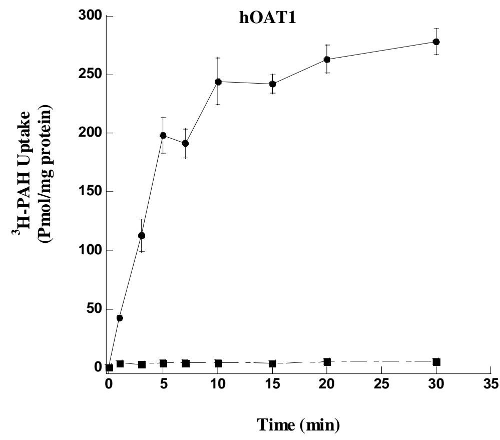
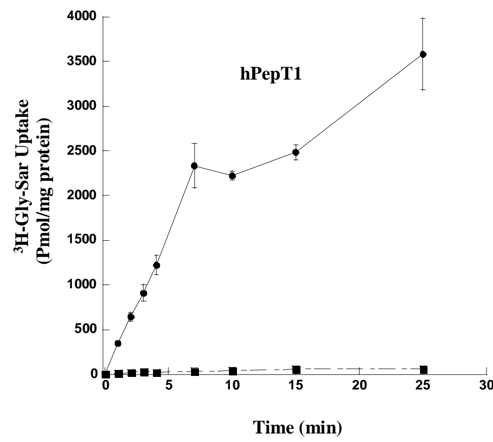
Figure 3

(A)



(B)



(C)

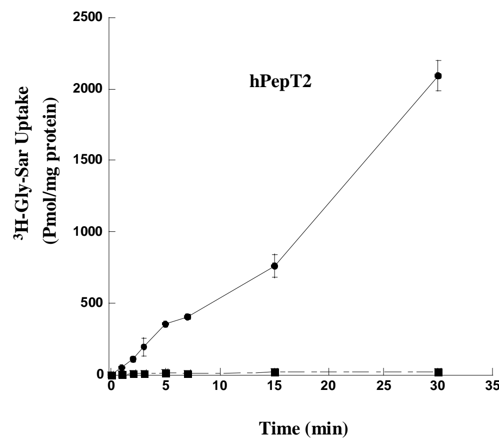


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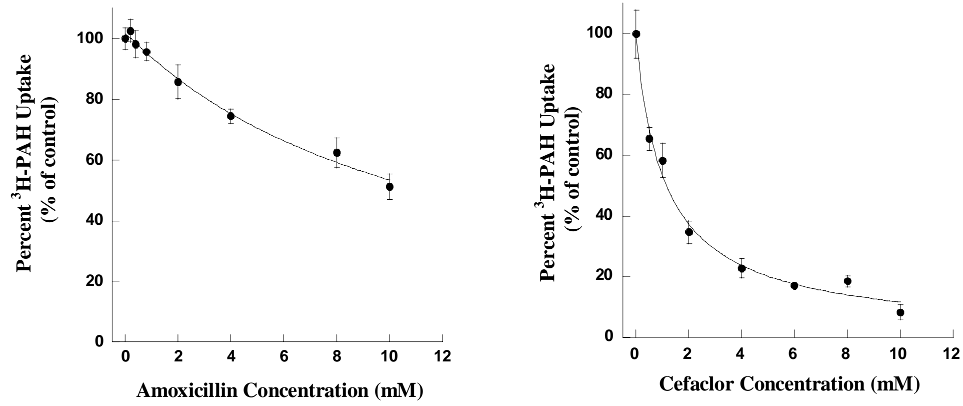
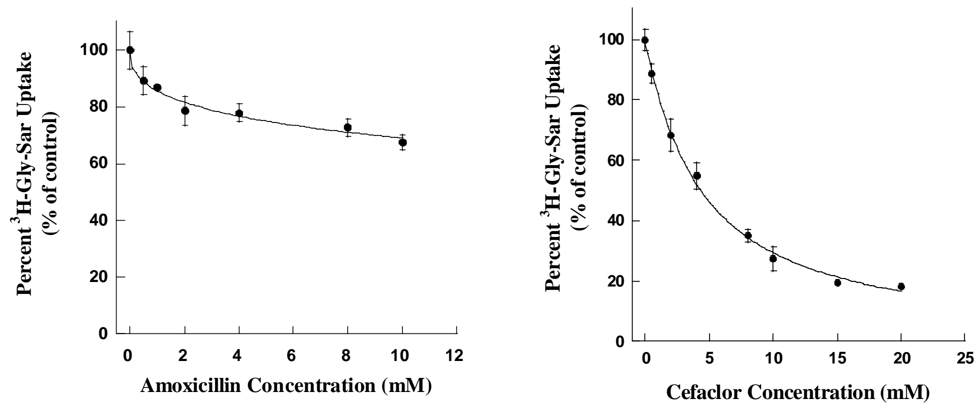
Figure 4

(A) hOAT1



(B) hPepT1



(C) hPepT2

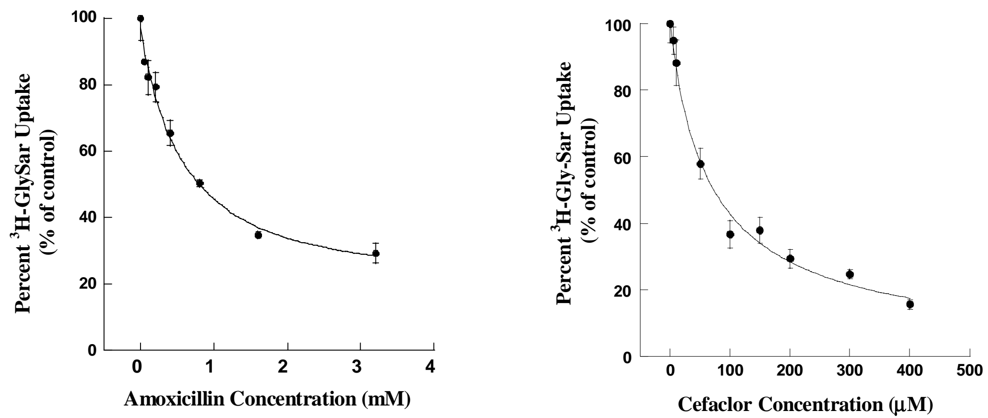


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Figure 5

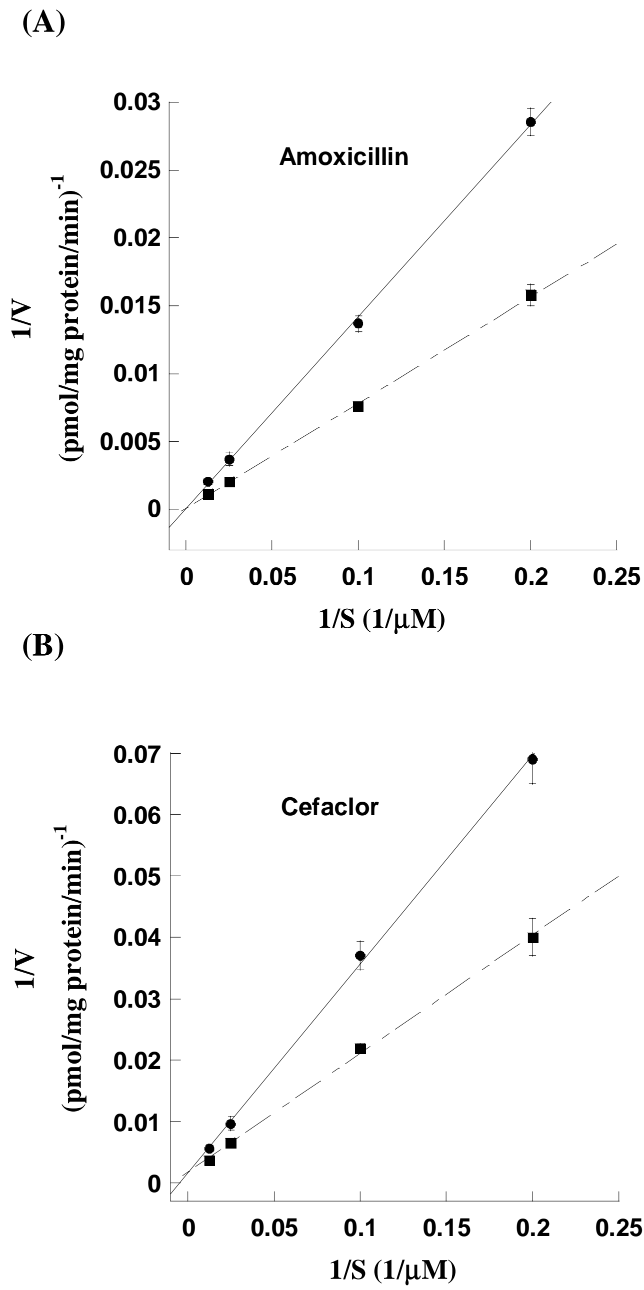

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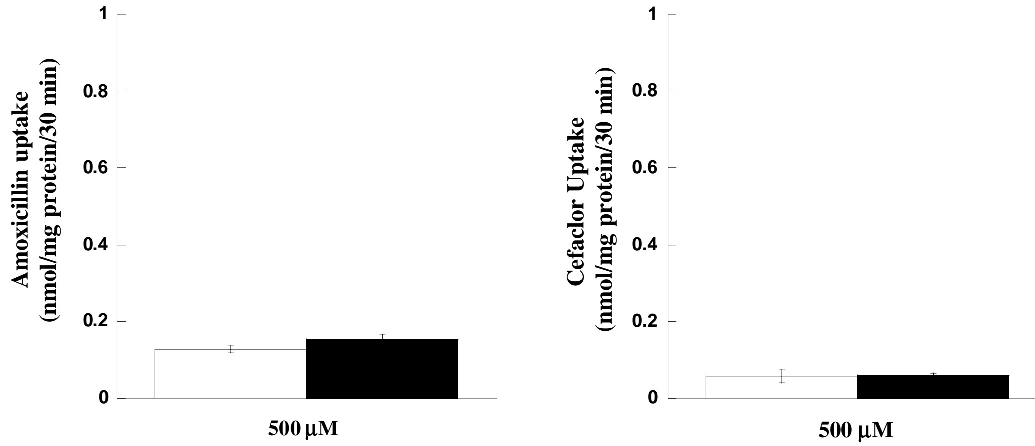
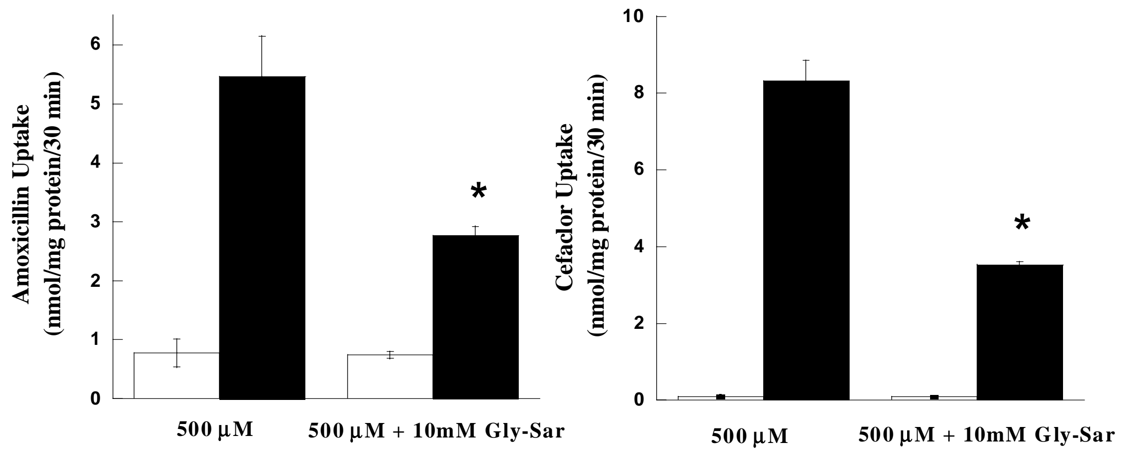
Figure 6

(A) hOAT1



(B) hPepT1



(C) hPepT2

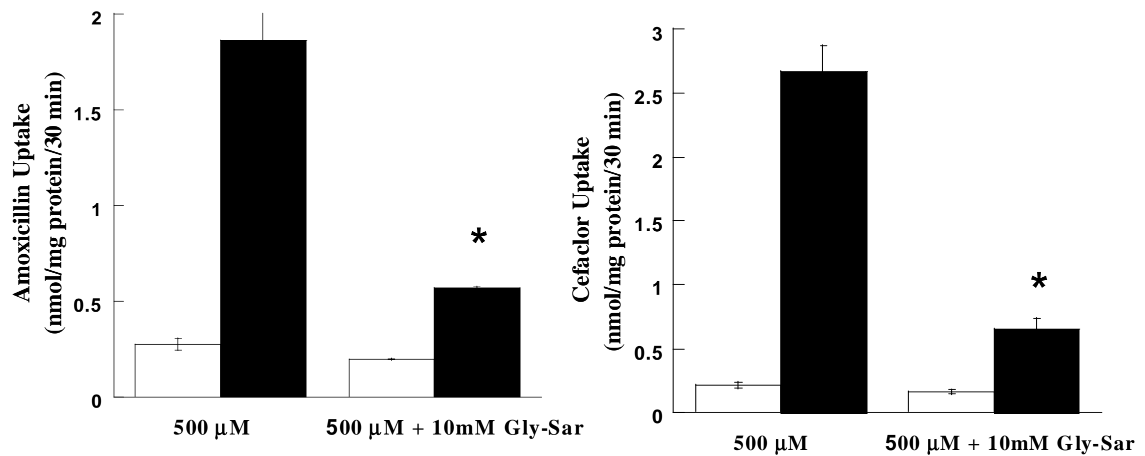


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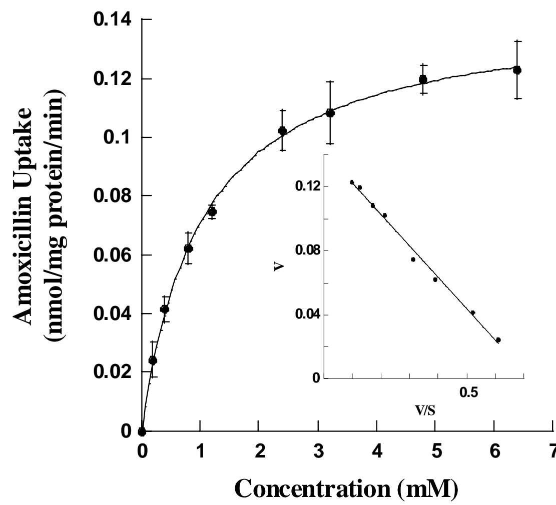
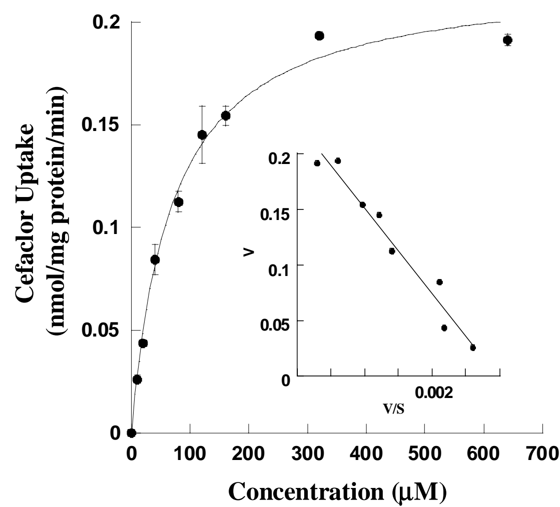
Figure 7

(A)



(B)



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