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Title Page

Prediction of Renal Transporter Mediated Drug-Drug Interactions for Pemetrexed Using Physiologically-Based Pharmacokinetic Modeling

Authors:

Maria M. Posada, James A. Bacon, Karen B. Schneck, Rommel G. Tirona, Richard B. Kim, J. William Higgins, Y. Anne Pak, Stephen D. Hall, Kathleen M. Hillgren.

Author Affiliation:

Eli Lilly and Company: Posada MM, Bacon JA, Schneck KB, Higgins JW, Pak YA, Hall SD, Hillgren KM.

University of Western Ontario: Tirona RG, Kim RB,

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Running Title Page

Running Title: Predicting Renal OAT-Mediated DDIs for Pemetrexed

Corresponding Author:

Maria M. Posada, Ph.D.

Email: posada_maria_mercedes@lilly.com.

Address: Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, 46285

Phone: 317-655-2054

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Abbreviations

AUC: area under the plasma-concentration time curve

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C_{max}: maximum plasma concentration

DDI: drug-drug interaction

DDI index: drug drug interaction index.

GFR: glomerular filtration rate

IC_{50}: concentration inhibiting 50% of the active transport

IVIVE: in vitro-in vivo extrapolation

Kd: passive permeability constant

K_m: Michaelis-Menten kinetic constant

NSAIDs: non-steroidal anti-inflammatory drugs

OAT: organic anion transporter

PBPK: physiologically-based pharmacokinetic modelling

P_{eff}: effective permeability

REF/ RAF: relative expression factor/ relative activity factor

V_{max}: maximal uptake rate

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Abstract

Pemetrexed, an anionic anti-cancer drug with a narrow therapeutic index, is eliminated mainly by active renal tubular secretion. The IVIVE approach used in this work was developed to predict possible DDIs that may occur following co-administration of pemetrexed and NSAIDs, and included *in vitro* assays, risk assessment models, and physiologically-based pharmacokinetic models. The pemetrexed transport and its inhibition parameters by several NSAIDs were quantified using HEK-PEAK cells expressing OAT3 or OAT4. The NSAIDs were ranked according to their DDI index, calculated as the ratio of their maximum unbound concentration in plasma over the concentration inhibiting 50% (IC_{50}) of active pemetrexed transport. A PBPK model for ibuprofen, the NSAID with the highest DDI index, was built incorporating active renal secretion in Simcyp®. The bottom-up model for pemetrexed under predicted the clearance by 2 fold. The model built using a scaling factor of 5.3 for the V_{max} of OAT3, estimated using plasma concentration profiles from patients given a 10-minute infusion of 500 mg/m^2 of pemetrexed supplemented with folic acid and vitamin B12, recovered the clinical data adequately. The observed/predicted increases in C_{max} and $AUC_{0\text{-}inf}$ of pemetrexed when ibuprofen was co-administered were 1.1 and 1.0, respectively. The co-administration of all other NSAIDs was predicted to have no significant impact on the $AUC_{0\text{-}inf}$ based on their DDI indexes. The PBPK model reasonably reproduced pemetrexed concentration time profiles in cancer patients and its interaction with ibuprofen.

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Introduction

Pemetrexed supplemented with folic acid and vitamin B12 is used to treat non-small cell lung cancer (NSCLC) and malignant mesothelioma, alone or in combination with cisplatin (Shih et al., 1997; Bajetta et al., 2003; Scagliotti et al., 2003; Latz et al., 2006; Takimoto et al., 2007). Pemetrexed is a hydrophilic anionic compound eliminated unchanged in the urine (70-90%) primarily by active tubular secretion, presumably by organic anion transporters (Rinaldi, 1999; Kurata et al., 2013). The structurally-related drug methotrexate was one of the first antifolates used for the treatment of cancer, and is predominantly eliminated unchanged in urine, with 80% of the dose being secreted actively by the kidney. The administration of inhibitors of the organic anion transporters, such as probenecid and NSAIDs reduces the clearance of methotrexate. For example, Tracy et al showed that naproxen, trisalicylate and ibuprofen decreased the plasma clearance by 1.28, 1.31, and 1.66 fold respectively (Cassano, 1989; Frenia and Long, 1992; Tracy et al., 1992). Additionally, the co-administration of high doses of methotrexate and ketoprofen was shown cause severe toxicities and even deaths (Thyss et al., 1986). Consequently the use of NSAIDs with high doses of methotrexate is not recommended. Since patients undergoing chemotherapy often take non-steroidal anti-inflammatory drugs (NSAIDS) for pain control and given that methotrexate and pemetrexed rely on renal transporters for their elimination, it was hypothesized that pemetrexed would experience the same drug interaction as methotrexate. Clinical studies demonstrated no interaction with aspirin, but a 20% increase in the $AUC_{0\text{-}inf}$ of pemetrexed was observed when patients with normal renal function (creatinine clearance > 80 mL/min) were co-treated with 400 mg of ibuprofen four times a day (Sweeney et al., 2006). While statistically significant, this increase does not require a dose adjustment in patients with normal renal function (creatinine clearance>80 mL/min). However, because renal insufficiency increases pemetrexed exposure, caution should be used when administering NSAIDs concurrently with pemetrexed to patients with mild and

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moderate renal insufficiency (creatinine clearance from 45 to 79 mL/min)(Mita et al., 2006). Although not studied clinically, it is recommended that NSAIDs with short elimination half-life should be avoided for a period of 2 days before, the day of, and 2 days after the administration of pemetrexed in patients with mild to moderate renal insufficiency. For NSAIDs with longer elimination half-lives, patient taking these NSAIDs should interrupt dosing for at least 5 days before, the day of and 2 days after pemetrexed administration (Pemetrexed label, http://labels.fda.gov/).

The organic anion transporters (OATs) are a group of membrane transporter proteins expressed in several epithelial tissues of the body, including the proximal tubular cells of the kidney (Erdman et al., 2006; Burckhardt, 2012). These transporters play an important role in renal elimination, by actively secreting a wide variety of endogenous and exogenous hydrophilic organic anions, including p-aminohippuric acid, prostaglandins, cephalosporins, antivirals, and anticancer drugs (Burckhardt, 2012). In the kidney, OAT1 (SLC22A6) and OAT3 (SLC22A8) are expressed on the basolateral membrane of the proximal tubular cells, where they import organic anions in exchange of dicarboxylates, such as succinate and α-ketoglutarate (Motohashi et al., 2002; Bakhiya et al., 2003; Sweet et al., 2003). OAT4 (SLC22A11) is expressed on the apical membrane of the apical tubular cells and functions as a bi-directional transporter, moving solutes from the cell into the tubular fluid and vice versa, in exchange of dicarboxylates, urate, and other organic anions (Motohashi et al., 2002; Hagos et al., 2007a; Hagos et al., 2007b). NSAIDs are a well characterized group of OAT inhibitors that are generally better inhibitors of OAT1 and 3, than OAT4. While ibuprofen and diclofenac are effective inhibitors of OAT1 and OAT3 with IC_{50} values in the lower micromolar range, acetaminophen, aspirin and salicylate are low affinity inhibitors (Khamdang et al., 2002).

Our objectives were to 1) identify the transporters responsible for the cellular transport of pemetrexed, 2) determine the kinetic parameters of pemetrexed transport, 3) quantify the inhibitory

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potency of a subset of NSAIDs towards these transporters, and finally 4) establish a mechanistic PBPK model of pemetrexed and its interaction with ibuprofen that could also be applied to other potential inhibitors of pemetrexed renal secretion.

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Materials and Methods

Chemicals

Pemetrexed was synthesized at Eli Lilly and Company. Radiolabeled ^{14}C-ibuprofen was purchased from American Radiochemicals (St. Louis, MO). Radiolabeled ^{3}H-estrone-3-Sulfate (E3S) was purchased from Perkin Elmer (Boston, MA). Internal standard ($^{13}C_5{}^{15}N$-pemetrexed) and celecoxib were synthesized at Eli Lilly and Company. All other chemicals were purchased from Sigma (St Louis, MO).

Preliminary *in Vitro* Substrate Transporter Screen

The preliminary uptake screen was performed according to a transient transporter expression method previously described using HeLa cells transfected with cDNAs of human transporters OAT1 (SLC22A6), OAT2 (SLC22A7), OAT3 (SCL22A8), OAT4 (SLC22A9), OCT1 (SLC22A1), OCT2 (SLC22A2), OCTN1 (SLC22A4), OATP1A2 (SLCO1A2), OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OATP2B1 (SLCO2B1), OATP4C1 (SLCO4C1), ASBT (SLC10A2), PEPT1 (SLC15A1), PEPT2 (SLC15A2) (Cvetkovic et al., 1999). Drug uptake was evaluated using ^{14}C- pemetrexed (60 μM) in medium for 10 minutes at 37°C. The reaction was terminated using ice cold buffer, the cells were lysed with 1% SDS. The samples were added to a vial containing Ultima Gold scintillation cocktail (Perkin Elmer) and the amount of pemetrexed in the cells was measured using a scintillation counter (Perkin Elmer, Tri-Carb 2900TR).

In vitro Transporter Kinetic Studies

HEK-PEAK cells were transfected with plasmids containing human OAT3 and OAT4 following previously published protocols (Godinot et al., 2003). In short, human OAT3 (NM_004254) and OAT4

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(NM_080866) genes were purchased from GeneArt (Regensburg, Germany), and inserted into EW1969 plasmid prior to transfection of HEK-PEAK cells using a Qiagene Effectene kit.

In vitro uptake experiments were performed based on reports published by Nozaki et al (Nozaki et al., 2004) with some modifications. HEK-PEAK cells from passages 36-75 were used 72 hours after seeding on CELLBIND 12-well plates at a density of 150,000cells/well. The reactions were performed in uptake buffer consisting of Krebbs-Henseleit buffer at pH 7.4 supplemented with 2.5 mM $CaCl_2$ and 10mM HEPES. Protein quantification was carried out using a BCA protein assay (Sigma-Aldrich Chemical Company, St Louis, MO) where bovine serum albumin was used as standard.

OAT3 and OAT4 qualification studies were performed to assess the functionality of the HEK-PEAK transfected cells. The cells were treated with 0.5 mL of buffer containing 1 μM ^{3}H-estrone-3-sulfate for 2 minutes in the presence or absence of 200 μM probenecid, a known inhibitor of the organic anion transporters, at room temperature. The incubations were stopped by the addition of ice cold buffer, followed by two washes with buffer. Cells were lysed in 0.3 mL of 0.2 N NaOH overnight at 4^{o}C. A 0.2 mL aliquot was added to a vial containing scintillation cocktail (ScintSafe 30%, Fisher Scientific) and the cellular uptake of ^{3}H-estrone-3-sulfate was measured using a scintillation counter. Protein quantification was performed as mentioned above with the remaining sample.

All pemetrexed uptake and inhibition studies were done at room temperature with 0.5 mL of pemetrexed in Krebs-Henseleit buffer. Before the beginning of the experiment, the vector control, OAT3, and OAT4 cells were washed twice with buffer and allowed to equilibrate for 5 minutes. Time-dependent uptake studies were performed with 39 μM pemetrexed and stopped at the specified times up to 15 minutes. Concentration-dependent uptake studies were carried out for 5 minutes with pemetrexed concentrations ranging from 0.01 to 117 μM. All reactions were stopped with the addition of cold buffer,

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and cells were lysed in a 1:1 methanol: water (v:v) for 3 minutes. The concentration of pemetrexed was quantified using LC-MS/MS.

In vitro Transporter Inhibition Studies

The inhibitory effects of individual NSAIDs and probenecid on pemetrexed uptake were evaluated using vector control, OAT3, and OAT4 transfected cells incubated at room temperature with 0.5 mL of pemetrexed (19.5 μM) in uptake buffer. Ibuprofen, diclofenac, naproxen, and celecoxib were added to the cells in concentrations ranging from 0.1 to 200 μM. The incubations were stopped at 5 minutes, the cells were rinsed twice with ice-cold buffer, and treated with 0.3 mL of 1:1methanol: water (volume: volume) to extract the pemetrexed for LC-MS/MS quantification.

Fraction Unbound in Incubation

The fraction unbound of ibuprofen in the incubations (fu_{inc}) was measured in vector control cells using ^{14}C-ibuprofen. The conditions for this experiment were the same ones as described before for the transport experiments. Briefly, the HEK-PEAK vector control cells were washed twice with buffer and incubated at room temperature for 5 minutes with 0.5 mL of 10 μM ibuprofen in buffer. Samples of the incubation media (200 μL) were collected at 0 and 5 minutes placed in a vial containing 10mL of scintillation cocktail and quantified using a scintillation counter. The fraction of pemetrexed unbound in the incubation was assumed to be 1.

Analytical Method

The concentration of pemetrexed in the cells was quantified by LC-MS/MS using $^{13}C_5{}^{15}N$-pemetrexed as an internal standard. Reverse-phase chromatography was used with a flow rate of 0.6 mL/min, an injection volume of 20 μL. The analytes were chromatographically separated using a Phenomenex Synergi MAX-RP 80A 5μ 100 X 2.0 mm HPLC column, with a gradient LC system

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composed of 0.2% formic acid in water, (Mobile Phase A), and 0.2% formic acid in methanol (Mobile Phase B). The gradient profile was 10% B from 0 to 0.30 min, 70% B at 0.70 to 2.00 min, and returned to 10% B at 2.20 min. The flow rate was 0.6 mL/min and the analysis was performed at ambient laboratory conditions with the effluent directed to the mass spectrometer between 0.10 and 2.40 minutes. Pemetrexed was detected in positive ion mode using a Sciex API 4000 triple quadruple mass spectrometer equipped with a Turbo Ion Spray interface and Analyst 1.4 software (Applied Biosystems/MDS, Foster City, CA). The mass:charge ratio for pemetrexed and $^{13}C_5{}^{15}N$-pemetrexed were 426 → 297 *m/z* and 432 → 297 *m/z*. The collision energies were -30 and -36 for pemetrexed and $^{13}C_5{}^{15}N$-pemetrexed, respectively. The dynamic range of the assay was between 0.77 and 395 ng/mL, and samples with pemetrexed concentrations above the upper limit of quantification were diluted with buffer to concentrations within the assay range. Three individual batches of quality control samples at four separate concentrations (0.98, 3.9, 125 and 500 ng/ml) were examined with 4-6 samples tested at each concentration. The results for 0.98, 3.9, 125 and 500 ng/ml samples showed an inter-batch accuracy of 4.3, 3.3, 4.7 and 7.7%, and an inter-batch precision of 11.1, 8.3, 7.9 and 8.4%, respectively.

Data Analysis

The Michaelis-Menten kinetic parameters for pemetrexed transport were estimated by fitting the pemetrexed uptake data using non-linear regression (WinNonlin v5.3 (Pharsight, Sunnyvale, CA), according to the following formula:

$$v = \frac{V_{max} \times S}{K_m + S} + K_d \times S \qquad (1)$$

where, υ is the initial uptake (pmol/min/mg of protein), V_{max} is the maximal uptake rate (pmol/min/mg of protein), S is the concentration of substrate (µM), K_m (µM) is the Michaelis-Menten kinetic constant and K_d (µL/min/mg of protein) is the passive permeability constant. The K_d for

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pemetrexed was calculated experimentally by fitting the uptake velocity of pemetrexed in the HEK-PEAK vector control cells. The passive intrinsic clearance ($Cl_{int\ passive}$) of pemetrexed used in the model was calculated by multiplying the K_d by the number of cells in one milligram of protein.

The inhibition constant (K_i) of the NSAIDs was calculated according to the following formula, assuming competitive inhibition:

$$K_i = \frac{IC_{50}}{1+\frac{[S]}{K_m}} \qquad (2)$$

Where IC_{50} is the concentration of NSAIDs necessary to inhibit 50% of the uptake of pemetrexed, [S] is the concentration of pemetrexed in the medium and K_m is the affinity of pemetrexed for the particular organic anion transporter. The IC_{50} for pemetrexed was calculated with the according to the following formula:

$$Percent\ of\ control = 100 * \frac{1}{1+\frac{[I]}{IC_{50}}} \qquad (3)$$

Where [I] is the concentration of the NSAID and percent of control is the % of pemetrexed taken up by the cells in the presence of the inhibitor compared to the cells treated with only pemetrexed.

Statistical Analysis

The *in vitro* experimental data are presented as mean ± standard error (S.E.) or mean (% CV). In order to test for significant differences between multiple groups, a one-way analysis of variance was used followed by either Dunnett's or Bonferroni's test. A p-value ≤ 0.05 was considered statistically significant (GraphPad Prism 6, La Jolla, CA).

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Modeling Strategy

The modeling strategy employed in this project was the following. 1) The active secretion of pemetrexed was defined to be via OAT3 and OAT4 on the basolateral and apical membrane of the proximal tubular cells, respectively. 2) A pemetrexed PBPK model was developed using *in vitro* and *in silico* data. 3) The model was optimized by using pemetrexed plasma concentration-time profiles from cancer patients. 4) The model was tested by changing the pemetrexed dose from 50 to 700 mg/m^2 and results were compared to other clinical studies. 5) A PBPK model for ibuprofen was developed using literature data and measured K_i and fu_{inc}. The model was verified using the results of the clinical interaction studies (Sweeney et al., 2006). This modeling strategy is described in detail below.

Pemetrexed Clinical Data

Human plasma concentration-time profiles after the administration of 10 minute infusion of 500 mg/m^2 of pemetrexed with vitamin B12 and folic acid from a published study (Sweeney et al., 2006) were used to build the model. In this study, serial blood samples were obtained pre-dose, immediately before the end of infusion and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hours after termination of the infusion. Results from published studies were used to test and verify the model (Latz et al., 2006; Mita et al., 2006; Nakagawa et al., 2006; Sweeney et al., 2006).

Pemetrexed PBPK Model

All simulations were performed using PKPD Profiles in Simcyp (Version 12.1). A full PBPK model for pemetrexed was built using measured and predicted physicochemical and biological data (Table 1) based on the Rodgers *et al* method, with rapid equilibrium between blood and tissues, except for the kidney which was set up as a permeability-limited tissue (Rodgers et al., 2005; Rodgers and Rowland, 2006). The multi-compartment kidney model built into Simcyp was used to incorporate the glomerular

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filtration and active renal secretion of pemetrexed. This mechanistic kidney model consists of 3 major compartments: blood, renal cells and tubular fluid, all sub-divided into several regions to approximate the anatomy and physiology of the kidney. Two active transport processes were enabled in the mechanistic kidney model; one on the basolateral membrane of the proximal tubular cells, to simulate OAT3-mediated uptake of pemetrexed, and a second one on the apical membrane of the proximal tubular cells, to simulate OAT4-mediated efflux of pemetrexed into the renal tubules. The values for the passive and active membrane transport of pemetrexed were assumed to be the equal in all 3 compartments of the proximal tubular cells and were taken from the *in vitro* experiments previously described. All other parameters used in the mechanistic kidney model were default Simcyp values with no modification, unless specifically stated. The pemetrexed model assumptions were the following: 1) pemetrexed was not metabolized and the only site of elimination was the kidney; 2) the passive permeability of pemetrexed in the vector control cell was representative of the passive permeability of pemetrexed in the kidney cells; 3) OAT3 is homogeneously expressed in the basolateral membrane of the three segments of the proximal tubular cells, and only transports pemetrexed in one direction, from the blood into the cells; 4) the efflux of pemetrexed from the proximal tubular cells is governed by only one transporter, OAT4, which is expressed uniformly on the apical membrane of the 3 segments of proximal tubular cells and transports pemetrexed only in one direction, from inside the cells out to into the tubular fluid compartment.

Ibuprofen PBPK Model

A full PBPK model was built for ibuprofen in Simcyp (Version 12.1) using the Rodgers *et al* method with rapid equilibrium between plasma and tissues (Rodgers and Rowland, 2006). The parameters used in the PBPK model for ibuprofen can be found in table 2. The dosing regimen of ibuprofen consisted of nine doses of 400 mg, taken every 6 hours for 2 days prior to the administration of pemetrexed, and a tenth dose of ibuprofen taken 1 hour prior to pemetrexed infusion (Sweeney et al., 2006). The model was

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based on the physicochemical properties and pharmacokinetic parameters of ibuprofen collected from published data (Yee, 1997; Davies, 1998; Chen et al., 2012). The absorption of ibuprofen was modeled using the advanced absorption and dissolution (ADAM) model in Simcyp, where the human effective jejunal permeability (P_{eff}) was predicted from Caco-2 permeability values (Yee, 1997). The intrinsic solubility of ibuprofen was 0.06 mg/mL and the model assumed an immediate release formulation (Shaw et al., 2005).

Virtual Population

The virtual population used in the simulations was built to replicate the cancer population in pemetrexed published clinical studies *(Sweeney et al., 2006)*. The population was based on the north European Caucasian with an average body weight of 76.8 kg, age ranging from 34 to 80 years old, and 30% of the subjects were female. The relative abundances and corresponding coefficient of variation (60%) of the transporters in the 3 segments of the proximal tubules were the same. No genetic polymorphisms were considered.

Parameter Sensitivity Analysis and Optimization

A parameter sensitivity analysis was performed to evaluate the effect of model parameter values on the $AUC_{0\text{-}inf}$ and C_{max} of pemetrexed. The first analysis was run for those parameters with high uncertainty or variability, such as the difference in expression or activity of the transporters in vitro versus in vivo. The model was optimized according to the results of the sensitivity analysis using the plasma concentration-time profiles of 25 patients that were administered 500 mg/m^2 of pemetrexed supplemented with folic acid and vitamin B12. The parameter estimation was performed using the hybrid minimization method in Simcyp and weighted least squares as the objective function.

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Model Verification

The PBPK model predictions for $AUC_{0\text{-}inf}$ and C_{max} over a range of pemetrexed doses from 50 to 600 mg/m^2 were compared to the observed ones in published clinical studies (Latz et al., 2006; Mita et al., 2006; Nakagawa et al., 2006; Sweeney et al., 2006). Predicted $AUC_{0\text{-}inf}$ was calculated dividing the dose by the predicted clearance. The observed pharmacokinetic parameters were calculated using non-compartmental analysis, according to the published clinical studies.

Drug-drug Interaction Predictions

The interaction between ibuprofen and pemetrexed occurred at both OAT3 and OAT4. The K_i and fu_{inc} values were obtained from *in vitro* experiments (see above) and are listed in table 2. To evaluate the effect of the interaction between pemetrexed and ibuprofen the observed and predicted $AUC_{0\text{-}inf}$ and C_{max} ratios were calculated and compared. The $AUC_{0\text{-}inf}$ ratio is defined as the pemetrexed $AUC_{0\text{-}inf}$ when pemetrexed is co-administered with ibuprofen, over the $AUC_{0\text{-}inf}$ of pemetrexed when pemetrexed is administered alone. Likewise, the C_{max} ratio is defined as the maximum concentration of pemetrexed when ibuprofen is co-administered over the pemetrexed C_{max} the when pemetrexed is administered alone. For the interaction model the assumptions were the following: 1) the interaction occurs via OAT3 and OAT4 in the proximal tubular cells of the kidney; 2) ibuprofen exerts its inhibition in a competitive manner for both OAT3 and OAT4; 3) both the S and R enantiomers of ibuprofen have the same affinity for OAT transporters; 5) ibuprofen metabolites do not interact with OAT3 and OAT4.

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Results

Preliminary *In vitro* Screen

^{14}C-Pemetrexed uptake was significantly higher in OAT3 and OAT4 transfected HeLa cells compared to the control (Figure 1). The uptake of ^{14}C-pemetrexed by all other transporters tested was not significantly different than the control. Based on this, all subsequent *in vitro* uptake and inhibition studies were performed in OAT3, OAT4 and vector control transfected cells.

In Vitro transporter Uptake and Inhibition Studies

The results of the qualification experiments, shown in figure 2A, demonstrated that the transfected HEK-PEAK hOAT3 and hOAT4 were functional and the uptake of estrone-3-sulfate, a known substrate for these transporters, is at least 100-fold higher than the vector control cells. When the OAT3 and OAT4 transfected cells were co-treated with probenecid, the uptake of estrone-3-sulfate via OAT3 and OAT4 was substantially reduced by 97 and 74%, respectively.

To probe the active and passive uptake of pemetrexed, kinetic studies were performed in HEK-PEAK vector control, OAT3, and OAT4 cells, over a concentration range of 1.6 and 117 μM of pemetrexed. As seen in Figure 2B the uptake of pemetrexed was linear up to 10 minutes in the conditions tested. Figure 2C shows saturable OAT3-mediated uptake of pemetrexed with a V_{max} and K_m of 185 pmol/mg/million cells and 18.2 μM, respectively. The passive intrinsic clearance of pemetrexed was estimated to be 3.98×10^{-3} μL/min/million cells. The uptake of pemetrexed in OAT4-transfected cells did not reach saturation in the conditions tested, and therefore the V_{max} and K_m values could not be calculated (Figure 2D). As a result, the intrinsic uptake clearance of OAT4 ($Cl_{int,T}$) was calculated by dividing the initial uptake of pemetrexed by its corresponding concentration, and was estimated to be 0.99 μL/min/million cells.

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The inhibition of pemetrexed uptake by several NSAIDs was studied over a concentration range 0.1 to 200 µM of the NSAIDs and 19.5 µM of pemetrexed. Table 4 shows the IC_{50} values for OAT3 and OAT4 for all the tested inhibitors. Table 5 shows the reported maximum plasma concentration of the tested inhibitors, fraction unbound in plasma (fu), and calculated Iu/ IC_{50} values for the NSAIDs (Goodman and Gilman). The 2012 FDA draft guideline on drug-drug interactions (http://fda.gov) concludes that an Iu/IC_{50} value higher than 0.1 indicates a high risk of clinical drug interaction and low potential for false negatives. Ibuprofen was the only tested NSAID with an Iu/IC_{50} value above 0.1, and therefore PBPK models were not built for the other NSAIDs.

Pemetrexed PBPK Model

The bottom-up pemetrexed PBPK model was built utilizing the measured in vitro transport kinetic data, without any scaling factors. The simulations predicted an $AUC_{0\text{-inf}}$ of 362 µg/mL·h (2.2 fold higher than the observed value), and a clearance of 2.5 L/h (55% smaller than the observed value). Since the difference between the *in vitro* and the *in vivo* amount and activity (REF/RAF) of the transporters is unknown, the model was optimized by estimating these parameters using clinical data. The REF/RAF value for OAT3 was estimated to 5.3 using human pemetrexed plasma concentration-time profiles published by Sweeney *et al* (Sweeney et al., 2006). The REF/RAF for OAT4 was set to 5.3 as well, which resulted in a lack of cellular accumulation and immediate excretion of pemetrexed in the urine. The optimized PBPK model was able to recover the observed plasma concentration-time profiles of pemetrexed. Figure 4A shows the mean plasma concentrations of the predicted and the observed individual plasma concentration-time profiles of pemetrexed after a single intravenous 10-minute infusion. The lines for the 5 and 95% confidence intervals are also shown in the graph. The observed/prediction ratios for the C_{max}, $AUC_{0\text{-inf}}$ and clearance were all within 0.91 and 1.04. For $AUC_{0\text{-inf,}}$ the observed and predicted geometric means were 164.7 and 158 µg/mL/h, respectively. The observed

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and predicted geometric means for C_{max} were 102.1 and 111.6 µg/mL, respectively. For plasma clearance, the observed and predicted geometric means were 5.6 and 5.7 L/h, respectively.

Impact of OAT3 and OAT4 on the PBPK Model Predictions

The impact of adding the kidney transporters to the PBPK model was evaluated by comparing predicted against the observed pemetrexed pharmacokinetic parameters (Rinaldi et al., 1999). As shown on table 5, when OAT3 is not added to the basolateral membrane of the proximal tubular cells in the model, the clearance of pemetrexed was under predicted by 74 % and the predicted $AUC_{0\text{-}inf}$ was 3.8 fold higher. When OAT3 was present, but OAT4 was not added to the apical membrane of the proximal tubular cells in the kidney model, the plasma concentrations were not affected, but the fraction excreted in urine unchanged ($fe_{0\text{-}24}$) was under-predicted by 70%. The predicted maximum intracellular drug concentration in the proximal tubular cells was 1000–fold in the absence of OAT4 compared to the model that had this transporter on the apical membrane.

Model Verification

The model was tested by comparing the predicted versus the observed C_{max} and $AUC_{0\text{-}inf}$ for a range of pemetrexed doses between 50 to 700 mg/m^2. The observed pharmacokinetic values were extracted from a phase 1 single dose escalation study (Rinaldi et al., 1999). Figure 4A shows the predicted and observed $AUC_{0\text{-}inf}$ versus pemetrexed dose in mg/m^2. In both the predicted and observed settings, the $AUC_{0\text{-}inf}$ increased linearly with dose. The slopes of the lines are very similar 0.4283 and 0.4896 for the predicted and observed, respectively (observed/predicted ratio = 1.14). Figure 4B shows the predicted and observed maximum plasma concentration vs. pemetrexed dose in mg/m^2. Again, the C_{max} changed linearly with the dose and the model predicts a similar slope of 0.243 and 0.219 for observed and predicted, respectively (observed/predicted ratio = 1.11).

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Drug-Drug Interaction Predictions

Ibuprofen plasma concentrations were not measured during the clinical study, therefore the predicted plasma concentration-time profiles of ibuprofen at the time of pemetrexed administration were compared against literature data(Aarons et al., 1983a; Aarons et al., 1983b; Grennan et al., 1983; Lockwood et al., 1983a; Lockwood et al., 1983b; Albert et al., 1984; Wagner et al., 1984; Davies, 1998). The pharmacokinetics of ibuprofen depend on several factors (i.e. formulation, fasted/fed state, physiology, disease state), a meta-analysis was performed to extract pharmacokinetic parameters from studies where ibuprofen was dosed at 400 mg in both healthy subjects and patients. Table 7 lists the reported and predicted steady state C_{max}, time of maximum concentration (T_{max}), and AUC values for ibuprofen dosed at 400 mg every 6 hours. The predicted T_{max} and C_{max} of ibuprofen at steady-state were 3% and 7% lower than the average of the values found in the literature (Aarons et al., 1983a; Grennan et al., 1983; Ragni et al., 1992; Davies, 1998). The predicted plasma exposure of ibuprofen between 42 and 48 hours ($AUC_{42\text{-}48h}$) was 1.01 fold-higher than the reported value. Figure 5A shows the predicted mean plasma concentrations and the 5 and 95% confidence intervals of ibuprofen. The results of the interaction model between ibuprofen and pemetrexed are shown in figure 5B. The pemetrexed AUC ratio was predicted to be 1.23 compared to 1.2 (1.11-1.31) observed and the predicted C_{max} ratio was 1.01 compared to the 1.15 (1.03-1.28) observed in the patients.

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Discussion

The goal of this study was to develop a strategy to predict clinically relevant transporter-mediated drug-drug interactions for pemetrexed using in vitro data, and to build confidence for the future prediction of drug interactions for renally cleared drugs. The first step in the strategy involved the in vitro identification of the transporters responsible for the active tubular secretion of pemetrexed. This was performed using a single time and single concentration assay, in transiently transfected HeLa cells. OAT3 and OAT4 were identified as the transporters likely to be involved in the active tubular secretion of pemetrexed. Under initial rate conditions in HEK-PEAK cells stably transfected with OAT3 and OAT4, we determined the V_{max}, K_m and Cl_{int}, and employed these parameters in a PBPK model to reproduce the disposition of pemetrexed in humans. The initial bottom-up PBPK model performed well and predicted the shape of the concentration-time curve, but the predicted systemic clearance was 55% lower than the observed. A parameter sensitivity analysis was conducted to identify possible causes of the under prediction of pemetrexed clearance. Based on the sensitivity analysis and the expected uncertainties, we hypothesized that the REF/RAF of OAT3 was the most likely contributor to this under prediction. The REF/RAF scaling factor was estimated to be 5.3 using a hybrid minimization method and weighted least squares as the objective function. The middle-out approach that employed the scaling factor recovered the shape of the concentration-time profile and systemic clearance accurately (Table 6). The need of scaling factors for transport parameters obtained *in vitro* is well-recognized in the prediction of drug absorption, distribution, and elimination via transporters (Bouzom et al., 2012; Tsamandouras et al., 2013; Hsu et al., 2014). In some cases the scaling factor may represent the differences in transporter protein abundance between *in vitro* and *in vivo*, but may also reflect the dissimilarities in activity per mg of protein, due to several factors including membrane environment or differences in post-translational modifications (Straumann et al., 2006; Croset et al., 2012). Is important to note that, in this study the V_{max}

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and K_m values were estimated from in vitro experiments performed at room temperature and not at 37°C. Rates of transport at room temperature are generally lower than those observed at 37°C and therefore the scaling factor should be lower when parameters are obtained at 37°C. This was demonstrated by (Jackson and Halestrap, 1996) for the active uptake of L-lactate into hepatocytes by the hepatic monocarboxylate transporter.

The model encompassed several important assumptions. First it was assumed that the OATs identified in the transporter screen were the only transporters involved in the tubular secretion of pemetrexed. We assumed that the uptake of pemetrexed into the tubular cells was unidirectional and driven by blood concentrations and OAT3 intrinsic clearance. It was also assumed that the concentrations of the co-transported dicarboxylates, such as alpha-ketoglutarate and succinate, were not rate limiting. We also assumed unidirectional flow of pemetrexed from the cells into the tubular fluid mediated by OAT4. Despite the fact that pemetrexed has been shown to be a substrate of MRP2 and MRP5, OAT4 was chosen as the only mechanism of active transport across the apical membrane given that its pemetrexed intrinsic clearance (2.5 µL/min/mg protein) is at least 10-fold higher than the reported ones for MRP2 (0.27 µL/min/mg protein) and MRP5 (0.04 µL/min/mg protein) (Pratt, 2002). It was also assumed that the passive permeability of pemetrexed was negligible based on the in vitro measurements. Under these conditions the OAT3-mediated step in the tubular secretion was an identifiable parameter and could be estimated using plasma curves. In comparison, the OAT4-mediated step in the model is non-identifiable because we do not have tubular concentrations nor do we have urinary excretion curves with samples collected over short intervals. Therefore OAT4 was not scaled, resulting in complete urinary excretion and no cellular accumulation of pemetrexed. This is consistent with the absence of renal cytotoxicity and the 70-90% observed urinary recovery of pemetrexed (Rinaldi, 1999; Rinaldi et al., 1999).

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One of the objectives of the current work was to test whether the *in vitro* inhibition potency of OATs could be confidently employed to predict the DDI observed *in vivo*. Using the transfected OAT3 and OAT4 HEK-PEAK cells we determined the IC_{50} values for commonly used NSAIDs. Of the NSAIDs tested, ibuprofen was identified as the most likely to cause a DDI with pemetrexed using the ratio of the unbound plasma C_{max} to the IC_{50} (Table 5). To foresee the effect of ibuprofen on the tubular secretion of pemetrexed, a PBPK model was constructed for ibuprofen based on literature pharmacokinetic data (Table 7). The combination of the predicted unbound ibuprofen plasma concentration-time profile and the *in vitro* inhibition constants (Ki) for OAT3 and OAT4 resulted in an accurate prediction of the increase in pemetrexed exposure. It is important to note that although the model included the inhibition of both OAT3 and OAT4, the predicted plasma clearance of pemetrexed was only affected by inhibition of OAT3. The inhibition of OAT4 by ibuprofen was shown to have no effect on the plasma clearance of pemetrexed (Figure 6). This is not surprising since the plasma clearance will be dependent on the uptake into the proximal tubular cells and not on the efflux of the drug from the cells into the tubules (Figure 6). Another important assumption of the current IVIVE approach was that only the parent drug was responsible for inhibiting the active transport, and that ibuprofen metabolites played no significant role in the inhibition. We believe this assumption is valid, because the levels of ibuprofen in plasma are considerably higher (approximately 10-fold) than the levels of its two primary metabolites (Mills et al., 1973).

An important strength of mechanistic PBPK modeling is the ability to simulate the outcome of different physiological or pathological changes that have not been specifically studied. This is illustrated in the case of pemetrexed, by examining the effect of renal impairment on the plasma clearance. Under the assumptions that glomerular filtration rate and active tubular secretion intrinsic clearance decrease proportionally in renal insufficiency and the plasma binding of pemetrexed is not altered, we predict the plasma clearance of 53.8 mL/min in patients with moderate renal insufficiency (GFR=40-59mL/min).

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However, in this case a clinical study has been performed to determine the effect of renal insufficiency on pemetrexed pharmacokinetics in cancer patients (Mita et al., 2006). In this study the observed plasma clearance of pemetrexed in the moderate renal insufficiency group was 54.7 mL/min (34% coefficient of variation), which is in good agreement with the prediction. The recruitment of severe renal insufficiency (GFR <20mL/min) group was problematic in this study, resulting in a single patient being studied. This is an example where mechanistic PBPK modeling could be used to estimate the effect of altered physiology/pathology in situations that are difficult to study. Similarly, scenarios involving the combinations of drug-drug interactions and disease states could be studied.

One the main challenges of mechanistic PBPK modeling of renal elimination is the lack of information on the intrinsic clearance of efflux transporters, such as OAT4, because plasma concentration-time curves are insensitive to this parameter (figure 6). To resolve this shortcoming, the measurement of pemetrexed concentrations in tubular cells or tubular fluid is necessary. A potential surrogate would be urine concentrations if a study design was customized for this purpose and urine is collected quantitatively, at sufficient frequency, and early time intervals, but this is rarely achieved in a clinical study. To date, the successful implementation of this approach is lacking and consequently the quantitation of renal tubular efflux transporter intrinsic clearance will usually be poorly defined.

In conclusion, we demonstrated for the first time that pemetrexed is transported by OAT3 and OAT4, and not by other solute carrier transporters that are involved in active secretion in the kidney. Importantly, we detail how the in vitro transport parameters for OAT3 and OAT, can define the structure of a PBPK model that will predict the renal clearance of pemetrexed within 2-fold of the observed value. Once the structure of the model was defined, we demonstrate for the first time that the decrease in the pemetrexed OAT3-mediated uptake and therefore the renal clearance could be predicted using in vitro inhibition parameters for OAT3. In this translational framework, bottom up PBPK models are a powerful

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tool for the prediction of pharmacokinetics in the presence of transporter-mediated drug interactions and will be enhanced when the accurate quantitation of protein concentration in in vitro and in vivo systems is available. However it is likely the in vitro systems will not completely reproduce the transport rates and affinities that exist in vivo and the middle out parameter optimization approach will be a necessary step in model verification. Ongoing work is designed to determine if the current model paradigm accurately predicts the pharmacokinetics of other OAT substrates and the interactions with inhibitors.

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Authorship Contribution

Participated in research design: Posada, Bacon, Kim, Hall, and Hillgren.

Conducted experiments: Posada, Bacon, Tirona, and Pak.

Performed data analysis: Posada, Bacon, Pak, Higgins, and Schneck.

Wrote or contributed to the writing of the manuscript: Posada, Bacon, Tirona, Hall, and Hillgren,

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Legends for Figures

Figure 1. Results of the *in vitro* screening assay. Expression of drug uptake transporters was done using a recombinant vaccinia system in HeLa cells. Uptake of ^{14}C-pemetrexed, 60 µM (n= 3-6) at 10 min time point. Relative uptake was compared to HeLa cells transfected with a vector lacking transporter cDNA (vector control). Data are presented as mean ± standard deviation (n=3). *** p<0.001

Figure 2. *In vitro* uptake studies. A) The uptake of 3H-Estrone-3-Sulfate (E3S) with or without probenecid, into vector control (VC), OAT3, and OAT4-transfected HEK PEAK cells. Cell cultures were treated with 1 µM [3H] E3S, alone (□), or in combination with 200 µM probenecid (■) for 2 minutes at room temperature. Data are presented as mean ± standard deviation (n =3). B) Time-dependent uptake of pemetrexed (39 µM) in VC, OAT3 and OAT4 transfected HEK-PEAK cells. Experiments were performed at room temperature. Data are presented as mean ± standard deviation (n = 3). C) Uptake of pemetrexed into OAT3- transfected HEK-PEAK cells treated with increasing concentrations of pemetrexed for 5 minutes. Squares represent mean ± standard deviation of the uptake in HEK OAT3, and triangles represent mean ± standard deviation of the uptake in vector control cells. D) Uptake of pemetrexed into OAT4-transfected HEK-PEAK cells treated with increasing concentrations of pemetrexed for 5 minutes. Squares represent mean ± standard deviation of the uptake in HEK OAT4, and triangles represent mean ± standard deviation of the uptake in vector control cells.

Figure 3. Results of the pemetrexed PBPK model simulation. Observed and predicted plasma concentration-time profiles after the administration of a single intravenous infusion of

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500 mg/m^2 of pemetrexed. The points represent the individual measured plasma concentrations of pemetrexed. The solid black line represents the predicted mean concentrations and the thin grey lines represent the 5 and 95% confidence intervals of the prediction.

Figure 4. A) Effect of increasing dose on pemetrexed plasma $AUC_{0\text{-inf}}$. The open circles represent the predicted mean $AUC_{0\text{-inf}}$ and the solid circles represent the observed values. Data are represented as mean and ± standard deviation. B) Effect of increasing dose on pemetrexed plasma C_{max}. The open circles represent the predicted mean plasma C_{max} values and the solid circles represent the observed values. Data are represented as mean and ± standard deviation.

Figure 5. Results of the PBPK simulations for ibuprofen and for interaction between pemetrexed and ibuprofen. A) Predicted plasma concentration-time profiles of ibuprofen after oral administration of multiple doses of 400 mg the NSAID. The thick black line represents the predicted mean concentrations in plasma, and the thin blue lines represent the 5 and 95% confidence intervals of the predictions. B) Predicted pemetrexed plasma concentration-time profiles after a single intravenous 10-minute infusion of 500 mg/m^2 of pemetrexed without (red lines) and with (blue lines) co-administration of ibuprofen in clinical subjects. The thick lines represent the mean values and the thin dotted lines represent the 5 and 95% confidence intervals, respectively. Blue closed diamonds represent the observed pemetrexed concentration-time points when pemetrexed was co-administered with ibuprofen. The red crosses represent the observed concentration-time points when pemetrexed was given alone.

Figure 6. Parameter sensitivity analyses for pemetrexed plasma clearance. A) Effect of the relative expression factors/activities of OAT3 and OAT4 on the plasma clearance of

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pemetrexed. B) Effect of the inhibition constant of Ibuprofen (Ki) OAT3 and OAT4 on the plasma clearance of pemetrexed.

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Tables

Table 1. Physicochemical and biochemical parameters used in the pemetrexed PBPK model.

Property	Value	Source
Compound name	Pemetrexed	
Compound type	Diprotic acid	Shih et al., 1997
Molecular weight	427.4	Shih et al., 1997
Molecular formula	$C_{20}H_{21}N_5O_6$	Shih et al., 1997
Fraction unbound plasma (fu)	0.19	Measured
Blood to plasma ratio (B:P)	0.55	Predicted
cLogP	- 0.797	Predicted chemaxon
pKa 1 (acid)	3.23	Measured
pKa 2 (acid)	4.4	Measured
Dose (mg/m^2)	500	
Route of administration	I.V. infusion	
Infusion time (min)	10	
Cl_{int} passive (mL/min/10^6 cells)	$8.0 * 10^{-6}$	Measured
K_m OAT3 (µM)	18.2	Measured
V_{max} OAT3 (pmol/min/10^6 cells)	185	Measured
$Cl_{int,T}$ OAT4 (µL/min/10^6 cells)	0.99	Measured
REF OAT3	5.3	Fitted

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Table 2. Ibuprofen physicochemical and biochemical parameters used for the PBPK model.

Property	Value	Source
Compound name	Ibuprofen	
Compound type	Monoprotic acid	(Davies, 1998)
Molecular weight	206.3	(Davies, 1998)
Molecular formula	$C_{13}H_{18}O_2$	(Davies, 1998)
Fraction unbound in plasma (fu)	0.012	(Lockwood et al., 1983b)
LogP	4.13	(Avdeef et al., 1999)
Blood to plasma ratio (B:P)	0.55	Default in Simcyp
pKa (acid)	4.42	(Avdeef et al., 1999; Avdeef et al., 2000)
Clearance/F (L/h)	3.84	(Davies, 1998)
Papp (cm/sec)	$52.2 * 10^{-6}$	(Yee, 1997)
Human Peff (cm/sec)	$5.69 * 10^{-4}$	Predicted based on Papp (Yee, 1997)
Fraction unbound in gut (fu_{gut})	1	Default in Simcyp
Intrinsic solubility (mg/mL)	0.049	(Avdeef et al., 1999)
Ki OAT3 (μM)	2.1	Measured
Ki OAT4 (μM)	18.6	Measured value
Fraction unbound incubation (fu_{inc})	0.89	Measured

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Table 3. Characteristics of the population used in the simulation. [a] Estimated from serum creatinine concentration using the Cockcroft-Gault equation (Cockcroft and Gault, 1976).

Property	**Mean (SD)**	**Range**
Age (years)	60.8 (12)	35 - 80
Body Surface area (m^2)	1.89 (0.222)	1.4 - 2.2
Weight (kg)	76.2 (15.8)	49 – 100.2
Creatinine Clearance (mL/min) [a]	115 (31.6)	66.1 - 192.5
% Female	37	NA

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Table 4. IC_{50} values of the inhibition of pemetrexed OAT-mediated uptake by different NSAIDs in OAT3 and OAT4 transfected HEK-PEAK cells. N=3, results are expressed as mean (%CV).

Inhibitor	IC_{50}					
	OAT3 Mean (µM)	OAT3 Mean (µg/mL)	(% CV)	OAT4 Mean (µM)	OAT4 Mean (µg/mL)	(% CV)
Ibuprofen	4.15	0.86	23.5	37.2	7.67	45
Diclofenac	3.7	0.76	21	2.63	0.54	9.4
Naproxen	27.9	5.76	24	39	8.05	10.3
Celecoxib	14.2	2.93	45	21	4.33	18.4

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Table 5. DDI indexes for OAT3 and OAT4 used to rank and predict the risk of the interaction between pemetrexed and different NSAIDs. Cmax values and unbound fractions were taken were taken from Goodman and Gilman's The Pharmacological Basis of Therapeutics (Goodman and Gilman).

Compound	C_{max} (μM)	$[I_u]$ Unbound C_{max} (μM)	DDI Index OAT3 $[I_u]/IC_{50}$	DDI Index OAT4 $[I_u]/IC_{50}$
Ibuprofen	155.1	1.55	0.38	0.042
Diclofenac	6.3	0.03	0.01	0.012
Naproxen	239.7	0.72	0.03	0.018
Celecoxib	1.85	0.06	0.004	0.003

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Table 6.Effect of the addition of OAT3 and OAT4 to the kidney model. Observed values are from Sweeney et al (Sweeney et al., 2006).

Parameter	Observed Value	Predicted		
		Both OAT3 and 4	No OAT3	No OAT4
C_{max} (µg/mL)	102.1	111.6	116.6	111.5
AUC_{0-inf} (µg/mL/h)	164.7	158	618.6	159.2
Cl plasma (L/h)	5.6	5.7	1.48	5.8
Fe_{0-24}	0.8 - 0.9	1	0.7	0.3
Maximum amount of drug in renal cells (mg)	NA	0.01	0.01	667.2

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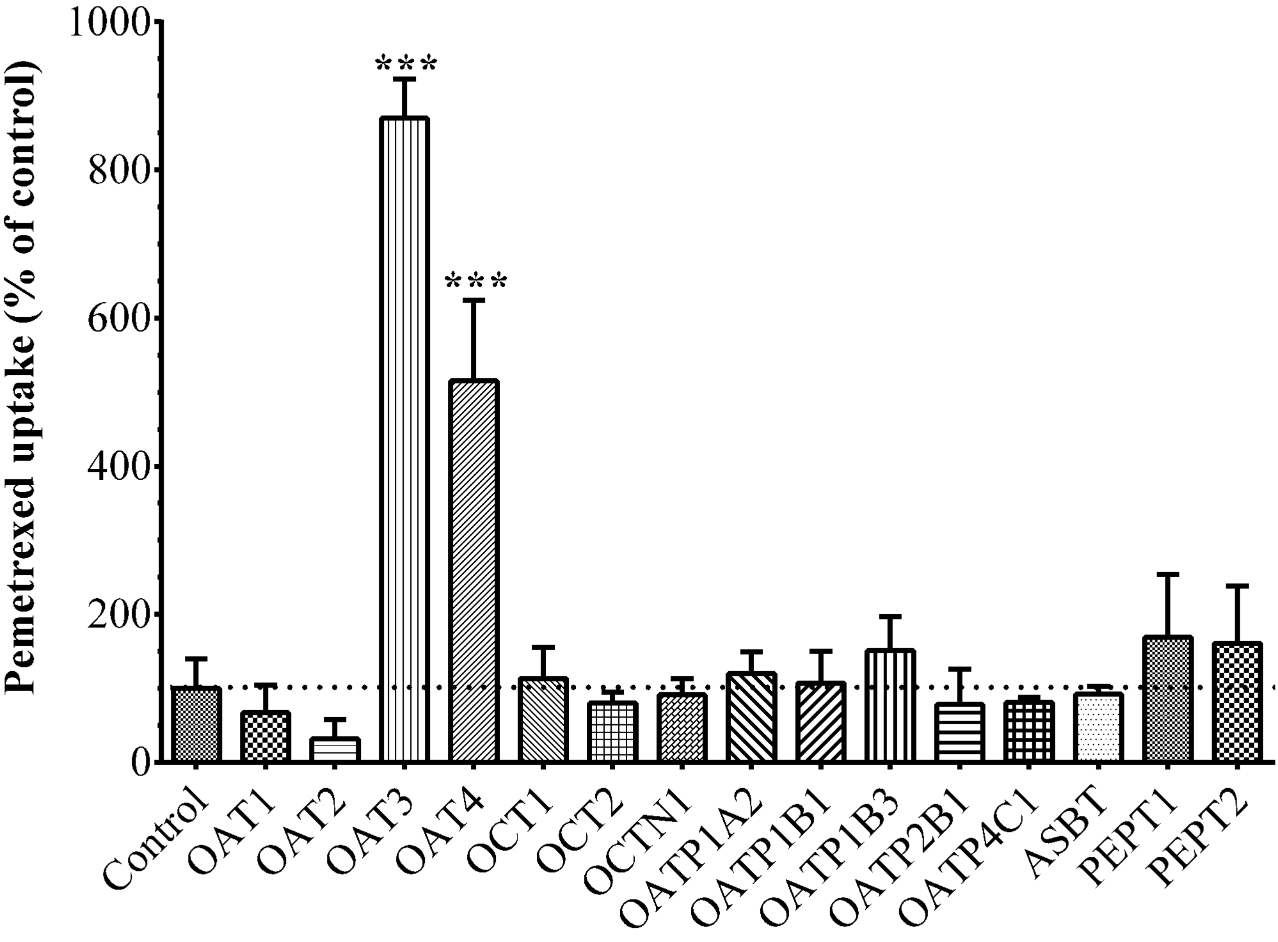
Table 7. Observed and predicted ibuprofen pharmacokinetic parameters at steady state (Aarons et al., 1983a).

Parameter	Observed Value Mean (% CV)	Predicted Value Mean (% CV)	Observed/Predicted
$T_{max,ss}$ (h)	1.3 (14)	1.3 (19.5)	1.05
$C_{max,ss}$ (µg/mL)	29.7 (19)	28.0 (22.7)	1.06
AUC_{ss} (µg/mL/h)	108.1 (12.3)	119.6 (35.3)	0.90

Figure 1



Pemetrexed uptake (% of control)
1000
800
600
400
200
0
***
***
Control
OAT1
OAT2
OAT3
OAT4
OCT1
OCT2
OCTN1
OATP1A2
OATP1B1
OATP1B3
OATP2B1
OATP4C1
ASBT
PEPT1
PEPT2

Figure 2

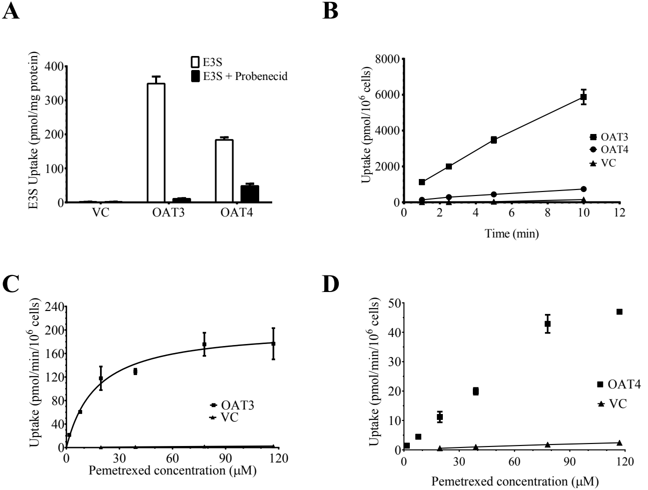
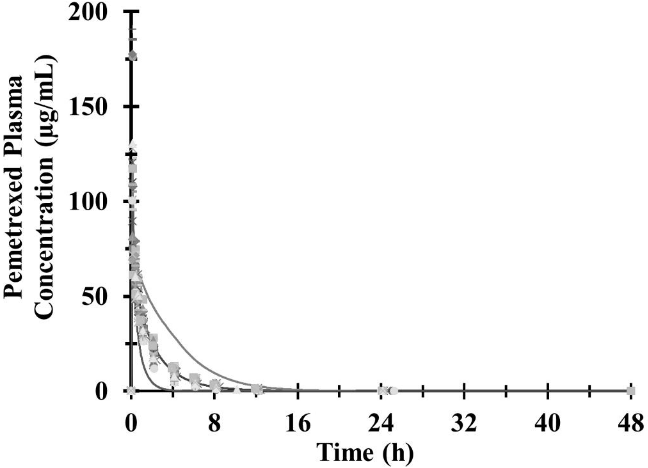
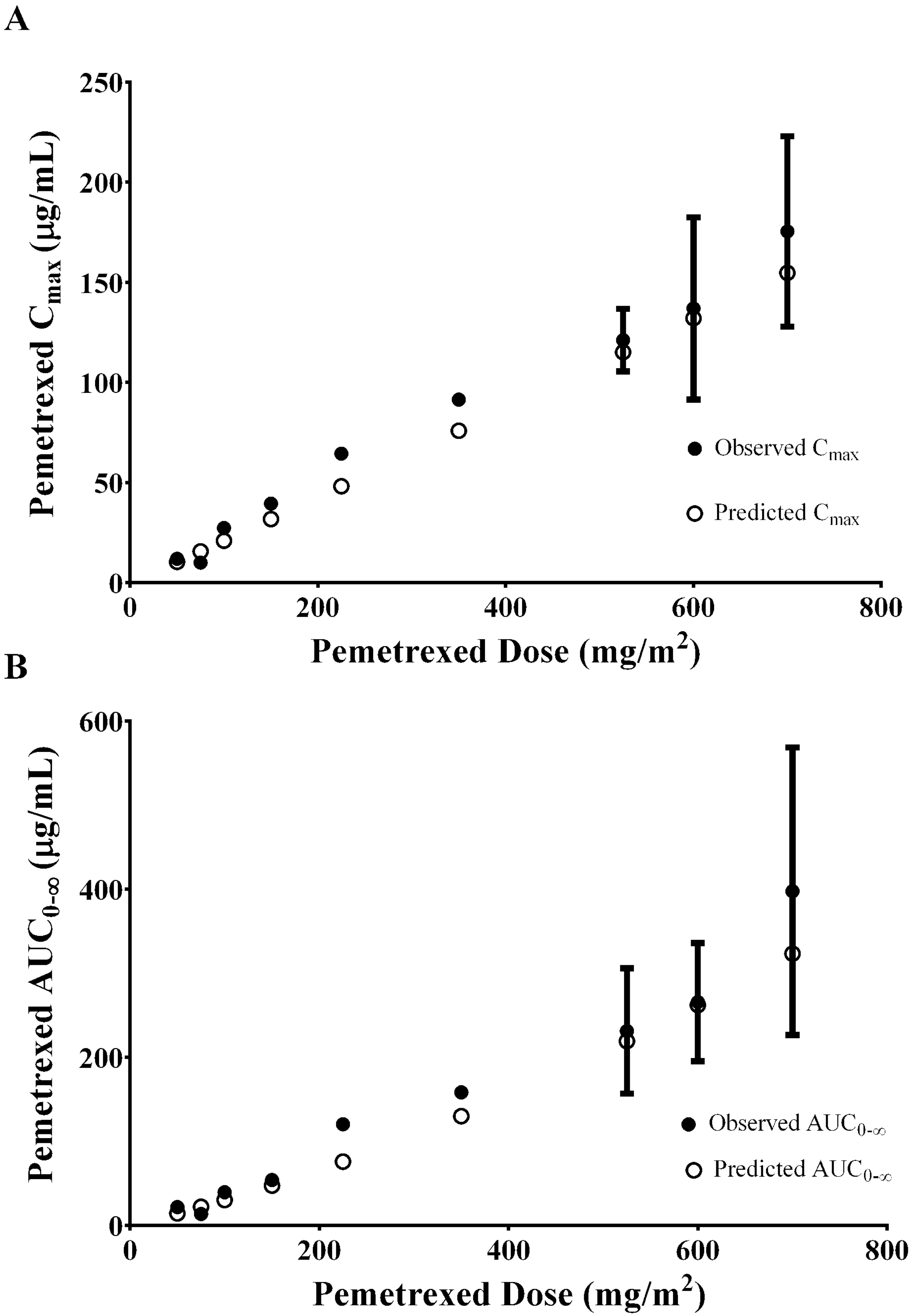

A
B
C
D
E3S Uptake (pmol/mg protein)
400
300
200
100
0
E3S
E3S + Probenecid
VC
OAT3
OAT4
8000
6000
4000
2000
0
Uptake (pmol/10^6 cells)
OAT3
OAT4
VC
0
2
4
6
8
10
12
Time (min)
Uptake (pmol/min/10^6 cells)
240
200
160
120
80
40
0
OAT3
VC
0
30
60
90
120
Pemetrexed concentration (μM)
Uptake (pmol/min/10^6 cells)
50
40
30
20
10
0
OAT4
VC
0
30
60
90
120
Pemetrexed concentration (μM)

Figure 3



200
150
100
50
0
Pemetrexed Plasma Concentration (μg/mL)
0
8
16
24
32
40
48
Time (h)

Figure 4



A
Pemetrexed C_{max} (µg/mL)
250
200
150
100
50
0
0
200
400
600
800
Pemetrexed Dose (mg/m^2)
Observed C_{max}
Predicted C_{max}
B
Pemetrexed $AUC_{0-\infty}$ (µg/mL)
600
400
200
0
0
200
400
600
800
Pemetrexed Dose (mg/m^2)
Observed $AUC_{0-\infty}$
Predicted $AUC_{0-\infty}$

Figure 5

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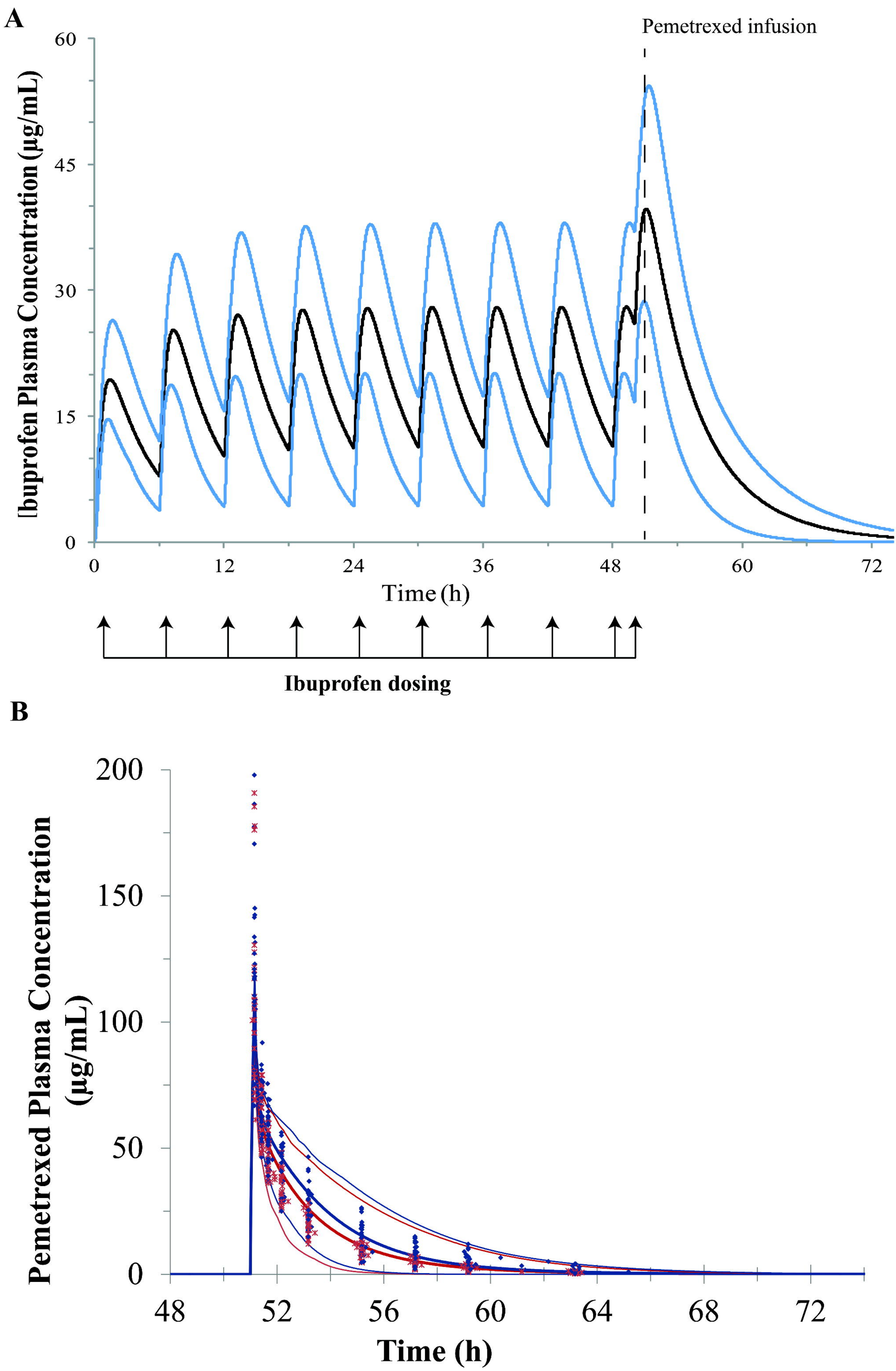
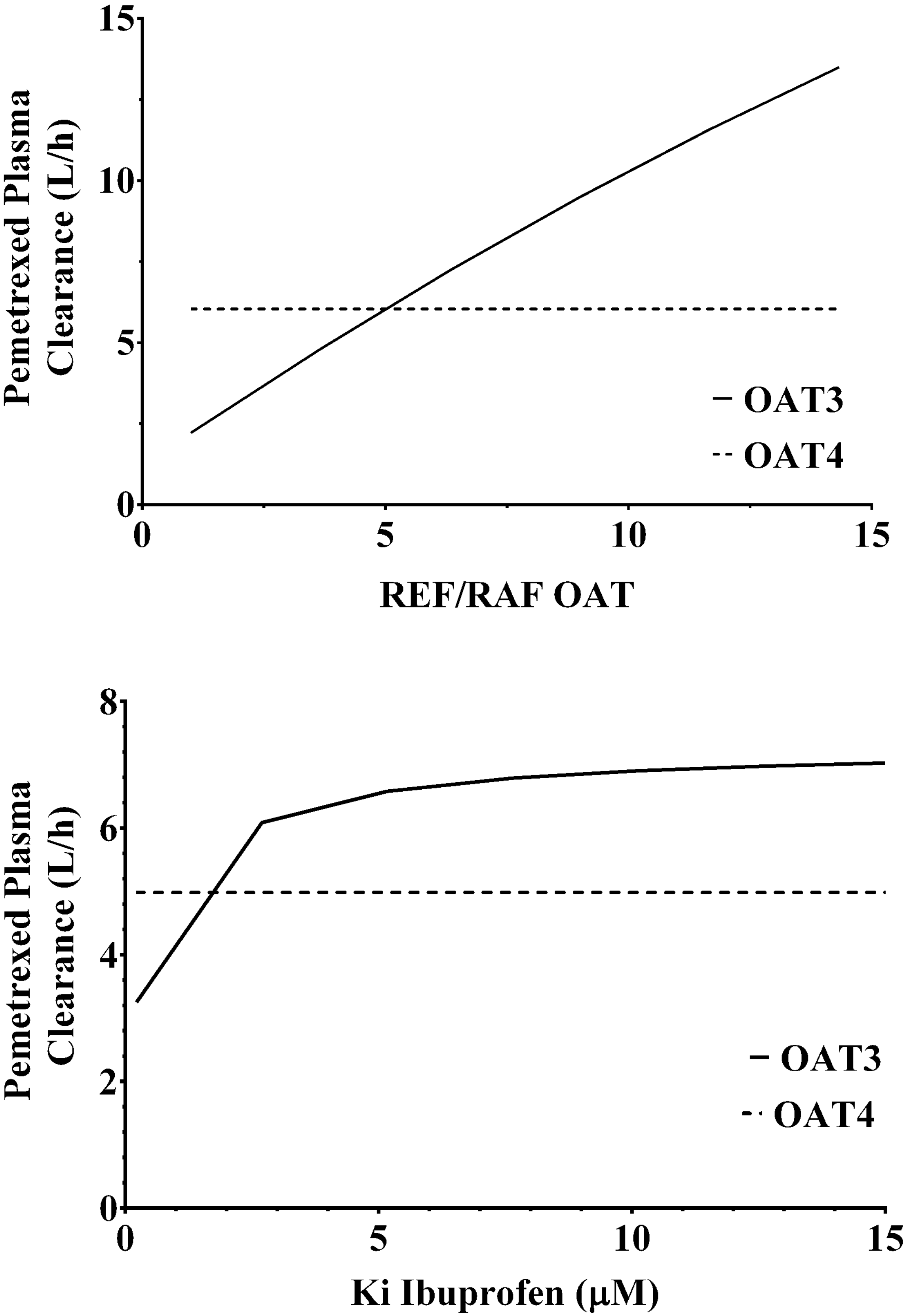


Figure 6



15
10
5
0
Pemetrexed Plasma Clearance (L/h)
OAT3
OAT4
0
5
10
15
REF/RAF OAT
8
6
4
2
0
Pemetrexed Plasma Clearance (L/h)
OAT3
OAT4
0
5
10
15
Ki Ibuprofen (μM)