Impact of Genetic Knockout of PEPT2 on Cefadroxil Pharmacokinetics, Renal Tubular Reabsorption and Brain Penetration in Mice

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Abbreviations: BCSFB, blood-cerebrospinal fluid barrier; CNS, central nervous system; CSF, cerebrospinal fluid; ER, excretion ratio; GFR, glomerular filtration rate; GlySar, glycylsarcosine; OAT, organic anion transporter; OCT, organic cation transporter; POT, proton-coupled oligopeptide transporter
ABSTRACT:

The aim of this study was to examine the role of PEPT2, a proton-coupled oligopeptide transporter of the SLC15 family, on the disposition of the antibiotic cefadroxil in the body, particularly the kidney and brain. Pharmacokinetic, tissue distribution and renal clearance studies were performed in wild-type and PEPT2 null mice after intravenous bolus administration of [3H]cefadroxil at 1, 12.5, 50 and 100 nmol/g body weight. Studies were also performed in the absence and presence of probenecid and quinine. Cefadroxil disposition kinetics was clearly nonlinear over the dose range studied (1-100 nmol/g), which was attributed to both saturable renal tubular secretion and reabsorption of the antibiotic. Following an intravenous bolus dose of 1 nmol/g cefadroxil, PEPT2 null mice exhibited a 3-fold greater total clearance and 3-fold lower systemic concentrations of drug compared to wild-type animals. Renal clearance studies further demonstrated that the renal reabsorption of cefadroxil was almost completely abolished in PEPT2 null versus wild-type mice (3% versus 70%, p<0.001). Out of the 70% of cefadroxil reabsorbed in wild-type mice, PEPT2 accounted for 95% and PEPT1 accounted for 5% of reabsorbed substrate. Tissue distribution studies indicated that PEPT2 had a dramatic effect on cefadroxil tissue exposure, especially in brain where the CSF-to-blood concentration ratio of cefadroxil was 6-fold greater in PEPT2 null mice compared to wild-type animals. These findings demonstrate that renal PEPT2 is almost entirely responsible for the reabsorption of cefadroxil in kidney and that choroid plexus PEPT2 limits the exposure of cefadroxil (and perhaps other aminoccephalosporins) in CSF.
Proton-coupled oligopeptide transporters (POTs) not only deliver peptide-bound amino nitrogen to cells but may also have a significant influence on the pharmacokinetics and pharmacodynamics of peptide-like drugs (Smith et al., 2004; Daniel and Kottra, 2004). To date, four POTs have been identified in a variety of mammalian species including human, rabbit, rat and mouse (i.e., PEPT1, PEPT2, PHT1, PHT2). Among these mammalian POTs, PEPT2 (also known as \textit{SLC15A2}) consists of 729 amino acids with an apparent molecular mass of 85 kDa, which is reduced to 81 kDa after deglycosylation. PEPT2 is specifically expressed on the apical (luminal) membrane of epithelial cells of proximal tubule in the kidney and transports a broad range of peptide-like compounds (e.g., cefadroxil, enalapril, bestatin, valacyclovir, 5-amino-levulinic acid) (Daniel and Kottra, 2004; Smith et al., 2004). Due to its high expression in kidney and high affinity for transport, it has been suggested that PEPT2 plays a primary role in renal reabsorption of some drugs (Takahashi et al., 1998; Shen et al., 1999). However, definitive \textit{in vivo} evidence for this assertion is lacking.

PEPT2 is thought to play an important role in the exposure and therapeutic outcome of peptide-like drugs in the central nervous system (CNS). Using immunohistochemistry, our group has recently demonstrated expression of PEPT2 in the apical membrane [cerebrospinal fluid (CSF)-facing] of rat choroid plexus epithelial cells, suggesting a possible role for PEPT2 in facilitating the efflux of substrates from CSF to blood (Shen et al., 2004). Primary cultures of rat choroid plexus epithelial cells, a model of the blood-cerebrospinal fluid barrier (BCSFB), also express PEPT2 on their apical surface (Shu et al., 2002). These cultures allow one to study the apical-to-basal (A-to-B) and basal-to-apical (B-to-A) permeability of drugs. Our results indicate a vectorial A-to-B directed and saturable transport of PEPT2 substrates like glycylsarcosine (GlySar), carnosine and cefadroxil (Shu et al., 2002; Teuscher et al., 2004; Shen et al., 2005),
suggesting that PEPT2 facilitates drug efflux from CSF to blood. However, in vivo experiments are needed in order to confirm this contention.

Cefadroxil is an aminoccephalosporin anti-infective agent with a broad spectrum of antibacterial activity. Clinical studies showed that cefadroxil is completely absorbed in the small intestine and distributed extensively to body tissues and fluids including kidney, lung, liver, muscle and tonsils (Tanrisever and Santella, 1986; Quintiliani, 1982). In addition, in vitro and in vivo studies have shown that cefadroxil is not metabolized (Tanrisever and Santella, 1986; AHFS Drug Information, 2006). Despite its favorable stability, the residence time of cefadroxil in vivo is relatively short (half-life ~ 1.5 hr in human). Rapid renal excretion is responsible for the short sojourn in vivo. Like other cephalosporin antibiotics, the elimination of cefadroxil is markedly decreased in patients with impaired renal function, resulting in a significant increase in the drug’s elimination half-life (Leroy et al., 1982). The renal secretion of most cephalosporins is believed to be mediated by organic anion transporters (OATs) located on the basolateral membrane of proximal tubule epithelia (Takeda et al., 2002; Shitara et al., 2005). Thus, the elimination rate of cefadroxil (and similar compounds) is significantly reduced by coadministration of probenecid, an inhibitor of OAT (Brown, 1993; Shitara et al., 2005). However, some cephalosporins are also subjected to renal reabsorption since they are substrates of PEPT1 and PEPT2 (Ganapathy et al., 1995), which are localized on the brush border membrane of proximal tubule. While PEPT1 is expressed in the early part of the proximal tubule (pars convoluta), PEPT2 is expressed further along the proximal tubule (pars recta). Moreover, like other β-lactam antibiotics, cefadroxil may fail in the treatment of CNS infections, including meningitis, because of poor penetration across the blood-brain barrier (BBB) and
BCSFB. It is reasonable to speculate that POTs and OATs in the choroid plexus might act as drug efflux proteins, resulting in subtherapeutic levels of antibiotics in CSF (Shen et al., 2005).

*In vivo* experiments that demonstrate the role and relevance of PEPT2 in affecting the disposition of β-lactam antibiotics in the body have yet to be performed. With this in mind, we examined the pharmacokinetics and tissue distribution of cefadroxil in wild-type and PEPT2 null mice. Our results are novel in demonstrating, for the first time, that PEPT2 has substantial impact on the *in vivo* disposition of an aminocephalosporin antibiotic drug, cefadroxil, particularly within the kidney and brain.
Material and Methods

Chemicals

[^3]H]Cefadroxil (3.8 Ci/mmol), [^14]C]carboxyl-inulin (1.3 mCi/g) and [^14]C]carboxyl-dextran (MW 70,000) (1.1 mCi/g) were purchased from Moravek Radiochemicals (Brea, CA). Radiochemical purity of all compounds was > 98% as determined by HPLC. Unlabeled cefadroxil (MW = 363.4) was obtained from Sigma-Aldrich (St. Louis, MO) and hyamine hydroxide was purchased from ICN (Irvine, CA). All other chemicals were obtained from standard sources.

Animals

Animal studies were conducted 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. Wild-type (PEPT2+/+) and PEPT2 null (PEPT2−/−) mice (> 99% C57BL/6 genetic background) between 8 and 15 weeks of age were used for these studies. Animals were kept in a temperature-controlled environment with a 12-hour light, 12-hour dark cycle and received a standard diet and water ad libitum (Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI).

Pharmacokinetic Studies with Cefadroxil

In all cases, gender- and weight-matched mice were fasted overnight before the onset of each experiment. Cefadroxil was dissolved in normal saline and administered to the mice in aqueous solution. Following sodium pentobarbital anesthesia (50 mg/kg ip), mice received cefadroxil through a tail vein injection (5 µl/g and 0.5 µCi/g body weight). Blood samples were
collected (~15 µl) via tail nicks at 0.25, 1, 2, 5, 15, 30, 45, 60, 90 and 120 min after the intravenous bolus dose. Heparinized blood samples were centrifuged immediately at 2,000 x g for 10 min to obtain plasma. Urine samples were collected, as indicated in the experiments below. The radioactivity of plasma and urine samples was measured by a dual-channel liquid scintillation counter (Beckman LS 3801; Beckman Coulter, Inc., Fullerton, CA).

A. Metabolic Stability of Cefadroxil

[^3H]Cefadroxil was administered intravenously to both genotypes at a dose of 50 nmol/g body weight (i.e., 0.5 µCi/g). Each animal was then placed in a metabolic cage with diuresis adapter (Nalge Nunc International, Naperville, IL) and urine was collected for 24 hr. Following several washes of the diuresis adapter with water, the diluted urine was collected and analyzed by reversed-phase high-performance liquid chromatography (HPLC). The system consisted of a Waters 515 pump (Waters, Milford, MA), a Rheodyne® injector port (Rohnert Park, CA), and a Packard 500TR Radiochemical detector (Packard Instrument Company, Downers Grove, IL). Cefadroxil was separated on a Discovery 5-µm (C18) column, 250 x 4.6 mm (Supelco, Bellefonte, PA), preceded by a µ-Bondapak C18 guard column (Waters). Isocratic elution was performed by mixing acetonitrile and water (6:94) at a constant flow rate of 1.0 ml/min. Peaks were recorded and integrated using FLO-ONE software for Windows Analysis (Packard Instrument Company, version 3.61). The retention time of cefadroxil was 4.8 min.

B. Dose Dependency of Cefadroxil

[^3H]Cefadroxil was administered intravenously to both genotypes at doses of 1, 12.5, 50 and 100 nmol/g body weight (i.e., 0.4, 4.5, 18.2 and 36.4 mg/kg) and serial blood samples were collected over 2 hours and the plasma harvested.
C. Renal Clearance of Cefadroxil

\[^3H\]Cefadroxil was administered intravenously to both genotypes at a dose of 1 nmol/g body weight along with \[^{14}C\]inulin (30.8 mg/kg) for the determination of GFR. Serial blood samples were then drawn over 2 hr and the plasma harvested. Urine was collected directly from the bladder at 120 min.

D. Inhibition of Cefadroxil Renal Secretion by Probenecid or Quinine

\[^3H\]Cefadroxil (1 nmol/g body weight) and \[^{14}C\]inulin (30.8 mg/kg) were administered by intravenous bolus injection to both genotypes. Three treatments of probenecid or quinine (at either 70 or 700 mg/kg) were administered subcutaneously at 60 min prior, at the same time, and at 60 min after the \[^3H\]cefadroxil and \[^{14}C\]inulin doses. Serial blood samples were obtained over 2 hr and urine was collected from the bladder at the end of blood sampling (i.e., at 2 hr).

E. Tissue Distribution of Cefadroxil

Wild-type and PEPT2 null mice were injected with \[^3H\]cefadroxil (1 nmol/g body weight) through the tail vein. \[^{14}C\]Dextran (MW = 70,000) (0.25 µCi/mouse) was administered intravenously 5 min prior to harvesting the tissues so that cefadroxil tissue concentrations could be corrected for the vascular space. A blood sample was obtained 120 min after intravenous administration of cefadroxil, after which a CSF sample was taken from the cisterna magna by puncture of the atlantooccipital membrane with a 28-gauge needle. The mouse was immediately decapitated and tissue samples from choroid plexuses (i.e., lateral and fourth ventricles), cerebral cortex, whole kidney, liver, eye, lung, heart, spleen, small and large intestines, skeletal muscle, testes and ovary were collected. Samples were weighed and then solubilized with 1 M hyamine hydroxide, as described by the manufacturer (ICN; Irvine, CA). After solubilization, the level of
radioactivity was determined in tissue homogenates after mixing with Ecolite (+) liquid scintillation cocktail (ICN; Irvine, CA).

Corrected tissue concentrations of cefadroxil ($C_{tiss, corr}$, nmol/g wet tissue) were calculated as (Keep et al., 1999; Ocheltree et al., 2005):

$$C_{tiss, corr} = C_{tiss} - DS \cdot C_b$$

where $C_{tiss}$ is the uncorrected cefadroxil tissue concentration (nmol/g), DS is the dextran space (i.e., blood vascular space) in the tissue (ml/g), and $C_b$ is the cefadroxil blood concentration (nmol/ml).

**Plasma Protein Binding of Cefadroxil**

The plasma unbound fraction of cefadroxil in wild-type and PEPT2 knockout mice was measured based on the ultrafiltration method reported by Ocheltree et al. (2005), with minor modification. Briefly, mouse plasma samples were spiked with cefadroxil to produce standard concentrations of 0.01, 0.1, 1 and 10 µM. A 1 µM cefadroxil plasma sample was also spiked with probenecid (0.1, 1 and 10 mM). After incubation at 37°C for 15 min, plasma samples (500 µl) were then loaded into Microcon YMT-30 devices and centrifuged at 1800 x g for 25 min at 37°C. Approximately 60% of the volume was recovered in the ultrafiltrate containing the unbound cefadroxil. The unbound fraction in plasma samples was calculated by dividing the ultrafiltrate cefadroxil concentration by that in the original plasma standard. Negligible binding of cefadroxil to the ultrafiltration device and lack of protein leakage during the ultrafiltration procedure were demonstrated by preliminary studies (data not shown).

**Data Analysis**

**A. Pharmacokinetics of Cefadroxil Plasma Concentrations**
Concentration-time curves, $C(t)$, were fit to a two-compartment model with a weighting factor of unity (WinNonlin version 5.0.1; Pharsight Inc.; Mountain View, CA) such that:

$$C(t) = C_1 \cdot e^{-\lambda_1 t} + C_2 \cdot e^{-\lambda_2 t}$$

Quality of the fit was judged by evaluating the standard error of parameter estimates, the coefficient of determination ($r^2$), and by the visual inspection of residual plots. Area under the plasma concentration-time curve (AUC; equivalent to $AUC_{0-\infty}$), total body clearance (CL), elimination rate constant from the central compartment ($K_{10}$), volume of the central compartment ($V_1$), volume of distribution steady-state ($V_{dss}$), terminal half-life ($t_{1/2}$), and mean residence time (MRT) were then determined by standard methods.

**B. Renal Clearance of Cefadroxil**

The renal clearance of cefadroxil ($CL_R$) and inulin (GFR) were calculated as:

$$CL_R = \frac{Ae_{cefadroxil}(0-120)}{AUC_{cefadroxil}(0-120)}$$

$$GFR = \frac{Ae_{inulin}(0-120)}{AUC_{inulin}(0-120)}$$

where $Ae_{cefadroxil}(0-120)$ or $Ae_{inulin}(0-120)$ is the cumulative amount of unchanged cefadroxil or inulin excreted in urine over 120 min. $AUC_{cefadroxil}(0-120)$ or $AUC_{inulin}(0-120)$ is the area under the cefadroxil or inulin plasma concentration-time curve from 0 to 120 min, as determined by trapezoidal rule.

Since cefadroxil undergoes glomerular filtration, tubular secretion (Bins and Mattie, 1988) and tubular reabsorption (Garcia-Carbonell et al., 1993) in kidney, and given the sequential expression of PEPT1 and PEPT2 in the renal proximal tubule (Smith et al., 1998; Shen et al., 1999), the drug’s renal clearance can be represented as:

$$CL_R = fu \cdot (GFR + CLs) \cdot (1 - Fr) = fu \cdot (GFR + CLs) \cdot (1 - Fr1) \cdot (1 - Fr2)$$
where $fu$ is the fraction of cefadroxil unbound in plasma, $CL_s$ is the secretion clearance, and $Fr$ is the fraction of available drug that is reabsorbed in which $Fr_1$ is the fraction of available drug reabsorbed by PEPT1 and $Fr_2$ is the fraction of available drug reabsorbed by PEPT2.

Since $Fr_2=0$ in PEPT2 null mice and $CL_s=0$ in the presence of saturating concentrations of probenecid, knowing the experimental values for renal clearance of cefadroxil, as well as $fu$ and GFR, will allow the direct calculation of $Fr_1$ in PEPT2$^{-/-}$ mice. $Fr_2$ can then be estimated directly in wild-type mice treated with cefadroxil plus probenecid ($CL_s=0$) since all other parameters will be known (i.e., $CLR$, $fu$, GFR and $Fr_1$).

The relative contribution of each transporter can be calculated as:

$$\%PEPT_1 = \left(\frac{Fr_1}{Fr}\right) \cdot 100 \quad \text{and} \quad \%PEPT_2 = \left[Fr_2 \cdot (1 - Fr_1)/Fr\right] \cdot 100$$

where $\%PEPT_1$ and $\%PEPT_2$ are the percentage of reabsorbed cefadroxil handled by PEPT1 and PEPT2, respectively.

The excretion ratio of cefadroxil (ER) was calculated as:

$$ER = \frac{CL_R}{fu \cdot GFR}$$

C. Statistical Analysis

Data are reported as mean ± SE. To test for statistically significant differences among multiple treatments for a given parameter, one-way analysis of variance (ANOVA) was performed. When the F ratio showed that there were significant differences among treatments, the Dunnett or Tukey method of multiple comparisons was used to determine which treatments differ. All statistical analyses were performed using Prism version 4.0 (GraphPad Software, Inc.; San Diego, CA).
Results

Metabolic Stability of Cefadroxil

Over 90% of cefadroxil radiolabel was recovered in the urine 24 hours after intravenous administration of the drug. No significant metabolism was found in that 96.8% and 95.0% of the urine sample was recovered as intact cefadroxil for wild-type and PEPT2 null mice, respectively. These results were consistent with the reported lack of metabolism for cefadroxil (Tanrisever and Santella, 1986; AHFS Drug Information, 2006). Therefore, cefadroxil instability was not a confounding issue in these studies and no further correction of the data was necessary.

Cefadroxil Dose-Dependent Pharmacokinetics

A preliminary pharmacokinetic analysis was performed in wild-type and PEPT2 null mice after administering intravenous doses of cefadroxil over a 1-100 nmol/g dose range (Table 1). Although clearance of cefadroxil increased steadily in PEPT2+/+ animals (1.5-fold) as the dose increased, the change was not statistically different. In addition, terminal half-life and volume of distribution steady-state values were relatively constant in these animals. Although the results suggest an “apparent” linearity in the clearance of cefadroxil in wild-type mice, it is more likely that statistical differences are not achieved because of a mitigating effect of renal tubular secretion (which would tend to reduce the clearance at higher doses) on renal tubular reabsorption (which would tend to increase the clearance at higher doses). In contrast, cefadroxil clearance decreased in PEPT2−/− animals (2-fold) as the dose increased from 1 to 100 nmol/g. The slower clearance at higher doses implies a capacity-limited elimination process and probably reflects that of renal tubular secretion. In PEPT2−/− mice, no significant differences were found in
the volume of distribution steady-state and terminal half-life of cefadroxil, although the latter term increased monotonically (1.5-fold) with higher dose.

In Figure 1 (A-D), plasma concentration-time curves of cefadroxil are shown for PEPT2+/+ and PEPT2-/- mice after intravenous administration of drug over the 100-fold dose range. When the 100 nmol/g dose was given, only minor differences were observed between genotypes in the plasma profiles of drug (Fig 1A). However, cefadroxil levels were substantially different between PEPT2+/+ and PEPT2-/- animals when the 1 nmol/g dose was administered (Fig 1D). These results suggest that the pharmacokinetics of cefadroxil are nonlinear and, in this case, probably reflects a change in renal tubular reabsorption.

Because the 1 nmol/g dose showed the largest difference in cefadroxil plasma concentrations between genotypes, this dose was studied in more detail (Table 2). As observed, the systemic exposure (i.e., AUC) of cefadroxil was 3-fold higher in wild-type mice, as compared to PEPT2 null mice, because its total plasma clearance was reduced by the same magnitude. PEPT2-/- animals exhibited a 3- to 4-fold increase in their central compartment elimination rate constant, as compared to PEPT2+/+. Significant differences were also observed between genotypes in drug half-life and mean residence time. In contrast, PEPT2 gene deletion did not influence the volume terms (i.e., V1 or Vdss) and initial cefadroxil plasma concentration.

**Effect of Probenecid and Quinine on Cefadroxil Pharmacokinetics**

Cefadroxil plasma concentration vs. time plots, in the absence and presence of probenecid (a classic inhibitor of organic anion transporters, OATs), are shown in Figure 2 (A and B). Administration of cefadroxil plus 70 mg/kg probenecid resulted in greater plasma concentrations of cefadroxil in both genotypes than after administration of cefadroxil alone. In contrast, quinine (a classic inhibitor of organic cation transporters, OCTs) had little effect on cefadroxil
disposition (Fig 2C). We also evaluated cefadroxil pharmacokinetics with coadministration of probenecid at a dose of 700 mg/kg. Cefadroxil plasma concentrations after coadministration with probenecid at the 70 and 700 mg/kg doses were not significantly different in either genotype, strongly suggesting that the renal secretion clearance of cefadroxil was completely abolished when probenecid was given at the lower probenecid dose.

**Renal Clearance of Cefadroxil**

As shown in Table 3 (and Figs 1D, 2A and 2B), PEPT2 deletion and probenecid coadministration caused profound changes in the pharmacokinetics and plasma concentration vs. time profiles of cefadroxil. In contrast, inulin plasma concentrations were similar in all studies, indicating that GFR was unchanged as a function of genotype or probenecid treatment (Fig 2D). Neither PEPT2 gene deletion nor treatment with probenecid had an effect on cefadroxil’s extent of protein binding, with about 80% of the drug being unbound in plasma. Approximately 55-60% of the administered dose of cefadroxil was excreted in the urine over 2 hr after an intravenous bolus dose of 1 nmol/g in both wild-type and PEPT2 null mice; the urinary recovery of cefadroxil was somewhat lower in probenecid treated mice (40-45%), which most likely reflects saturation of renal tubular secretion and the slower excretion of drug.

It was observed (Table 3) that cefadroxil renal clearance increased by 3-fold in PEPT2−/− versus PEPT2+/+ mice (i.e., 0.554 vs. 0.182 mL/min after cefadroxil alone and 0.215 vs. 0.063 mL/min after cefadroxil plus probenecid), but that cefadroxil renal clearance decreased by 3-fold in probenecid-treated versus untreated animals (i.e., 0.063 vs. 0.182 mL/min in PEPT2+/+ mice and 0.215 vs. 0.554 mL/min in PEPT2−/− mice). When cefadroxil renal clearance was normalized for fraction unbound and functional nephron mass (i.e., GFR), the excretion ratio in wild-type mice was 0.95, suggesting that neither renal secretion nor reabsorption of the drug had occurred.
(i.e., filtration was the sole mechanism of renal excretion). However, the excretion ratio of cefadroxil was substantially greater in PEPT2 null mice (i.e., ER of 2.68), indicating a net secretory mechanism for drug clearance. In the absence of renal tubular secretion (i.e., in the presence of probenecid), the excretion ratio of cefadroxil was significantly reduced for both genotypes. Thus, the excretion ratio fell from 0.95 to 0.30 in wild-type mice and from 2.68 to 0.97 in PEPT2 null animals (Fig 3). Renal clearance studies further demonstrated that the tubular reabsorption of cefadroxil was almost completely abolished in PEPT2 null mice compared to wild-type animals (i.e., 3% vs. 70%, respectively; p < 0.001). Out of the 70% of cefadroxil reabsorbed in wild-type mice, PEPT2 accounted for 95% and PEPT1 accounted for 5% of reabsorbed substrate (Table 3).

**Tissue Distribution of Cefadroxil at 120 Minutes**

Figure 4A shows the distribution of cefadroxil in selected tissues at 120 min post-administration. A comparison of PEPT2 null mice to wild-type controls clearly demonstrates that PEPT2 has a major effect on the tissue distribution of cefadroxil. In particular, mice with an intact PEPT2 gene had 21 times more cefadroxil in kidney than in PEPT2-deficient animals. Except for CSF, the concentration of cefadroxil in other tissues and blood was two to eight times greater in wild-type than in PEPT2 null mice. Because the blood concentration of cefadroxil was significantly different between genotypes, tissue concentrations of drug were also corrected by their corresponding values in blood (Fig 4B). The tissue-to-blood concentration ratio in kidney was significantly lower in PEPT2⁺⁻ mice than that in PEPT2⁺⁺ mice (about 3-fold), however, the concentration ratio in CSF was significantly higher in PEPT2 null animals (about 6- to 7-fold). No statistically significant differences were observed in the blood-corrected ratios of other tissues, including the choroid plexus and cerebral cortex. However, when drug concentrations in
these two brain tissues were normalized by CSF concentration (Fig 4C), PEPT2−/− mice had significantly lower ratios than those in PEPT2+/+ mice (12-fold and 3-fold in choroid plexus and cerebral cortex, respectively).
Discussion

The antibacterial activity of an antibiotic depends on its exposure at pharmacological target sites and, as such, it is important to characterize the molecular mechanism(s) that affect the pharmacokinetics and tissue distribution of cephalosporins. Cefadroxil has a significantly slower rate of excretion and a more persistent concentration in blood and tissue, compared to other cephalosporins, which is a pharmacokinetic advantage in terms of its antibacterial activity (Tanrisever and Santella, 1986). Nevertheless, the exact mechanism for the delivery and removal of cefadroxil from tissue has not yet been fully elucidated. In this study, we examined the in vivo function of the proton-coupled oligopeptide transporter PEPT2 by analyzing the disposition of cefadroxil in wild-type and PEPT2 knockout mice. Our data clearly show that PEPT2 had an overwhelmingly predominant role in the renal tubular reabsorption of cefadroxil. The decreased renal reabsorption in PEPT2 null mice resulted in substantially decreased levels of cefadroxil in plasma and tissues, indicating that this protein can reduce the systemic and tissue exposure of drugs that are PEPT2 substrates. PEPT2 also impacted the efflux of cefadroxil from CSF into choroid plexus, thereby having a major effect on drug exposure in brain. This finding suggests that PEPT2 may also impact the drug’s effectiveness in treating infections of the CNS.

Unexpectedly, we found little difference in cefadroxil plasma concentrations between wild-type and PEPT2 null mice at a dose of 100 nmol/g (Fig 1A). However, the plasma levels of cefadroxil were substantially different between wild-type and PEPT2 null mice at the 1 nmol/g dose (Fig 1D). The data suggest that the dose-dependency between genotypes was due to a change in the renal tubular reabsorption of cefadroxil in PEPT2-competent animals. The Km of cefadroxil is about 10–40 µM for PEPT2, depending upon the experimental system being studied.
Thus, as the dose of cefadroxil increased, along with drug concentrations in plasma (and presumably glomerular filtrate), PEPT2 became capacity-limited in its ability to transport drug back to the peritubular capillaries. This is particularly evident at the three higher doses of drug (i.e., 100, 50 and 12.5 nmol/g), in which the maximal concentrations of cefadroxil in plasma (i.e., 1,000, 300 and 100 µM, respectively) are significantly higher than its Km value. This type of saturation would not affect the renal tubular reabsorption of cefadroxil in PEPT2-deficient animals since the remaining PEPT1 transporter is of low-affinity (i.e., Km values in the mM range) and represents a minor pathway relative to that of PEPT2. A monotonic increase was also observed in the total plasma clearance of cefadroxil in wild-type mice at increasing dose levels of drug (Table 1), a finding consistent with saturable tubular reabsorption. This observation agrees with other pharmacokinetic studies where an increase in the plasma clearance of cefadroxil was observed following high doses of drug in both human and rat (Garrigues et al., 1991; Garcia-Carbonell et al., 1993).

Cefadroxil undergoes three mechanisms of renal excretion, namely glomerular filtration, renal tubular secretion and renal tubular reabsorption. The latter two processes could become saturated at higher plasma concentrations of drug. While cefadroxil and other β-lactam aminocephalosporin antibiotics are secreted by organic anion transporters located in the basolateral membrane of proximal tubule (i.e., OAT1 and OAT3 in mouse), the drugs are reabsorbed by brush border membrane POTs (i.e., PEPT1 and PEPT2) in proximal tubule (Terada et al., 1997; Takeda et al., 2002; Luckner and Brandsch, 2005; Shitara et al., 2005). In comparison to the relatively high affinity of cefadroxil for PEPT2 (i.e., Km=10-40 µM, as noted before), the affinity of cefadroxil for rodent OATs appears to be much lower, with IC50 and Ki
values in the mM range (Jung et al., 2002; Khamdang et al., 2003). Therefore, it appears likely that the PEPT2-mediated reabsorption of cefadroxil may exhibit more saturability than the OAT1/3-mediated secretion of drug at therapeutic plasma concentrations.

In the renal clearance experiments, PEPT2-mediated reabsorption of cefadroxil was knocked out by gene deletion while the drug’s tubular secretion was functionally knocked out by probenecid, a potent inhibitor of rodent OAT1 and OAT3 (Shirata et al., 2005). In contrast, quinine, a potent inhibitor of rodent OCT1 and OCT2 (Shirata et al., 2005), had no effect on the renal tubular secretion of cefadroxil indicating a lack of organic cation transport in the uptake process. As a result, only filtration and PEPT1-associated reabsorption of cefadroxil remained intact in the PEPT2−/− animals with probenecid present. The fact that cefadroxil’s excretion ratio was almost unity (i.e., ER 0.97) suggests that PEPT1 played a very minor role in the drug’s renal reabsorption. When these results are combined with the fractional reabsorption calculations (Table 3), it is clear that PEPT2 is the dominant peptide transporter in kidney. Out of the 70% of cefadroxil reabsorbed in PEPT2+/+ mice, 95% of this value was reabsorbed by PEPT2 and only 5% by PEPT1. In a previous study by our laboratory (Ocheltree et al., 2005), we reported that out of the 46% of GlySar reabsorbed in wild-type mice, PEPT2 accounted for 86% and PEPT1 accounted for 14% of reabsorbed substrate. There is little doubt that PEPT2 is much more important than PEPT1 in the reabsorption of both cefadroxil and GlySar. However, it is less obvious as to why the fractional reabsorption of GlySar (Fr=0.46) is lower than that of cefadroxil (Fr=0.70). One possible explanation is that cefadroxil was studied at plasma concentrations of ≤ 10 µM (this study) while GlySar was studied at plasma concentrations ≤ 500 µM (Ocheltree et al., 2005), concentrations at which GlySar may be experiencing capacity-limited renal tubular reabsorption. Regardless, it should be appreciated that cefadroxil plasma concentrations
produced at the 1 nmol/g dose (i.e., about 0.01-10 µM) in this study are clinically relevant since they are in the minimal inhibitory concentration range of most bacteria (Courtieu and Drugeon, 1983).

Strikingly higher concentrations of cefadroxil were observed in all the tissues of wild-type mice as compared to PEPT2-deficient animals, suggesting a dramatic effect of PEPT2 on the tissue distribution of drug (Fig 4A). The shift in concentration between genotypes could be due to differences in systemic drug concentrations or cellular uptake. To rule out differences being due to systemic exposure alone, the tissue concentrations of cefadroxil for each genotype were corrected by their corresponding blood concentrations. When this correction was made, only the kidney (3-fold reduction in null mice) and CSF (6- to 7-fold increase in null mice) had statistically significant differences in their tissue-to-blood concentration ratios between genotypes (Fig 4B). These findings substantiate our hypothesis that the renal reabsorption and CSF efflux of cefadroxil are mediated by PEPT2, a protein which is absent from the apical membranes of renal proximal tubular and choroid plexus epithelial cells in PEPT2−/− mice (Shen et al., 2003). Although the tissue-to-blood concentration ratios in choroid plexus and cerebral cortex were not significantly different between genotypes, the tissue-to-CSF concentration ratios were substantially lower in PEPT2−/− mice (i.e., 12-fold for choroid plexus and 3-fold for cerebral cortex; Fig 4C). These findings suggest that the CNS exposure of cefadroxil is largely controlled by PEPT2’s functional activity in brain, and, in particular, its role in effluxing drug from CSF into choroid plexus.

Recently, the PEPT2 gene was found to be polymorphically expressed in humans with single nucleotide polymorphisms (Terada et al., 2004; Pinsonneault et al., 2004). The GlySar transport activity of variant R57H was completely abolished (Terada et al., 2004). Genetic
deficiencies for the gene may have both positive and negative consequences for drug therapy. In the case of a PEPT2 deficiency, reduced uptake of drugs into the kidney may result in a decreased efficacy of drugs that have their therapeutic action in the kidney. On the other hand, reduced uptake of drugs into the kidney could be beneficial for drugs that have adverse effects in the kidney. Moreover, the loss of PEPT2 expression in choroid plexus may have significant influences on CSF and brain concentrations of peptides and peptide-like drugs. Based on our results, we expect that humans with a deficiency in PEPT2 will have impaired renal reabsorption of some drugs and that this may result in decreased systemic exposure. We also expect that genetic deficiencies may reduce the clearance of some drugs from CSF to blood. Therefore, it is important to test whether or not these transport differences will translate into pharmacological or toxicological phenotypes in our transgenic mouse colony. Moreover, it will be interesting to determine whether polymorphisms in the human PEPT2 genes also correlate with altered drug disposition and dynamics in patients. If our findings can indeed be extrapolated to humans, the PEPT2 knockout mouse model will provide a unique tool for predicting and explaining peptide/mimetic sensitivity and/or toxicity, which may ultimately help in the development of new peptide-based pharmaceuticals.
References


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Footnotes

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Legends for figures

Fig. 1. Plasma concentration-time profiles of cefadroxil after intravenous bolus administration of drug at 100 nmol/g (A), 50 nmol/g (B), 12.5 nmol/g (C) and 1 nmol/g (D). Data are expressed as mean ± SE (n=5-7).

Fig. 2. Plasma concentration-time profiles of cefadroxil (CEF) after intravenous bolus administration of drug at 1 nmol/g in the absence and presence of probenecid (PRO; 70 or 700 mg/kg) in PEPT2+/+ (A) and PEPT2−/− (B) mice, or in the absence and presence of quinine (QUI; 700 mg/kg) in both genotypes (C). Data are expressed as mean ± SE (n=4-7). Plasma concentration-time profiles of inulin after intravenous administration (30.8 mg/kg) in the absence and presence of probenecid (PRO; 70 mg/kg) in PEPT2+/+ and PEPT2−/− mice (D). Data are expressed as mean ± SE (n=5-7).

Fig. 3. Excretion ratio of cefadroxil after intravenous administration of 1 nmol/g drug in the absence and presence of probenecid (70 mg/kg) in PEPT2+/+ and PEPT2−/− mice. Data are expressed as mean ± SE (n=5-7). The dashed line (i.e., excretion ratio of unity) represents the scenario of drug filtration alone or drug filtration with equivalent contributions of both tubular secretion and reabsorption.

Fig. 4. Tissue concentrations (A), tissue-to-blood concentration ratios (B) and CNS tissue-to-CSF concentration ratios (C) of cefadroxil in PEPT2+/+ and PEPT2−/− mice, observed 120 min after intravenous bolus administration of drug at 1 nmol/g. Data are expressed as mean ± SE (n=6-12). *p < 0.05, **p < 0.01, and ***p < 0.001 compared with wild-type mice.
### TABLE 1

**Pharmacokinetics of cefadroxil after a range of intravenous bolus doses of drug**

Values represent the mean ± SE (n=4-7). Statistics were conducted by ANOVA followed by Dunnett’s test.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Parameter</th>
<th>Dose (nmol/g)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>12.5</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PEPT2+/+</td>
<td>CL (mL/min)</td>
<td>0.302 ± 0.026</td>
<td>0.377 ± 0.099</td>
<td>0.393 ± 0.023</td>
<td>0.461 ± 0.057</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t₁/₂ (min)</td>
<td>30.4 ± 3.2</td>
<td>26.0 ± 9.1</td>
<td>25.9 ± 8.7</td>
<td>26.6 ± 5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vdₚₛ (mL)</td>
<td>11.9 ± 1.9</td>
<td>13.7 ± 2.6</td>
<td>12.5 ± 2.6</td>
<td>10.8 ± 5.2</td>
<td></td>
</tr>
<tr>
<td>PEPT2⁻/⁻</td>
<td>CL (mL/min)</td>
<td>0.921 ± 0.098</td>
<td>0.643 ± 0.148</td>
<td>0.509 ± 0.070</td>
<td>0.480 ± 0.096</td>
<td></td>
</tr>
<tr>
<td></td>
<td>t₁/₂ (min)</td>
<td>19.5 ± 1.5</td>
<td>24.8 ± 3.4</td>
<td>26.6 ± 6.9</td>
<td>29.2 ± 4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vdₚₛ (mL)</td>
<td>15.7 ± 1.7</td>
<td>12.7 ± 3.3</td>
<td>14.0 ± 3.6</td>
<td>13.2 ± 4.5</td>
<td></td>
</tr>
</tbody>
</table>

\[a\text{ p < 0.05 compared to the control group at 1 nmol/g of cefadroxil.}\]
**TABLE 2**

*Pharmacokinetics of cefadroxil after a 1 nmol/g intravenous bolus dose of drug*

Values represent the mean ± SE (n=7). Statistics were conducted using the two-sided unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PEPT2&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>PEPT2&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (min•µmol/L)</td>
<td>67.9 ± 11.7</td>
<td>21.9 ± 4.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CL (mL/min)</td>
<td>0.30 ± 0.03</td>
<td>0.92 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V&lt;sub&gt;1&lt;/sub&gt; (mL)</td>
<td>3.3 ± 0.2</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>K&lt;sub&gt;10&lt;/sub&gt; (min&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>0.09 ± 0.01</td>
<td>0.33 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>6.3 ± 1.0</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>30.4 ± 3.2</td>
<td>19.5 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>31.4 ± 1.5</td>
<td>19.8 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (mL)</td>
<td>11.9 ± 1.9</td>
<td>15.2 ± 1.7</td>
</tr>
<tr>
<td>r&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.999 ± 0.001</td>
<td>0.998 ± 0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.01, <sup>b</sup> p < 0.001 compared with PEPT2<sup>+/+</sup> (wild-type) mice.
TABLE 3

Renal pharmacokinetics of cefadroxil after a 1 nmol/g intravenous bolus dose of drug

Values represent the mean ± SE (n=5-7). Statistics were conducted by ANOVA followed by Tukey’s test. For a given parameter, means with the same superscript letter are not significantly different (α = 0.05).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PEPT2+/+</th>
<th>PEPT2-/-</th>
<th>PEPT2+/+ + probenecid</th>
<th>PEPT2-/- + probenecid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLR (mL/min)</td>
<td>0.182 ± 0.002 A</td>
<td>0.554 ± 0.016 B</td>
<td>0.063 ± 0.004 C</td>
<td>0.215 ± 0.008 B</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>0.235 ± 0.004 A</td>
<td>0.258 ± 0.007 A, B</td>
<td>0.249 ± 0.024 A, C</td>
<td>0.278 ± 0.012 B, C</td>
</tr>
<tr>
<td>fu</td>
<td>0.82 ± 0.01 A</td>
<td>0.80 ± 0.01 A</td>
<td>0.80 ± 0.02 A</td>
<td>0.79 ± 0.01 A</td>
</tr>
<tr>
<td>fe₁₂₀</td>
<td>0.55 ± 0.02 A</td>
<td>0.60 ± 0.02 B</td>
<td>0.41 ± 0.01 C</td>
<td>0.43 ± 0.01 C</td>
</tr>
<tr>
<td>ER</td>
<td>0.95 ± 0.02 A</td>
<td>2.68 ± 0.10 B</td>
<td>0.30 ± 0.03 C</td>
<td>0.97 ± 0.03 A</td>
</tr>
<tr>
<td>Fr</td>
<td>0.70 A</td>
<td>0.03 B</td>
<td>0.70 ± 0.03 A</td>
<td>0.03 ± 0.03 B</td>
</tr>
<tr>
<td>Fr₁</td>
<td>0.03 A</td>
<td>0.03 A</td>
<td>0.03 A</td>
<td>0.03 ± 0.03 A</td>
</tr>
<tr>
<td>Fr₂</td>
<td>0.69 A</td>
<td>0.00 B</td>
<td>0.69 ± 0.03 A</td>
<td>0 B</td>
</tr>
<tr>
<td>%PEPT1</td>
<td>4.6 A</td>
<td>100 B</td>
<td>4.6 ± 0.2 A</td>
<td>100 B</td>
</tr>
<tr>
<td>%PEPT2</td>
<td>95.4 A</td>
<td>0 B</td>
<td>95.4 ± 0.2 A</td>
<td>0 B</td>
</tr>
</tbody>
</table>
Fig. 1A
Fig. 1B

(B) 50 nmol/g

Cefadroxil Plasma Concentration (μM)

Time (min)

PEPT2^{+/+}
PEPT2^{-/-}
(C) 12.5 nmol/g

Fig. 1C
Fig. 1D

Cefadroxil Plasma Concentration (µM)

Time (min)

(D) 1 nmol/g

PEPT2^{+/+} [closed circles]
PEPT2^{-/-} [open circles]
Fig. 2A
(B) Null: CEF ± PRO

- ○ CEF alone
- ● CEF + PRO (70 mg/kg)
- □ CEF + PRO (700 mg/kg)

Time (min)

Cefadroxil Plasma Concentration (µM)
Fig. 2C
Fig. 3
Fig. 4A
Fig. 4C