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Species-Dependent Uptake of Glycylsarcosine but Not Oseltamivir in *Pichia Pastoris* Expressing the Rat, Mouse, and Human Intestinal Peptide Transporter PEPT1

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Running Title: Species-Dependent PEPT1 Transport of GlySar and Oseltamivir

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ABBREVIATIONS: GlyPro, glyclyproline; GlySar, glycylsarcosine; Mrp4, multidrug resistance-associated protein 4; Oat3, organic anion transporter 3; PEG, polyethylene glycol; PEPT1, peptide transporter 1; Pgp, P-glycoprotein; POT, proton-coupled oligopeptide transporter; PPB, potassium phosphate buffer pH 6.5; SITS, 4-acetamido-4′-isothiocyanostilbene-2, 2′-disulfonic acid; TEA, tetraethylammonium.

Abstract

The purpose of this study was to determine if glycylsarcosine (a model dipeptide) and oseltamivir (an antiviral prodrug) exhibited a species-dependent uptake in yeast *Pichia pastoris* expressing the rat, mouse, and human homologues of PEPT1. Experiments were performed with [^{3}H]glycylsarcosine (GlySar) in yeast *Pichia pastoris* expressing human, mouse and rat PEPT1, in which uptake was examined as a function of time, concentration, potential inhibitors and the dose-response inhibition of GlySar by oseltamivir. Studies with [^{14}C]oseltamivir were also performed under identical experimental conditions. We found that GlySar exhibited a saturable uptake in all three species, with *Km* values for human (0.86 mM) > mouse (0.30 mM) > rat (0.16 mM). GlySar uptake in the yeast transformants was specific for peptides (GlyPro) and peptide-like drugs (cefadroxil, cephradine, valacyclovir), but was unaffected by glycine, L-histidine, cefazolin, cephalothin, cephapirin, acyclovir, SITS, TEA and elacridar. Although oseltamivir caused a dose-dependent inhibition of GlySar uptake [*IC50* values for human (27.4 mM) > rat (18.3 mM) > mouse (10.7 mM)], the clinical relevance of this interaction would be very low in humans. Importantly, oseltamivir was not a substrate for the intestinal PEPT1 transporter in yeast expressing the three mammalian species tested. Instead, the prodrug exhibited nonspecific binding to the yeast vector and PEPT1 transformants. Finally, the mouse appeared to be a better animal model than rat for exploring the intestinal absorption and pharmacokinetics of peptides and peptide-like drugs in human.

Introduction

Oseltamivir phosphate (Tamiflu®), an ethyl ester prodrug of the active metabolite Ro 64-0802 (oseltamivir carboxylate), is used for the treatment and prophylaxis of influenza A or B in adults and children ≥ one year of age (Moscona, 2005; Davies 2010). The prodrug is well absorbed (75-80%) from the gastrointestinal tract and is efficiently converted by human liver carboxylesterase 1 to Ro 64-0802 (Shi et al., 2006). It is this drug moiety that acts as a potent neuraminidase inhibitor, thereby, preventing the release of virions from infected host cells and viral replication. Oseltamivir has dose-proportional absorption and a linear pharmacokinetic profile with respect to the active metabolite. Ro 64-0802 accumulates less than 2-fold after oral dosing of oseltamivir over a dose range of 50 to 500 mg twice daily. The pharmacokinetics of both oseltamivir and oseltamivir carboxylate are seemingly uncomplicated due to low protein binding (i.e., 42% for prodrug and 3% for active metabolite), a lack of cytochrome P450 interactions, and no other metabolic species being formed (He et al., 1999; Dutkowski et al., 2003). Renal clearance of both compounds exceeds their filtration clearance because of active tubular secretion via the organic anionic pathway (Hill et al., 2002). This mechanism, however, has low potential for drug-drug interactions given the weak affinity of Ro 64-0802 for human OAT1 (Ki of 45.1 mM).

Oseltamivir has a good safety record in which the most common side effects are transient nausea, vomiting, and abdominal pain, which occur in about 5-10% of the patient population (Moscona, 2005). More recently, however, there have been post-marketing reports (mostly from Japan) of neuropsychiatric side effects in younger patients taking oseltamivir and in some cases resulting in fatal outcomes (http://www.gene.com/gene/products/information/tamiflu/pdf/pi.pdf).

This finding has led to a number of studies evaluating the mechanism by which oseltamivir and oseltamivir carboxylate are transported across capillary endothelial cells forming the blood-brain barrier (Moritmoto et al., 2008; Ose et al., 2008; Ose et al, 2009). It was demonstrated that oseltamivir and its active metabolite were actively effluxed by luminally expressed P-glycoprotein (Pgp) and multidrug resistance-associated protein 4 (Mrp4), respectively, thereby limiting their penetration in brain. The mechanism by which prodrug and active metabolite could enter the brain was uncertain but, at least for Ro 64-0802, may be related to the expression of organic anion transporter 3 (Oat3) on both luminal and abluminal membranes of capillary endothelia. Moreover, a recent PET study (Takashima et al., 2011) suggested that age-related developmental changes in Pgp function in the blood-brain barrier, as assessed with R-^{11}C-verapamil and ^{11}C-oseltamivir in rhesus monkeys, might be related to the observed differences in CNS drug responses in children and adult humans.

The proton-coupled oligopeptide transporter (POT) PEPT1 is abundantly expressed on apical membranes of the small intestine (Jappar et al., 2010) and has been shown to have high-capacity, low-affinity carrier properties for the electrogenic symport of di/tripeptides or peptide-like drugs with a proton (Brandsch et al., 2008; Rubio-Aliaga and Daniel, 2008). In particular, it is believed that PEPT1 significantly improves the oral availability of the antiviral agent acyclovir by transforming the drug into a valine-ester prodrug which can then take advantage of the intestinally localized POT protein (Han et al., 1998). Because of this structure-function relationship, and the concern regarding transporter-drug interactions, Ogihara et al., (2009) explored the possibility that oseltamivir may also be a substrate for PEPT1. These authors, using cell culture methods and *in vivo* rat studies, reported that oseltamivir was not only a substrate of PEPT1, but that its intestinal absorption after oral dosing was markedly reduced when

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administered with milk. The same authors subsequently reported (Morimoto et al., 2011), in a crossover clinical study in healthy volunteers, that milk had no affect on the systemic exposure of oseltamivir after oral dosing (i.e., area under the plasma concentration-time curve from time zero to infinity) although a small 31% change in the maximum plasma concentration was observed. The discrepancy in oseltamivir oral absorption between rat and human, as a function of milk co-administration, was attributed to a possible species difference. Because of these conflicting reports and the lack of a systematic study evaluating if a species-dependent difference exists in PEPT1-mediated transport of peptides/mimetics, in general, the following two objectives were proposed: 1) to characterize the uptake properties of glycylsarcosine, a model dipeptide, in yeast *Pichia pastoris* expressing the rat, mouse, and human homologues, and 2) to determine if oseltamivir exhibits a species-dependent uptake by PEPT1, as evaluated in *Pichia cells* expressing these mammalian species.

Materials and Methods

Materials. [^{3}H]Glycylsarcosine (98 mCi/mmol) and [^{14}C]polyethylene glycol (PEG) 200 (1.1 mCi/g) were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). [^{14}C]Oseltamivir phosphate (103 μCi/mg) and unlabeled oseltamivir phosphate were gifts of F. Hoffmann-La Roche Ltd (Basel, Switzerland). Biotin, AccuTaq LA DNA Polymerase, and unlabeled glycylsarcosine (GlySar) was purchased from Sigma-Aldrich (St. Louis, MO). Superscript III reverse transcriptase, E. *coli* DH5α competent cells, *Pichia pastoris* GS115 strain and vector pPIC3.5K were purchased from Invitrogen Corp (Carlsbad, CA). The human Pept1 cDNA was a gift from Matthias Hediger (University of Bern, Switzerland). All other chemicals were acquired from standard sources.

PCR Amplification of Pept1 cDNA and Construction of Expression Vector pPIC3.5K-Pept1. Rat, mouse, and human Pept1 cDNA were cloned by proof-reading PCR with species specific primers (Table 1A) using the reverse transcripts of rat or mouse small intestine total RNA, or from a vector containing the human cDNA. Each species cDNA was digested with the appropriate restriction enzymes (*Bam*HI/*Avr*II for human and rat; *Bgl*II/*Xba*I for mouse), ligated into the plasmid DNA vector pPIC3.5K, and then transformed into E. *coli* DH5α competent cells. Positive colonies were screening by PCR with a pair of primers designed for an internal fragment of the Pept1 gene (Table 1B). Once positive colonies were obtained, plasmid DNA was purified using the PureYield Plasmid Midiprep System (Promega, Madison, WI) after which each species specific plasmid DNA was sequenced on both strands of the entire Pept1 gene by the DNA Sequencing Core, University of Michigan.

Transformation of Plasmid DNA pPIC3.5K-Pept1 into *Pichia Pastoris* GS115.   The procedure was performed as described in Manual Version M of the Pichia Expression Kit (Invitrogen, San Diego, USA).   In brief, plasmid DNA containing each species of Pept1 cDNA was linearized by the restriction enzyme *Sal*I and then purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).   A 10 ng aliquot of linearized plasmid DNA was used to transform *Pichia pastoris* GS115 by electropermeabilization using a MicroPulser Electroporator (Bio-Rad, Hercules, CA).   The GS115 yeast cells were then cultured on MM (1.34% YNB, 4 x 10^{-5}% biotin, 0.5% methanol, 1.5% agarose) and MD (1.34% YNB, 4 x 10^{-5}% biotin, 2% dextrose, 1.5% agarose) plates for distinguishing the His^+Mut^+ from His^+Mut^s transformants.   Following isolation of genomic DNA from the His^+Mut^+ GS115 transformants, real-time PCR was performed with species specific primers (Table 1C) to measure the gene copy number of Pept1 cDNA in yeast cells (Abad et al., 2010).   The ARG4 gene of yeast was set as the internal control and the plasmid DNA pPIC3.5K/Pept1 was set as the positive control.

Expression of Recombinant *Pichia* Strains and PEPT1 Protein.   The procedure was performed as described in Manual Version M of the Pichia Expression Kit.   Briefly, His^+Mut^+ transformants containing pPIC3.5K (vector alone), pPIC3.5K-hPept1 (human), pPIC3.5K-mPept1 (mouse), and pPIC3.5K-rPept1 (rat) were inoculated on MM and MD plates and incubated at 30°C for 2 days.   A single colony of each specific plasmid was picked from the MD plate, transferred into a 100-mL baffled flask containing 10 mL of BMGY media (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 1% glycerol), and grown overnight in a shaking incubator at 30°C.   After centrifuging at 3000 g x 5 min at room temperature, the cell pellet was suspended in 100 mL of BMMY media (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, 0.5%

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methanol), and grown overnight in a shaking baffled flask at 30°C for inducing PEPT1 protein expression.

Transport Assay in Yeast. Uptake studies were performed with radiolabeled GlySar or oseltamivir (after 20-24 hr induction of PEPT1 expression) using a method described previously (Döring et al., 1997; Döring et al., 1998). Cell cultures were harvested by centrifugation at 3000 g x 5 min at room temperature, washed with the same volume of 100 mM potassium phosphate buffer pH 6.5 (PPB), centrifuged and re-suspended in one-half the original volume of 100 mM PPB, centrifuged and re-suspended in one-tenth the volume of 100 mM PPB, and stored on ice.

All uptake measurements were carried out at 24°C unless otherwise indicated. Uptake was initiated by rapidly mixing 20 μl of yeast cell suspension and 30 μl of PPB containing 0.05 μCi of [^{3}H]GlySar (final concentration of 5.0 μM), and then incubating for the designated time period. For concentration-dependent studies (0.005-10 mM GlySar), the reaction was terminated at 30 sec, a time shown in preliminary experiments to reflect linear uptake kinetics. For specificity studies, [^{3}H]GlySar (5.0 μM) was incubated for 30 sec in the presence of potential inhibitors (10 mM) such as amino acids (glycine, L-histidine), a dipeptide (glyclyproline; GlyPro), cephalosporins with (cefadroxil, cephradine) and without (cefazolin, cephalothin, cephapirin) an α-amino group, an antiviral (acyclovir and its ethyl ester prodrug valacyclovir), organic anion and cation (4-acetamido-4′-isothiocyanostilbene-2, 2′-disulfonic acid; SITS and tetraethylammonium; TEA), and a dual ABCB1/ABCG2 inhibitor (elacridar). For dose-response inhibitory studies, [^{3}H]GlySar (5.0 μM) was incubated for 30 sec in the presence of increasing concentrations of unlabeled oseltamivir (0.5-200 mM). Other studies were also performed with [^{14}C]oseltamivir (final concentration of 5.0 μM) under identical experimental conditions.

At the appropriate time, uptake was terminated by the addition of 1 mL ice-cold 100 mM PPB. The cell suspension was then filtered using a rapid filtration technique with HATF filters (0.45 µM pore size, Millipore, Billerica, MA) and the filters washed four times with 1.5 mL ice-cold PPB. The filters were then transferred into glass vials and 6 mL of Cytoscint cocktail (MP Biomedicals, Solon, OH) was added to each vial. The samples were left to stand for 24 hr at room temperature and then measured for radioactivity on a dual-channel liquid scintillation counter (Beckman LS 6000SC; Beckman Coulter Inc., Fullerton, CA).

Oseltamivir Stability. The stability of oseltamivir was evaluated in yeast cells as a function of time by incubating [^{14}C]oseltamivir (5.0 µM) in the presence of an internal standard, [^{14}C]PEG200 (5.0 µM). After the designated incubation period, a 50-µL aliquot of 20% trichloroacetic acid was added to the cell suspension, mixed thoroughly, and centrifuged at 15,000 g x 5 min at 4°C. The supernate was then analyzed immediately by HPLC (Waters 515 pump; Milford, MA) with radiochemical detection (Packard 500TR; PerkinElmer Life and Analytical Sciences, Boston, MA) using a reversed-phase C_{18} column (Discovery 5 µm, 250 x 4.6 mm; Supelco, Bellefonte, PA), preceded by a µ-Bondapak C_{18} guard column (Waters). The mobile phase consisted of 60% acetonitrile:0.5% trifluoroacetic acid, run at 1.0 mL/min, ambient temperature. Chromatogram peaks were recorded and integrated using FLO-ONE software for Windows Analysis (PerkinElmer Life and Analytical Sciences, version 3.61). Retention times of [^{14}C]oseltamivir and [^{14}C]PEG200 were 14.9 and 3.6 min, respectively.

Data Analysis. Data are reported as mean ± SE of at least three independent experiments, with each cellular uptake experiment (expressed as pmol/OD or pmol/OD/min, where OD represents the optical density of cells measured at 600 nm) being run in triplicate.

Concentration-dependent cellular uptake results were fitted to an equation containing both saturable and nonsaturable transport components such that:

$$v = \frac{Vmax * C}{Km + C} + Kd * C \qquad (Eq.1)$$

where v represents the observed cellular uptake rate, $Vmax$ the maximum uptake rate, Km the Michaelis constant, Kd the first-order nonsaturable rate constant, and C the substrate (GlySar) concentration. Inhibition results were fitted to the equation:

$$\% \, Control \, Uptake = 100 * \left(1 - \frac{C^N}{IC50^N + C^N} \right) \qquad (Eq.2)$$

where $\% \, Control \, Uptake$ represents the ratio of observed cellular uptake of substrate in the presence and absence of inhibitor (expressed as percentage), $IC50$ the concentration of inhibitor (oseltamivir) that results in 50% inhibition, N is the slope factor, and C the substrate (GlySar) concentration. The unknown parameters in $Eq. 1$ (i.e., $Vmax, Km, \, Kd$) and $Eq. 2$ ($IC50, N$) were estimated by nonlinear regression using the software package Prism v5.0 (GraphPad Software, Inc., La Jolla, CA). Quality of the fit was evaluated by standard error of the parameters, the coefficient of determination (r^2), and by visual inspection of the residuals. Statistical differences were determined using a one-way analysis of variance followed by the Dunnett's test for pairwise comparisons with the control group (Prism v5.0). A p value ≤ 0.05 was considered statistically significant.

Results

Time Course of [³H]GlySar Uptake in *Pichia Pastoris* Cells Expressing PEPT1. As shown in Fig. 1, the uptake of 5.0 µM GlySar in yeast-transformed PEPT1 species far exceeded that of vector alone (about 30-fold for hPEPT1, and 100-fold for mPEPT1 and rPEPT1 at 30 min). It was also observed that the control vector displayed a linear uptake of GlySar over time, whereas the yeast-transformed PEPT1 species rapidly accumulated GlySar reaching a plateau (or near plateau) value by 30 min. Given the linearity of GlySar uptake in vector and all three species of PEPT1 over one min, a 30-sec incubation time was chosen to represent the initial rate uptake of GlySar in subsequent experiments. During this time (30 sec), the uptake of GlySar in *P. pastoris* was very low in pPIC3.5K (0.038 pmol/OD/min), but substantially greater in pPIC3.5K-hPept1 (23-fold), pPIC3.5K-mPept1 (61-fold) and pPIC3.5K-rPept1 (136-fold). There was no evidence of a functionally active peptide transporter in the yeast *P. pastoris*.

Concentration-Dependent Uptake of [³H]GlySar in *Pichia Pastoris* Cells Expressing PEPT1. *P. pastoris* cells were incubated with increasing concentrations of GlySar (0.005-10 mM) to determine the transport kinetics of GlySar in yeast transformants expressing mammalian PEPT1. As observed in Fig. 2, GlySar exhibited a saturable uptake in human, mouse and rat clones along with a nonsaturable component. In contrast, the uptake of GlySar in vector was linear, with a slope very similar to the nonsaturable component observed in mammalian transformants. Using the fitted value for nonsaturable uptake in yeast expressing vector alone (i.e., Kd=2.21 µL/OD/min), Michaelis-Menten parameters were estimated simultaneously for *P. pastoris* cells expressing human, mouse and rat PEPT1 (Table 2). The rank order of Km values was human (0.86 mM) > mouse (0.30 mM) > rat (0.16 mM), in which the saturable component

dominated the totality of uptake in all three mammalian species (i.e., 92.9% for human, 98.9% for mouse, and 99.6 for rat).

Specificity of [^{3}H]GlySar Uptake in *Pichia Pastoris* Cells Expressing PEPT1. To define the specificity of our system, GlySar uptake was challenged in yeast transformants using a wide variety of potential inhibitors (Table 3). We found that yeast expressing PEPT1 from each of the three mammalian species had a similar inhibitory profile. In particular, the uptake of GlySar in *Pichia* cells expressing human, mouse and rat PEPT1 was substantially reduced by the dipeptide GlyPro (over 95%), by the aminocephalosporins cefadroxil and cephradine (over 75%), and by the antiviral ester prodrug valacyclovir (over 94%). All of these compounds are known substrates and/or inhibitors of PEPT1. In contrast, GlySar uptake was not altered by the presence of amino acids (i.e., glycine and L-histidine), by cephalosporins lacking an α-amino group (i.e., cefazolin, cephalothin, and cephapirin), by the active antiviral drug acyclovir, by the organic anion SITS and organic cation TEA, and by the dual Pgp/Bcrp inhibitor elacridar. Moreover, the results were not confounded by a potential overlap with the peptide/histidine transporters PHT1/2 since L-histidine had no effect on PEPT1 functional activity.

Dose-Response Inhibition of [^{3}H]GlySar Uptake by Oseltamivir in *Pichia Pastoris* Cells Expressing PEPT1. The interaction of oseltamivir with PEPT1 was first evaluated by its inhibitory potential of GlySar uptake in transformed *Pichia* cells. As shown in Fig. 3, oseltamivir caused a dose-dependent reduction in the uptake of GlySar. The rank order of *IC50* values was human (27.4 mM) > rat (18.3 mM) > mouse (10.7 mM), whereas the slope factor (*N*) was approximately 1.0 for all species (Table 4). Given this result, the PEPT1-mediated transport of oseltamivir was tested directly in the three mammalian species heterologously expressed in yeast.

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Characterization of [^{14}C]Oseltamivir Binding in *Pichia Pastoris* Cells Expressing PEPT1.

The results in Fig. 4A clearly demonstrated that very little oseltamivir was associated with *Pichia* yeast, regardless of species, and that values in *Pichia* transformants expressing human, mouse and rat PEPT1 were no different than in *Pichia* transformed with vector alone. Moreover, oseltamivir did not accumulate over time as would be expected for any passive and/or active uptake process. This lack of accumulation can be further illustrated by comparing the uptake of 5.0 µM [^{14}C]oseltamivir versus 5.0 µM [^{3}H]GlySar, a positive control, at early (0.5 min) and later (5.0 min) time points (Fig 4B). Whereas GlySar exhibited both a species- and time-dependent uptake in *Pichia* cells, oseltamivir values were flat at both times for all four yeast transformants. Suspecting that the oseltamivir values might "really" reflect nonspecific binding, experiments were performed at 0°C and zero time (i.e., oseltamivir incubation was quenched immediately after its addition to the yeast cell suspension). As shown in Fig. 4C, there was no difference between vector control values and *Pichia* cells expressing the human, mouse and rat clones. Moreover, these values were unremarkable (0.20 - 0.25 pmol/OD) and essentially the same as oseltamivir when incubated at 24°C for 30 min (see Fig. 4A).

Stability of [^{14}C]Oseltamivir in *Pichia Pastoris* Cells Expressing PEPT1. To rule out the possibility of our findings inadvertently being the result of prodrug degradation, the stability of oseltamivir in *Pichia pastoris* transformants was measured by HPLC/radiodetection for up to 30 min of incubation. As demonstrated in Fig. 5, oseltamivir was completely stable in *Pichia* cells expressing vector as well as human, mouse and rat PEPT1. It should be noted, however, that prodrug values were about 25% lower when oseltamivir was incubated with yeast as compared to buffer alone (data not shown). This finding was consistent with our previous results, indicating a

nonspecific binding of oseltamivir to yeast, which happens immediately upon admixture and

remains constant over time.

Discussion

It has been clearly demonstrated that plasma membrane transporters may exhibit species differences in their substrate affinity. For example, LLC-PK$_1$ cells transfected with *MDR1/Mrdr1* from human, monkey, canine, rat and mouse exhibited a 16.5-fold difference in their apparent *Km* values (Katoh et al., 2006). Interspecies differences were also reported for the liver efflux transporters *MRP/Mrp* (Ito et al., 2008; Li et al., 2008) and *BCRP/Bcrp* (Li et al., 2008), and for the bile salt export pump *BSEP/Bsep* (Yabuuchi et al., 2008). However, there is sparse information on potential species differences in PEPT1-mediated transport, especially when studied in the same experimental system. As shown in Table 5, the affinity of PEPT1 for GlySar differed by as much as 10.8-fold in the nine animal species listed, and 5.5-fold in the same species (chicken) when studied by the same investigators using two different methods (i.e., cRNA injected oocytes vs. cDNA-transfected CHO cells). This finding highlights the difficulty in comparing species-dependent uptake of membrane transporters when studied by different investigators and laboratories, and especially under different experimental conditions.

For this reason, we initially examined the species-dependent uptake of GlySar in a single system, that is, yeast *Pichia pastoris* expressing human, mouse and rat PEPT1. Once *Pichia* cells were validated for these three mammalian species, subsequent experiments focused on whether or not oseltamivir exhibited a difference in transport between human, mouse and rat. This particular comparison was brought to the forefront because of recent conflicting studies suggesting a species difference between rodents and humans in the PEPT1-mediated intestinal absorption of oseltamivir (Ogihara et al., 2009; Morimoto et al., 2011). In the present study, we found that: 1) GlySar was transported into yeast transformants expressing human, mouse and rat

PEPT1, and that dipeptide uptake was inhibited by known substrates of PEPT1 for all three mammalian species; 2) GlySar uptake was saturable for the mammalian species tested although some differences were observed in substrate affinity for PEPT1 (i.e., *Km* values); 3) oseltamivir inhibited the uptake of GlySar, in a dose-dependent manner, for human, rat and mouse PEPT1, with some differences in inhibitory potential (i.e., *IC50* values); and 4) oseltamivir was not a substrate of human, mouse and rat PEPT1, but instead exhibited nonspecific binding to the yeast vector and PEPT1 transformants.

The question, therefore, is how can one reconcile the differences observed between our study and that of other investigators (Ogihara et al., 2009; Morimoto et al, 2011) regarding the PEPT1-mediated intestinal absorption of oseltamivir? In the first study (Ogihara et al., 2009), *in vitro* results in Caco-2 cells and hPEPT1-transfected HeLa cells indicated a time- and temperature-dependent uptake of oseltamivir, along with inhibition by typical PEPT1 inhibitors such as GlySar. They also reported that oseltamivir uptake was saturable with *Km* values of 6.5 and 8.6 mM, respectively, in Caco-2 and HeLa/hPEPT1 cells. However, upon closer inspection of the results (and experimental methods), it is clear that substantial binding of oseltamivir occurred in these studies, as judged by the significant cell-to-medium ratios observed at time zero. Moreover, it does not appear that adequate controls were in place since mannitol was not co-incubated with drug during the cell culture experiments and, as a result, corrections were not made for extracellular content including nonspecific binding.

In the second study (Morimoto et al., 2011), *in vivo* results in adult male human volunteers indicated significant reductions in the maximum plasma concentration of oseltamivir and its active metabolite Ro 64-0802 in milk versus water following 75 mg oral doses of oseltamivir (milk/water ratios of 69% for both compounds), but no changes in area under the plasma

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concentration-time curve ($AUC_{0-\infty}$) for either drug moiety (i.e., milk/water ratios of 97% and 84%, respectively, for oseltamivir and Ro 64-0802). Nevertheless, there were significant reductions in the oseltamivir AUC_{0-2hr} (milk/water ratio of 34%), and in the Ro 64-0802 AUC_{0-2hr} (milk/water ratio of 14%) and AUC_{2-4hr} (milk/water ratio of 44%) in milk as compared to water controls. *In vivo* results in adult male rats by the same investigators indicated that milk co-administration with 30 mg/kg oral doses of oseltamivir resulted in marked changes in the oseltamivir maximum plasma concentration (milk/water ratio of 20%), AUC_{0-1hr} (milk/water ratio of 22%), AUC_{1-2hr} (milk/water ratio of 36%) and AUC_{0-6hr} (milk/water ratio of 38%). Based on these findings, the authors reported that milk inhibited the intestinal absorption of oseltamivir in humans, and that this interaction was limited in humans as compared with rats because of possible species differences in regional expression of PEPT1 in the small intestine. However, our interpretation of these results would suggest an effect of milk on oseltamivir absorption rate and not extent, the mechanism of which is unclear. Moreover, their study design was lacking in that plasma samples were not stabilized to prevent the possible hydrolysis of prodrug to active metabolite, and a limited number of humans (n=6) and rats (n=3) were evaluated.

A broader aspect to consider involves the value of using mice and rats as surrogates for humans in preclinical studies on peptide/mimetic intestinal absorption via PEPT1. Based on the *Km* values in this study, the mouse better reflects the affinity of GlySar for human PEPT1 than rat (i.e., hPEPT1/mPEPT1 ratio of 2.9 vs. hPEPT1/rPEPT1 ratio of 5.4). However, to properly make a species comparison it is important to look at several factors. In this context, it appears that human, mouse and rat have similar peptide transport mechanisms (i.e., pH- and membrane potential-dependent uptake), substrate specificity, and substrate affinity for cloned PEPT1 (Liang

et al., 1995; Saito et al., 1995; Fei et al., 2000; Zhu et al., 2000). In addition, all three species express PEPT1 in the apical membrane of epithelial cells lining the intestine (Walker et al., 1998; Shen et al., 1999; Hu et al., 2008) and kidney (Shen et al., 1999; Hu et al., 2008; Ahlin et al., 2009). Human, mouse and rat PEPT1 are also expressed throughout the small intestine (i.e., duodenum, jejunum and ileum) with little or no expression in colon (Ogihara et al., 1996; Walker et al., 1998; Groneberg et al., 2001; Jappar et al., 2010). With respect to homology, there was an 82.2% (rat and human) and 83.4% (mouse and human) cDNA identity and an 88.6% (rat and human) and 89.3% (mouse and human) amino acid identity between species. Still, it appears that differences may exist in the levels of gene expression among these species. In this regard, human and mouse, but not rat, had comparable levels of PEPT1 mRNA expression in duodenal samples (Kim et al., 2007). In kidney samples, PEPT1 mRNA was moderately expressed in rat with very low expression levels in mouse (Lu and Klaassen, 2006). In human kidney, expression levels of PEPT1 were very low (Hilgendorf et al., 2007), being more similar to that of mouse than rat.

In concluding, the present study and that of our companion paper (Poirier et al., 2012) provide definitive and overwhelming evidence that oseltamivir is not a substrate for the intestinal peptide transporter PEPT1 in human, mouse and rat. Although oseltamivir does inhibit GlySar uptake in *Pichia* cells expressing these mammalian transformants, the clinical relevance of this interaction is doubtful. Thus, based on a typical 75 mg dose and 250 mL volume, the gastrointestinal concentration of oseltamivir in human is estimated at 1 mM and, as a result, would have a very low inhibitory potential (less than 5% as calculated by: $1+ I/Ki$ using a human $Ki = IC50 = 27.4$ mM). Finally, whereas both mouse and rat are reasonable surrogates for exploring the intestinal

absorption and pharmacokinetics of peptides and peptide-like drugs, the mouse should more

faithfully reflect these events in humans.

Authorship Contributions

Participated in research design: Hu and Smith

Conducted experiments: Hu and Chen

Contributed new reagent or analytical tools:

Performed data analysis: Hu

Wrote or contributed to the writing of the manuscript: Hu and Smith

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References

Abad S, Kitz K, Hörmann A, Schreiner U, Hartner FS, and Glieder A (2010) Real-time PCR-based determination of gene copy numbers in Pichia pastoris. *Biotechnol J* **5**:413-420.

Ahlin G, Hilgendorf C, Karlsson J, Szigyarto CA, Uhlén M, and Artursson P (2009) Endogenous gene and protein expression of drug-transporting proteins in cell lines routinely used in drug discovery programs. *Drug Metab Dispos* **37**:2275-2283.

Brandsch M, Knütter I, Bosse-Doenecke E (2008) Pharmaceutical and pharmacological importance of peptide transporters. *J Pharm Pharmacol* **60**:543-585.

Chen H, Pan Y, Wong EA, Bloomquist JR, and Webb KE (2002) Molecular cloning and functional expression of a chicken intestinal peptide transporter (cPepT1) in Xenopus oocytes and Chinese hamster ovary cells. *J Nutr* **132**:387-393.

Davies BE (2010) Pharmacokinetics of oseltamivir: an oral antiviral for the treatment and prophylaxis of influenza in diverse populations. *J Antimicrob Chemother* **65**(Suppl 2):ii5-ii10.

Döring F, Theis S, and Daniel H (1997) Expression and functional characterization of the mammalian intestinal peptide transporter PepT1 in the methylotropic yeast Pichia pastoris. *Biochem Biophys Res Commun* **232**:656-662.

Döring F, Michel T, Rösel A, Nickolaus M, and Daniel H (1998) Expression of the mammalian renal peptide transporter PEPT2 in the yeast Pichia pastoris and applications of the yeast system for functional analysis. *Mol Membr Biol* **15**:79-88.

Dutkowski R, Thakrar B, Froehlich E, Suter P, Oo C, and Ward P (2003) Safety and pharmacology of oseltamivir in clinical use. *Drug Saf* **26**:787-801.

Fei YJ, Kanai Y, Nussberger S, Ganapathy V, Leibach FH, Romero MF, Singh SK, Boron WF, and Hediger MA (1994) Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* **368**:563-566.

Fei YJ, Sugawara M, JLiu JC, Li HW, Ganapathy V, Ganapathy ME, and Leibach FH (2000) cDNA structure, genomic organization, and promoter analysis of the mouse intestinal peptide transporter PEPT1. *Biochim Biophys Acta* **1492**:145-154.

Groneberg DA, Doring F, Eynott PR, Fischer A, and Daniel H (2001) Intestinal peptide transport: ex vivo uptake studies and localization of peptide carrier PEPT1. *Am J Physiol Gastrointest Liver Physiol* **281**:G697-G704.

Han H, de Vrueh RL, Rhie JK, Covitz KM, Smith PL, Lee CP, Oh DM, Sadée W, and Amidon GL (1998) 5'-Amino acid esters of antiviral nucleosides, acyclovir, and AZT are absorbed by the intestinal PEPT1 peptide transporter. *Pharm Res* **15**:1154-1159.

He G, Massarella J, and Ward P (1999) Clinical pharmacokinetics of the prodrug oseltamivir and its active metabolite Ro 64-0802. *Clin Pharmacokinet* **37**:471-484.

Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell AL, and Karlsson J (2007) Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. *Drug Metab Dispos* **35**:1333-1340.

Hill G, Cihlar T, Oo C, Ho ES, Prior K, Wiltshire H, Barrett J, Liu B, and Ward P (2002) The anti-influenza drug oseltamivir exhibits low potential to induce pharmacokinetic drug interactions via renal secretion-correlation of in vivo and in vitro studies. *Drug Metab Dispos* **30**:13-19.

Hu Y, Smith DE, Ma K, Jappar D, Thomas W, and Hillgren KM (2008) Targeted disruption of peptide transporter Pept1 gene in mice significantly reduces dipeptide absorption in intestine. *Mol Pharm* **5**:1122-1130.

Ito K (2008) ABCC2/Abcc2 transport property in different species and its modulation by heterogeneous factors. *Drug Metab Pharmacokinet* **23**:394-405.

Jappar D, Wu SP, Hu Y, and Smith DE (2010) Significance and regional dependency of peptide transporter (PEPT) 1 in the intestinal permeability of glycylsarcosine: in situ single-pass perfusion studies in wild-type and Pept1 knockout mice. *Drug Metab Dispos* **38**:1740-1746.

Katoh M, Suzuyama N, Takeuchi T, Yoshitomi S, Asahi S, and Yokoi T (2006) Kinetic analyses for species differences in P-glycoprotein-mediated drug transport. *J Pharm Sci* **95**:2673-2683.

Kim HR, Park SW, Cho HJ, Chae KA, Sung JM, Kim JS, Landowski CP, Sun D, Abd El-Aty AM, Amidon GL, and Shin HC (2007) Comparative gene expression profiles of intestinal transporters in mice, rats and humans. *Pharmacol Res* **56**:224-236.

Klang JE, Burnworth LA, Pan YX, Webb KE Jr, and Wong EA (2005) Functional characterization of a cloned pig intestinal peptide transporter (pPepT1). *J Anim Sci* **83**:172-181.

Li M, Yuan H, Li N, Song G, Zheng Y, Baratta M, Hua F, Thurston A, Wang J, and Lai Y (2008) Identification of interspecies difference in efflux transporters of hepatocytes from dog, rat, monkey and human. *Eur J Pharm Sci* **35**:114-126.

Liang R, Fei YJ, Prasad PD, Ramamoorthy S, Han H, Yang-Feng TL, Hediger MA, Ganapathy V, and Leibach FH (1995) Human intestinal H+/peptide cotransporter. Cloning, functional expression, and chromosomal localization. *J Biol Chem* **270**:6456-6463.

Lu H and Klaassen C (2006) Tissue distribution and thyroid hormone regulation of Pept1 and Pept2 mRNA in rodents. *Peptides* **27**:850-857.

Morimoto K, Nakakariya M, Shirasaka Y, Kakinuma C, Fujita T, Tamai I, and Ogihara T (2008) Oseltamivir (Tamiflu) efflux transport at the blood-brain barrier via P-glycoprotein. *Drug Metab Dispos* **36**:6-9.

Morimoto K, Kishimura K, Nagami T, Kodama N, Ogama Y, Yokoyama M, Toda S, Chiyoda T, Shimada R, Inano A, Kano T, Tamai I, and Ogihara T (2011) Effect of milk on the pharmacokinetics of oseltamivir in healthy volunteers. *J Pharm Sci* **100**:3854-3861.

Moscona A (2005) Neuraminidase inhibitors for influenza. *N Engl J Med* **353**:1363-1373.

Ogihara H, Saito H, Shin BC, Terado T, Takenoshita S, Nagamachi Y, Inui K, and Takata K (1996) Immuno-localization of H+/peptide cotransporter in rat digestive tract. *Biochem Biophys Res Commun* **220**:848-852.

Ogihara T, Kano T, Wagatsuma T, Wada S, Yabuuchi H, Enomoto S, Morimoto K, Shirasaka Y, Kobayashi S, and Tamai I (2009) Oseltamivir (Tamiflu) is a substrate of peptide transporter 1. *Drug Metab Dispos* **37**:1676-1681.

Ose A, Kusuhara H, Yamatsugu K, Kanai M, Shibasaki M, Fujita T, Yamamoto A, Sugiyama Y (2008) P-glycoprotein restricts the penetration of oseltamivir across the blood-brain barrier. *Drug Metab Dispos* **36**:427-434.

Ose A, Ito M, Kusuhara H, Yamatsugu K, Kanai M, Shibasaki M, Hosokawa M, Schuetz JD, and Sugiyama Y (2009) Limited brain distribution of [3R,4R,5S]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate (Ro 64-0802), a pharmacologically active form of oseltamivir, by active efflux across the blood-brain barrier mediated by organic

anion transporter 3 (Oat3/Slc22a8) and multidrug resistance-associated protein 4 (Mrp4/Abcc4). *Drug Metab Dispos* **37**:315-321.

Pan Y, Wong EA, Bloomquist JR, and Webb KE (2001) Expression of a cloned ovine gastrointestinal peptide transporter (oPepT1) in Xenopus oocytes induces uptake of oligopeptides in vitro. J *Nutr* **131**:1264-1270.

Poirier A, Belli S, Funk C, Otteneder M, Portmann R, Heinig K, Lazic SE, Rayner CR, Hoffmann G, Smith DE, and Schuler F (2012) Role of the gastrointestinal transporter PEPT1 in oseltamivir absorption: *in vitro* and *in vivo* studies. *Drug Metab Dispos* (in review).

Rønnestad I, Murashita K, Kottra G, Jordal AE, Narawane S, Jolly C, Daniel H, and Verri T (2010) Molecular cloning and functional expression of atlantic salmon peptide transporter 1 in Xenopus oocytes reveals efficient intestinal uptake of lysine-containing and other bioactive di- and tripeptides in Teleost fish. *J Nutr* **140**:893-900.

Rubio-Aliaga I and Daniel H (2008) Peptide transporters and their roles in physiological processes and drug disposition. *Xenobiotica* **38**:1022-1042.

Saito H, Okuda M, Terada T, Sasaki S, and Inui K (1995) Cloning and characterization of a rat H+/peptide cotransporter mediating absorption of beta-lactam antibiotics in the intestine and kidney. J *Pharmacol Exp Ther* **275**:1631-1637.

Shen H, Smith DE, Yang T, Huang YG, Schnermann JB, and Brosius FC (1999) Localization of PEPT1 and PEPT2 proton-coupled oligopeptide transporter mRNA and protein in rat kidney. *Am J Physiol Renal Physiol* **276**:F658-F665.

Shi D, Yang J, Yang D, LeCluyse EL, Black C, You L, Akhlaghi F, and Yan B (2006) Anti-influenza prodrug oseltamivir is activated by carboxylesterase human carboxylesterase 1, and

the activation is inhibited by antiplatelet agent clopidogrel. *J Pharmacol Exp Ther* **319**:1477-1484.

Takashima T, Yokoyama C, Mizuma H, Yamanaka H, Wada Y, Onoe K, Nagata H, Tazawa S, Doi H, Takahashi K, Morita M, Kanai M, Shibasaki M, Kusuhara H, Sugiyama Y, Onoe H, and Watanabe Y (2011) Developmental changes in P-glycoprotein function in the blood-brain barrier of nonhuman primates: PET study with R-11C-verapamil and 11C-oseltamivir. *J Nucl Med* **52**:950-957.

Walker D, Thwaites DT, Simmons NL, Gilbert HJ, and Hirst BH (1998) Substrate upregulation of the human small intestinal peptide transporter, hPepT1. *J Physiol* **507**:697-706.

Yabuuchi H, Tanaka K, Maeda M, Takemura M, Oka M, Ohashi R, and Tamai I (2008) Cloning of the dog bile salt export pump (BSEP; ABCB11) and functional comparison with the human and rat proteins. *Biopharm Drug Dispos* **29**:441-448.

Zhang EY, Fu DJ, Pak YA, Stewart T, Mukhopadhyay N, Wrighton SA, and Hillgren KM (2004) Genetic polymorphisms in human proton-dependent dipeptide transporter PEPT1: implications for the functional role of Pro586. *J Pharmacol Exp Ther* **310**:437-445.

Zhu T, Chen XZ, Steel A, Hediger MA, and Smith DE (2000) Differential recognition of ACE inhibitors in Xenopus laevis oocytes expressing rat PEPT1 and PEPT2. *Pharm Res* **17**:526-532.

Footnotes

Figure Legends

Figure 1. Uptake of 5.0 µM [^{3}H]GlySar versus time in *Pichia pastoris* expressing human (pPIC3.5K-hPept1), mouse (pPIC3.5K-mPept1) and rat (pPIC3.5K-rPept1) PEPT1, and vector control (pPIC3.5K). The insert shows GlySar uptake over one minute. Uptakes were performed at 24°C using pH 6.5. buffer. Data are expressed as mean ± SE (n=6).

Figure 2. Concentration dependent uptake of [^{3}H]GlySar (0.005-10 mM) in *Pichia pastoris* expressing human (pPIC3.5K-hPept1), mouse (pPIC3.5K-mPept1) and rat (pPIC3.5K-rPept1) PEPT1, and vector control (pPIC3.5K). The 30-sec uptakes were obtained at 24°C using pH 6.5. buffer. Data are expressed as mean ± SE (n=6). Solid lines were generated using the fitted parameters in Table 2.

Figure 3. Dose-response inhibition of 5.0 µM [^{3}H]GlySar uptake by oseltamivir (0.5-200 mM) in *Pichia pastoris* expressing human (pPIC3.5K-hPept1), mouse (pPIC3.5K-mPept1) and rat (pPIC3.5K-rPept1) PEPT1, and vector control (pPIC3.5K). The 30-sec uptakes were obtained at 24°C using pH 6.5. buffer. Data are expressed as mean ± SE (n=6). Solid lines were generated using the fitted parameters in Table 4, and the dashed line represents 100% of control.

Figure 4. Binding of 5.0 µM [^{14}C]oseltamivir versus time in *Pichia pastoris* expressing human (pPIC3.5K-hPept1), mouse (pPIC3.5K-mPept1) and rat (pPIC3.5K-rPept1) PEPT1, and vector control (pPIC3.5K) (A); comparative studies evaluating the binding of 5.0 µM [^{14}C]oseltamivir and uptake of 5.0 µM [^{3}H]GlySar, at 30 sec and 5 min, in PEPT1 mammalian species and vector control (B); nonspecific binding of oseltamivir as determined at 0°C and zero time (C). In contrast, the studies in panels A and B were performed at 24°C. In panel A, GlySar uptake was run as a positive control in all studies and, in panel B, the uptake of GlySar and binding of oseltamivir were conducted in parallel at the same time. pH 6.5 buffer was used throughout the experiments. Data are expressed as mean ± SE (n=6). The dashed line in panel A represents the average value for nonspecific binding of oseltamivir to yeast (i.e., at 0°C and zero time).

Figure 5. Stability of 5.0 µM [^{14}C]oseltamivir as a function of incubation time in *Pichia pastoris* expressing human (pPIC3.5K-hPept1), mouse (pPIC3.5K-mPept1) and rat (pPIC3.5K-rPept1) PEPT1, and vector control (pPIC3.5K). The incubations were performed at 24°C using pH 6.5. buffer. Data are expressed as mean ± SE (n=6).

TABLE 1A

Primers for Pept1 cDNA cloning

Yeast Transformant	PCR Primers	Product (bp)
Human	*Forward:* GCCGGATCCGCCATGGGAATGTCCAAA	2258
	Reverse: AAGCCTAGGTCATCAGGGGCCATCCAATG	
Mouse	*Forward:* GCCAGATCTATGGGGATGTCCAAGTCTCG	2158
	Reverse: TCTTCTAGAACTTGCCTTCTGCCCTTCAC	
Rat	*Forward:* AGCGGATCCAATGGGGATGTCCAAGTCTC	2143
	Reverse: TGACCTAGGCATGTTTGTCTGTGAGACAGG	

TABLE 1B

Primers for Pept1 transformant screening

Yeast Transformant	PCR Primers	Product (bp)
Human	*Forward:* CCTGTCCACCGCCATCTACC	254
	Reverse: CGAGAGCTATCAGGGCCAGG	
Mouse	*Forward:* CTTTGTGGTGGCTGCAATTG	345
	Reverse: TCCCCTTTCTCTGGCTTTTG	
Rat	*Forward:* GTTGCTTTGGCTACCCATTG	533
	Reverse: AGTGATGATCGTGGAGAGCA	

TABLE 1C

Primers for measuring Pept1 integration

Yeast Transformant	PCR Primers
Vector	*Forward:* TGGCGAGGTTCATGTTTGTTTAT
	Reverse: TCTGGAGTGATGTTCGGGTGTA
Human	*Forward:* TGACCTCACAGACCACAACCA
	Reverse: GCCAGGCCGATCAAGGA
Mouse	*Forward:* CCACGGCCATTTACCATACG
	Reverse: TGCGATCAGAGCTCCAAGAA
Rat	*Forward:* CCTCGGCAGTGGAATGTACAA
	Reverse: GGCAAACCGAATGCACTTG
ARG4	*Forward:* TCCTCCGGTGGCAGTTCTT
	Reverse: TCCATTGACTCCCGTTTTGAG

TABLE 2

Transport kinetics of [^{3}H]GlySar uptake in Pichia pastoris cells expressing different

mammalian species of Pept1 gene

Parameter estimates were expressed as mean ± SE (n=9). The data were fitted to *Eq. 1* using a

weighting factor of unity. GlySar concentrations ranged from 0.005-10 mM.

Parameter	Yeast Transformant			
	pPIC3.5K	pPIC3.5K-hPept1	pPIC3.5K-mPept1	pPIC3.5K-rPept1
Vmax (pmol/OD/min)	NA	24.7 ± 2.4	61.5 ± 1.7	88.8 ± 1.5
Km (mM)	NA	0.86 ± 0.25	0.30 ± 0.03	0.16 ± 0.01
Kd (μL/OD/min)	2.21 ± 0.14	2.21 ± 0.14	2.21 ± 0.14	2.21 ± 0.14
Vmax/Km (μL/OD/min)	NA	28.7	205.0	555.0
r^2	0.894	0.933	0.950	0.934
Gene Copy Number*	1.28 ± 0.01	1.05 ± 0.14	1.17 ± 0.23	0.97 ± 0.07

*Gene copy number was measured by real-time PCR using species-specific *Pept1* gene primers

and the ARG4 gene as a control; AOX1 gene primers were used for vector alone (Table 1C).

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TABLE 3

Effect of potential inhibitors on [³H]GlySar uptake in Pichia pastoris cells expressing different

mammalian species of Pept1 gene

Data are expressed as mean ± SE (n=3). The results were vector-subtracted and shown as percent of control (buffer). ***p ≤ 0.001 compared to control. GlySar was studied at 5.0 µM and the potential inhibitors at 10 mM.

Inhibitory	Yeast Transformant		
Compound	pPIC3.5K-hPept1	pPIC3.5K-mPept1	pPIC3.5K-rPept1
Control	100 ± 28	100 ± 7	100 ± 10
Glycine	130 ± 17	104 ± 6	91. ± 6.4
L-Histidine	122 ± 13	103 ± 6	113 ± 8
GlyPro	3.9 ± 1.7***	1.9 ± 1.0***	0.6 ± 0.3***
Cefadroxil	6.4 ± 13.7***	16.7 ± 1.7***	23.1 ± 1.2***
Cephradine	23. ± 5.4***	9.8 ± 0.7***	23. ± 1.8***
Cefazolin	81. ± 2.6	87. ± 0.7	93. ± 6.3
Cephalothin	84. ± 3.3	84. ± 4.6	119 ± 1
Cephapirin	100 ± 14	83. ± 6.3	122 ± 5
Acyclovir	114 ± 8	99. ± 5.6	109 ± 10
Valacyclovir	3.5 ± 3.3***	4.5 ± 1.1***	5.5 ± 0.5***
SITS	127 ± 12	86. ± 3.6	107 ± 10
TEA	113 ± 9	83. ± 2.0	94. ± 5.4
Elacridar	100 ± 6	89. ± 3.6	119 ± 3

TABLE 4

Inhibitory effect of oseltamivir on [^{3}H]GlySar uptake in Pichia pastoris cells expressing

different mammalian species of Pept1 gene

Parameter estimates were expressed as mean ± SE (n=6). The data were fitted to *Eq. 2* using a

weighting factor of 1/y. GlySar was studied at 5.0 µM while oseltamivir concentrations ranged

from 0.5-200 mM.

Parameter	Yeast Transformant		
	pPIC3.5K-hPept1	pPIC3.5K-mPept1	pPIC3.5K-rPept1
IC50 (mM)	27.4 ± 2.3	10.7 ± 0.5	18.3 ± 0.9
N	0.90 ± 0.06	1.07 ± 0.03	1.11 ± 0.04
r^2	0.888	0.978	0.963

Note: GlySar uptakes in vector alone were reduced by 10% or less over the oseltamivir

concentrations studied.

TABLE 5

Species-dependent affinity of PEPT1 for glycylsarcosine

Animal Species	GlySar Km (mM)	Experimental Conditions for Uptake	Reference
Rabbit	1.9	cRNA-injected XLO, TEVC -60 mV, pH 5.5	Fei et al., 1994
Human	0.29	cDNA-transfected HeLa cells, ^{14}C-label, pH 6.0	Liang et al., 1995
Rat	0.24	cRNA-injected XLO , ^{14}C-label, pH 6.0	Zhu et al., 2000
Mouse	0.75	cRNA-injected XLO, TEVC -60 mV, pH 5.5	Fei et al., 2000
Sheep	0.61	cRNA-injected XLO, TEVC -60 mV, pH 5.5	Pan et al., 2001
Chicken	0.47 2.6	cRNA-injected XLO, TEVC -60 mV, pH 6.0 cDNA-transfected CHO cells, ^{3}H-label, pH 6.0	Chen et al., 2002
Monkey	0.35	cDNA-transfected HeLa cells, ^{14}C-label, pH 6.0	Zhang et al., 2004
Pig	0.94	cDNA-transfected CHO cells, ^{3}H-label, pH 6.0	Klang et al., 2005
Salmon	0.5	cRNA-injected XLO, TEVC -60 mV, pH 6.5	Rønnestad et al., 2010

XLO refers to Xenopus *laevis* oocytes; HeLa to human epithelial cervical adenocarcinoma cells;

CHO to Chinese hamster ovary cells; TEVC to two-electrode voltage-clamp electrophysiology;

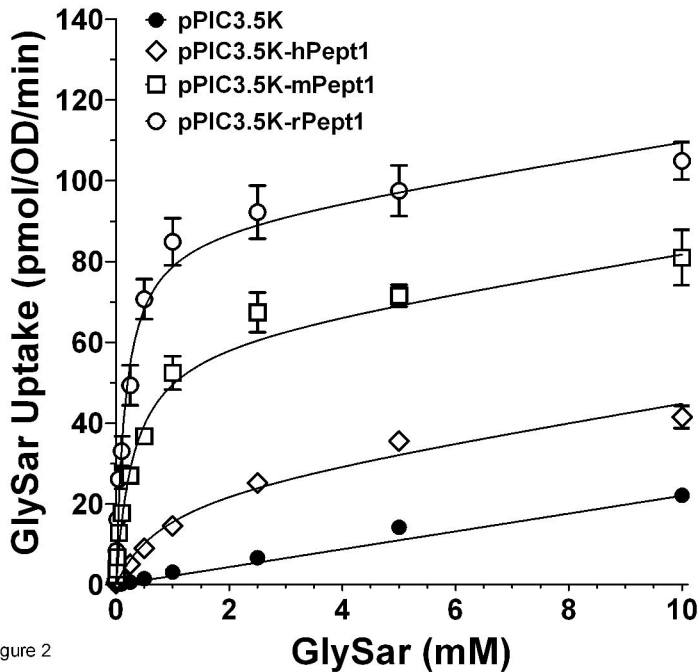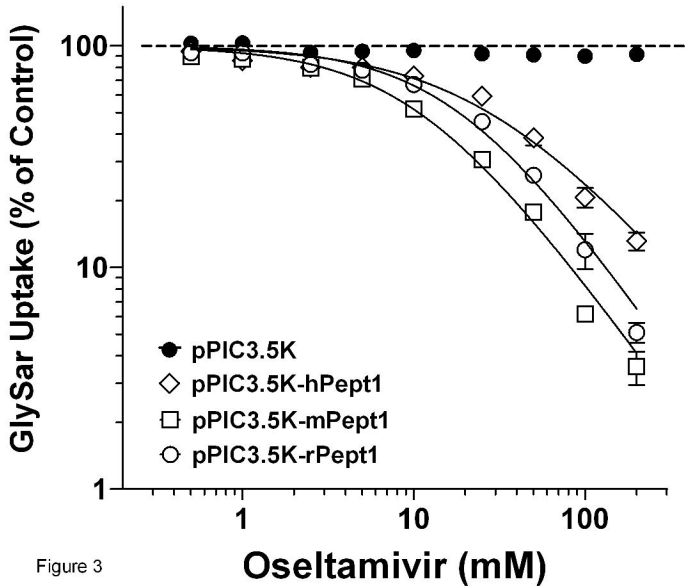and [14]C- and ^{3}H-labels to the radioisotope of GlySar.

Figure 1

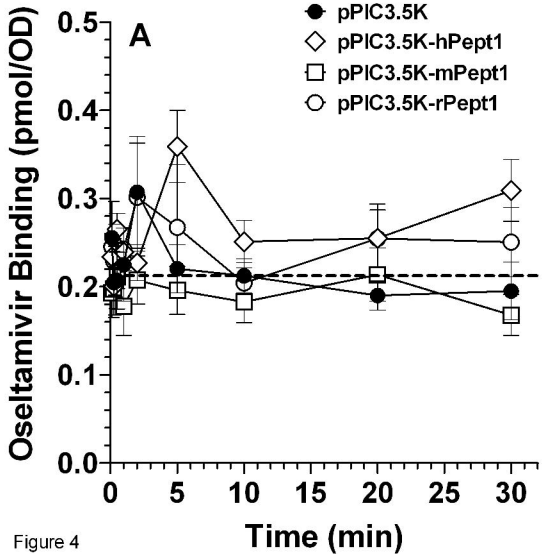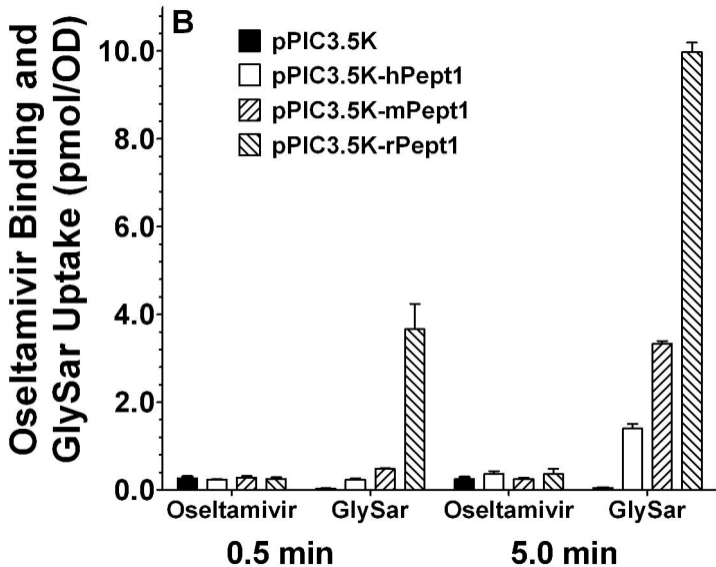Figure 2

Figure 3

pPIC3.5K

pPIC3.5K-hPept1

pPIC3.5K-mPept1

pPIC3.5K-rPept1

Figure 4

Figure 4

Figure 4

Figure 5