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Species Differences in the Pharmacokinetics of Cefadroxil as Determined in Wildtype and Humanized *PepT1* Mice

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Abstract

PepT1 (SLC15A1) is a high-capacity low-affinity transporter that is important in the absorption of digested di/tripeptides from dietary protein in the small intestine. PepT1 is also crucial for the intestinal uptake and absorption of therapeutic agents such as the β-lactam aminocephalosporins and antiviral prodrugs. Species differences, however, have been observed in PepT1-mediated intestinal absorption and pharmacokinetics, thereby, making it more difficult to predict systemic drug exposure. In the present study, we evaluated the *in situ* intestinal permeability of the PepT1 substrate cefadroxil in wildtype and humanized *PepT1* (*huPepT1*) mice, and the *in vivo* absorption and disposition of drug after escalating oral doses. The *in situ* perfusions indicated that cefadroxil had a two-fold higher affinity (i.e., two-fold lower K_m) for jejunal PepT1 in *huPepT1* mice, lower but substantial permeability in all regions of the small intestine, and low but measureable permeability in the colon as compared to wildtype animals. The *in vivo* experiments indicated almost superimposable pharmacokinetic profiles between the two genotypes after intravenous bolus dosing of cefadroxil. In contrast, after oral dose escalation, the systemic exposure of cefadroxil was reduced in *huPepT1* mice as compared to wildtype animals. Moreover, the AUC and C_{max} versus dose relationships were nonlinear for *huPepT1* but not wildtype mice, and similar to that observed from human subjects. In conclusion, our findings indicate that *huPepT1* mice may provide a valuable tool in the drug discovery process by better predicting the oral pharmacokinetic profiles of PepT1 substrates in humans.

Graphical abstract

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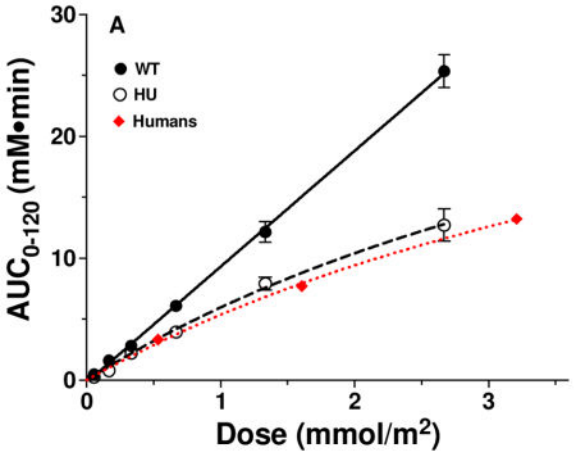

Keywords

Species differences; Pharmacokinetics; Cefadroxil; Humanized mice; PEPT1

1. Introduction

The peptide transporter PepT1 (SLC15A1) is expressed predominately at the apical side of enterocytes in the small intestine with 50% abundance as compared to the total protein content of clinically relevant transporters [1]. As a consequence, PepT1 is mainly responsible for the uptake of di/tripeptides and peptide-like drugs from the intestinal lumen [2–5]. However, differences in specific protein activities have been observed between mammalian species which, in turn, may affect the absorption, disposition, metabolism and excretion of drugs [6]. Using a yeast system expressing mouse, rat and human *PepT1* cDNA, a species difference in PepT1 activity was demonstrated for glycylsarcosine (GlySar) where the uptake was saturable in all three species and 3- to 5-fold differences were observed in their K_m values [7]. Recognizing the need to improve prediction of human pharmacokinetics, drug-drug interactions and safety concerns because of species differences, genetically humanized and chimeric liver humanized mouse models were proposed by Scheer and Wilson [8]. At present, most humanized mouse models have focused on addressing the species differences in drug metabolizing enzymes [9], xenobiotic receptors [10, 11] and, to a lesser extent, drug transporters [12].

Cefadroxil, (6*R*,7*R*)-7-{[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino}-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, is a first generation aminocephalosporin with good patient compliance [13], a long-acting therapeutic effect, high solubility and relatively broad spectrum of anti-bacterial activity [14, 15]. It is used to treat urinary tract infections [16], skin and soft tissue infections [17, 18], pharyngitis [19, 20] and tonsillitis [21]. Cefadroxil has low plasma protein binding (~20%) and good oral bioavailability of at least 90% [22, 23]. Renal excretion is the primary route of elimination, with more than 90% of the orally administered drug being excreted unchanged in urine over 24 hours [22, 24]. Cefadroxil is also a substrate of the intestinal peptide transporter PepT1,

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which is primarily responsible for the drug's uptake across the apical membrane of small intestine [25–29].

Species differences in PepT1-mediated permeability were first observed for the synthetic dipeptide GlySar during *in situ* jejunal perfusions in which the K_m was reduced two- to four-fold in humanized *PepT1* (*huPepT1*) mice as compared to wildtype animals [12]. In addition, during oral dose escalation studies with cefadroxil, there was no evidence of nonlinear intestinal absorption of drug in both wildtype and *PepT1* knockout mice as demonstrated by dose-proportional increases in area under the plasma concentration-time curve (AUC) and maximum plasma concentration (C_{max}) [28]. The latter finding in mice, however, was contrary to other studies in humans where non-proportional increases in AUC were reported after increasing oral doses [23,24,30]. These studies clearly demonstrated that a species difference existed in the intestinal absorption and/or systemic exposure of peptides/mimetics as attributable to mouse and human PepT1.

In the present study, we hypothesized that cefadroxil would have a greater affinity (i.e., lower K_m) for intestinal PEPT1 when present in *huPepT1* mice as compared to wildtype mice. We further hypothesized that, given these differences in PEPT1 affinity, a nonlinear intestinal absorption should be more evident in the humanized mice. With this in mind, *in situ* permeability studies were performed with cefadroxil during small and large intestinal perfusions, along with *in vivo* absorption and disposition studies of drug after intravenous bolus injection at low and high doses, and after oral dose escalation. Our findings indicated that the humanized *PepT1* mouse model could provide a valuable tool in the drug discovery process, as well as better predict the pharmacokinetic profiles of PepT1 substrates in humans.

2. Materials and methods

2.1. Chemicals

[^{3}H]Cefadroxil (0.7 Ci/mmol) and [^{14}C]inulin 5000 (1.1 mCi/g) were purchased from Moravek Biochemicals and Radiochemicals (Brea, CA). Unlabeled cefadroxil, glycylproline (GlyPro), glycyl-glycyl-histidine (GlyGlyHis), glycine, L-histidine, probenecid, p-aminohippuric acid (PAH), tetraethylammonium (TEA), quinidine, N^1-methylnicotinamide (NMN), carnosine, cephalexin, cephalothin, dimethylamiloride (DMA) and inulin 5000 were purchased from Sigma-Aldrich (St. Louis, MO). CytoScint™ scintillation solution and hyamine hydroxide were purchased from MP Biomedicals (Solon, OH). All other chemicals were acquired from standard sources.

2.2. Animals

In-house breeding of gender- and weight-matched, 8–10 week, *mPepT1*$^{+/+}$ (wildtype), *mPepT1*$^{-/-}$/*hPepT1*$^{-/-}$ (*PepT1* knockout) and *mPepT1*$^{-/-}$/*hPepT1*$^{+/-}$ (humanized *PepT1*, *huPepT1*] mice, on a C57BL/6 background, were used for these experiments as reported previously [12]. Wildtype, *PepT1* knockout and humanized *PepT1* mice were identified by genotyping and culled from the same litter. The mice were housed in a temperature-controlled environment with 12-hr light and 12-hr dark cycles, receiving a standard diet and water ad libitum (Unit for Laboratory Animal Medicine, University of Michigan, Ann

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Arbor, MI). All mouse studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

2.3. In situ single-pass intestinal perfusion studies

Wildtype, *PepT1* knockout and *huPepT1* mice were fasted overnight (~ 12 hour) with free access to water and then anesthetized with sodium pentobarbital (40–60mg/kg ip). Perfusion studies of the jejunum, as well as all regional segments, were carried out according to methods described previously [5, 31]. In brief, after sterilizing the abdominal area with 70% ethanol and keeping the mice on top of a heating pad to maintain body temperature, the intestines were exposed by a mid-line incision of the abdomen. When studying regional segments, 2 cm of the duodenum, 8 cm of the proximal jejunum (i.e., ~ 2 cm distal to the ligament of Treitz), 6 cm of the ileum (i.e., ~ 1 cm proximal to the cecum) and 4 cm of the colon (i.e., ~ 0.5 cm distal to the cecum) were isolated, and incisions then made at both the proximal and distal ends. For jejunal studies, only the 8-cm segment of proximal jejunum was isolated. The segments were rinsed with 0.9% isotonic saline solution, and a glass cannula (2.0 mm outer diameter) was inserted at each end of the intestinal segment and secured in place with silk suture. The isolated intestinal segment(s) were covered with saline-wetted gauze and parafilm to prevent dehydration. After cannulation, the animals were transferred to a temperature-controlled chamber, at 31°C, to maintain body temperature during the entire perfusion procedure. The cannulas were then connected to inlet tubing, which was attached to a 10-mL syringe (BD, Franklin Lakes, NJ USA) placed on a perfusion pump (Model 22: Harvard Apparatus, South Natick, MA), and to outlet tubing, which was placed in a collection vial.

The perfusate buffer contained 135 mM NaCl, 5 mM KCl and 10 mM MES/Tris (pH 6.5) plus 10 μM of [^{3}H]cefadroxil (0.5 μCi) and 0.01% (w/v) [^{14}C]inulin 5000 (0.25 μCi) (which served as a non-absorbable marker to correct for water flux). The buffer was perfused through the intestinal segments at a flow rate of 0.1 mL/min, and the exiting perfusate was collected every 10 min for 90 min. A 100-μL aliquot of each perfusate collection was added to a vial containing 6.0 mL of scintillation solution, and the samples measured for radioactivity using a dual-channel liquid scintillation counter (Beckman LS 6000 SC, Beckman Coulter Inc., Fullerton, CA). At the end of experimentation, the actual length of intestinal segments was measured.

For the inhibition studies in jejunum, 10 mM of potential inhibitors was added to the perfusate except for DMA (0.1 mM). For the concentration-dependent studies in jejunum, cefadroxil varied from 0.01–25 mM in perfusate buffer containing [^{3}H]cefadroxil (0.5 μCi) and 0.01% (w/v) [^{14}C]inulin 5000 (0.25 μCi).

2.4. In vivo intravenous pharmacokinetic studies

Wildtype and *huPepT1* mice were anesthetized with sodium pentobarbital (40–60 mg/kg ip) prior to an intravenous bolus injection of [^{3}H]cefadroxil (11 and 528 nmol/g body weight, 5.0 μCi per dose) in 100 μL of saline. Serial blood samples were collected at 1, 2.5, 5, 10, 20, 30, 45, 60, 90 and 120 min after dosing via tail transections. Blood samples (15–20 μL)

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were placed into tubes containing 1.0 μL of EDTA-K3 and centrifuged for 3 min × 3000 g to obtain the plasma (10 μL). A 30-μL aliquot of 30% H_2O_2 was then added, followed by 6.0 mL of scintillation solution and 20 μL of 0.5 M acetic acid. Radioactivity in the plasma samples was measured using a dual-channel liquid scintillation counter.

For the biodistribution studies, 0.2 μCi of [^{14}C]inulin in 100 μL of saline was given by bolus intravenous injection, 2.0 min prior to the time at which the tissue samples were harvested (i.e., 120 min). Following decapitation, the tissues (including a blood sample) were weighed and 300 μL of hyamine hydroxide was added to the samples and then incubated at 37°C until the entire tissue was dissolved. After the samples cooled down to room temperature, 30 μL of 30% H_2O_2 was added, followed by 6.0 mL of scintillation solution and 20 μL of 0.5 M acetic acid. Radioactivity in these samples was measured using a dual-channel liquid scintillation counter. The cefadroxil tissue-to-plasma concentration ratios were also determined at 120 min.

2.5. In vivo oral pharmacokinetic studies

Following an overnight fast (~ 12 hr), wildtype and *huPepT1* mice were anesthetized briefly with isoflurane prior to oral administration of [^{3}H]cefadroxil (11, 33, 66, 132, 264 and 528 nmol/g body weight, 10 μCi per dose) by gavage in 200 μL of saline. Serial blood samples were collected at 2.5, 5, 10, 20, 30, 45, 60, 90 and 120 min after dosing via tail transections. Animals were returned to their cages in between blood sampling where they had free access to water. Blood samples (15–20 μL) were then placed into tubes as described previously and the plasma harvested. Radioactivity in the plasma samples was measured using a dual-channel liquid scintillation counter.

2.6. Data analysis

A steady-state loss of drug was observed from the perfusate approximately 30 min after the start of intestinal perfusion. The effective permeability (P_{eff}) of drug was calculated according to a complete radial mixing parallel-tube model [32, 33] such that: $P_{eff} = [-Q \bullet \ln(C_{out}/C_{in})]/(2\pi RL)$, where Q represents the perfusion flow rate (0.1 mL/min), C_{out} the outlet drug concentration after correcting for changes in water flux, C_{in} the inlet drug concentration, R the internal radius (0.1 cm for small intestine and 0.2 cm for colon), and L the length of intestinal segment. The concentration-dependent flux (J) of cefadroxil in jejunum was best fit to a single Michaelis-Menten term such that: $J = P_{eff} \bullet C_{in} = J'_{max} \bullet C_{in}/(K'_m + C_{in}) = J_{max} \bullet C_w/(K_m + C_w)$, where the parameters J'_{max} and K'_m were referenced to inlet drug concentrations (C_{in}), and the parameters J_{max} and K_m were referenced to intestinal wall drug concentrations (C_w) after correcting for the unstirred aqueous layer permeability. Pharmacokinetic parameters were calculated by noncompartmental analysis using Phoenix WinNonlin 6.3 (Certara, St. Louis, MO USA).

Data were reported as mean ± standard deviation (SD), unless otherwise noted. Statistical differences between two groups were determined using an unpaired t-test. Multiple treatment groups were compared using one-way analysis of variance followed by either a Tukey's or Dunnett's test, with wildtype mice serving as the control group (GraphPad Prism 6.0; GraphPad software, Inc., La Jolla, CA). A value of $p \leq 0.05$ was considered significant.

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3. Results

3.1. In situ concentration-dependent transport kinetics of cefadroxil in jejunum of wildtype and humanized PepT1 mice

As shown in Fig. 1, both wildtype and humanized *PepT1* mice demonstrated a saturable transport of cefadroxil. When referenced to inlet concentrations (C_{in}), wildtype mice had a maximal flux (J'_{max}) = 0.380 ± 0.001 nmol/cm^2/sec and a Michaelis constant (K'_m) = 6.01 ± 0.46 mM for cefadroxil (r^2=0.982, Fig. 1A). In comparison, the values for cefadroxil in humanized *PepT1* mice were 0.057 ± 0.006 nmol/cm^2/sec and 2.69 ± 0.93 mM, respectively (r^2=0.658, Fig. 1A). When adjusted for the unstirred water layer and referenced to intestinal wall concentrations (C_w), wildtype mice had a maximal flux (J_{max}) = 0.392 ± 0.010 nmol/cm^2/sec and a Michaelis constant (K_m) = 4.80 ± 1.00 mM for cefadroxil (r^2=0.996, Fig. 1B). The values in humanized *PepT1* mice were 0.056 ± 0.009 nmol/cm^2/sec and 2.37 ± 1.21 mM, respectively (r^2=0.728, Fig. 1B). Thus, the affinity of cefadroxil for PepT1 was about two-fold lower (i.e., two-fold higher K_m) in wildtype as compared to humanized *PepT1* mice.

3.2. In situ permeability of cefadroxil in regional intestinal segments of wildtype, humanized PepT1 and PepT1 knockout mice

Given a K_m for cefadroxil on the order of 2.4–6.0 mM, subsequent perfusion studies were performed at 10 μM cefadroxil to maintain conditions of transport linearity. As shown in Fig. 2A, the permeability of cefadroxil was considerable in the duodenum, jejunum and ileum of wildtype and humanized *PepT1* mice, although it was 50–60% lower in humanized mice. In contrast, the permeability of cefadroxil in *PepT1* knockout mice was negligible in all regions of small intestine, with a residual permeability of 5% or less as compared to wildtype animals. Still, the permeability of cefadroxil in humanized *PepT1* mouse colon was measurable and 14-fold higher than that of wildtype mice.

3.3. In situ permeability and substrate specificity of cefadroxil in jejunum of humanized PepT1 mice

In the presence of excess concentrations of potential inhibitors, the permeability of cefadroxil was reduced 95% by GlyPro, 80% by GlyGlyHis and about 70% by carnosine or cephalexin during jejunal perfusions in humanized *PepT1* mice (Fig. 2B). In contrast, amino acid (glycine and L-histidine), OAT (probenecid and PAH) and OCT (TEA, quinidine and NMN) substrates, and cephalosporins lacking an α-amino group (i.e., cephalothin) had no effect on the jejunal permeability of cefadroxil. However, DMA (an inhibitor of the sodium-proton exchanger) was able to reduce the jejunal permeability of cefadroxil by 55% as compared to the control group. Thus, the permeability of cefadroxil was specific for PepT1 and primarily due to intestinal PepT1 expression in the humanized mouse model.

3.4. In vivo pharmacokinetic studies of cefadroxil in wildtype and humanized PepT1 mice following intravenous bolus doses

To rule out potential differences in the systemic disposition of cefadroxil, wildtype and humanized *PepT1* mice were evaluated following intravenous bolus injections of drug at

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both low (11 nmol/g) and high doses (528 nmol/g). As shown in Fig. 3, the plasma concentration-time profiles of cefadroxil were virtually superimposable between the two genotypes for each dose. Noncompartmental analyses indicated that the pharmacokinetic parameters of cefadroxil (i.e., CL, $T_{1/2}$, Vss and MRT) were not significantly different between wildtype and humanized *PepT1* mice or as a function of dose (Table 1). As expected, dose-dependent increases were observed in the AUC for the low versus high doses of cefadroxil, but no differences were observed between the two genotypes. In agreement with this finding, no significant differences were found in the tissue distribution of cefadroxil between wildtype and humanized *PepT1* mice (Fig. 4).

3.5. In vivo pharmacokinetic studies of cefadroxil in wildtype and humanized PepT1 mice following oral dose escalation

The *in vivo* functional activity of PepT1 was evaluated in wildtype and humanized *PepT1* mice after increasing oral doses of cefadroxil. The choice of mouse doses (11–528 nmol/g) was based on adjusting human adult doses (250–2000 mg) so that similar concentrations of cefadroxil were produced in the stomach as well as in the systemic circulation. As shown in Fig. 5, the plasma concentrations of cefadroxil were much lower in humanized *PepT1* mice than in wildtype animals for all doses (i.e., 11, 33, 66, 132, 264 and 528 nmol/g). According to noncompartmental analyses (Table 2), the AUC and C_{max} values of cefadroxil were about two-fold lower and the T_{max} about two-fold longer in the humanized *PepT1* mice. The $T_{1/2}$, however, was not significantly different between the two genotypes, in agreement with the results following single intravenous bolus doses.

3.6. AUC and C_{max} versus dose relationships of cefadroxil in wildtype, humanized PepT1 and human subjects after oral dose escalation

Because of the higher affinity of cefadroxil for human over mouse PepT1, as determined by the *in situ* perfusion studies, the oral absorption of cefadroxil was more likely to be saturated in humanized *PepT1* mice than in wildtype animals. As shown in Fig. 6, a nonlinear relationship was observed for the $AUC_{0–120}$ versus dose (Fig. 6A) and C_{max} versus dose (Fig. 6B) profiles of cefadroxil in humanized *PepT1* mice during the oral dose escalation studies. Moreover, these profiles were strikingly similar between the humanized *PepT1* mice and human values obtained from the literature [24]. In contrast, the $AUC_{0–120}$ versus dose (Fig. 6A) and C_{max} versus dose (Fig. 6B) relationships were linear in wildtype mice and far from congruent with human values.

4. Discussion

The generation and application of humanized mouse models to translate animal pharmacokinetics, metabolism, toxicity and target validation to humans have gained significant favor over the past 5–10 years [8–11]. Because of species differences noted previously in the PepT1-mediated uptake kinetics of GlySar in transformed yeast [7], Hu and coworkers [12] approached this challenge by developing and characterizing a mouse line humanized for the intestinal peptide transporter *PepT1*. In the present investigation, these humanized *PepT1 (huPepT1)* mice were further validated with respect to a relevant therapeutic drug, the β-lactam antibiotic cefadroxil.

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Several major new findings were observed during the study of cefadroxil: 1) the *in situ* permeability of cefadroxil was comparable but smaller in the small intestine of *huPepT1* versus wildtype mice, and cefadroxil colonic permeability was measurable in *huPepT1* mice but not in wildtype or *PepT1* knockout animals; 2) a species difference was found in the *in situ* affinity of mouse and human PepT1 for cefadroxil in which Km differed by two-fold between species (i.e., 4.8–6.0 mM for wildtype versus 2.7–2.4 mM for *huPepT1* mice); 3) the uptake of cefadroxil *in situ* was specific for PepT1 and proton gradient-dependent; 4) the *in vivo* pharmacokinetics and tissue distribution of cefadroxil after intravenous dosing were not different between wildtype and *huPepT1* mice; 5) the *in vivo* pharmacokinetic of cefadroxil after oral dose escalation was different between the two genotypes (i.e., lower C_{max} and AUC, and longer T_{max} in *huPepT1* mice), along with nonlinear AUC and C_{max} versus dose relationships being observed in *huPepT1* but not wildtype mice; and 6) more similar profiles were observed between the AUC and C_{max} versus dose relationships of *huPepT1* mice and human subjects than between wildtype mice and human subjects. Taken as a whole, it appears that humanized *PepT1* mice were able to more accurately predict the oral absorption and disposition of cefadroxil in humans than were wildtype mice.

Less than proportional increases in AUC and C_{max} were observed in *huPepT1* mice following increasing oral doses of cefadroxil, a finding also observed in human subjects [24]. In contrast, linear absorption kinetics of cefadroxil were observed in wildtype mice in this study and under similar oral dose escalations in a previous study [28]. Possible reasons to explain the interspecies discrepancy in the dose-dependent systemic exposure of cefadroxil after oral dosing would include changes in the drug's intestinal absorption profile and/or systemic clearance. Mechanisms consistent with these changes might be: 1) the saturable PepT1-mediated uptake of cefadroxil in the small intestine of *huPepT1* but not wildtype mice; and 2) the saturable OAT-mediated secretion and/or PepT2-mediated reabsorption of cefadroxil in the renal tubules of *huPepT1* but not wildtype mice. The second possibility, however, is unlikely because the disposition of cefadroxil was unchanged between genotypes following the 11 and 528 nmol/g intravenous bolus doses of drug. In this regard, wildtype and *huPepT1* mice had very similar plasma concentration-time profiles (Fig. 3) and pharmacokinetics (Table 1) of cefadroxil, along with no significant difference in the drug's tissue distribution (Fig. 4). The first possibility is more likely given the two-fold higher affinity of human PepT1 for cefadroxil (i.e., lower Km), thereby, resulting in a saturable intestinal absorption of cefadroxil in *huPepT1* but not wildtype mice after oral dose escalation.

The ability of *huPepT1* mice to accurately predict the pharmacokinetics of drug in humans depends upon many factors including the faithful expression and functional activity of PepT1 in the small and large intestines, and orally administered doses that are clinically relevant, thereby, reflecting the concentrations of drug in both the stomach and plasma. PepT1 is a high-capacity low-affinity transporter with K_m values ranging from 0.2 to 10 mM [34–36]. During our *in situ* jejunal perfusion studies, the affinity of cefadroxil was two-fold higher in *huPepT1* mice (K_m=2.7 mM) than in wildtype animals (K_m=6.0 mM). These values were similar to the K_m values of 2–4 mM in wildtype mice [25] and to the K_m of 5.9 mM [37] in rat, both observed during *in situ* jejunal perfusions of cefadroxil. No information is currently available on the permeability of cefadroxil in humans although a value of 1.56 ×

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10^{-4} cm/s was reported for cephalexin, another β-lactam antibiotic, in human jejunum [38]. Even though both compounds are from the same class of orally administered aminocephalosporin drugs, they have different chemical structures that could impart different affinities between species and, as a result, elicit different intestinal permeabilities. Finally, we did not quantitatively measure the absolute protein concentrations of hPepT1 in humanized mouse intestines or kidney. Notwithstanding this uncertainty, our previous results [12] demonstrate that the relative density of hPepT1 in *huPepT1* mouse intestine was about 10–20 times higher in small intestine as compared to colon, a finding very similar to the difference observed by Drozdzik et al [1] in their LC-MS/MS analysis of hPepT1 in human intestinal biopsies. Although hPepT1 was not measured in *huPepT1* mouse kidney, only 5% of cefadroxil's renal reabsorption occurs via PepT1 as opposed to 95% by PepT2 [39]. Subsequent studies should measure the absolute quantitation of hPepT1 in *huPepT1* mouse tissues to further validate this animal model as being useful for predicting drug absorption in humans.

Studies in mouse, rat and human show that PepT1 is apically located and abundantly expressed in all regions of the small intestine [5,40–47]. However, expression of PepT1 in the large intestine is controversial and may be species- and/or region-dependent [43]. In the current study, cefadroxil permeability was high in the duodenum, jejunum and ileum in both wildtype and *huPepT1* mice, agreeing with the protein expression pattern of PepT1 in these two genotypes [5,12]. In contrast, the colonic permeability of cefadroxil was negligible to non-existent in wildtype mice and not significantly different from permeability values in *PepT1* knockout animals, a finding reported in similar studies with cefadroxil [25] and other PepT1 substrates such as GlySar [5] and valacyclovir [48]. Nonetheless, as demonstrated in this study with cefadroxil and a previous study with GlySar [12], and in agreement with qPCR and immunoblot results [12], low but notable permeabilities were observed for the colonic permeabilities of cefadroxil and GlySar in *huPepT1* mice. Collectively, it appears that the functional activities of PepT1 substrates in *huPepT1* mice are consistent with the protein expression patterns of PepT1 in the small intestine and colon of humans, as reported previously [1].

For the oral studies, cefadroxil was administered to mice at doses ranging from 11 to 528 nmol/g so that their initial stomach concentrations would reflect the values observed in humans after clinical oral doses of 250 to 2000 mg. Thus, given a stomach fluid volume of 0.4 mL for mice [49] and 250 mL for humans [50], the initial stomach concentrations of cefadroxil were similar between species, ranging from 0.6–26.4 mM in mice and from 2.8–22.1 mM in humans. These estimated stomach concentrations were higher than the K_m values of cefadroxil for PepT1, as determined during the *in situ* jejunal perfusions of drug in wildtype and *huPepT1* mice (i.e., 6.0 and 2.7 mM, respectively) (Fig. 1). However, given its lower K_m, it was hypothesized that *huPepT1* mice would be more likely to exhibit saturable PepT1-mediated intestinal absorption of cefadroxil *in vivo* than wildtype animals. This contention was confirmed, where both the AUC (Fig. 6A) and C_{max} (Fig. 6B) of cefadroxil increased linearly after oral dose escalation in wildtype but not *huPepT1* mice. Instead, less than proportional increases were observed for AUC and C_{max} in *huPepT1* animals as a function of increasing oral dose administration. Moreover, the findings in *huPepT1* mice were more predictive of the systemic profiles of cefadroxil after oral dosing in human

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subjects [24] than were wildtype mice. It is unclear why the wildtype mice did not also show a dose-dependent (i.e., nonlinear) intestinal absorption of cefadroxil, although this finding was consistent with a previous study by our laboratory with the same drug [28]. Although speculative, it is possible that, in wildtype mice, as cefadroxil travels down the proximal to distal regions of the small intestine, the concentration of drug remains below its K_m value and exhibits a linear uptake. This phenomenon would be less likely in the *huPepT1* mice.

Specificity studies were performed previously by our laboratory in wildtype mice demonstrating that, during *in situ* intestinal perfusions of drug ± inhibitor, the jejunal permeabilities of GlySar [5], cefadroxil [25] and valacyclovir [48] were specific for PepT1. In the present study, the jejunal permeability of cefadroxil in *huPepT1* mice was unaffected by excess concentrations of the amino acids glycine and L-histidine, the organic anions probenecid and PAH, the organic cations TEA, quinidine and NMN, and cephalothin, a β-lactam drug lacking an α-amino group (Fig. 2B). Thus, the apical uptake of cefadroxil was not influenced by glycine-dependent amino acid transporters and the L-histidine-dependent peptide transporters PhT1/2, as well as by the OAT and OCT transporters. Instead, the interaction between cefadroxil and PepT1 at the apical membrane of enterocytes was specific, a finding supported by the proton-dependent uptake of drug as demonstrated by its reduced uptake in the presence of the sodium-proton exchange inhibitor DMA (Fig. 2B). The negligible permeability of cefadroxil in the jejunum of *PepT1* knockout mice further supports the lack of a meaningful role for other transporters in the drug's intestinal uptake across apical membranes (Fig. 2A).

In concluding, the *in situ* perfusion studies revealed a clear species difference between the intestinal permeabilities of cefadroxil in wildtype and humanized *PepT1* mice. In particular, the drug K_m was two-fold lower in *huPepT1* animals, thereby, making it more likely to exhibit nonlinear PepT1-related intestinal absorption in this genotype. Moreover, the colonic permeability of cefadroxil in *huPepT1* mice, although low, was measurable and consistent with the protein expression pattern of PepT1 in *huPepT1* animals and humans. The oral dose escalation studies confirmed these species differences *in vivo* and demonstrated that the nonlinear AUC and C_{max} versus dose relationships of cefadroxil in *huPepT1* mice were quite similar to these relationships in human subjects. In contrast, linear AUC and C_{max} versus dose relationships were observed in wildtype mice, which were poorly correlated with that of human subjects. The present studies extend our previous validation of the synthetic dipeptide GlySar (12) to the clinically useful antibiotic cefadroxil, and support our contention that these *huPepT1* mice provide a valuable animal model in PepT1-targeted drug design for improved biopharmaceutics. Studies are currently underway with PepT1 prodrugs to further validate the usefulness of *huPepT1* mice in the drug discovery process.

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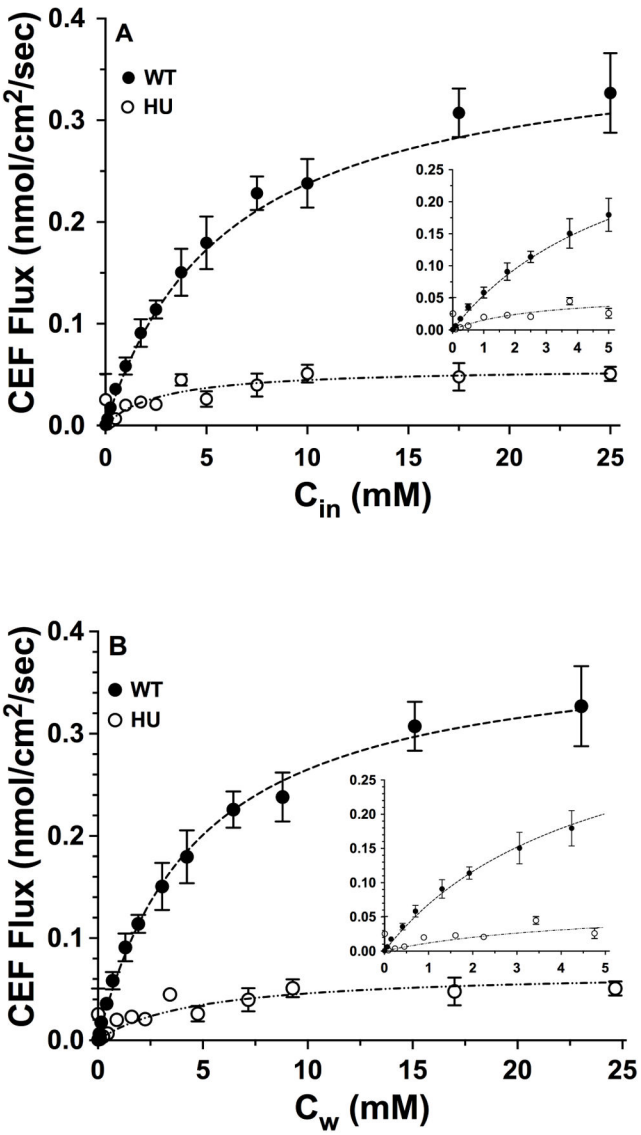

Figure 1.

Concentration-dependent flux of [^{3}H]cefadroxil (0.01 to 25 mM) during jejunal perfusions in wildtype (WT) and humanized *PepT1* (HU) mice, where C_{in} is the inlet concentration of cefadroxil in perfusate (A) and C_w is the estimated concentration of cefadroxil at the membrane wall (B). The inserts show these relationships at lower concentrations (0.01–5 mM). All studies were performed in pH 6.5 buffer. Data are reported as mean ± standard error (SE) (n=4).

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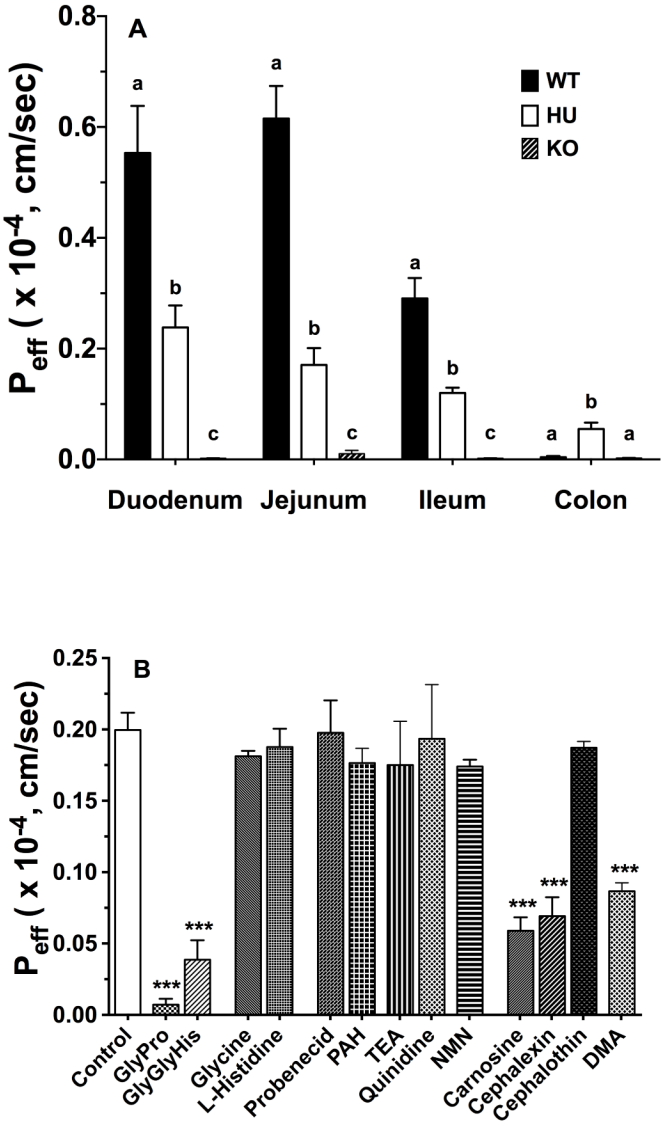

Figure 2.

Effective permeability (P_{eff}) of 10 μM [^{3}H]cefadroxil in different regions of the small and large intestines in wildtype (WT), humanized *PepT1* (HU) and *PepT1* knockout (KO) mice (A). All studies were performed in pH 6.5 buffer. Data are reported as mean ± SE (n=4). Treatment groups with different letters are significantly different, as evaluated by analysis of variance followed by Tukey post hoc comparisons. Specificity studies were performed in the jejunum of humanized *PepT1* mice in which P_{eff} was determined for 10 μM [^{3}H]cefadroxil in the absence and presence of 10 mM potential inhibitors (0.1 mM for DMA) (B). All studies were performed in pH 6.5 buffer. Data are reported as mean ± SE (n=3). $^{***}p < 0.001$, as evaluated by analysis of variance followed by Dunnett post hoc comparisons with the control group. Glycyl-proline (GlyPro), glycyl-glycyl-histidine (GlyGlyHis), p-aminohippuric acid (PAH), tetraethylammonium (TEA), N^1-methylnicotinamide (NMN), and dimethylamiloride (DMA).

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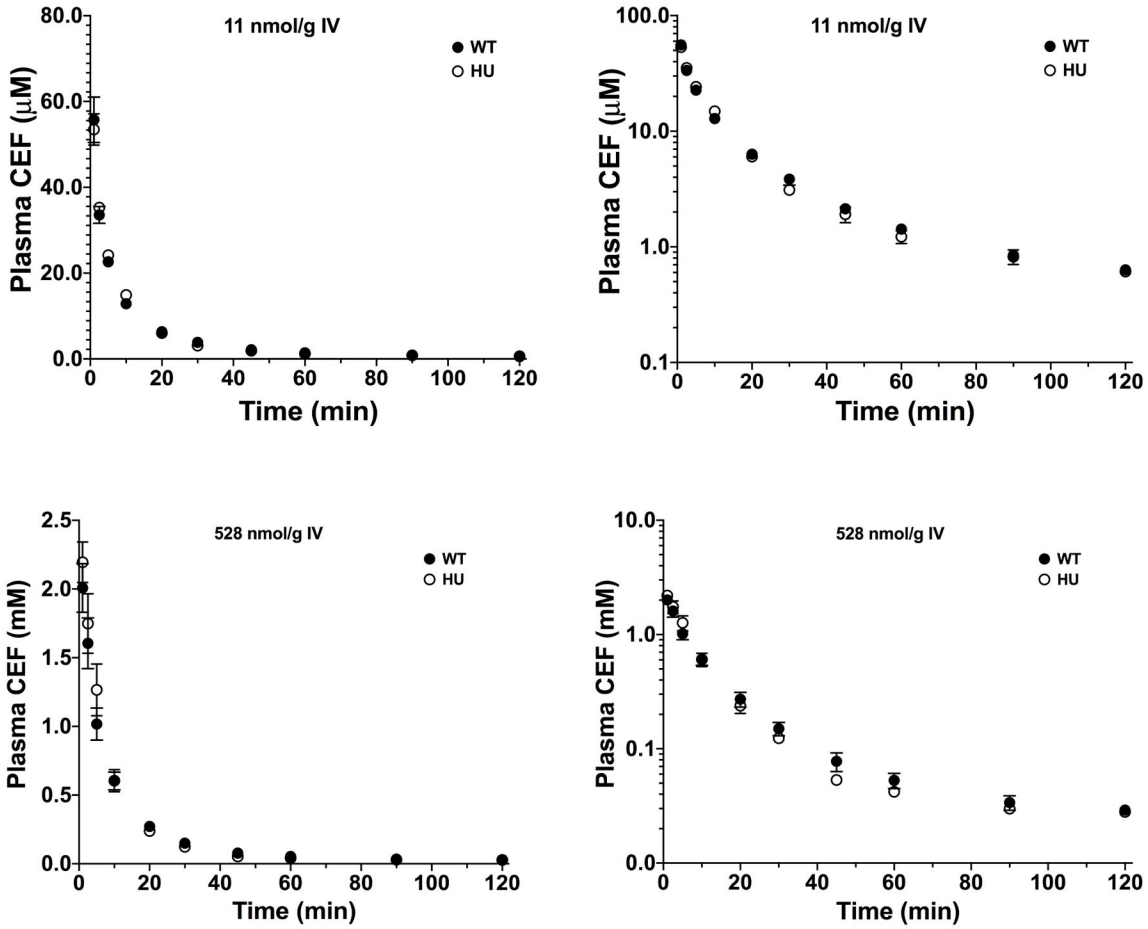

Figure 3.

Plasma concentration-time profiles of [^{3}H]cefadroxil in wildtype (WT) and humanized *PepT1* (HU) mice following low and high intravenous bolus injections. Data are reported as mean ± SE (n=4–5) in which the y-axis is displayed on a linear scale (left-sided panels) and on a logarithmic scale (right-sided panels).

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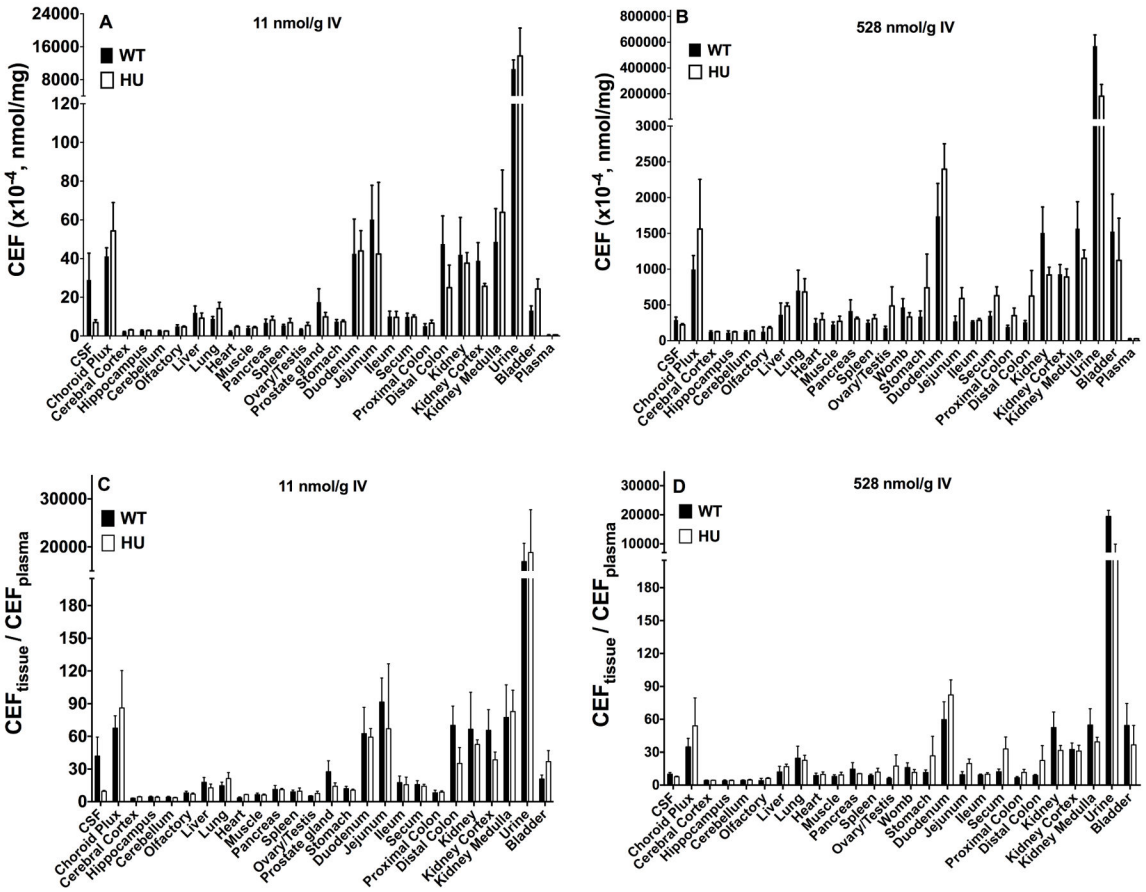

Figure 4.

Tissue distribution of [^{3}H]cefadroxil, and plasma-normalized values, in wildtype (WT) and humanized *PepT1* (HU) mice following low and high intravenous bolus injections, in which the tissues were collected 120 min after dosing. Data are reported as mean ± SE (n=4–5). No significant differences were observed between the two genotypes, as evaluated by unpaired t-tests.

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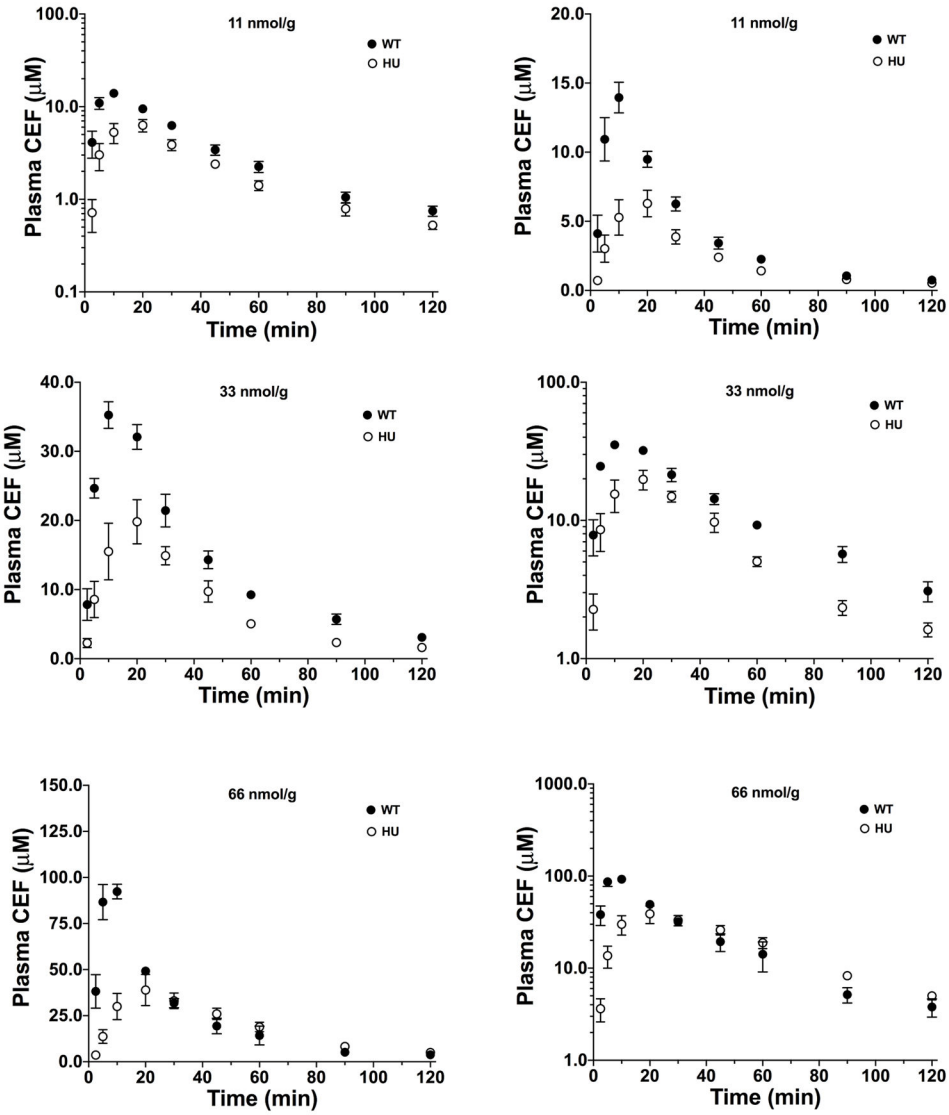

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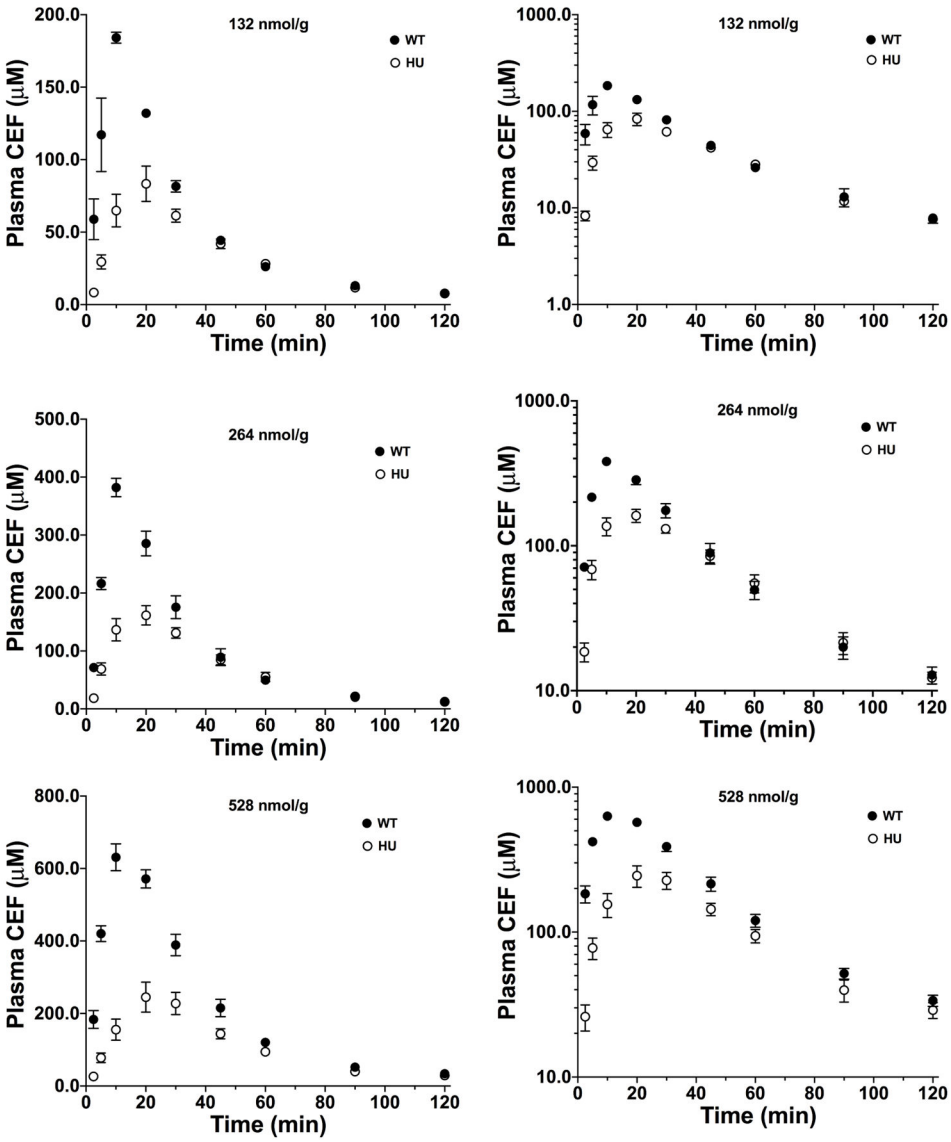

Figure 5.

Plasma concentration-time profiles of [^{3}H]cefadroxil in wildtype (WT) and humanized *PepT1* (HU) mice following oral dose escalation. Data are reported as mean ± SE (n=6–7) in which the y-axis is displayed on a linear scale (left-sided panels) and on a logarithmic scale (right-sided panels).

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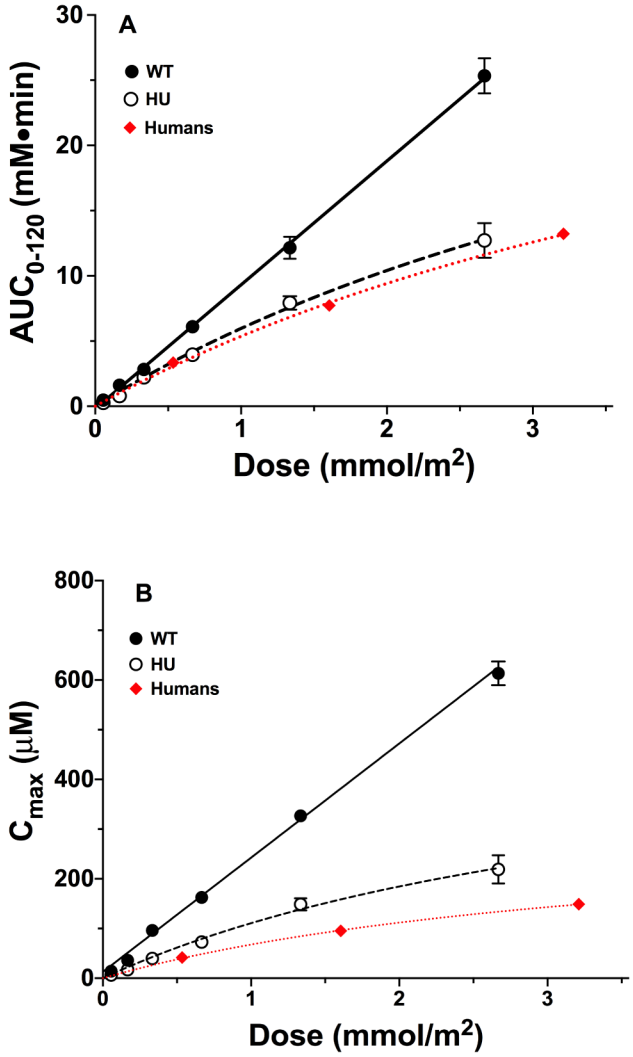

Figure 6.

Area under the plasma concentration-time curve from time 0 to 120 min ($AUC_{0–120}$) versus oral dose (A) and maximum plasma concentration (C_{max}) versus oral dose (B) of [^{3}H]cefadroxil in wildtype (WT, n=6), humanized *PepT1* (HU, n=7) and clinical data (humans, n=3) obtained from the literature [24]. The body surface areas (BSA) of mice were calculated as: $BSA_{mice} = (BW_{mice}/BW_{humans})^{0.75} \bullet BSA_{humans}$, where the body weights (BW) and BSA of humans were 70 kg and 1.8 m^2, respectively. Data are reported as mean ± SE.

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Table 1

Noncompartmental analysis of [^{3}H]cefadroxil pharmacokinetics after intravenous bolus dosing in wildtype and humanized *PepT1* mice

Parameters	Low Dose (11 nmol/g)		High Dose (528 nmol/g)	
	WT	HU	WT	HU
CL (mL/hr)	21.6 ± 2.1	21.6 ± 1.8	25.5 ± 4.5	24.0 ± 3.4
$T_{1/2}$ (min)	51.7 ± 6.8	56.2 ± 2.8	44.1 ± 21.9	72.4 ± 46.4
V_{ss} (mL)	12.0 ± 2.5	11.9 ± 1.6	17.2 ± 9.8	13.4 ± 3.9
MRT (min)	19.6 ± 1.5	18.5 ± 2.9	19.1 ± 0.7	16.9 ± 0.9
$AUC_{0–120}$ (mM•min)	0.57 ± 0.06	0.56 ± 0.05	23.6 ± 4.6	23.8 ± 3.8
$AUC_{0–8}$ (mM•min)	0.62 ± 0.06	0.61 ± 0.05	25.5 ± 5.1	26.7 ± 3.6

Data are expressed as mean ± SD (n=3–5) in wildtype (WT) and humanized *PepT1* (HU) mice. No significant differences were observed between the pharmacokinetics of cefadroxil as a function of genotype or dose, as evaluated by a two-way analysis of variance (except for differences in AUC between the low and high doses in each genotype; $p < 0.001$).

CL is the total clearance; $T_{1/2}$ the log-linear terminal half-life; V_{SS} the volume of distribution steady-state; MRT the mean residence time; $AUC_{0–120}$ the area under the plasma concentration-time curve from time 0 to 120 min; and $AUC_{0-\infty}$ the area under the plasma concentration-time curve from time zero to infinity.

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Table 2

Noncompartmental analysis of [^{3}H]cefadroxil pharmacokinetics after oral dose escalation in wildtype and humanized *PepT1* mice

	C_{max} (μM)		T_{max} (min)		$T_{1/2}$ (min)		$AUC_{0–120}$ (mM•min)		$AUC_{0-\infty}$ (mM•min)	
Dose (nmol/g)	**WT**	**HU**	**WT**	**HU**	**WT**	**HU**	**WT**	**HU**	**WT**	**HU**
11	14.2±2.9	6.9±2.6***	9.3±1.9	20.7±11.7***	32.1±8qq.7	32.4±10.7	0.47±0.07	0.26±0.05***	0.51±0.08	0.29±0.04***
33	37.0±3.3	17.8±8.8***	13.3±5.2	27.5±9.8***	36.3±16.1	38.4±12.8	1.60±0.28	0.79±0.21***	1.78±0.32	0.89±0.27***
66	96.3±10.4	41.3±18.9***	7.5±2.7	30.0±12.2***	28.0±2.6	33.6±7.5	2.82±0.73	2.22±0.48***	2.98±0.82	2.47±0.45***
132	185±11	84.4±28.0***	9.2±2.0	21.7±4.1***	26.1±3.2	28.9±2.7	6.07±0.59	3.95±0.58***	6.36±0.62	4.27±0.61***
264	382±38	174±39***	10.0±0.0	18.3±4.1***	24.0±4.7	27.2±5.8	12.1±2.1	7.92±1.30***	12.6±2.2	8.41±1.30***
528	637±85	263±92***	13.3±5.2	24.3±5.3***	29.8±7.6	31.5±7.2	25.3±3.3	12.7±3.5***	26.7±3.8	14.1±3.5***

Data are expressed as mean ± SD (n=6–7) in wildtype (WT) and humanized *PepT1* (HU) mice.

*** $p < 0.001$, as evaluated by an unpaired t-test between genotypes for each dose level.