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Species Differences in Human and Rodent PEPT2-Mediated Transport of Glycylsarcosine and Cefadroxil in *Pichia Pastoris* Transformants

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ABSTRACT

The proton-coupled oligopeptide transporter PEPT2 (SLC15A2) plays an important role in the disposition of di/tripeptides and peptide-like drugs in kidney and brain. However, unlike PEPT1 (SLC15A1), there is little information about species differences in the transport of PEPT2-mediated substrates. The purpose of this study was to determine whether PEPT2 exhibited a species-dependent uptake of glycylsarcosine (GlySar) and cefadroxil using yeast *Pichia pastoris* cells expressing cDNA from human, mouse, and rat. In such a system, the functional activity of PEPT2 was evaluated with [^{3}H]GlySar as a function of time, pH, substrate concentration, and specificity, and with [^{3}H]cefadroxil as a function of concentration. We observed that the uptake of GlySar was pH-dependent with an optimal uptake at pH 6.5 for all three species. Moreover, GlySar showed saturable uptake kinetics, with K_m values in human (150.6 μM) > mouse (42.8 μM) ≈ rat (36.0 μM). The PEPT2-mediated uptake of GlySar in yeast transformants was specific, being inhibited by di/tripeptides and peptide-like drugs, but not by amino acids and nonsubstrate compounds. Cefadroxil also showed a saturable uptake profile in all three species, with K_m values in human (150.8 μM) > mouse (15.6 μM) ≈ rat (11.9 μM). These findings demonstrated that the PEPT2-mediated uptake of GlySar and cefadroxil was specific, species dependent, and saturable. Furthermore, based on the K_m values, mice appeared similar to rats but both were less than optimal as animal models in evaluating the renal reabsorption and pharmacokinetics of peptides and peptide-like drugs in humans.

Introduction

The peptide transporter PEPT2 (SLC15A2) is a member of the mammalian proton-coupled oligopeptide transporter family consisting of PEPT1, PEPT2, PHT1, and PHT2 (Herrera-Ruiz and Knipp, 2003). PEPT2 is characterized as a high-affinity (i.e., K_m values in the μM range) low-capacity transporter mediating the uptake of a broad range of di/tripeptides [e.g., glycylsarcosine (GlySar), glycyl-L-glutamine, and carnosine] and peptide-like drugs (e.g., cefadroxil, bestatin, valacyclovir, and 5-aminolevulinic-acid) (Daniel and Herget, 1997; Daniel and Kottra, 2004; Smith et al., 2004, 2013). PEPT2 is abundantly expressed in the apical membrane of renal proximal tubule and choroid plexus epithelial cells (Shen et al., 1999, 2004), where it plays an important role in the reabsorption of di/tripeptides and peptidomimetics from glomerular filtrate and in the removal of degraded neuropeptides from the cerebrospinal fluid, respectively (Brandsch et al., 2008; Rubio-Aliaga and Daniel, 2008; Smith et al., 2013). Studies on the distribution of GlySar, cefadroxil, and the peptidomimetic heme precursor 5-aminolevulinic acid in the brain have shown that cerebrospinal fluid-to-blood concentration ratios were 4- to 8-fold higher in PEPT2 knockout mice than in wild-type animals (Ocheltree et al., 2004b; Hu et al., 2007; Shen et al., 2007; Kamal et al., 2008). In addition, the renal tubular reabsorption of cefadroxil was almost completely abolished in mice during PEPT2 ablation (Shen et al., 2007). These findings demonstrate that PEPT2 plays an indispensable role in the systemic and regional exposure of peptide/mimetic substrates.

PEPT1 (SLC15A1), in contrast to PEPT2, is a low-affinity (i.e., K_m values in the mM range) high-capacity transporter that is mainly expressed at the apical side of epithelial cells in the small intestine, where it absorbs di/tripeptides and peptide-like compounds across the luminal membrane into enterocytes (Hu et al., 2008; Jappar et al., 2010; Yang et al., 2013). Species differences have been observed in the PEPT1-mediated uptake of the synthetic dipeptide GlySar and the β-lactam antibiotic cefadroxil (Hu et al., 2012, 2014; Hu and Smith, 2016). In in vitro studies, yeast cells transfected with human (h), mouse (m), and rat (r) PEPT1 cDNA exhibited saturable uptake of GlySar with K_m values estimated over a 5-fold range (Hu et al., 2012). Subsequent in situ perfusion studies in jejunum confirmed this species difference and reported 2- to 4-fold higher affinities (i.e., lower K_m values) for both GlySar and cefadroxil in humanized PEPT1 mice compared with wild-type mice (Hu et al., 2014; Hu and Smith, 2016). Such observations, along with significant examples involving species-dependent pharmacokinetic profiles of other transporters, such as P-glycoprotein, MRP1, and BCRP (Katoh et al., 2006; Ito, 2008; Li et al., 2008; Chu et al., 2013), demand that transporter studies be performed in different species during drug discovery and preclinical and clinical development.

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ABBREVIATIONS: GlySar, glycylsarcosine; h, human; m, mouse; OD, optical density; PCR, polymerase chain reaction; PPB, potassium phosphate buffer; r, rat; 4-acetamido-49-isothiocyanostilbene-2,29-disulfonic acid, SITS; tetraethylammonium, TEA; YNB, yeast nitrogen base.

TABLE 1

Primers for cloning PEPT2 cDNA

Species	Direction	PCR Primer	Product
			bp
Human	Forward	AGCTACGTACATGAATCCTTTCCAGAAAA	2208
	Reverse	TTAGCGGCCGCGAGTTTTGTCTTCTTGGTCTCTAGT	
Mouse	Forward	ATAGGATCCGCCACCATGCTTGAGGAGAGAGAGAG	2244
	Reverse	TATCCTAGGGAGCCTTGTATTCTTGGTTTC	
Rat	Forward	CAGTACGTAATGCTTGAGGAGAGAGAGAG	2240
	Reverse	TTAGCGGCCGCGAGCCTTGTATTCTTGGTTTC	

bp, base pairs.

Convincing data on the species-dependent functional activity of PEPT2 is generally lacking, in large part because comparisons are made across different laboratories using different experimental systems. Since GlySar and cefadroxil were both found to have species-dependent PEPT1 uptake—and given the overlapping substrate specificity between PEPT1 and PEPT2—it was logical to propose that PEPT2 might also have species differences in the uptake of di/tripeptides and peptide-like drugs. Furthermore, the amino acid homology of PEPT2 between mice, rats, and humans is 83%–92%, thereby introducing a molecular basis for potential species differences in this transporter. Still, no studies have as yet systematically evaluated whether a species difference exists in the PEPT2-mediated transport of peptides/mimetics. With this in mind, the present study evaluated the uptake properties of GlySar and cefadroxil in yeast *Pichia pastoris* cells expressing human, mouse, and rat orthologs of PEPT2. *P. pastoris* cells were chosen as a model system, compared with other heterologous expression systems (e.g., HeLa, LLC-PK_1, and *Xenopus* oocytes), because of the lack of endogenous transport activity along with high PEPT2 functional activity (Döring et al., 1998). Collectively, our finding demonstrated that the PEPT2-mediated uptake of GlySar and cefadroxil was species dependent. However, whereas both mice and rats displayed similar affinities of GlySar and cefadroxil for PEPT2, their K_m values were approximately 4- and 12-fold lower, respectively, than hPEPT2 yeast transformants.

Materials and Methods

Materials. [^{3}H]GlySar (2.8 Ci/mmol) and [^{3}H]cefadroxil (5 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). HotStar HiFidelity polymerase chain reaction (PCR) polymerase and OmniScript reverse transcriptase were purchased from Qiagen (Valencia, CA), and *Escherichia coli* strain XL10-Gold Ultracompetent cells were purchased from Agilent Technologies (Santa Clara, CA). Biotin, unlabeled GlySar, and cefadroxil were obtained from Sigma-Aldrich (St. Louis, MO), and HATF filters (0.45 μm) were purchased from Millipore Corporation (Billerica, MA). *P. pastoris* GS115 strain, vector pPIC3.5K, and yeast nitrogen base (YNB) were obtained from Invitrogen (Carlsbad, CA). The hPEPT2 and rPEPT2 cDNA were purchased from GE Dharmacon (Lafayette, CO). All other chemicals were obtained from standard sources.

Cloning PEPT2 cDNA. mPEPT2 cDNA was amplified by proofreading the PCR using the reverse transcript of mouse kidney total RNA. The hPEPT2 and rPEPT2 cDNAs were subcloned from a pCMV-SPORT6 vector containing the full-length human or rat cDNA. Species-specific primers were designed for amplifying the full-length PEPT2 cDNA (Table 1).

Construction of pPIC3.5K-PEPT2. The full-length cDNA of PEPT2 was digested by the restriction enzymes SnaB I and Not I for human and rat, and by BamH I and Avr II for mouse, purified and ligated into the enzyme-digested pPIC3.5K vectors, and then transformed into *E. coli* XL10-Gold competent cells. The cDNA sequences of all inserts were screened by PCR with primers for amplification of an internal *PEPT2* gene fragment (Table 2). Once positive colonies were selected, plasmid DNA was isolated by using the QIAprep Spin Miniprep Kit (Qiagen) and the sequence of the expression constructs were confirmed by the DNA Sequencing Core, University of Michigan.

Transformation of pPIC3.5K-PEPT2 Plasmid into *P. Pastoris* GS115. Each species of PEPT2 plasmid was linearized by the restriction enzyme Sal I and purified with the MinElute Reaction Cleanup Kit (Qiagen). Transformations of yeast GS115 cells were performed according to the electroporation method as described in the manual of the MicroPulser Electroporator (BioRad, Hercules, CA). The yeast cells were then cultured on minimal methanol medium (1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, and 1.5% agar) and minimal dextrose medium (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose, and 1.5% agar) plates incubated at 30°C for 2 days, and screening the His^+Mut^+ from His^+Mut^s transformants.

Cell Culture. The recombinant *P. Pastoris* clones were cultured as described in the Pichia Expression Kit (https://tools.thermofisher.com/content/sfs/manuals/pich_man.pdf; Invitrogen). In brief, the species-specific recombinants were cultured in a 50-ml baffled flask containing 5 ml minimal glycerol medium (1.34% YNB, 4×10^{-5}% biotin and 1.0% glycerol) and grown at 30°C in a shaking incubator (250 rpm) for 18 hours. Cells were pelleted at 3000*g* for 5 minutes at room temperature, suspended in 50 ml minimal methanol medium (1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol) and grown at 30°C in a shaking incubator (250 rpm) for 24 hours. Cell density was determined in the culture medium by measuring the optical density (OD) at 600 nm.

Amplification of PEPT2 Genomic DNA. Genomic DNA was isolated from the recombinant *P. pastoris* clones as described in the Pichia Expression Kit (https://tools.thermofisher.com/content/sfs/manuals/pich_man.pdf; Invitrogen). After isolating the genomic DNA from human, mouse, rat, and vector transformants, real-time PCR was performed with species-specific primers (Table 3) to measure the gene integration copy number of PEPT2 cDNA in yeast cells (Abad et al., 2010). The *ARG4* gene was used as an internal control and the gene copy number was calculated as: C_t (target gene) divided by C_t (*ARG4*).

Uptake in Yeast Transformants. For the uptake studies, cell cultures were harvested by centrifugation at 3000*g* for 5 minutes at room temperature, washed once with an equal volume of 100 mM potassium phosphate buffer (PPB) (132 ml 100 mM K_2PO_4, 868 ml 100mM KH_2PO_4, pH 6.5), centrifuged, resuspended to one-fifth the volume of 100 mM PPB, and stored on ice. Uptake measurements were performed at 24°C using rapid filtration with HATF filters, as described previously (Döring et al., 1997, 1998). Briefly, uptake was initiated by rapidly mixing 20 μl of cell suspension with 30 μl of 100 mM PPB (pH 6.5) containing 0.02 μCi [^{3}H]GlySar for a final concentration of 1.0 μM, and then incubating up to 180 minutes; the optimal time point for subsequent studies was 10 minutes as determined by linear regression. The pH-dependent uptake of 1.0 μM [^{3}H]GlySar

TABLE 2

Primers for screening PEPT2 transformants

Species	Direction	PCR Primer	Product
			bp
Human	Forward	TATGGATGTAAAGGCACTGA	323
	Reverse	TTTTTATCTCTACAGCTGCC	
Mouse	Forward	GTTTTGGCGAAGACTGCTAT	302
	Reverse	CATGGGCAGTGGGATATAAA	
Rat	Forward	AGTCTTCTACCTCGCCATCAAT	307
	Reverse	TTTTTCTGCTGCCCAGTCTA	

bp, base pairs.

TABLE 3
Primers for measuring PEPT2 integration

Species	Direction	PCR Primer
Vector (*AOX 1*)	Forward	TGGCGAGGTTCATGTTTGTTTAT
	Reverse	TCTGGAGTGATGTTCGGGTGTA
Human	Forward	GGTACAGTGGGCCGAATTCA
	Reverse	GCCCATGATGGAGAAGATCAG
Mouse	Forward	TGGCTGGGAAAATTCAAGACA
	Reverse	ATGGCACCCAAAGACTTGAATAC
Rat	Forward	GGCCGAGTTCGTTTTGTTTTC
	Reverse	GAGGAACGTAGTAGTAGGCCATGAC
ARG4	Forward	TCCTCCGGTGGCAGTTCTT
	Reverse	TCCATTGACTCCCGTTTTGAG

was evaluated over a pH range of 5.5–8.0. Concentration-dependent uptake studies of [^{3}H]GlySar were performed over substrate concentrations of 1.0–1000 μM (pH 6.5 buffer), and inhibition studies were performed with 1.0 μM [^{3}H]GlySar in the absence and presence of 1 mM concentrations of potential inhibitors (pH 6.5 buffer). The concentration-dependent uptake of [^{3}H]cefadroxil was determined over the concentration range of 1.0–250 μM (pH 6.5 buffer) using 10-minute incubations; the time that was optimal for initial rates as determined by preliminary studies. All uptake studies were terminated by the addition of 2 ml of ice-cold 100 mM PPB, followed by filtration. The filter was washed three times with 2 ml of ice-cold buffer, transferred into glass vials into which 6 ml of Cytoscint cocktail (MP Biomedicals, Solon, OH) was added, and then the radioactivity was counted on a dual-channel liquid scintillation counter (Beckman LS 6000SC; Beckman Coulter, Fullerton, CA).

Data Analysis. For the concentration-dependent studies, the kinetic parameters of GlySar and cefadroxil uptake were best fit to a Michaelis-Menten equation:

$$V = \frac{V_{max} \cdot C}{K_m + C}$$

where V is the observed uptake rate; V_{max} is the maximum uptake rate; K_m is the Michaelis constant; and C is the substrate (GlySar or cefadroxil) concentration, after being corrected for uptake in pPIC3.5K vector control cells.

All data are reported as mean ± S.E. of three different experiments with each experiment being carried out in triplicate. Statistical comparisons between multiple treatment groups were determined by one-way analysis of variance followed by either Tukey's or Dunnett's test (GraphPad Prism, v6.0; GraphPad Software, Inc., La Jolla, CA). A probability of $P \leq 0.05$ was considered to be statistically significant. The quality of fit for nonlinear regression analysis was evaluated by the coefficient of determination (r^2), S.E. of the parameters, and visual inspection of the residuals.

Results

Uptake of GlySar versus Time. For determination of the optimal time for incubation (i.e., linear uptake or initial rates), the uptake of 1.0 μM GlySar was measured in yeast transformants over 180 minutes. As shown in Fig. 1, the accumulation of GlySar in PEPT2-transformed yeast cells was far greater than that of vector alone during the entire 180-minute period. While the uptake of GlySar by vector was linear for 180 minutes, the three mammalian transformants showed saturable uptake profiles of GlySar, with 30-minute accumulations over vector controls of 106-fold for pPIC3.5K-hPEPT2, 169-fold for pPIC3.5K-rPEPT2, and 52-fold for pPIC3.5K-mPEPT2. GlySar uptake was linear for about 20 minutes, and as a result an incubation time of 10 minutes was selected for subsequent experiments in all three species. At the 10-minute incubation time, the uptake of GlySar was very low in the pPIC3.5K vector (0.00173 $pmol^{-1} \cdot OD^{-1} \cdot min^{-1}$) but substantially greater in pPIC3.5K-mPEPT2 (36-fold), pPIC3.5K-rPEPT2 (109-fold), and pPIC3.5K-hPEPT2 (60-fold).

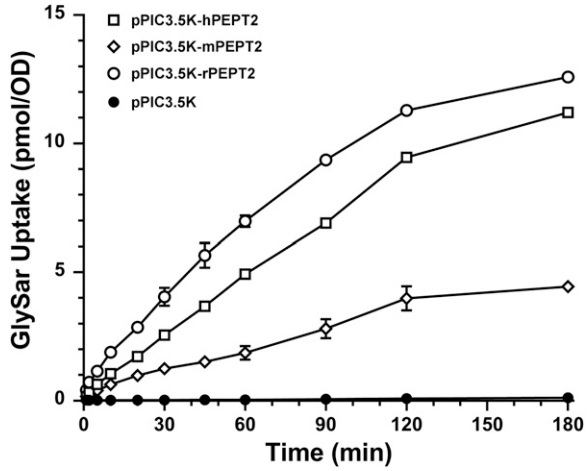

Fig. 1. Time-dependent uptake of [^{3}H]GlySar in *P. pastoris* expressing the human (pPIC3.5K-hPEPT2), mouse (pPIC3.5K-mPEPT2), and rat (pPIC3.5K-rPEPT2) PEPT2 transformants and vector controls (pPIC3.5K). Uptake studies were performed with 1.0 μM [^{3}H]GlySar in PPB buffer (pH 6.5) at 24°C. Data are expressed as mean ± S.E. (n = 3).

pH-Dependent Uptake of GlySar. Since the PEPT2-mediated uptake of GlySar was favored by a proton gradient as the driving force for transport, the pH-dependent uptake of 1.0 μM GlySar was evaluated at pH values ranging from 5.5 to 8.0. The results showed that the uptake of GlySar was influenced greatly by the pH value, with an optimal uptake occurring at around pH 6.5 for all three species (Fig. 2).

Concentration-Dependent Uptake of GlySar. The uptake kinetics of GlySar in yeast transformants expressing hPEPT2, mPEPT2, and rPEPT2 was determined over the concentration range of 1–1000 μM. PEPT2-mediated uptake of GlySar was determined after subtracting the uptake of GlySar in vector controls. As shown in Fig. 3, all three species of PEPT2 displayed a similar saturable uptake of GlySar and were fit simultaneously to a single Michaelis-Menten term. PEPT2 showed the lowest affinity for GlySar in human (K_m = 150.6 ± 10.3 μM) followed by mouse (K_m = 42.8 ± 7.3 μM) and rat (K_m = 36.0 ± 3.5 μM); no significant differences in affinity were observed between mouse and rat (Table 4).

Statistical analyses indicated a significant difference in K_m (and V_{max}) values between mice or rats and humans, but not between mice and rats. In contrast, the uptake of GlySar by vector alone was linear over this concentration range and negligible compared with the mammalian PEPT2 transformants (data not shown). The intrinsic clearance of GlySar (i.e., V_{max}/K_m) was significantly different between all three species, with rPEPT2 (0.411 ± 0.041 μl/OD/min) > hPEPT2 (0.098 ± 0.007 μl/OD/min) > mPEPT2 (0.040 ± 0.007 μl/OD/min).

Effect of Inhibitors on GlySar Uptake. The specificity of GlySar uptake in *P. pastoris* cells expressing PEPT2 was established using a wide range of potential inhibitors, such as amino acids, di/tripeptides, valacyclovir (prodrug) and acyclovir (active drug), cephalosporins, the angiotensin-converting enzyme inhibitor captopril, and organic cationic and anionic compounds (Fig. 4). All three mammalian species showed a similar inhibitory profile in the yeast expression system. The amino acids glycine and L-histidine had no effect on the uptake of GlySar, indicating that GlySar was stable during the period of incubation and that the peptide/histidine transporters PHT1/PHT2 (or similar endogenous genes with overlapping substrate specificities) were not expressed at the plasma membrane of yeast since L-histidine was without effect. As expected, GlySar uptake by hPEPT2, mPEPT2, and rPEPT2 transformants was significantly reduced by the dipeptides glycylsarcosine (≥ 90%), glycyl-L-glutamine (≥ 95%), and L-homocarnosine (≥ 50%),

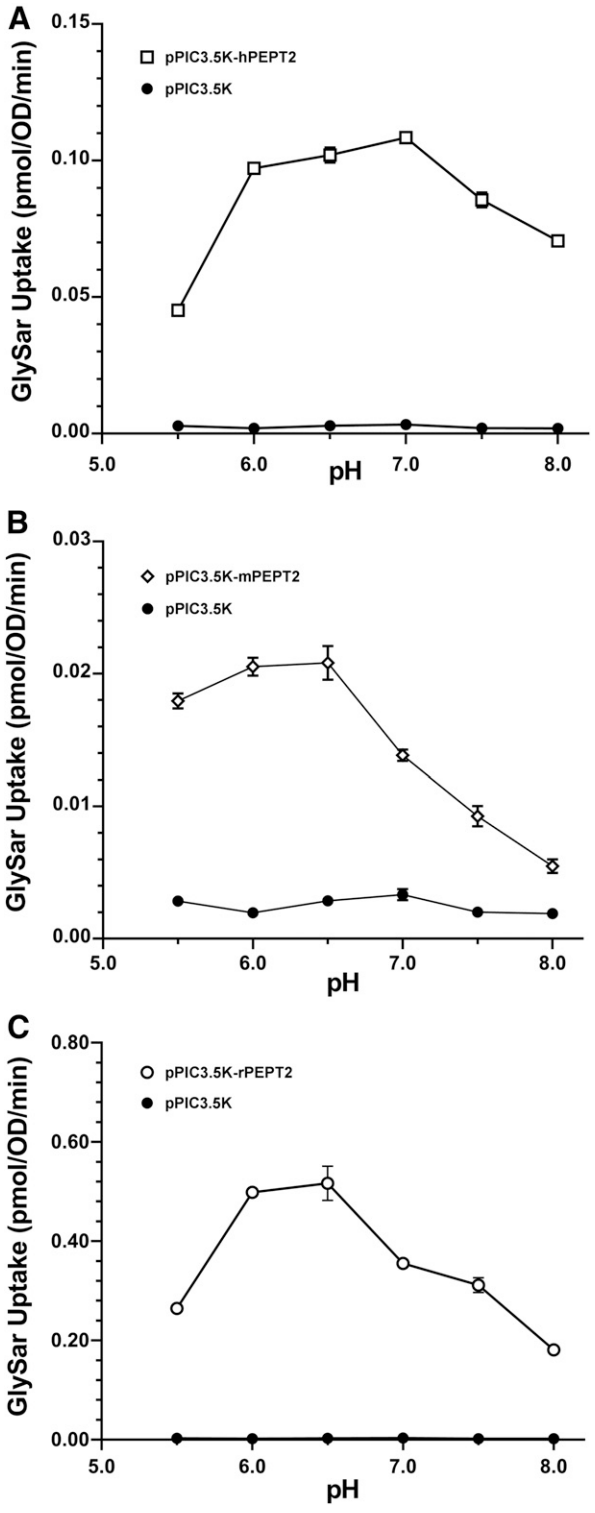

Fig. 2. pH-Dependent uptake of [^{3}H]GlySar in *P. pastoris* expressing (A) human (pPIC3.5K-hPEPT2), (B) mouse (pPIC3.5K-mPEPT2), and (C) rat (pPIC3.5K-rPEPT2) PEPT2 transformants and vector controls (pPIC3.5K). Uptake studies were performed over 10 minutes with 1.0 μM [^{3}H]GlySar at 24°C. Data are expressed as mean ± S.E. (n = 3).

along with inhibition by the tripeptide glycylglycyl-L-histidine (≥ 75%). With respect to peptidomimetics, the antiviral ester prodrug valacyclovir showed significant inhibition of GlySar uptake (≥ 90%); however, this uptake was not inhibited by the active moiety acyclovir. Moreover,

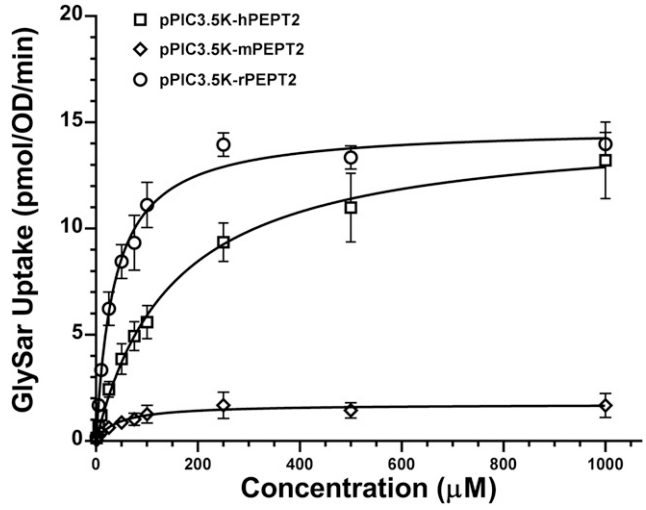

Fig. 3. Concentration-dependent uptake of [^{3}H]GlySar in *P. pastoris* expressing human (pPIC3.5K-hPEPT2), mouse (pPIC3.5K-mPEPT2), and rat (pPIC3.5K-rPEPT2) PEPT2 transformants. Uptake studies were performed over 10 minutes with 1–1000 μM GlySar in PPB buffer (pH 6.5) at 24°C. Uptake rates of vector controls (pPIC3.5K) were subtracted from the corresponding rates of PEPT2-expressing cells. Data are expressed as mean ± S.E. (n = 3).

GlySar was inhibited by the aminocephalosporins cefadroxil, cephalexin, and cephradine (≥ 90%), whereas the cephalosporins lacking an α-amino group (i.e., cephalothin and cefazolin) were without effect. Likewise, neither captopril nor the organic anion/cation 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS)/tetraethylammonium (TEA) had an effect on GlySar uptake. Thus, it appeared that the uptake of GlySar was specific for PEPT2 and was inhibited by di/tripeptides and peptide mimetics, known to be substrates of PEPT2.

Concentration-Dependent Uptake of Cefadroxil. The transport kinetics of a therapeutic agent, cefadroxil, was also evaluated by *P. pastoris* cells expressing the hPEPT2, mPEPT2, and rPEPT2 transformants. In these studies, uptake was determined after 10-minute incubations with cefadroxil concentration ranging from 1 to 250 μM. The incubation time of 10 minutes represented the linear uptake (or initial rate) of cefadroxil, as judged by preliminary experiments. As before, vector uptake rates of cefadroxil were subtracted from uptake rates in yeast cells expressing the three mammalian species of PEPT2. As shown in Fig. 5, all three species demonstrated saturable transport profiles of cefadroxil, which were fit simultaneously to a single Michaelis-Menten term (Table 5). The ranking of K_m values for the three mammalian species of PEPT2 was human (K_m = 150.8 ± 11.8 μM) > mouse (K_m = 15.6 ± 1.8 μM) > rat (11.9 ± 1.5 μM), in which no significant differences in affinity were observed between mouse and rat.

Interestingly, the PEPT2 transport capacity for cefadroxil (as judged by the V_{max} value) was much greater in humans (8.71 ± 0.37 pmol/OD/min) than in rats (2.18 ± 0.08 pmol/OD/min) or mice (0.22 ± 0.01 pmol/OD/min) (Table 5). However, the V_{max} value for GlySar (Table 4) was the same in humans and rats (i.e., 14.8 ± 0.4 pmol/OD/min) and much greater than the value in mice (1.7 ± 0.1 pmol/OD/min), suggesting a substrate dependence in PEPT2 capacity among the three mammalian species. The differences in the V_{max} value do not appear to be the result of varying PEPT2 protein expression, as evaluated by immunoblot analysis (data not shown). Notwithstanding potential differences in antibody affinity between human, mouse, and rat proteins, and the lack of an internal standard, the relative protein expression was ± 25% between the three mammalian species.

TABLE 4

Transport kinetics of [^{3}H]GlySar uptake in *P. pastoris* cells expressing different mammalian *PEPT2* genes

Parameters are expressed as mean ± S.E. (n = 3). Following vector subtraction, the data were fit to a Michaelis-Menten equation using a weighting factor of unity. GlySar concentrations ranged from 1 to 1000 μM. Statistical analyses were performed using one-way analysis of variance followed by Tukey's test. Parameters with common capital letters (i.e., A, B, or C) were not significantly different between genotypes.

Parameters (unit)	Yeast Transformant			
	pPIC3.5K	pPIC3.5K-hPEPT2	pPIC3.5K-mPEPT2	pPIC3.5K-rPEPT2
V_{max} (pmol/OD/min)	N.A.	14.8 ± 0.4^A	1.7 ± 0.1^B	14.8 ± 0.4^A
K_m (μM)	N.A.	150.6 ± 10.3^A	42.8 ± 7.3^B	36.0 ± 3.5^B
V_{max}/K_m (μl/OD/min)	N.A.	0.098 ± 0.007^A	0.040 ± 0.007^B	0.411 ± 0.041^C
r^2	0.998	0.997	0.974	0.992
Gene copy number[a]	0.98 ± 0.01	0.99 ± 0.01	0.98 ± 0.02	0.97 ± 0.01

N.A., not applicable.

[a]Gene copy number was measured by real-time PCR using human, mouse, and rat PEPT2 primers, and the *ARG4* gene as a control; *AOX1* gene primers were used for vector alone (Table 3).

Discussion

Species differences in enzymes, receptors, and transporters complicate our understanding of drug action during discovery, development, and clinical use (Jiang et al., 2011). In particular, it is important to investigate if potential species differences exist in transporters since any disparities can impact the absorption, distribution, metabolism, and excretion of many drugs, thereby making the prediction of human pharmacokinetics and drug-drug interactions more difficult. Recently, it has been clearly demonstrated that some membrane transporters of the solute carrier and ATP-binding cassette superfamilies exhibit species-dependent effects on drug disposition (Chu et al., 2013; Scheer and Wolf, 2014). In particular, *Xenopus laevis* oocytes injected with PEPT1 cRNA from rabbit, rat, mouse, sheep, chicken, and salmon exhibited 8-fold differences in their K_m values for GlySar uptake (Hu et al., 2012). With respect to PEPT2, it was observed that HeLa, HEK293, and LLC-PK1 cells (or *Xenopus* oocytes), transfected with the cDNA (or cRNA) of human, rat, monkey, and pig also exhibited 7- to 8-fold differences in K_m values for the uptake of GlySar (Ramamoorthy et al., 1995; Terada et al., 1997; Sawada et al., 1999; Zhu et al., 2000; Zhang et al., 2004; Søndergaard et al., 2008; Guo et al., 2012). Still, it should be appreciated that substantial differences in substrate affinity have been observed, not only between species but within a single species when studied by the same investigators using different methods of study (e.g., cRNA-injected oocytes versus cDNA-transfected Chinese hamster ovary cells for GlySar uptake by PEPT1) (Hu et al., 2012). These examples demonstrate that although species differences might exist for PEPT2 this conclusion is tenuous because the findings reported for GlySar were from different investigators using a variety of experimental systems.

To date, no studies have systematically evaluated whether species differences exist in the transport kinetics of PEPT2 substrates. Therefore, we used the yeast *P. pastoris* expression system to compare the uptake of GlySar and cefadroxil by hPEPT2, mPEPT2, and rPEPT2. Our results have demonstrated the following: 1) the PEPT2-mediated transport of GlySar and cefadroxil was saturable, and the three mammalian species of PEPT2 had different affinities for these two substrates (K_m of GlySar in human > mouse ≈ rat; K_m of cefadroxil in human >> mouse ≈ rat 2) PEPT2-mediated transport was specific in yeast transfected with the mammalian species, studied with the uptake of GlySar being inhibited by known substrates of PEPT2; 3) PEPT2 functional activity showed a pH dependence with an optimal uptake at 6.5 for all three species; and 4) the transport capacity of PEPT2 was substrate dependent among the three species (GlySar V_{max}: human = rat >> mouse; cefadroxil V_{max}: human > rat >> mouse).

In concentration-dependent studies with GlySar, we compared the dipeptide's affinities in hPEPT2, mPEPT2, and rPEPT2 transformants. In all species, the μM values for K_m were observed, which were consistent with the high-affinity properties of PEPT2 (Brandsch et al., 2008; Rubio-Aliaga and Daniel, 2008; Smith et al., 2013). Specifically, the GlySar K_m value for PEPT2 was about 4-fold higher in human (K_m = 151 μM) than in mouse (K_m = 42.8 μM) or rat (K_m = 36.0 μM). These results were consistent with the K_m values reported for GlySar in HEK293 cells expressing hPEPT2 (K_m = 170 μM) (Terada et al., 2004), and in primary choroid plexus cell cultures expressing mouse (K_m = 70 μM) (Ocheltree et al., 2004b) and rat (K_m = 60 μM) (Shu et al., 2002) PEPT2. Moreover, these K_m values were remarkably similar to the findings from our yeast studies, where mPEPT2 and rPEPT2 had similar affinities for GlySar and much higher affinities (i.e., lower K_m values) than that observed in humans. To rule out an effect of different gene copy numbers being present in the human, mouse, and rat transformants, *PEPT2* gene integration into the yeast genome was tested and determined to be one copy in all three mammalian species (Table 4).

The functional characteristics of PEPT2, such as substrate specificity and pH dependence, were similar between the three species. The constitutive amino acid glycine had no effect on the PEPT2 uptake of GlySar, confirming the stability of GlySar during the incubation process as well as the absence of relevant amino acid transporters in non-transfected yeast. Moreover, the lack of GlySar inhibition by excess L-histidine ruled out the presence of the peptide/histidine transporters PHT1 and PHT2 in the yeast cell plasma membrane. Neither the organic cation tetraethylammonium (TEA) nor the organic anion 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) had any influence on GlySar, indicating that organic cation transporters (OCT)s and organic anion transporters (OAT)s were not affecting the uptake of dipeptide in the three yeast transformant species. Since PEPT2 activity was known to be stimulated by a proton gradient, the pH-dependent studies indicated an optimal pH of 6.5 for GlySar uptake in human, mouse, and rat transformants, which was consistent with data from previous studies in *P. pastoris*, HeLa, and LLC-PK1 cells (Terada et al., 1997; Döring et al., 1998; Guo et al., 2012) transfected with different species of PEPT2.

GlySar is a synthetic dipeptide typically used as a model substrate for the PEPT2 transporter, and as a result species differences in PEPT2 activity were also evaluated using the clinical drug cefadroxil. Our kinetic analysis of cefadroxil uptake in yeast cells expressing hPEPT2, mPEPT2, and rPEPT2 indicated there were no significant differences in cefadroxil affinity between mouse (K_m = 15.6 μM) and rat (K_m = 11.9 μM), although an approximate 12-fold difference existed between the rodents and human (K_m = 150.8 μM). The K_m value of cefadroxil observed in mPEPT2 transformants (K_m = 15.6 μM) was close to values reported for cefadroxil uptake in isolated mouse choroid plexus

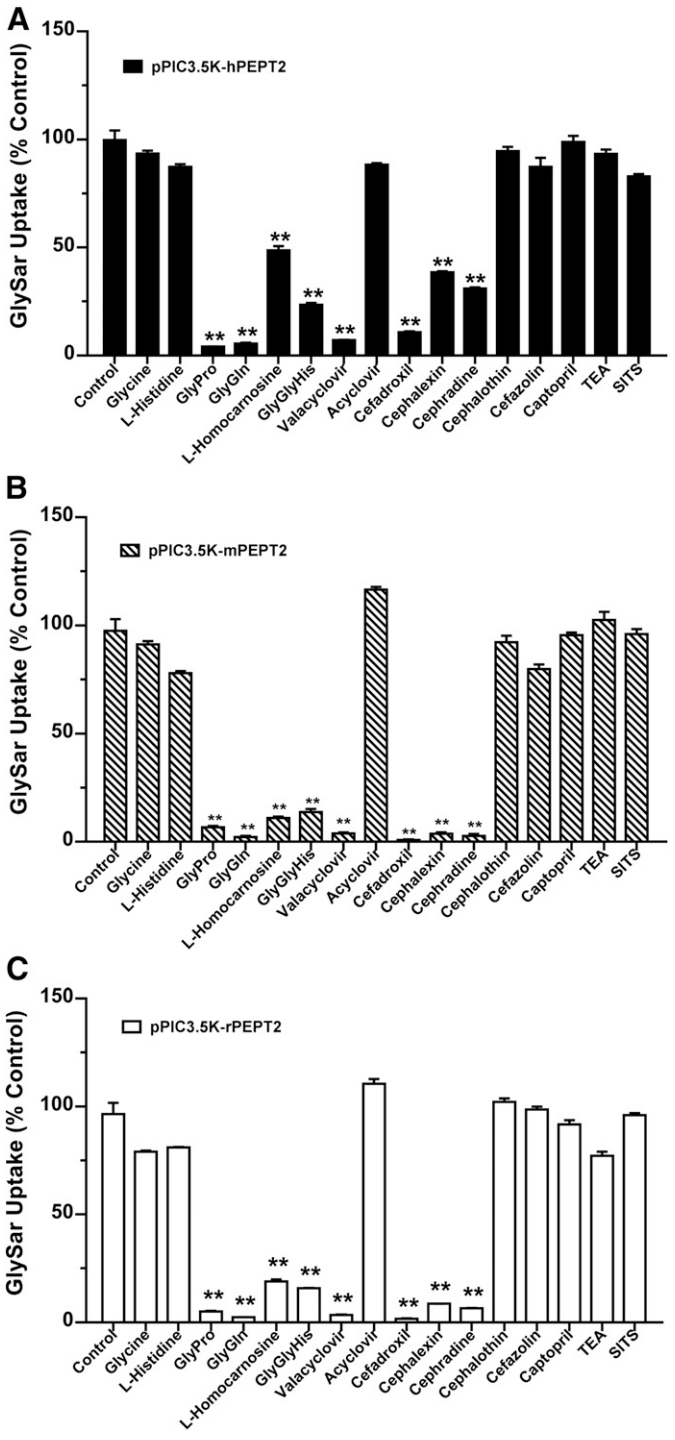

Fig. 4. Effect of inhibitors on the uptake of [^{3}H]GlySar in *P. pastoris* expressing (A) human (pPIC3.5K-hPEPT2), (B) mouse (pPIC3.5K-mPEPT2), and (C) rat (pPIC3.5K-rPEPT2) PEPT2 transformants. Uptake studies were performed over 10 minutes with 1.0 μM GlySar in the absence and presence of 1.0 mM of potential inhibitors at 24°C in PPB buffer (pH 6.5). Data are expressed as mean ± S.E. (n = 3). Uptake rates of vector controls (pPIC3.5K) were subtracted from the corresponding rates of PEPT2-expressing cells and are shown as percentage of control (i.e., GlySar alone). Statistical analyses were performed using one-way analysis of variance followed by Dunnett's test, where $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with control values.

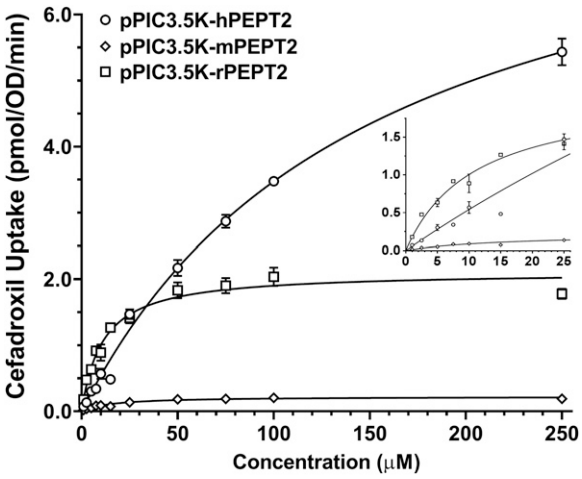

Fig. 5. Concentration-dependent uptake of [^{3}H]cefadroxil in *P. pastoris* expressing human (pPIC3.5K-hPEPT2), mouse (pPIC3.5K-mPEPT2), and rat (pPIC3.5K-rPEPT2) PEPT2 transformants. Uptake studies were performed over 10 minutes with 1–250 μM cefadroxil in PPB buffer (pH 6.5) at 24°C. Uptake rates of vector controls (pPIC3.5K) were subtracted from the corresponding rates of PEPT2-expressing cells. Data are expressed as mean ± S.E. (n = 3).

(K_m = 27 μM) (Ocheltree et al., 2004a). Moreover, the K_m value of cefadroxil observed in rPEPT2 transformants (K_m = 11.9 μM) was comparable to values reported in *Xenopus* oocytes injected with rPEPT2 cRNA (K_m = 23.8 μM) (Boll et al., 1996), in rat isolated choroid plexus (K_m = 17.1 μM) (Shen et al., 2005), and in rat renal brush-border membrane vesicles (K_m = 8.8 μM) (Ries et al., 1994).

At the present time, it is uncertain how well the species-dependent differences in substrate affinity for PEPT2 in the yeast transformant model reflect that of PEPT2 in vivo. One might infer a faithful translation of results based on the observation that the in vitro affinity of mPEPT2 for cefadroxil in yeast (K_m = 15.6 μM) was very similar to the in vivo affinity of PEPT2 observed in mice (K_m = 27.1 μM) during a population pharmacokinetic analysis of cefadroxil renal transport in wild-type and knockout animals (Xie et al., 2016). A species-dependent uptake of PEPT1 substrates in yeast *P. pastoris* was also observed in vivo when comparing the area under the plasma concentration-time curve and maximum plasma concentration (C_{max}) of orally administered cefadroxil in wild-type mice, humanized PEPT1 mice, and clinical data obtained from the literature (Hu and Smith, 2016). Since PEPT2 dominates the tubular reabsorption of peptides/mimetics in the kidney, as well as maintaining endogenous peptide homeostasis in the brain via efflux from the cerebrospinal fluid into the choroid plexus, the availability of a humanized PEPT2 mouse model might have significant utility in being able to translate PEPT2 substrate pharmacokinetics from rodent to man.

In conclusion, our studies characterized the species differences in PEPT2-mediated transport of GlySar and cefadroxil using a yeast *P. pastoris* expression system. In particular, the K_m values of GlySar and cefadroxil were similar in both mouse and rat, but significantly lower than that observed in human transformants. Still, the specificity and pH dependence of PEPT2 were maintained in all three mammalian species. Given that mouse and rat exhibited 4- and 8- to 9-fold lower K_m values for GlySar and cefadroxil, respectively, it appears that these rodent models are less than optimal for translating the renal reabsorption and pharmacokinetics of peptides and peptide-like drugs in humans. Although mechanistically valuable, transporter studies in cell cultures (naïve or transfected), and other in vitro or in situ experimental systems, might not accurately reflect the outcome in vivo during human studies (Hu et al., 2014). The establishment of humanized PEPT2 and humanized PEPT1/PEPT2 mice could provide a valuable bridge in predicting drug-drug interactions and in utilizing physiologically based pharmacokinetic models to better predict drug performance in humans.

TABLE 5

Transport kinetics of [^{3}H]cefadroxil uptake in P. pastoris cells expressing different mammalian PEPT2 genes

Parameters are expressed as mean ± S.E. (n = 3). Following vector subtraction, the data were fit to a Michaelis-Menten equation using a weighting factor of unity. Cefadroxil concentrations ranged from 1 to 250 μM. Statistical analyses were performed using one-way analysis of variance followed by Tukey's test. Parameters with common capital letters (i.e., A, B, or C) were not significantly different between genotypes.

Parameters (unit)	Yeast Transformant			
	pPIC3.5K	pPIC3.5K-hPEPT2	pPIC3.5K-mPEPT2	pPIC3.5K-rPEPT2
V_{max} (pmol/OD/min)	N.A.	8.71 ± 0.37^A	0.22 ± 0.01^B	2.18 ± 0.08^C
K_m (μM)	N.A.	150.8 ± 11.8^A	15.6 ± 1.8^B	11.9 ± 1.5^B
V_{max}/K_m (μl/OD/min)	N.A.	0.058 ± 0.005^A	0.014 ± 0.002^B	0.183 ± 0.024^C
r^2	0.996	0.989	0.923	0.892

N.A., not applicable.

Authorship Contributions

Participated in research design: Song, Hu, Jiang, Smith.

Conducted experiments: Song, Hu.

Performed data analysis: Song, Hu.

Wrote or contributed to the writing of the manuscript: Song, Hu, Jiang, Smith.

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