

In Silico Prediction of the Absorption and Disposition of Cefadroxil in Humans using an Intestinal Permeability Method Scaled from Humanized *PepT1* Mice[§]

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ABSTRACT

It is difficult to predict the pharmacokinetics and plasma concentration-time profiles of new chemical entities in humans based on animal data. Some pharmacokinetic parameters, such as clearance and volume of distribution, can be scaled allometrically from rodents, mammals, and nonhuman primates with good success. However, it is far more challenging to predict the oral pharmacokinetics of experimental drug candidates. In the present study, we used *in situ* estimates of intestinal permeability, obtained *in silico* and from rat, wild-type (WT), and humanized *PepT1* (*huPepT1*) mice, to predict the systemic exposure of cefadroxil, an orally administered model compound, under a variety of conditions. Using the GastroPlus simulation software program (Simulations Plus, Lancaster, CA), we found that the C_{max} and area under the plasma concentration-time curve from time zero to the last measurable concentration

of cefadroxil were better predicted using intestinal permeability estimates (both segmental and jejunal) from *huPepT1* than from WT mice, and that intestinal permeabilities based on *in silico* and rat estimates gave worse predictions. We also observed that accurate predictions were possible for cefadroxil during oral dose escalation (i.e., 5, 15, and 30 mg/kg cefadroxil), a drug-drug interaction study (i.e., 5 mg/kg oral cefadroxil plus 45 mg/kg oral cephalixin), and an oral multiple dose study [i.e., 500 mg (6.7 mg/kg) cefadroxil every 6 hours]. Finally, the greatest amount of cefadroxil was absorbed in duodenal and jejunal segments of the small intestine after a 5 mg/kg oral dose. Thus, by combining a humanized mouse model and *in silico* software, the present study offers a novel strategy for better translating preclinical pharmacokinetic data to oral drug exposure during first-in-human studies.

Introduction

The translation of animal pharmacokinetics and plasma concentration-time profiles to humans is critical for the safe and effective development of new chemical entities. Allometric scaling is a valuable approach in predicting, from preclinical studies, primary pharmacokinetic parameters of candidate drugs in humans such as clearance (CL) and the volume of distribution (V_d) (Tang and Mayersohn, 2006). However, other pharmacokinetic parameters, such as the absorption rate constant (K_a) and bioavailability (F) and related effects on the maximum plasma concentration (C_{max}) and systemic exposure [area under the curve (AUC)] of candidate drugs, are more unpredictable. This unpredictability is due, in part, to species differences in intestinal physiology, along with differences among species in the quantity and quality of intestinal transporters and/or enzymes that impact systemic availability.

Oral drug absorption is a complex process, and as a result its predictive modeling and simulation continue to be a challenge in humans. Thus, several mechanistic approaches have emerged to better predict oral absorption and bioavailability (Huang et al., 2009), including quasiequilibrium models, steady-state models, and dynamic models, differing largely by their dependence on spatial and temporal variables. Dynamic

models, developed and extended from the mid-1990s, include the compartmental absorption and transit model (Yu et al., 1996), Grass model (Grass, 1997), gastrointestinal transit absorption model (Sawamoto et al., 1997), advanced compartmental absorption and transit (ACAT) model (Yu and Amidon, 1999), and advanced dissolution, absorption, and metabolism model (Jamei et al., 2009). All of these models treat the gastrointestinal tract as a series of linked sequential compartments in which drug absorption occurs from each compartment as a function of time.

The ACAT model, as implemented in the Gastroplus software program (Simulations Plus, Lancaster, CA), takes into account physicochemical factors (e.g., pK_a , solubility, and permeability), physiologic factors (e.g., gastric emptying, intestinal transit, and presystemic metabolism and transport), and formulation factors (e.g., dosage form and dose) in predicting oral drug absorption. In doing so, Gastroplus was successful in predicting the oral absorption profiles of several drugs in which transporters and/or enzymes were involved (Tubic et al., 2006; Bolger et al., 2009; Abuasal et al., 2012), as well as in predicting food effects (Henze et al., 2018), formulation effects (Cvijic et al., 2018), and drug-drug interactions (Pedersen et al., 2017; Chung and Kesisoglou, 2018). However, even though Gastroplus has a function for the optimization of select parameters, accuracy in predicting plasma concentration-time profiles of a drug is still limited by the quality of data that are parameterized into the program (as are other programs). In particular, one must rely on the fidelity of *in silico* estimates for some parameters such as intestinal permeability, or obtain these estimates experimentally from *in vitro*

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ABBREVIATIONS: ACAT, advanced compartmental absorption and transit; AUC, area under the curve; AUC_{0-t} , area under the plasma concentration-time curve from time zero to the last measurable concentration; BW, body weight; CL, clearance; CEP, cephalixin; *huPepT1*, humanized *PepT1*; P_{eff} , effective permeability; V_d , volume of distribution; WT, wild-type.

TABLE 1
Predicted physicochemical properties of cefadroxil

| Property | Cefadroxil | Source |
|------------------------------|---|-----------------------------|
| Molecular formula | C ₁₆ H ₁₇ N ₃ O ₅ S | |
| Molecular weight | 363.39 | |
| Predicted log P (neutral) | -2.08 | ADMET Predictor version 8.5 |
| pK _a ¹ | 2.55 | Shalaeva et al. (2008) |
| pK _a ² | 7.21 | Shalaeva et al. (2008) |
| pK _a ³ | 9.71 | Shalaeva et al. (2008) |
| Aqueous solubility | 2.68 mg/ml (pH 5.13) | ADMET Predictor version 8.5 |
| Diffusion coefficient | 0.72 × 10 ⁻⁵ cm ² /s | ADMET Predictor version 8.5 |
| Mean precipitation time | 900 s | GastroPlus Default |
| Drug particle density | 1.2 g/ml | GastroPlus Default |
| Particle size (radius) | 25.0 μm | GastroPlus Default |
| Dosage form (human) | Solution | |
| Dose volume (human) | 250 ml | |

Caco-2 cells, parallel artificial membrane permeability assays, or in situ intestinal perfusions of mice or rats. The ability of intestinal permeability to predict oral bioavailability is made even more difficult by differences between species, regional differences along the length of the small and large intestines, and the presence and potential saturability of enzymes and transporters (Cao et al., 2006).

Membrane transporters have demonstrated an essential role in the absorption, distribution, metabolism, and excretion of many drugs, although sometimes they are accompanied by species differences in functional activity and specificity (Hu et al., 2012; Chu et al., 2013; Hu and Smith, 2016). To overcome these species differences, humanized mouse models were developed in which the human genomic DNA was introduced into mice lacking the target gene, thereby avoiding overlapping functional activities between the endogenous murine gene and the human transgene (Cheung and Gonzalez, 2008; Hu et al., 2014). For example, previous oral dose escalation studies by our group (Hu and Smith, 2016) demonstrated a linear AUC (or C_{max}) relationship with cefadroxil dose in wild-type (WT) mice. However, in humanized *PepT1* (*huPepT1*) mice as well as in human subjects (Garrigues et al., 1991), a nonlinear relationship was observed between the AUC (or C_{max}) value and cefadroxil dose. In situ jejunal perfusions indicated that this species difference was due to the greater affinity (i.e., lower K_m) of cefadroxil for human *PepT1* compared with mouse *PepT1*, such that saturable intestinal absorption occurred in *huPepT1* (and human subjects) but not WT mice. Humanized mouse models have also been generated to overcome species differences in drug metabolism, disposition, and regulation (Miksys et al., 2005; Ma et al., 2007; Cheung and Gonzalez, 2008; Patterson et al., 2008; Liu et al., 2015).

In the present study, we hypothesized that the in situ intestinal permeability of cefadroxil obtained from *huPepT1* mice compared

with in silico or rat values would better predict the in vivo plasma concentration-time profiles of cefadroxil in humans. This approach was successfully applied to the in vivo performance of cefadroxil in humans after oral dose escalation, an oral drug-drug interaction study, and multiple oral dosing.

Materials and Methods

Physicochemical Properties of Cefadroxil. Details on the physicochemical properties of cefadroxil, including dose and dosage form information, are given in Table 1. The values provided are based on literature information, default values provided in GastroPlus version 9.5 (Simulations Plus), and ADMET Predictor version 8.5 (Simulations Plus).

In Vivo Pharmacokinetics of Intravenous Cefadroxil. The plasma concentration-time profiles of cefadroxil in mice, following an 11 nmol/g (4 mg/kg) intravenous dose, were reported previously (Hu and Smith, 2016) and fit to a two-compartment body model using Phoenix WinNonlin version 8.0 (Certara USA, Inc., Princeton, NJ) and a weighing scheme of 1/y². Other models (e.g., one- and three-compartment body models) were tested but were found less suitable as judged by Akaike's information criterion. The goodness of fit was evaluated by *r*², the S.E. of parameter estimates, and visual inspection of the residual plots. The total clearance and central volume of distribution values were then adjusted for humans using an allometric scaling approach (as described subsequently).

Intestinal Permeability of Cefadroxil. The effective permeability (*P*_{eff}) of cefadroxil in human intestine was unknown, and as a result four different methods were used to estimate this parameter. They included values based on: 1) in silico human *P*_{eff} of 0.35 × 10⁻⁴ cm/s (ADMET Predictor); 2) rat *P*_{eff} of 0.75 × 10⁻⁴ cm/s (Caldwell et al., 2004); 3) WT mouse *P*_{eff}; and 4) *huPepT1* mouse *P*_{eff} (Hu and Smith, 2016). The rodent values were then adjusted for humans using an allometric scaling approach (as described subsequently).

Allometric Scaling. The predicted values of cefadroxil CL and V_d were estimated in human subjects by eq. 1 (Santella and Hennessy, 1982;

TABLE 2
Observed and allometric scaling of primary pharmacokinetic parameters of cefadroxil

Plasma concentration-time profiles were fit to a two-compartment disposition model in mice after an 11 nmol/g (4 mg/kg) intravenous bolus dose of cefadroxil (Hu and Smith, 2016). For the total plasma clearance, K10 = CL/V1. For the clearance between the central and peripheral compartments, CLD = CL12, K12 = CL12/V1, and K21 = CL21/V2.

| Parameter | Mouse (Observed) | CV | Mouse (Optimized) | Error | Human (Predicted) |
|------------------------|------------------|------|-------------------|-------|-------------------|
| | | % | | % | |
| CL (l/h per kilogram) | 0.92 | 10.5 | 0.88 | -4.3 | 0.079 |
| CLD (l/h per kilogram) | 0.38 | 29.8 | | | 0.041 |
| V1 (l/kg) | 0.18 | 20.6 | 0.16 | -15.5 | 0.063 |
| V2 (l/kg) | 0.31 | 42.9 | | | 0.122 |

CLD, clearance between the central and peripheral compartments; V1, volume of distribution in the central compartment; V2, volume of distribution in the peripheral compartment.

TABLE 3

Allometric scaling of cefadroxil P_{eff} to humans when estimated by the in situ permeability of small and large intestines from WT and humanized PepT1 mice

Intestinal segments include duodenum, jejunum, ileum, and ascending colon. The P_{eff} values of cefadroxil in humans were predicted after single oral doses of 5, 15, and 30 mg/kg, after 500 mg (6.7 mg/kg) oral doses every 6 hours, and after the drug-drug interaction of 5 mg/kg cefadroxil plus 45 mg/kg cephalixin. The intestinal permeability (P_{eff}) is in units of centimeter/second ($\times 10^{-4}$). The method for obtaining these values is described in the Supplemental Material.

| Segment | 5 mg/kg Dose | | 15 mg/kg Dose | | 30 mg/kg Dose | | 500 mg (6.7 mg/kg) Dose | | CEF + CEP Dose (hu) |
|-----------|--------------|------|---------------|------|---------------|------|-------------------------|------|---------------------|
| | WT | hu | WT | hu | WT | hu | WT | hu | |
| Stomach | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Duo | 3.95 | 1.69 | 2.19 | 0.96 | 1.40 | 0.58 | 3.46 | 1.49 | 0.35 |
| Jej 1 | 4.48 | 1.24 | 2.49 | 0.70 | 1.58 | 0.42 | 3.93 | 1.09 | 0.26 |
| Jej 2 | 4.32 | 1.19 | 2.40 | 0.68 | 1.53 | 0.41 | 3.78 | 1.05 | 0.25 |
| Ile 1 | 1.95 | 0.80 | 1.08 | 0.45 | 0.69 | 0.27 | 1.71 | 0.71 | 0.17 |
| Ile 2 | 1.86 | 0.76 | 1.03 | 0.43 | 0.66 | 0.26 | 1.63 | 0.67 | 0.16 |
| Ile 3 | 1.73 | 0.71 | 0.96 | 0.40 | 0.61 | 0.24 | 1.52 | 0.63 | 0.15 |
| Cecum | 0.01 | 0.20 | 0.01 | 0.11 | 0.01 | 0.07 | 0.01 | 0.17 | 0.04 |
| Asc colon | 0.02 | 0.24 | 0.01 | 0.13 | 0.01 | 0.08 | 0.02 | 0.21 | 0.05 |

Asc, ascending; CEF, cefadroxil; CEP, cephalixin; Duo, duodenum; hu, humanized; Ile, ileum; Jej, jejunum.

Ito and Houston, 2005; Hosea et al., 2009) and eq. 2 (Huh et al., 2011; Sanoh et al., 2015):

$$CL_{\text{human}} = CL_{\text{rodent}} \cdot (BW_{\text{human}}/BW_{\text{rodent}})^{0.72} \quad (1)$$

$$V_{d,\text{human}} = V_{d,\text{rodent}} \cdot (BW_{\text{human}}/BW_{\text{rodent}})^{0.89} \quad (2)$$

where BW is the body weight of human (70 kg) and rodent (0.25 kg for rat and 0.02 kg for mouse). The final estimates for CL and V_d are listed in Table 2.

Given that the absorption rate constant (K_a) = $2 \times P_{\text{eff}}/R$ (Yu et al., 1996; Yang and Smith, 2017) and assuming that the absorption rate constant was the same between human subjects and *huPepT1* mice (i.e., $K_{a,\text{human}} = K_{a,\text{huPepT1}}$), the predicted P_{eff} in human subjects ($P_{\text{eff},\text{human}}$) was estimated as

$$P_{\text{eff},\text{human}} = P_{\text{eff},\text{huPepT1}} \cdot (R_{\text{human}}/R_{\text{huPepT1}}) \quad (3)$$

where R is the intestinal radius. Here, the jejunal P_{eff} of cefadroxil was obtained using the method presented in the Supplemental Material (see Supplemental Fig. 1; Supplemental Table 1), with the P_{eff} values in other regions of the mouse intestines estimated accordingly (see Supplemental Table 2). The results were then scaled allometrically to human subjects (Table 3).

Finally, in studies in which cefadroxil was coadministered with cephalixin (CEP), a *PepT1* inhibitor, the predicted P_{eff} in human subjects [$P_{\text{eff},\text{human}} + \text{CEP}$] was estimated as

$$P_{\text{eff},\text{human}+\text{CEP}} = P_{\text{eff},\text{huPepT1}+\text{CEP}} \cdot (R_{\text{human}}/R_{\text{huPepT1}}) \quad (4)$$

Here, the P_{eff} value of cefadroxil was obtained during in situ jejunal perfusions of *huPepT1* mice when coperfused with 10 mM cephalixin (Hu and Smith, 2016). The P_{eff} values in other regions of the mouse intestines were then estimated accordingly (see Supplemental Material and Supplemental Table 2), and scaled allometrically to human subjects (Table 3). A flowchart outlining our overall approach is shown in Fig. 1.

Parameter Sensitivity Analysis. Accurate input parameters are crucial for obtaining meaningful predictions of the oral performance of cefadroxil using the ACAT model. Therefore, several physiologic (i.e., intestinal transit time, length, radius, pH, permeability, and fluid volume) and pharmacokinetic (i.e., clearances and volumes of distribution) properties of cefadroxil were examined to determine which parameters, if any, might most influence the *in silico* predictions. Specifically, the effect of parameter sensitivity on the maximum plasma concentration (C_{max}) and area under the plasma concentration-time curve from time zero to the last measurable concentration ($\text{AUC}_{0-\infty}$) was determined for cefadroxil at a human dose of 5 mg/kg. Test factors used in the parameter sensitivity analysis were scaled by 10-fold in each direction.

In Silico Predictions of Oral Cefadroxil Performance. All simulations for the plasma concentration-time profiles of cefadroxil were performed using the GastroPlus version 9.5 software program. The ACAT model conditions included

“human-physical-fasted” and “Opt logD Model SA/V 6.1.” Input parameters produced by ADMET Predictor version 8.5 remained unchanged except for that of allometric scaling. Cefadroxil was administered as immediate release solution in 250 ml of water, regardless of dose. The oral plasma concentration-time data in humans were obtained from the literature after dose escalation of cefadroxil

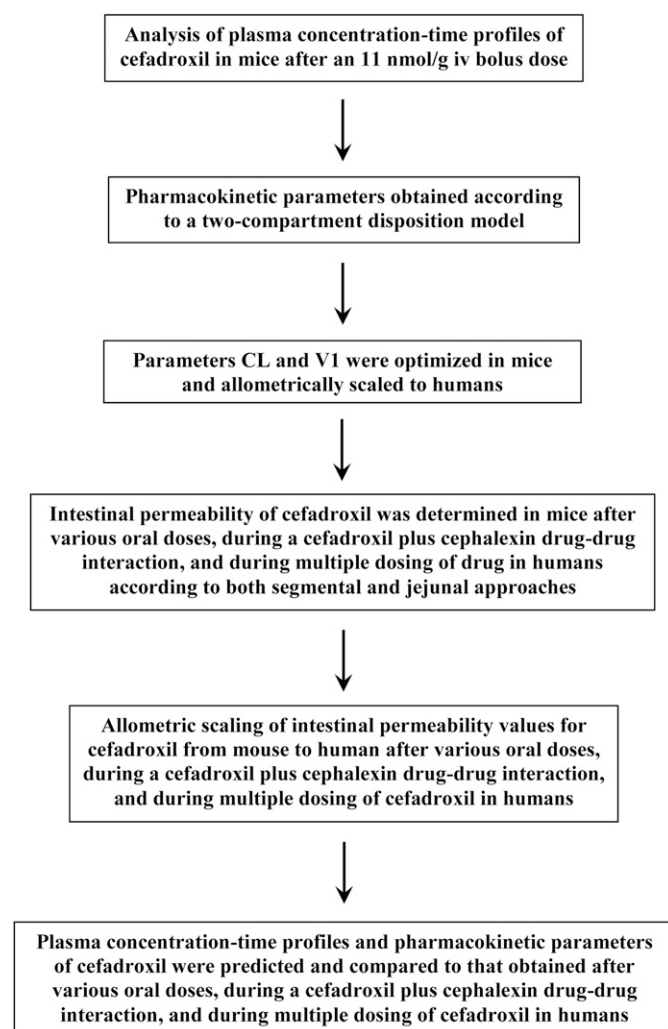


Fig. 1. Schematic strategy of the simulations.

(5, 15, and 30 mg/kg) and during a drug-drug interaction study of 5 mg/kg cefadroxil + 45 mg/kg cephalixin (Garrigues et al., 1991), and from an oral multiple dose study of 500 mg (6.7 mg/kg) cefadroxil every 6 hours (Santella and Henness, 1982). Population estimates (i.e., mean, 90% confidence interval, and 95% probability) were also obtained from 25 bootstrap analyses and the predicted values compared with observed values in humans for C_{max} and AUC_{0-t} .

Results

Parameter Sensitivity Analysis. As shown in Fig. 2A, the C_{max} value of cefadroxil was most sensitive to changes in CL, central compartment volume of distribution, and P_{eff} , followed by modest changes caused by small intestinal radius and little to no change by the other physiologic

parameters. Figure 2B shows that cefadroxil AUC_{0-t} is most sensitive to changes in CL, with modest changes caused by small intestinal radius, transit time, and P_{eff} . Collectively, it appeared that intestinal permeability had the greatest effect on cefadroxil, in which intestinal permeability was positively correlated with drug exposure, resulting in 3.5- and 3.0-fold changes in C_{max} and AUC_{0-t} , respectively, over a 100-fold range of P_{eff} values.

Effect of Species-Dependent Intestinal Permeability on Predicting the Systemic Oral Exposure of Cefadroxil in Human Subjects.

The predicted plasma concentration-time profiles of cefadroxil in human subjects were generated using *in silico* estimates of intestinal P_{eff} , rat P_{eff} (jejunal), WT mouse P_{eff} (jejunal vs. segmental), and *huPepT1* mouse P_{eff} (jejunal vs. segmental) at an oral cefadroxil dose of 5 mg/kg.

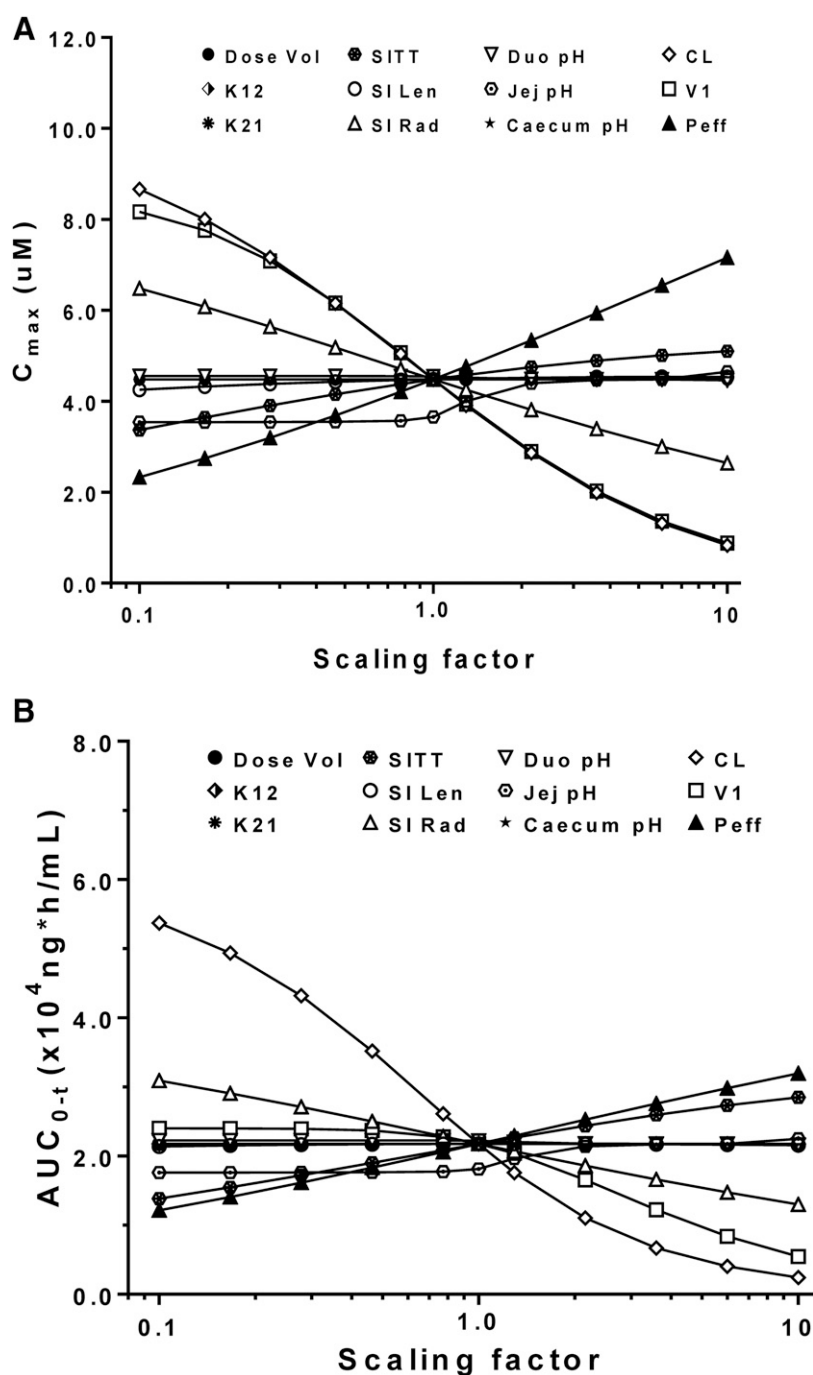


Fig. 2. Sensitivity of predicted C_{max} (A) and AUC_{0-t} (B) to input parameters after a human oral dose of 5 mg/kg cefadroxil. Parameters were changed by multiplying the initial input values by the scaling factors in the range of 0.1–10. K12 and K21 are the distribution rate constants between the central and peripheral compartments, respectively, and V1 is the volume of distribution for the central compartment. Duo, duodenum; Jej, jejunum; Len, length; Rad, radius; SI, small intestine; SITT, small intestine transit time; Vol, volume.

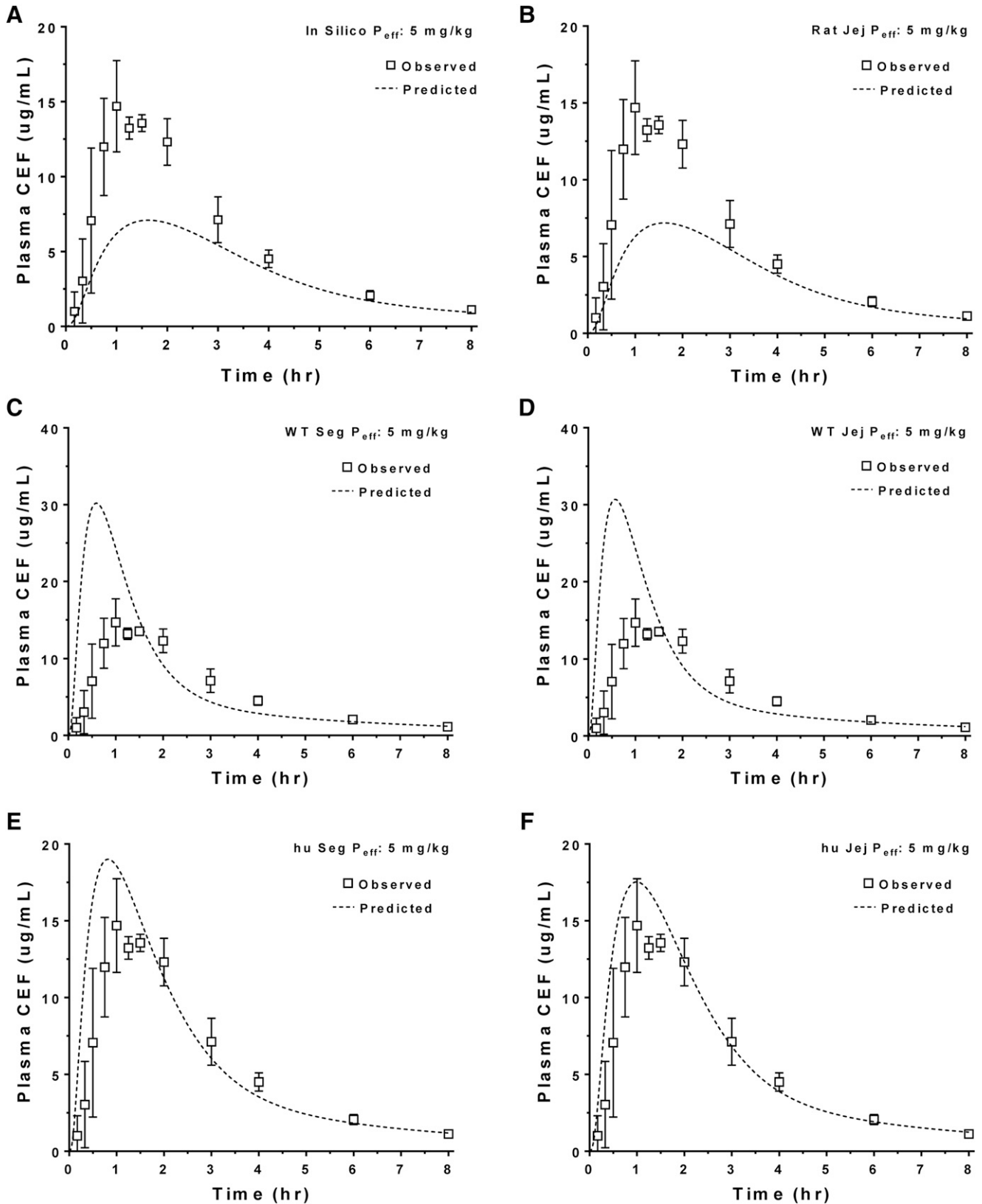


Fig. 3. Model predicted plasma concentration-time profiles of cefadroxil (CEF) after a human oral dose of 5 mg/kg using permeability estimates (P_{eff}) obtained in silico (A), and from rats (B) and WT (C and D) and humanized (E and F) mice. Both segmental (Seg) and jejunal (Jej) approaches were applied in mice. Human data were obtained from the literature (Garrigues et al., 1991); hu, humanized *PepT1* mice.

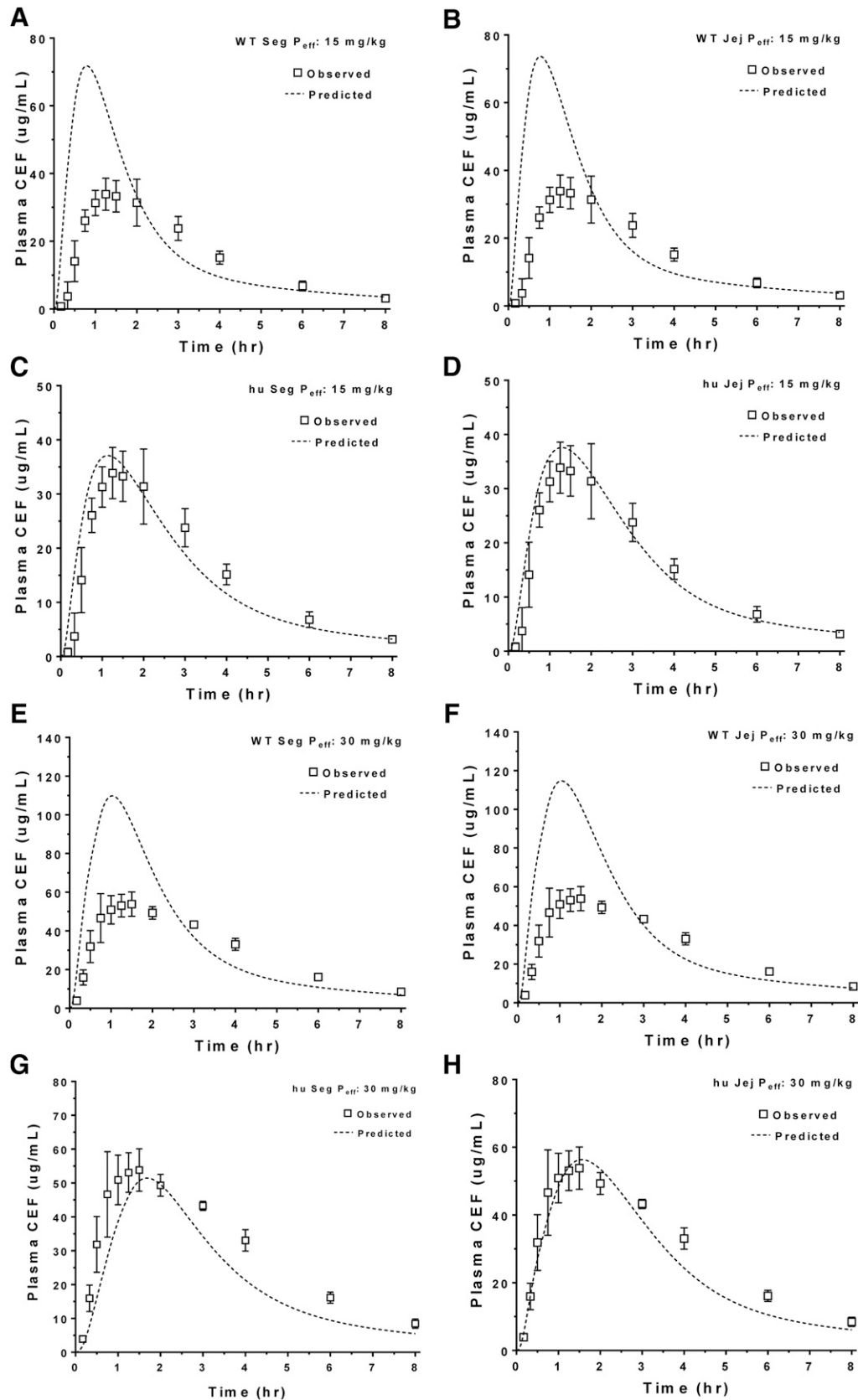


Fig. 4. Model predicted plasma concentration-time profiles of cefadroxil (CEF) after human oral doses of 15 and 30 mg/kg using permeability estimates (P_{eff}) obtained from WT (A, B, E, and F) and humanized (C, D, G, and H) mice. Both segmental (Seg) and jejunal (Jej) approaches were applied. Human data were obtained from the literature (Garrigues et al., 1991); hu, humanized *PepT1* mice.

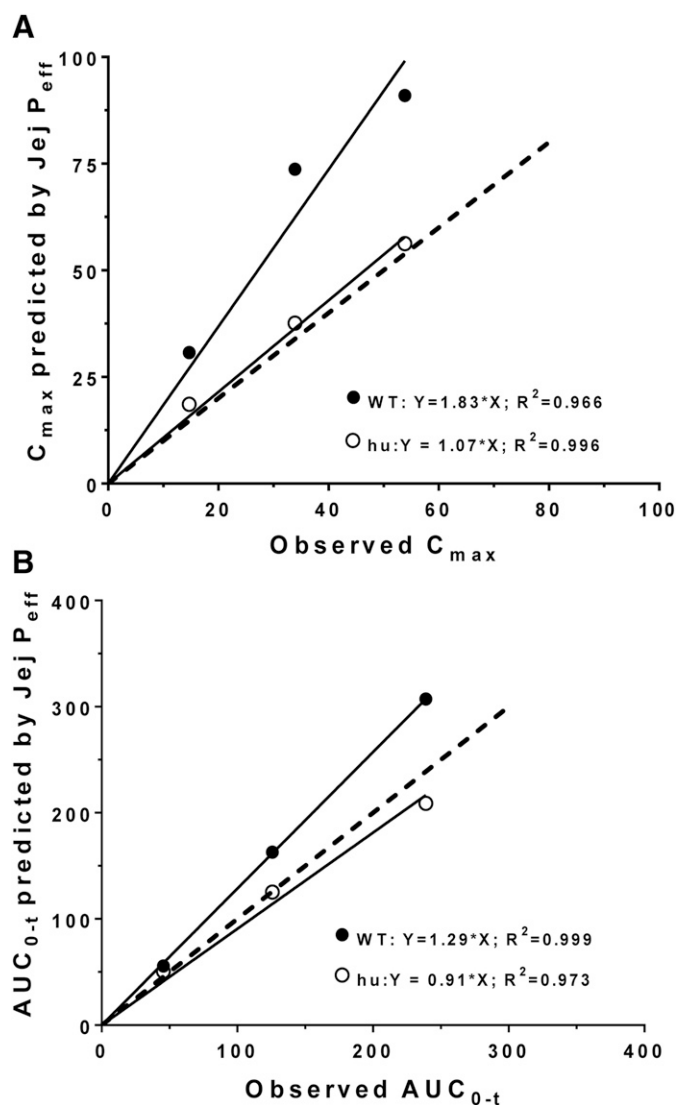


Fig. 5. Correlation between the segmental (Seg P_{eff}) and jejunal permeability (Jej P_{eff}) estimates in predicting the C_{max} (A) and AUC_{0-t} (B) values of cefadroxil after human oral doses of 5, 15, and 30 mg/kg. The dashed line represents a slope of unity; hu, humanized *PepT1* mice. C_{max} has units of $\mu\text{g}/\text{ml}$ and AUC_{0-t} has units of $\mu\text{g}\cdot\text{h}/\text{ml}$. Human data were obtained from the literature (Garrigues et al., 1991).

species in drug capacity (V_{max}) and affinity (K_m) of related transporters (Hu et al., 2012; Song et al., 2017).

Humanized mouse models have been developed in an attempt to improve the predictability of pharmacokinetics, metabolic contributions, drug toxicity, and receptor response when translating results from animals to human subjects (Kato et al., 2004; Gonzalez and Yu, 2006; Scheer and Roland Wolf, 2013; Scheer and Wilson, 2016). In particular, our laboratory generated *huPepT1* mice (Hu et al., 2014) and demonstrated that the correlation between systemic exposure (or C_{max}) of cefadroxil with oral dose escalation in humans was more similar to that of *huPepT1* mice compared with WT animals (Hu and Smith, 2016). This current study extended these results and addressed the ability of *huPepT1* mouse intestinal permeability to predict the oral dose nonlinear pharmacokinetics of cefadroxil in humans without the need for using transport parameters (i.e., V_{max} and K_m) scaled for humans. In doing so, we made the following major observations: 1) the C_{max} and AUC_{0-t} values of cefadroxil were better predicted using intestinal permeability estimates (both segmental and jejunal) from *huPepT1* than from WT

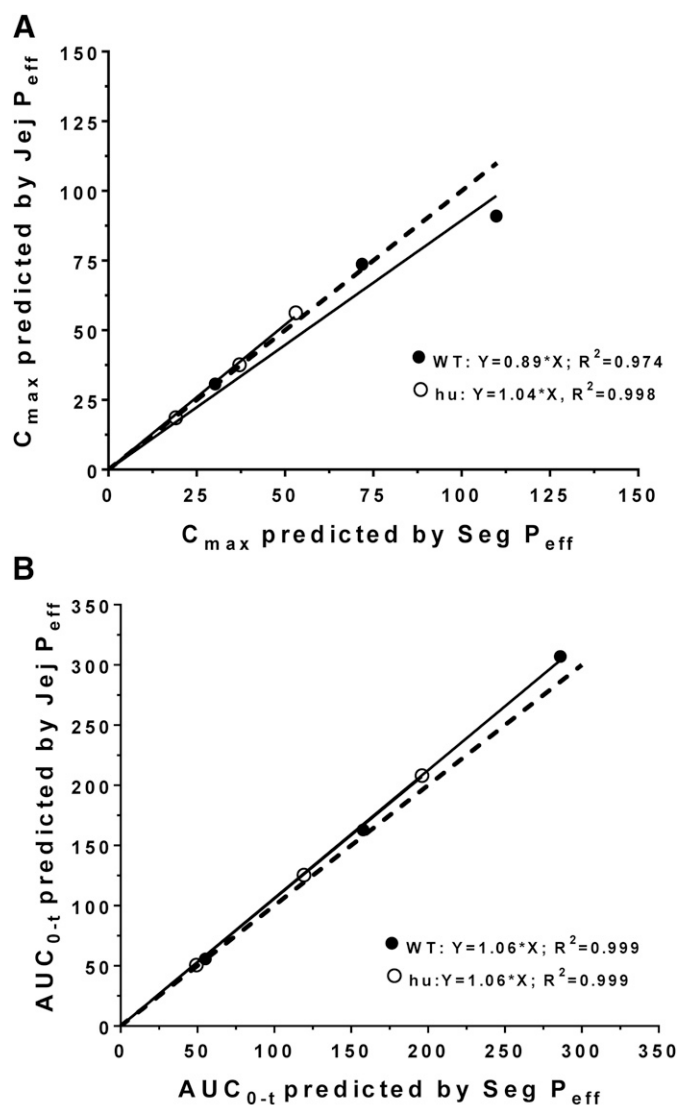


Fig. 6. Correlation between the observed and predicted C_{max} (A) and AUC_{0-t} (B) values of cefadroxil after human oral doses of 5, 15, and 30 mg/kg. The pharmacokinetic parameters were predicted using jejunal permeability (Jej P_{eff}), as estimated from WT and humanized (hu) *PepT1* mice. The dashed line represents a slope of unity. C_{max} has units of $\mu\text{g}/\text{ml}$ and AUC_{0-t} has units of $\mu\text{g}\cdot\text{h}/\text{ml}$.

mice; 2) intestinal permeabilities based on in silico and rat estimates gave worse predictions; 3) accurate predictions were possible for cefadroxil during oral dose escalation, a drug-drug interaction with cephalixin, and multiple oral dosing; and 4) the greatest amount of cefadroxil was absorbed in the duodenal and jejunal segments of the small intestine.

CL and central compartment volume of distribution showed the greatest effect on C_{max} and AUC_{0-t} (Fig. 2), and as a result these two parameters were optimized in our analysis (Table 2). The P_{eff} value also showed a significant effect on the plasma concentration-time profile of orally administered cefadroxil, indicating that an accurate assessment of this parameter was essential for improved predictions (Fig. 2). Thus, significant effort was applied to ascertaining the best way to estimate intestinal permeability, first by comparing in silico and rodent estimates and then by comparing jejunal versus multiple intestinal segments.

It should be noted that the substitution of human *PepT1* for mouse *PepT1* had no effect on the total clearance (or renal clearance) of cefadroxil since this parameter did not differ between WT and *huPepT1*

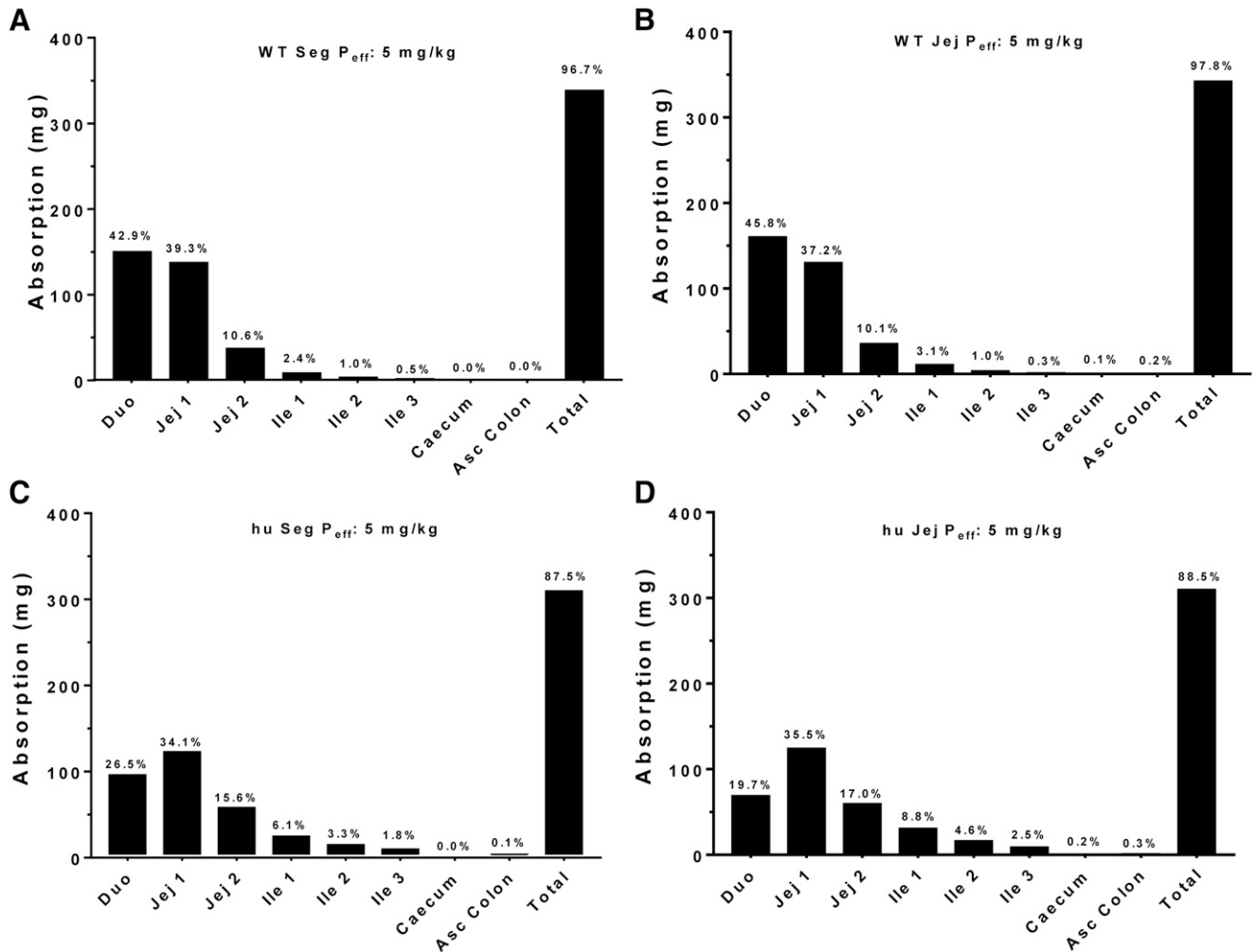


Fig. 7. Contribution of specific intestinal regions in the absorption of cefadroxil after a human oral dose of 5 mg/kg. Oral absorption was predicted using both segmental (Seg P_{eff}) and jejunal (Jej P_{eff}) permeability, as estimated from WT (A and B, respectively) and humanized (C and D, respectively) mice; hu, humanized *PepT1* mice.

mice after both low (i.e., 11 nmol/g or 4 mg/kg) and high (528 nmol/g or 192 mg/kg) intravenous bolus administrations of drug (Hu and Smith, 2016). Moreover, renal *PepT1* plays a very minor role in the tubular reabsorption of cefadroxil in kidney, accounting for only 5% of this process compared with 95% being reabsorbed by *PepT2* (Shen et al., 2007). Based on the volume of distribution steady state of cefadroxil, the drug would be restricted to extracellular fluid. Given V_d (extracellular fluid) = $7 + 8 \cdot \text{unbound fraction} = 7 + 8 \cdot 0.8$, the volume of distribution steady state equals 13.4 l (the unbound fraction for cefadroxil is 0.8) (Shen et al., 2007). Based on a 75-kg human, the 0.185 l/kg value (the central compartment volume of distribution plus the volume of distribution in the peripheral compartment) (Table 2) equals 13.9 l.

There is scant information on the in vitro-in vivo extrapolation of scaling factors for intestinal transport proteins, especially with respect to kinetic data (e.g., V_{max} and K_m) describing the active uptake and oral absorption of transporter substrates or drugs. Literature-obtained relative expression factors (i.e., human protein expression divided by *Caco-2* protein expression for a given transporter) have been reported to range from 0.4 to 5.1 for *P-glycoprotein* and from 1.1 to 90 for breast cancer resistance protein (Harwood et al., 2016). This variability, especially from different laboratories, has made it difficult to apply in vitro-in vivo extrapolation for successful pharmacokinetic outcomes in human subjects. This difficulty may be due to a variety of reasons, including in vitro accuracy and reproducibility of cell culture systems, culture

conditions, inconsistent intestinal expression of transporters and expression quantification, and post-translational effects on transporter activity. As shown by Harwood et al. (2016), a 4.3-fold increase (optimization) in the V_{max} value of *P-glycoprotein* was required to account for the drug-drug interaction between orally administered digoxin and rifampin in eight healthy volunteers. Because of the difficulty in scaling kinetic data such as V_{max} , whether estimated in vitro from cell cultures or parallel artificial membrane permeability assays or in situ from single-pass intestinal perfusions, we elected to use a concentration-dependent permeability approach and to then allometrically scale the results from mouse to human based on intestinal radius.

TABLE 5

Population analysis for drug-drug interaction of 5 mg/kg cefadroxil plus 45 mg/kg cephalixin in human subjects when estimated by the intestinal permeability from *huPepT1* mice

Observed values were obtained from the literature (Garrigues et al., 1991).

| Parameter | Observed | Segmental P_{eff} | | | Jejunal P_{eff} | | |
|---|----------|---------------------|------|-------|-------------------|------|-------|
| | | Predicted | CV | Error | Predicted | CV | Error |
| | | | % | % | | % | % |
| C_{max} ($\mu\text{g/ml}$) | 8.4 | 6.8 | 27.4 | -19.3 | 7.5 | 16.4 | -10.5 |
| AUC_{0-4} ($\mu\text{g}\cdot\text{h/ml}$) | 36.8 | 29.7 | 31.4 | -19.2 | 30.6 | 21.7 | -16.8 |

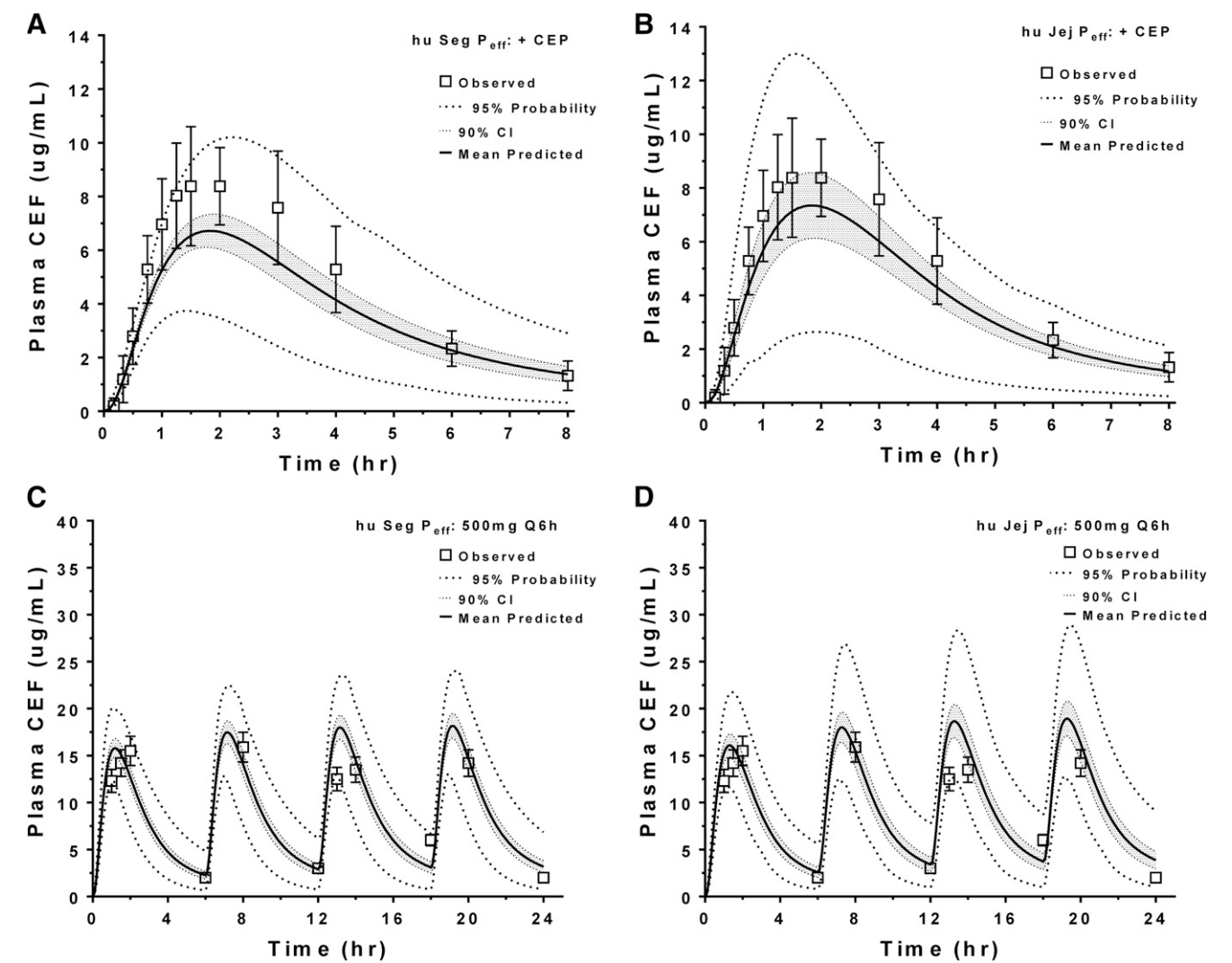


Fig. 8. Population analysis of the predicted plasma concentration-time profiles of cefadroxil during a drug-drug interaction study of 5 mg/kg oral cefadroxil plus 45 mg/kg oral cephalixin (A and B) [human data were obtained from Garrigues et al. (1991)], and during a multiple dose study of 500 mg (6.7 mg/kg) cefadroxil administered orally every 6 hours (Q6h) [human data were obtained from Santella and Henness (1982)] (C and D). Analyses were performed using segmental (Seg P_{eff}) and jejunal (Jej P_{eff}) permeability, as estimated from humanized (*hu*) *PepT1* mice. CEF, cefadroxil; CEP, cephalixin.

During our analysis, we found that in silico rat and WT mouse estimates of jejunal permeability were inadequate predictors of cefadroxil oral pharmacokinetics (Figs. 3 and 4). However, segmental and jejunal estimates of *huPepT1* mouse permeability both gave improved estimates of the plasma concentration-time profiles of orally administered cefadroxil (Figs. 3 and 4), the former approach being more physiologically correct. However, it may not make much of a difference in this specific case because the duodenal and jejunal permeabilities were similar for cefadroxil (Table 3), representing intestinal regions where most of the drug was predicted to be absorbed (Fig. 7). It was also observed, using *huPepT1* mouse permeabilities (both segmental and jejunal), that the population predictions of oral cefadroxil pharmacokinetics were well characterized during a drug-drug interaction study with cephalixin (Fig. 8, A and B) and multiple oral dosing of cefadroxil (Fig. 8, C and D). Thus, our approach in applying concentration-dependent permeabilities based on *huPepT1* mice gave improved predictions of oral cefadroxil pharmacokinetics (i.e., C_{max} and AUC_{0-t}) under nonlinear conditions and for a number of study designs. One caveat is that we assumed in our analyses that the absorption rate constant of cefadroxil was the same between human

subjects and *huPepT1* mice (i.e., $K_{a, human} = K_{a, huPepT1}$). We feel this is a reasonable assumption since valacyclovir, another *PepT1* substrate therapeutic, had similar K_a values in humans (0.68 hour⁻¹) and *huPepT1* mice (0.86 hour⁻¹) (Epling et al., 2018).

In summary, the current studies have demonstrated that simulation software (i.e., GastroPlus), in combination with intestinal permeability estimates from *huPepT1* mice, can be used to predict the oral

TABLE 6

Population analysis for multiple dosing regimen of 500 mg (6.7 mg/kg) cefadroxil administered orally every 6 hours in human subjects when estimated by the intestinal permeability from *huPepT1* mice

Observed values were obtained from the literature (Santella and Henness, 1982).

| Parameter | Observed | Segmental P_{eff} | | | Jejunal P_{eff} | | |
|---|----------|---------------------|------|-------|-------------------|------|-------|
| | | Predicted | CV | Error | Predicted | CV | Error |
| | | | % | % | | % | % |
| | | | | | | | |
| C_{max} ($\mu\text{g/ml}$) | 15.9 | 19.5 | 26.3 | 22.9 | 19.5 | 26.3 | 22.4 |
| AUC_{0-t} ($\mu\text{g}\cdot\text{h/ml}$) | 223 | 225 | 31.3 | −1.0 | 237 | 34.0 | 4.2 |

pharmacokinetic behavior of a therapeutic agent in humans without the need for artificial scaling of V_{\max} . Moreover, our approach was applied for the first time to the nonlinear intestinal absorption of a model PepT1 substrate, cefadroxil. This approach may have great practical value in the accurate prediction of the plasma concentration-time profiles during oral single and multiple dosing and for drug-drug interaction studies of new chemical entities in humans that are primarily absorbed in the intestines by PepT1. The possibility of extending this approach to compounds that are absorbed by other intestinal uptake and/or efflux transporters would have to be tested and validated experimentally.

Authorship Contributions

Participated in research design: Hu, Smith.

Conducted experiments: Hu.

Performed data analysis: Hu.

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

References

- Abusal BS, Bolger MB, Walker DK, and Kaddoumi A (2012) In silico modeling for the nonlinear absorption kinetics of UK-343,664: a P-gp and CYP3A4 substrate. *Mol Pharm* **9**:492–504.
- Bolger MB, Lukacova V, and Woltosz WS (2009) Simulations of the nonlinear dose dependence for substrates of influx and efflux transporters in the human intestine. *AAPS J* **11**:353–363.
- Caldwell GW, Masucci JA, Yan Z, and Hageman W (2004) Allometric scaling of pharmacokinetic parameters in drug discovery: can human CL_{ss} and $t_{1/2}$ be predicted from in-vivo rat data? *Eur J Drug Metab Pharmacokinet* **29**:133–143.
- Cao X, Gibbs ST, Fang L, Miller HA, Landowski CP, Shin HC, Lennernas H, Zhong Y, Amidon GL, Yu LX, et al. (2006) Why is it challenging to predict intestinal drug absorption and oral bioavailability in human using rat model. *Pharm Res* **23**:1675–1686.
- Cheung C and Gonzalez FJ (2008) Humanized mouse lines and their application for prediction of human drug metabolism and toxicological risk assessment. *J Pharmacol Exp Ther* **327**:288–299.
- Chu X, Bleasby K, and Evers R (2013) Species differences in drug transporters and implications for translating preclinical findings to humans. *Expert Opin Drug Metab Toxicol* **9**:237–252.
- Chung J and Kesiosoglou F (2018) Physiologically based oral absorption modelling to study gut-level drug interactions. *J Pharm Sci* **107**:18–23.
- Cvijic S, Ibric S, Parojcic J, and Djuris J (2018) An in vitro-in silico approach for the formulation and characterization of ranitidine gastroretentive delivery systems. *J Drug Deliv Sci Technol* **45**:1–10.
- Epling D, Hu Y, and Smith DE (2018) Evaluating the intestinal and oral absorption of the prodrug valacyclovir in wildtype and huPepT1 transgenic mice. *Biochem Pharmacol* **155**:1–7.
- Fagerholm U, Johansson M, and Lennernas H (1996) Comparison between permeability coefficients in rat and human jejunum. *Pharm Res* **13**:1336–1342.
- Garrigues TM, Martin U, Peris-Ribera JE, and Prescott LF (1991) Dose-dependent absorption and elimination of cefadroxil in man. *Eur J Clin Pharmacol* **41**:179–183.
- Gonzalez FJ and Yu AM (2006) Cytochrome P450 and xenobiotic receptor humanized mice. *Annu Rev Pharmacol Toxicol* **46**:41–64.
- Grass GM (1997) Simulation models to predict oral drug absorption from in vitro data. *Adv Drug Deliv Rev* **23**:199–219.
- Harwood MD, Achour B, Neuhoof S, Russell MR, Carlson G, Warhurst G, and Rostami-Hodjegan A (2016) In vitro-in vivo extrapolation scaling factors for intestinal P-glycoprotein and breast cancer resistance protein: part II. The impact of cross-laboratory variations of intestinal transporter relative expression factors on predicted drug disposition. *Drug Metab Dispos* **44**:476–480.
- Henze LJ, Griffin BT, Christiansen M, Bundgaard C, Langguth P, and Holm R (2018) Exploring gastric emptying rate in minipigs: effect of food type and pre-dosing of metoclopramide. *Eur J Pharm Sci* **118**:183–190.
- Hosea NA, Collard WT, Cole S, Maurer TS, Fang RX, Jones H, Kakar SM, Nakai Y, Smith BJ, Webster R, et al. (2009) Prediction of human pharmacokinetics from preclinical information: comparative accuracy of quantitative prediction approaches. *J Clin Pharmacol* **49**:513–533.
- Hu Y, Chen X, and Smith DE (2012) Species-dependent uptake of glycylsarcosine but not oseltamivir in *Pichia pastoris* expressing the rat, mouse, and human intestinal peptide transporter PEPT1. *Drug Metab Dispos* **40**:1328–1335.
- Hu Y and Smith DE (2016) Species differences in the pharmacokinetics of cefadroxil as determined in wildtype and humanized PepT1 mice. *Biochem Pharmacol* **107**:81–90.
- Hu Y, Xie Y, Wang Y, Chen X, and Smith DE (2014) Development and characterization of a novel mouse line humanized for the intestinal peptide transporter PEPT1. *Mol Pharm* **11**:3737–3746.
- Huang W, Lee SL, and Yu LX (2009) Mechanistic approaches to predicting oral drug absorption. *AAPS J* **11**:217–224.
- Huh Y, Smith DE, and Feng MR (2011) Interspecies scaling and prediction of human clearance: comparison of small- and macro-molecule drugs. *Xenobiotica* **41**:972–987.
- Ito K and Houston JB (2005) Prediction of human drug clearance from in vitro and preclinical data using physiologically based and empirical approaches. *Pharm Res* **22**:103–112.
- Jamei M, Marciniak S, Feng K, Barnett A, Tucker G, and Rostami-Hodjegan A (2009) The Simcyp population-based ADME simulator. *Expert Opin Drug Metab Toxicol* **5**:211–223.
- Katoh M, Matsui T, Nakajima M, Tatenko C, Kataoka M, Soeno Y, Horie T, Iwasaki K, Yoshizato K, and Yokoi T (2004) Expression of human cytochromes P450 in chimeric mice with humanized liver. *Drug Metab Dispos* **32**:1402–1410.
- Liu Z, Megaraj V, Li L, Sell S, Hu J, and Ding X (2015) Suppression of pulmonary CYP2A13 expression by carcinogen-induced lung tumorigenesis in a CYP2A13-humanized mouse model. *Drug Metab Dispos* **43**:698–702.
- Ma X, Shah Y, Cheung C, Guo GL, Feigenbaum L, Krausz KW, Idle JR, and Gonzalez FJ (2007) The PREGnane X receptor gene-humanized mouse: a model for investigating drug-drug interactions mediated by cytochromes P450 3A. *Drug Metab Dispos* **35**:194–200.
- Mahmood I (1999) Prediction of clearance, volume of distribution and half-life by allometric scaling and by use of plasma concentrations predicted from pharmacokinetic constants: a comparative study. *J Pharm Pharmacol* **51**:905–910.
- Mahmood I (2002) Prediction of clearance in humans from in vitro human liver microsomes and allometric scaling. A comparative study of the two approaches. *Drug Metabol Drug Interact* **19**:49–64.
- Mahmood I, Martinez M, and Hunter RP (2006) Interspecies allometric scaling. Part I: prediction of clearance in large animals. *J Vet Pharmacol Ther* **29**:415–423.
- Martinez M, Mahmood I, and Hunter RP (2006) Interspecies allometric scaling: prediction of clearance in large animal species: Part II: mathematical considerations. *J Vet Pharmacol Ther* **29**:425–432.
- Miksys SL, Cheung C, Gonzalez FJ, and Tyndale RF (2005) Human CYP2D6 and mouse CYP2D5: organ distribution in a humanized mouse model. *Drug Metab Dispos* **33**:1495–1502.
- Musther H, Olivares-Morales A, Hatley OJ, Liu B, and Rostami-Hodjegan A (2014) Animal versus human oral drug bioavailability: do they correlate? *Eur J Pharm Sci* **57**:280–291.
- Patterson D, Graham C, Cherian C, and Matherly LH (2008) A humanized mouse model for the reduced folate carrier. *Mol Genet Metab* **93**:95–103.
- Pedersen JM, Khan EK, Bergström CAS, Palm J, Hoogstraate J, and Artursson P (2017) Substrate and method dependent inhibition of three ABC-transporters (MDR1, BCRP, and MRP2). *Eur J Pharm Sci* **103**:70–76.
- Pfeffer M, Jackson A, Ximenes J, and de Menezes JP (1977) Comparative human oral clinical pharmacology of cefadroxil, cephalixin, and cephradine. *Antimicrob Agents Chemother* **11**:331–338.
- Sanoh S, Naritomi Y, Fujimoto M, Sato K, Kawamura A, Horiguchi A, Sugihara K, Kotake Y, Ohshita H, Tatenko C, et al. (2015) Predictability of plasma concentration-time curves in humans using single-species allometric scaling of chimeric mice with humanized liver. *Xenobiotica* **45**:605–614.
- Santella PJ and Henness D (1982) A review of the bioavailability of cefadroxil. *J Antimicrob Chemother* **10** (Suppl B):17–25.
- Sawamoto T, Haruta S, Kurosaki Y, Higaki K, and Kimura T (1997) Prediction of the plasma concentration profiles of orally administered drugs in rats on the basis of gastrointestinal transit kinetics and absorbability. *J Pharm Pharmacol* **49**:450–457.
- Scheer N and Roland Wolf C (2013) Xenobiotic receptor humanized mice and their utility. *Drug Metab Rev* **45**:110–121.
- Scheer N and Wilson ID (2016) A comparison between genetically humanized and chimeric liver humanized mouse models for studies in drug metabolism and toxicity. *Drug Discov Today* **21**:250–263.
- Shalaeva M, Kenseth J, Lombardo F, and Bastin A (2008) Measurement of dissociation constants (pK_a values) of organic compounds by multiplexed capillary electrophoresis using aqueous and cosolvent buffers. *J Pharm Sci* **97**:2581–2606.
- Shen H, Ocheltree SM, Hu Y, Keep RF, and Smith DE (2007) Impact of genetic knockout of PEPT2 on cefadroxil pharmacokinetics, renal tubular reabsorption, and brain penetration in mice. *Drug Metab Dispos* **35**:1209–1216.
- Song F, Hu Y, Jiang H, and Smith DE (2017) Species differences in human and rodent PEPT2-mediated transport of glycylsarcosine and cefadroxil in *Pichia pastoris* transformants. *Drug Metab Dispos* **45**:130–136.
- Sun D, Lennernas H, Welage LS, Barnett JL, Landowski CP, Foster D, Fleisher D, Lee KD, and Amidon GL (2002) Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. *Pharm Res* **19**:1400–1416.
- Tang H and Mayersohn M (2006) A global examination of allometric scaling for predicting human drug clearance and the prediction of large vertical allometry. *J Pharm Sci* **95**:1783–1799.
- Tanrisever B and Santella PJ (1986) Cefadroxil. A review of its antibacterial, pharmacokinetic and therapeutic properties in comparison with cephalixin and cephradine. *Drugs* **32** (Suppl 3):1–16.
- Tubic M, Wagner D, Spahn-Langguth H, Bolger MB, and Langguth P (2006) In silico modeling of non-linear drug absorption for the P-gp substrate talinolol and of consequences for the resulting pharmacodynamic effect. *Pharm Res* **23**:1712–1720.
- Yang B and Smith DE (2017) In silico absorption analysis of valacyclovir in wildtype and *Pept1* knockout mice following oral dose escalation. *Pharm Res* **34**:2349–2361.
- Yu LX and Amidon GL (1999) A compartmental absorption and transit model for estimating oral drug absorption. *Int J Pharm* **186**:119–125.
- Yu LX, Lipka E, Crison JR, and Amidon GL (1996) Transport approaches to the biopharmaceutical design of oral drug delivery systems: prediction of intestinal absorption. *Adv Drug Deliv Rev* **19**:359–376.

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Supplemental Data

***In Silico* Prediction of the Absorption and Disposition of Cefadroxil in Humans using an Intestinal Permeability Method Scaled from Humanized *PepT1* Mice**

Yongjun Hu, David E. Smith

Method to Estimate the Concentration-Dependent Permeability of Cefadroxil in Mouse Intestinal Segments during Single Escalating Doses and during Multiple Dosing. The jejunal permeability values of cefadroxil were obtained from wildtype and *huPepT1* mice during *in situ* perfusions of the drug over a concentration range of 0.01-25 mM (Hu and Smith, 2016). The concentration-dependent permeability of cefadroxil (shown in Figure S1) was then fit to the equation:

$$P_{eff} = P_{eff,max} \left(1 - \frac{I}{IC_{50} + I} \right)$$

where $P_{eff,max}$ is the jejunal permeability of cefadroxil at 10 μ M, a concentration that is at least 270x lower than the drug's K_m value in both genotypes, I is the inhibitor concentration of cefadroxil in this self-inhibition study, and IC_{50} is the concentration of inhibitor at which the P_{eff} of cefadroxil is one-half its maximum value. The parameter estimates are shown in Table S1.

The observed jejunal P_{eff} values of 10 μM cefadroxil in wildtype and *huPepT1* mice are presented in Table S2, along with P_{eff} estimates made in mouse jejunum from human single escalating doses (i.e., 4.1 mM at 5 mg/kg, 12.4 mM at 15 mg/kg, 24.8 mM at 30 mg/kg) and during multiple dosing [i.e., 5.5 mM at 500 mg (6.7 mg/kg) every 6 hours].

The P_{eff} values of 10 μM cefadroxil in other intestinal segments were obtained from wildtype and *huPepT1* mice during *in situ* perfusions of the drug (Hu and Smith, 2016). P_{eff} estimates at other dose levels were then determined by proportionality (Table S2). For example, the duodenal P_{eff} of cefadroxil at 5 mg/kg was estimated in wildtype mice as: $0.40/0.62 \times 0.55 = 0.36 \times 10^{-4}$ cm/sec. These values in mice were then scaled for human subjects by: $P_{\text{eff, human}} = P_{\text{eff, mouse}} \cdot (R_{\text{human}}/R_{\text{mouse}})$ where R is radius of intestine (Tables 3 and S2).

Method to Estimate the Permeability of Cefadroxil in Intestinal Segments of *huPepT1* Mice during the 5 mg/kg Cefadroxil \pm 45 mg/kg Cephalexin Drug-Drug Interaction Study.

Since the *in situ* intestinal permeability of cephalexin was unknown, we assumed that cefadroxil and cephalexin had the same IC_{50} values. Thus, cephalexin equivalents (i.e., 38.9 mM at 45 mg/kg) were added to cefadroxil (i.e., 4.1 mM at 5 mg/kg) and, at a total concentration of 43.0 mM, the jejunal permeability of cefadroxil in *huPepT1* mice was estimated according to the above equation as 0.023×10^{-4} cm/sec. P_{eff} estimates in the other intestinal segments were then determined by proportionality (Table S2). For example, the duodenal P_{eff} in *huPepT1* mice was estimated as: $0.023/0.111 \times 0.154 = 0.032 \times 10^{-4}$ cm/sec. As before, these values in mice were then scaled for human subjects by: $P_{\text{eff, human}} = P_{\text{eff, mouse}} \cdot (R_{\text{human}}/R_{\text{mouse}})$ where R is radius of intestine. (Tables 3 and S2).

TABLE S1

Model fitting of permeability parameters in wildtype (WT) and humanized (hu) PepT1 mice

| Parameter | WT | hu |
|-------------------------------------|-----------------|-----------------|
| $P_{eff,max}$ (cm/sec x 10^{-4}) | 0.71 ± 0.07 | 0.18 ± 0.04 |
| IC_{50} (mM) | 4.85 ± 1.73 | 6.52 ± 4.26 |

TABLE S2

Observed and predicted intestinal P_{eff} of cefadroxil in wildtype (WT) and humanized (hu) *PepT1* mice when estimated by the *in situ* permeability of small and large intestines

| | Mouse | Human | WT | hu | WT | hu | WT | hu | WT | hu | WT | hu | hu |
|--------------------|-------------|-------|------------------------------------|-------------|---------|-------|----------|-------|----------|-------|-----------------------|-------|-----------------|
| | Radius (cm) | | 10 μM | | 5 mg/kg | | 15 mg/kg | | 30 mg/kg | | 500 mg (6.7 mg/kg) | | CEF + CEP |
| Stomach | 0.40 | 9.67 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Duodenum | 0.14 | 1.53 | 0.55 | 0.24 | 0.361 | 0.154 | 0.201 | 0.088 | 0.128 | 0.053 | 0.317 | 0.136 | 0.032 |
| Jejunum 1 | 0.13 | 1.45 | 0.62 | 0.17 | 0.402 | 0.111 | 0.223 | 0.063 | 0.142 | 0.038 | 0.352 | 0.098 | 0.023 |
| Jejunum 2 | 0.12 | 1.29 | 0.62 | 0.17 | 0.402 | 0.111 | 0.223 | 0.063 | 0.142 | 0.038 | 0.352 | 0.098 | 0.023 |
| Ileum 1 | 0.11 | 1.13 | 0.29 | 0.12 | 0.190 | 0.078 | 0.106 | 0.044 | 0.067 | 0.027 | 0.167 | 0.069 | 0.016 |
| Ileum 2 | 0.10 | 0.98 | 0.29 | 0.12 | 0.190 | 0.078 | 0.106 | 0.044 | 0.067 | 0.027 | 0.167 | 0.069 | 0.016 |
| Ileum 3 | 0.09 | 0.82 | 0.29 | 0.12 | 0.190 | 0.078 | 0.106 | 0.044 | 0.067 | 0.027 | 0.167 | 0.069 | 0.016 |
| Caecum | 0.62 | 3.39 | 0.00 | 0.06 | 0.003 | 0.036 | 0.001 | 0.020 | 0.001 | 0.012 | 0.002 | 0.032 | 0.007 |
| Ascending Colon | 0.33 | 2.41 | 0.00 | 0.06 | 0.003 | 0.032 | 0.001 | 0.018 | 0.001 | 0.011 | 0.002 | 0.029 | 0.007 |

P_{eff} , intestinal permeability in units of cm/sec ($\times 10^{-4}$).

Observed P_{eff} values of 10 μM cefadroxil in the small and large intestines of WT and *huPepT1* mice were obtained previously by our laboratory (Hu and Smith, 2016), and shown in bold text.

Predicted P_{eff} values of cefadroxil in the small and large intestines of WT and *huPepT1* mice are shown after human single oral doses of 5, 15 and 30 mg/kg, after 500 mg (6.7 mg/kg) oral doses every six hours, and after 5 mg/kg cefadroxil plus 45 mg/kg cephalexin (CEF + CEP).

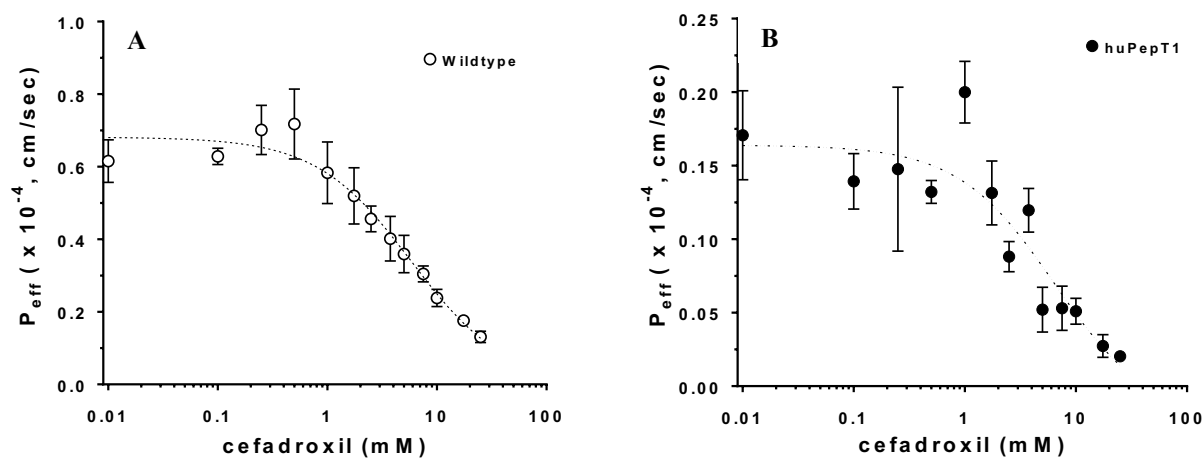


Figure S1. The concentration-dependent jejunal permeability of cefadroxil in wildtype (panel A) and humanized mice (panel B).