Preclinical and Clinical Evidence for the Collaborative Transport and Renal Secretion of an Oxazolidinone Antibiotic by Organic Anion Transporter 3 (OAT3/SLC22A8) and Multidrug and Toxin Extrusion Protein 1 (MATE1/SLC47A1)

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ABSTRACT

N-({(5S)-3-[4-(1,1-dioxidothiomorpholin-4-yl)-3,5-difluorophenyl]-2-oxo-1,3-oxazolidin-5-yl}methyl)acetamide (PNU-288034), an oxazolidinone antibiotic, was terminated in phase I clinical development because of insufficient exposure. Analysis of the drug pharmacokinetic and elimination profiles suggested that PNU-288034 undergoes extensive renal secretion in humans. The compound was well absorbed and exhibited approximately linear pharmacokinetics in the oral dose range of 100 to 1000 mg in human. PNU-288034 was metabolically stable in liver microsomes across species, and unchanged drug was cleared in the urine by an apparent active renal secretion process in rat and monkey (two to four times glomerular filtration rate) but not dog. In vitro studies conducted to characterize the transporters involved demonstrated PNU-288034 uptake by human organic anion transporter 3 (OAT3; $K_m = 44 \pm 5~\mu M$) and human multidrug and toxin extrusion protein 1 (hMATE1; $K_m = 340 \pm 55~\mu M$). The compound was also transported by multidrug resistance P-glycoprotein and breast cancer resistance protein. In contrast, human organic cation transporter 2, human OAT1, and hMATE2-K did not transport PNU-288034. Coadministration of PNU-288034 and the OAT3 inhibitor probenecid significantly increased PNU-288034 plasma area under the curve (170%) and reduced both plasma and renal clearance in monkey. Coadministration of PNU-288034 and cimetidine, a MATE1 inhibitor, also reduced plasma clearance in rat to a rate comparable with probenecid coadministration. Collectively, our results demonstrated a strong in vitro–in vivo correlation for active renal secretion coordinated through the vectorial transport process of OAT3 and MATE1, which ultimately resulted in limiting the systemic exposure of PNU-288034.

Introduction

Linezolid (Zyvox) was introduced to clinical practice in 2000 as the first oxazolidinone antibacterial, which led to the successful treatment of serious Gram-positive infections (Koh et al., 2009). Linezolid is rapidly and completely absorbed after oral administration with T_{max} (time to C_{max}) within 2 h. The majority of linezolid (>50%) is metabolized by oxidation of the morpholine ring, and less than 30% of parent drug appears in the urine (Slatter et al., 2001). In an effort to improve the safety, pharmacology, and pharmacokinetics of the next generation of oxazolidinone antibiotics, N-({(5S)-3-[4-(1,1-dioxidothiomorpholin-4-yl)-3,5-difluorophenyl]-2-oxo-1,3-oxazolidin-5-yl}methyl)acetamide (PNU-288034) (Fig. 1) was advanced through preclinical studies. Based on the favorable safety and pharmacology profile, the compound subsequently entered phase I clinical development to assess safety and pharmacokinetics in healthy volunteers. Results from the clinical study demonstrate that PNU-288034 is cleared primarily in the urine as unchanged drug. The renal clearance was approximately 3-fold greater than the glomerular filtration rate (GFR), suggesting

ABBREVIATIONS: PNU-288034, N-({(5S)-3-[4-(1,1-dioxidothiomorpholin-4-yl)-3,5-difluorophenyl]-2-oxo-1,3-oxazolidin-5-yl}methyl)acetamide; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; OCT, organic cation transporter; OAT, organic anion transporter; hOAT, human OAT; MATE, multidrug and toxin extrusion protein; hMATE, human MATE; GFR, glomerular filtration rate; C_{max}, maximum concentration; T_{max}, time to C_{max}; CL, clearance; AUC, area under the curve; DDI, drug–drug interaction; WT, wild type; LC-MS/MS, liquid chromatography/tandem mass spectrometry; ACN, acetonitrile; SVC, superior vena cava; HEK, human embryonic kidney; HBSS, Hanks' balanced salt solution; MEM, minimum essential medium; FBS, fetal bovine serum; MDCK, Madin-Darby canine kidney; P_{app}, apparent permeability; MRT, mean residence time; PNU-100766, (S)-N-[[3-[3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl]-acetamide.
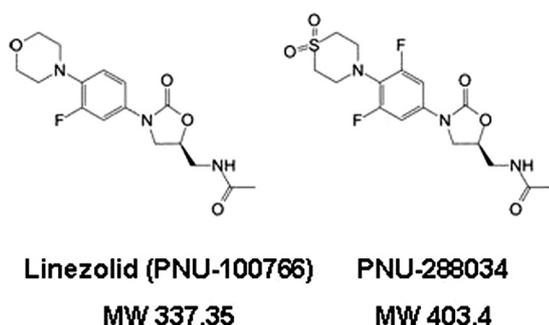
Fig. 1. Chemical structure of linezolid [PNU-100766; (*S*)-*N*-[[3-[3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl]-acet-amide (left) and PNU-288034 (right). MW, molecular weight.

that renal secretion is the major pathway for elimination in humans. Thus, an investigation was undertaken using both in vitro and in vivo approaches to identify the mechanism underlying the high renal clearance.

The kidney plays an important role in the urinary excretion of endogenous substances, xenobiotics, and their water-soluble metabolites. Several processes determine urinary drug excretion including glomerular filtration, tubular secretion, and selective or passive reabsorption. The active renal secretion of drugs is accomplished through a vectorial transport process in renal proximal tubules, which consists of uptake from systemic circulation via the basolateral membrane and subsequent efflux into urine via the luminal membrane by efflux transporters or passive permeability (Dresser et al., 2001; Chu et al., 2007). Various membrane transporters involved in renal secretion have been identified and include organic cation transporters (OCTs) and organic anion transporters (OATs). OAT1, OAT3, and OCT2 are located on the basolateral membrane of renal proximal tubule cells (Hosoyamada et al., 1999; Motohashi et al., 2002) and mediate the uptake of a variety of organic cations or organic anions from systemic circulation (Dresser et al., 2001). Substrates taken up from the systemic circulation may subsequently undergo efflux across the brush-border membrane of proximal tubule cells by various efflux transporters such as the multidrug resistance P-glycoprotein (P-gp) or the breast cancer resistance protein (BCRP) (Deeley et al., 2006). Recently, multidrug and toxin extrusion proteins 1 (MATE1) and 2 (MATE2-K) have been identified in the brush-border membrane of proximal tubular cells in human and preclinical species (Omote et al., 2006) and play an important role in the renal secretion of xenobiotics with highly diverse chemical structures (Tanihara et al., 2007; Ohta et al., 2009). Given that transporters can ultimately alter plasma levels of their drug substrates, it was hypothesized that drug transporters are involved in the mechanism underlying the rapid clearance of PNU-288034. This article describes preclinical and clinical studies that were performed to evaluate the renal clearance of PNU-288034.

Materials and Methods

Chemicals and Reagents. PNU-288034 (Fig. 1) and [³H]PNU-288034 (21 Ci/mmol, 2.0 mCi/ml) were synthesized at Pfizer Global Research and Development (Groton, CT). High-performance liquid chromatography-grade acetonitrile (ACN) and water were purchased from Honeywell Burdick & Jackson (Muskegon, MI) and EMD Chemicals, Inc. (Gibbstown, NJ), respectively. Dulbecco's modified

Eagle's medium, minimum essential medium-α (MEM-α), fetal bovine serum (FBS), nonessential amino acids, penicillin/streptomycin, L-glutamine, geneticin, and Hanks' balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Hygromycin B was purchased from Calbiochem (San Diego, CA).

Single-Dose Clinical Pharmacokinetic Studies. The phase 1 clinical trial was performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects adopted by the 18th World Medical Assembly (1964 and later revisions), the International Conference on Harmonization Guidelines for Good Clinical Practices, and Food and Drug Administration regulations. The Institutional Review Board at the participating investigation center approved the study protocol, and all participants were given full and adequate verbal and written information regarding the objective and procedures of the trial and the possible risks involved before inclusion in the trial.

The study was a randomized, double-blind, placebo-controlled, single-dose escalation in healthy male and female volunteers. Subjects who signed an informed consent form to participate in the study underwent prestudy screening within 4 weeks of the study start date. Volunteers with a history or evidence of clinically significant disease or conditions potentially affecting absorption, distribution, metabolism, or excretion of the drug were excluded. The exclusion criteria included: 1) clinically relevant abnormal findings at the screening physical examination, specifically prolonged QTc interval (>440 ms) and/or abnormal safety laboratory tests; 2) history or evidence of idiopathic hematuria; 3) administration of any investigational medication or medications capable of inducing hepatic enzyme metabolism within 30 days or any prescription or nonprescription medication and/or herbal preparations within 7 days or five half-lives (whichever was longer); 4) history of substance abuse within 1 year before the start of this study; 5) major surgery within 6 months of the start of the study; and 6) inherited or acquired long QT syndrome.

Six groups of subjects (*n* = 8 subjects per group) received a single oral dose of either placebo (*n* = 2) or PNU-288034 (*n* = 6) capsules in an escalating dose design. Pharmacokinetic samples for quantitation of PNU-288034 in plasma were obtained over 48 h after the single-dose administration of PNU-288034 or placebo. Urine was collected at designated intervals over a 24-h period before dosing and over a 48-h period after dosing. Dosing did not proceed to the next higher dosing group until pharmacokinetics, safety, and tolerability were assessed by the investigator and study management. Blood was collected via direct venipuncture or indwelling catheter. Blood samples were collected before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, and 48 h after single-dose administration of PNU-288034. Blood was collected into EDTA vacutainers and centrifuged at 3000 rpm for 10 min, and the plasma was transferred to sample vials. Urine samples were collected over 24 h before dosing (for creatinine clearance determination) and over 48 h after dose at the following intervals: 0 to 4, 4 to 8, 8 to 12, 12 to 24, and 24 to 48 h (for determination of both creatinine clearance and PNU-288034 clearance). All voided urine was measured for volume. Plasma and urine were stored at −20°C until analysis by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

Pharmacokinetics of PNU-288034 in Mouse, Rat, and Dog. The Pharmacia and Pfizer Institutional Animal Care and Use Committees reviewed and approved the animal use in these studies. These animal care and use programs are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Female CF-1 HSD mice, male Sprague-Dawley rats, and male purebred beagle dogs were used for preclinical pharmacokinetic assessments. All animals were fasted overnight before dosing the next morning, and food was withheld for 4 h after dosing. Water was allowed ad libitum throughout the studies. For pharmacokinetic assessments in female CF-1 HSD mice, intravenous doses were administered in either of the two lateral tail veins by using a 27-gauge, ½-inch needle and a 1-ml syringe (BD Bioscience, San

Jose, CA). Oral doses for mice were administered with a 20-gauge stainless-steel intubation tube and a 1-ml syringe. Blood samples from mice were obtained by decapitation. Male Sprague-Dawley rats (200–300 g) were surgically implanted with a cannula in the superior vena cava (SVC) via the jugular vein and in the femoral vein under light aesthesia (Durapen 0.1 ml/rat) 4 days before the study. During the study the animals were kept in individual metabolism cages and wore collars that held the infusion tubing. Rats were dosed with PNU-288034 through the SVC catheter or oral gavage. Blood samples (0.25 ml) were drawn by syringe via the SVC. Male beagle dogs were administered a single intravenous dose of PNU-288034 into the saphenous vein. After a washout period (7 days), the same animals received an oral solution dose of PNU-288034 administered by gavage tube. Blood samples were drawn by syringe using the saphenous vein. For each study, blood samples were collected at intervals from 3 min to 24 h after dose and centrifuged to obtain plasma. The urine of intravenously dosed rats and dogs was also collected up to 24 h. The plasma and urine samples were stored at −80°C until LC-MS/MS analysis.

Metabolic Stability Assessment in Liver Microsomes from Rat, Dog, Monkey, and Human. The metabolic stability of PNU-288034 was examined in discrete experiments using pooled liver microsomes from rat, dog, monkey, or human (BD Bioscience). Assays were performed in a total incubation volume of 0.2 ml containing 100 mM potassium phosphate (pH 7.4), 3.3 mM magnesium chloride, 0.5 mg/ml liver microsomal protein, and 5 μM substrate. After a 5-min preincubation at 37°C, the reactions were initiated by the addition of 2 mM NADPH. After 30 min of incubation, the reactions were sequentially stopped by the addition of cold ACN (including internal standard). After centrifugation at 5000g for 5 min, supernatants were transferred to 96-well assay plates for analysis of parent compound by LC-MS/MS.

Cell Culture and in Vitro Transporter-Mediated Uptake or Efflux Assays. Caco-2 cell permeability was used for the estimation of absorption of PNU-288034, conducted according to a previously reported method (Lai et al., 2007). To evaluate the involvement of drug transporters in the renal secretion of PNU-288034, control and human renal transporter gene-transfected HEK293 cell lines (Feng et al., 2008; Matsushima et al., 2009) were used to conduct the drug uptake assays. HEK cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS (nondialyzed), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and either 50 μg/ml hygromycin B (hOAT1, hOAT3, and hOCT2) or 200 μg/ml geneticin (hMATE1 and hMATE2-K). The assays were carried out in 96-well poly-D-lysine-coated plates (BD Bioscience) with cells seeded at 300,000 cells/cm^2 and grown for 24 h before each experiment. The OAT- and OCT-transfected cells were washed and preincubated with 100 μl of HBSS for 15 min followed by incubation with 100 μl of HBSS buffer containing 10 μM PNU-288034 without or with inhibitors at 37°C for 1 min. For experiments with MATE transporters, cells were washed and preincubated with 100 μl of uptake buffer (145 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5 mM glucose, 5 mM HEPES, pH 7.4) either alone or with 30 mM ammonium chloride (for stimulation of uptake through intracellular acidification) for 20 min, followed by incubation with 100 μl of uptake buffer containing 10 μM PNU-288034 without or with inhibitors at 37°C for 2 min. Cellular uptake was terminated by washing the cells three times with 100 μl of ice-cold incubation buffer. The cells were lysed directly on the plate by using a 50% methanol solution containing the internal standard (50 nM carbamazepine). After centrifugation the supernatant and PNU-288034 standards were subjected to LC-MS/MS analysis as described below. The protein concentration was determined with the Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) with bovine serum albumin as a standard.

Concentration-dependent uptake was also characterized in HEK-hOAT3 cells (5–160 μM PNU-288034) and HEK-hMATE1 cells (6–400 μM PNU-288034) to determine the kinetics of drug transport.

The incubations were performed as described above and terminated after 1 min, which was found to be an optimal time point for initial rate determination for both transporters. The transporter-mediated uptake was obtained by subtracting the uptake velocity in control cells from that in transfected cells. Kinetic parameters were determined by nonlinear regression analysis (fit to the Michaelis-Menten equation) performed by using Prism version 5.01 for Windows (GraphPad Software Inc., San Diego, CA).

The involvement of P-gp and BCRP were examined in MDCK gene-transfected cells. The MDCK-wild-type (WT), cells MDCK-MDR1 cells (P. Borst, Netherlands Cancer Institute, Amsterdam, The Netherlands), and MDCK-BCRP cells (Xiao et al., 2006) were plated onto 24-well insert plates (BD Biosciences) at 200,000 cells/cm^2 and grown for 4 days in MEM-α containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.1 mM MEM nonessential amino acids. On the day of the study, the cell monolayers were rinsed twice with buffer B (HBSS containing 25 mM D-glucose and 20 mM HEPES, pH 7.4) and preincubated for 30 min at 37°C. Buffer B containing 2 μM PNU-288034 was applied to apical or basal (donor) chambers to initiate the transport. The incubation was performed for 2 h at 37°C. Aliquots were removed from both donor and receiver chambers at the end of the incubation and mixed with one volume of ACN containing internal standard. The concentration of PNU-288034 was determined by LC-MS/MS. The apparent permeability (P_{app}, cm/s) was determined for both A-to-B and B-to-A directions, and the efflux ratio was calculated from the $P_{app(B\ to\ A)}/P_{app(A\ to\ B)}$ ratio. To eliminate the potential contribution from endogenous canine transport proteins in the MDCK cells, a ratio of ratios, [transfected cell ratio]/[MDCK-WT ratio] was used to determine the involvement of P-gp or BCRP (Xiao et al., 2006).

Distribution and Clearance of PNU-288034 in Oat3 Gene Knockout Mice. Oat3 gene knockout mice [Oat3(−/−)] were generated at Pfizer Global Research and Development after microinjection of *slc22a8*-targeted embryonic stem cells (Deltagen Inc., San Carlos, CA) into C57BL/6 blastocyst-stage embryos (Charles River Laboratories Inc., Wilmington MA) and subsequent breeding using previously described methods (Zaher et al., 2008). The homozygous knockout mice and WT age-matched C57BL mice ($n = 4$/group) were used to investigate the impact of Oat3 deficiency on PNU-288034 exposure. Mice were allowed food and water ad libitum. PNU-288034 was formulated in 10% 2-hydroxypropyl-β-cyclodextrin to a 1 mg/ml concentration. Dosing solutions were sonicated 5 to 10 min and transferred to Alzet 2001D pumps and surgically inserted under the skin in male WT and Oat3(−/−) mice. The Alzet pumps delivered compound at 8 μl/h subcutaneously. Animals were sacrificed 24 h after pump insertion, and blood samples and kidneys were collected for bioanalysis. Blood was transferred to EDTA tubes and centrifuged, and plasma was transferred to sample plates. Kidneys were homogenized with five volumes of water. Plasma and kidney homogenate samples were processed for LC-MS/MS analysis by protein precipitation with a 2-fold excess of ACN containing the internal standard (linezolid). After centrifugation, samples were diluted 1:1 with water in a fresh sample plate and subjected to LC-MS/MS analysis.

Chemical Inhibition of PNU-288034 Clearance in Rat and Monkey. To investigate the impact of renal transporter inhibition on PNU-288034 clearance, studies were conducted in rat and monkey with and without codosed inhibitors. A continual infusion of [^{14}C]inulin was administered to four adult male Sprague-Dawley rats via the femoral vein for 0 to 12 h at a rate of 0.2 μCi/9.6 μl/min. At 4 h, [^{3}H]PNU-288034 was added to the infusion at the same rate, along with a loading dose of 2.17 μCi/kg via the SVC. Probenecid or cimetidine were administered to two of the rats as an intravenous bolus at 8 h via the SVC at 70 or 50 mg/kg, respectively, and dosed again five times at 40 mg/kg over the 8- to 12-h time period. Blood samples (0.25 ml) were collected at various time points throughout the study via the SVC and immediately transferred into EDTA

polypropylene centrifuge tubes and centrifuged, and the plasma was harvested. Radioactivity of inulin and PNU-288034 was determined by a conventional double isotope liquid scintillation method (Packard TR2300; PerkinElmer Life and Analytical Sciences , Waltham, MA). Plasma clearance (CL_p) was determined from plasma samples collected before and during treatment with inhibitors such that each rat served as its own control. The plasma clearance of inulin in rats was taken as equal to the GFR. Similar renal transporter inhibition studies were also conducted in male cynomolgus monkeys. At day 1, the single intravenous dose pharmacokinetics of PNU-288034 at 10 mg/kg was determined for three monkeys. After a 2-week washout period, probenecid was orally administered to each animal every 6 h at 30 mg/kg starting 24 h before the intravenous dose of 10 mg/kg PNU-288034. At designated time points, blood was collected via an indwelling catheter, and the plasma was subjected to LC-MS/MS analysis.

LC-MS/MS Analysis for PNU-288034. Quantitation of PNU-288034 was conducted by LC-MS/MS analysis using either an API-3000 or API-4000 triple quadrupole mass spectrometer with an atmospheric pressure electrospray ionization source (MDS Sciex, Concord, ON, Canada) and two LC-10ADvp pumps with a SCL-10ADvp controller (Shimadzu, Columbia, MD). A 5- or 10-μl sample was injected onto a Aquasil-C18 column (2.1×30 mm, 3.0 μ; Thermo Fisher Scientific) and eluted by a mobile phase with initial conditions of 5% solvent B for 0.5 min, followed by a gradient of 5% solvent B to 95% solvent B over 2 min (solvent A: 100% H_2O with 0.1% formic acid; solvent B: 100% ACN with 0.1% formic acid), 95% solvent B held for 0.5 min, followed by an immediate return to initial conditions maintained for 0.5 min, at a flow rate of 0.4 ml/min. Using the positive ionization mode (5.5-kV spray voltage), mass spectral analyses were performed by using multiple reaction monitoring for the PNU-288034 transition of m/z 404.1 to 362.0, with a source temperature of 500°C, declustering potential of 96 eV, entrance potential of 10 eV, and collision energy of 27 eV. The peak areas of the analyte and internal standard were quantified by using Analyst 1.4.2 (MDS Sciex). The lower limit of quantitation was reported as 1.7, 4.88, 8.2, and 10 ng/ml in the CF-1 HSD mouse, Oat3(−/−) mouse, monkey, and human sample analyses, respectively.

Statistical Analysis. Statistical differences were determined by using the unpaired or paired Student's t test. Differences were considered significant at the level of $p \leq 0.05$.

Pharmacokinetic Calculations. Concentration–time profiles were analyzed by using standard noncompartmental methods by Watson LIMS (version 6.4.0.02; IrmaPhase Corporation, Philadelphia, PA) or WinNonlin v3.2 (Pharsight, Mountain View, CA). Maximum concentration (C_{max}) and T_{max} were derived directly from the observed concentrations. Areas under the concentration–time curves from time 0 to infinity ($AUC_{0-\infty}$) and from time 0 to the last time (T_{last}) with a quantifiable concentration, AUC_{last}, were calculated by using the linear-log trapezoidal rule. The apparent terminal phase half-life ($t_{1/2}$) of PNU-288034 was calculated as $\ln(2)/\lambda_z$, where λ_z is the apparent terminal rate constant estimated from the terminal log-linear phase. The fraction of the PNU-288034 dose recovered in urine was obtained by dividing drug in urine by dose. In the human study, renal clearance (CL_r) was estimated as total amount recovered in urine divided by AUC_{last}. In preclinical studies, CL_r was estimated from the equation: CL_r = [amount in urine/intravenous dose] $\times$ CL_p. Creatinine clearance, as the indicator of GFR and hence renal function in human, was calculated from the urinary excretion rate of creatinine (mg/h) divided by the serum creatinine concentration sampled the same day of the urine collection period. In the rat transporter inhibition study, the clearance of inulin was set as renal GFR.

Results

In Vitro ADME Properties of PNU-288034. Several in vitro studies were conducted to estimate the absorption and metabolism of PNU-288034 in both preclinical species and

humans. Drug absorption was assessed by using the well characterized Caco-2 cell model. Based on these results, PNU-288034 was classified as having low to moderate permeability with Caco-2 apical to basolateral permeability of 2.7×10^{-6} cm/s and basolateral to apical permeability of 8.75×10^{-6} cm/s (efflux ratio = 3.3). To evaluate the contribution of hepatic metabolism to overall drug clearance, 5 μM PNU-288034 was incubated with liver microsomes from rat, dog, monkey, and human. The percentage of parent PNU-288034 remaining was measured at 30 min after incubation. The metabolism-dependent disappearance of PNU-288034 was found to be negligible during the 30-min incubation with liver microsomes from all species tested (data not shown). These results suggested that the compound would not be significantly cleared by hepatic P450-dependent metabolism.

Human Pharmacokinetics. At all oral doses of a hand-filled capsule formulation, PNU-288034 was found to be relatively well absorbed (>64%) with a T_{max} of 2 h (Fig. 2A). The
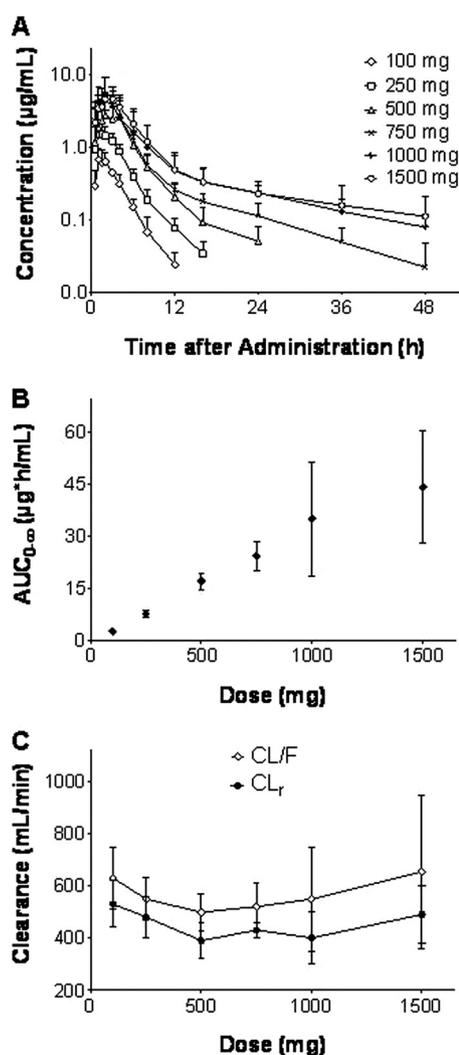

Fig. 2. Human pharmacokinetics after the single-dose administration of PNU-288034. Six groups of healthy volunteers received a single oral dose of PNU-288034 capsules in an escalating fashion from 100 to 1500 mg, and blood samples were taken at specified intervals for 48 h after dose. Concentrations of PNU-288034 in plasma were quantitated by using LC-MS/MS. Pharmacokinetic parameters (AUC and CL) were calculated as described under *Materials and Methods*. Values are mean ± S.D., n = 6/dose. A, concentration-time profile. B, changes in AUC with increased dose. C, comparison of CL/F and CL_r.

TABLE 1

Mean (± S.D.) pharmacokinetic parameters of PNU-288034 in humans after oral administration to six healthy volunteers per dose level

Plasma and urine samples were collected over a 48-h period and analyzed by LC-MS/MS. Pharmacokinetic parameters were derived from concentration vs. time profiles as described under *Materials and Methods*.

Dose	C_{max}	CL_r	$AUC_{0-\infty}$	Unchanged in Urine
mg	*µg/ml*	*ml/min*	*µg × h/ml*	*%*
100	0.8 ± 0.2	530 ± 90	2.7 ± 0.5	93 ± 2
250	2.0 ± 0.4	480 ± 80	7.7 ± 1.1	87 ± 3
500	3.9 ± 1.2	390 ± 70	17.1 ± 2.4	78 ± 9
750	4.7 ± 0.8	430 ± 30	24.4 ± 4.1	81 ± 10
1000	6.4 ± 3.0	400 ± 100	35.0 ± 16.3	70 ± 15
1500	6.0 ± 1.4	490 ± 110	44.2 ± 16.3	64 ± 13

$AUC_{0-\infty}$ and C_{max} were approximately dose proportional over the dose range of 100 to 1000 mg (Fig. 2B; Table 1). The urinary recovery of unchanged compound was decreased at higher doses, but was never less than 64% (Table 1). Oral bioavailability (F) was not assessed during the clinical trials; however, high renal clearance (CL_r >73% of CL/F), ranging from 390 to 530 ml/min (approximately three to four times GFR), was observed in all dose groups (Fig. 2C; Table 1), thereby suggesting renal secretion is the major route for PNU-288034 elimination.

Pharmacokinetics in Preclinical Species. The intravenous and oral pharmacokinetics of PNU-288034 were evaluated in mouse, rat, and dog, whereas the monkey intravenous data were obtained from a control group in the chemical inhibition study. As shown in Table 2, plasma clearance in mouse was 32.9 ml/min/kg with a volume of distribution (V_{ss}) of 1.36 l/kg and mean residence time (MRT) of 0.69 h. Intravenous plasma clearance in rat, dog, and monkey was 18.9, 5.58, and 6.55 ml/min/kg, respectively, with a distributional volume ranging from 0.57 to 1.3 l/kg. After oral administration of PNU-288034, rapid absorption was observed (T_{max} of 0.17 to 0.5 h) in the mouse, rat, and dog with high oral bioavailability ranging from 73 to 96%, which is consistent with the observations in humans. More than 73% of unchanged compound was recovered in the urine of rat and dog, whereas only 42% was recovered in monkey (Table 2). The ratio of CL_r/GFR is shown in Fig. 3. The renal clearance values were between 2- and 4-fold over GFR in rat, monkey, and human, suggesting active tubular secretion of the compound. Although the unchanged PNU-288034 was predominantly eliminated in the urine in dog (95% in intravenous cases and 88.7% in oral cases), the ratio of CL_r/GFR was close to 1, suggesting that dog does not display this apparent active renal secretion phenomenon (Fig. 3). The results of the

pharmacokinetic linearity experiments for PNU-288034 in rat are given in Table 3. The $AUC_{0-\infty}$ and C_{max} increased proportionally with increasing dose, with the recovery of unchanged compound in rat urine declining to 50.8% at the highest dose tested (107 mg/kg).

In Vitro Renal Transport of PNU-288034. The uptake of PNU-288034 was examined in human OAT1, OAT3, OCT2, MATE1, and MATE2-K gene-transfected HEK293 cells. As shown in Fig. 4, uptake was significantly increased in HEK-hOAT3 cells compared with that in control cells, suggesting that PNU-288034 is a substrate of OAT3, but not a substrate of OAT1 or OCT2. Moreover, the OAT3-mediated PNU-288034 uptake was significantly inhibited by 10 µM probenecid. The uptake of PNU-288034 in HEK-hOAT3 cells was further characterized and found to be saturable with a K_m = 44 ± 5 µM (Fig. 5A). Transport by hMATE1 and hMATE2-K was also examined in an in vitro setting. Uptake assays conducted in the presence of an oppositely directed H⁺-gradient demonstrated PNU-288034 is a substrate of hMATE1, but not hMATE2-K (Fig. 4), with a K_m of 340 ± 55 µM (Fig. 5B).

The P-gp- and BCRP-mediated efflux was confirmed in bidirectional transport assays using human MDR1 or BCRP gene-transfected MDCK cell lines. The ratio of efflux ratios in MDCK-MDR1 and MDCK-BCRP cells over that in MDCK-WT cells was 2.49 ± 0.41 and 3.27 ± 0.39, respectively, suggesting that PNU-288034 is a P-gp and BCRP substrate (Table 4).

PNU-288034 Plasma Concentration Assessments with Oat3-Gene Knockout Mice. Given that PNU-288034 is a hOAT3 substrate in in vitro assays, this renal transporter could contribute significantly to the active tubular secretion across species. To elucidate the impact of drug transport on substrate disposition in vivo, Oat3 knockout [Oat3(−/−)] and WT mice were pump-infused with 1 mg/ml PNU-288034 at 8 µl/h, and the plasma concentration was evaluated upon termination of the infusion. As shown in Fig. 6, the plasma concentration of PNU-288034 in Oat3(−/−) mice was increased to 160% of the concentration in WT mice ($p < 0.05$), suggesting that the Oat3 function deficiency in the knockout mice reduces active renal secretion of PNU-288034. The disposition of PNU-288034 in the kidney of pump-infused mice was also investigated. An accumulation of PNU-288034 in kidney tissue was not observed in Oat3(−/−) mice, compared with that in WT mice (data not shown).

Pharmacokinetics of PNU-288034 Coadministered with Probenecid in Monkey and Probenecid or Cimetidine in Rat. The effect of renal transporter inhibition on
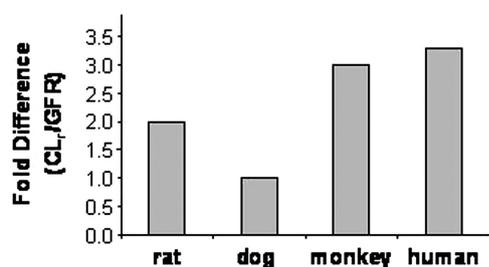
TABLE 2

Pharmacokinetic parameters of PNU-288034 in mouse, rat, dog, and monkey

Plasma and urine samples were collected, and concentration-time curves were determined. Pharmacokinetic parameters were derived as described under *Materials and Methods*. Absolute oral bioavailability (F) was calculated by using dose and AUC values.

	Route	Dose	C_{max}	T_{max}	$t_{1/2}$	MRT	V_{ss}	CL	Unchanged in Urine	F
		mg/kg	*µg/ml*	*h*	*h*	*h*	*l/kg*	*ml/min/kg*	*%*	*%*
CF-1 mouse	IV	4	5.31 ± 0.1		0.76	0.69	1.36	32.9	ND	
	PO	4	2.49 ± 0.1	0.17	0.92	1.1	—	—	ND	96
Sprague-Dawley rat	IV	9.6	19.9 ± 1.5		8.05 ± 0.00	1.15 ± 0.09	1.30 ± 0.26	18.9 ± 2.4	81.9 ± 0.2	
	PO	21.3	9.10 ± 2.0	0.50	5.90 ± 1.4	2.63 ± 0.26	—	—	73.2 ± 6.4	73.3 ± 9.3
Beagle dog	IV	9.7	49.5 ± 2.0		6.51 ± 0.28	1.72 ± 0.00	0.57 ± 0.02	5.58 ± 0.23	95.0 ± 16	
	PO	10	7.60 ± 1.4	0.50	2.70 ± 1.8	2.29 ± 0.18	—	—	88.7 ± 5.7	72.9 ± 6.9
Monkey	IV	10	35.7 ± 8.8		5.49 ± 2.05	2.18 ± 0.29	0.855 ± 0.089	6.55 ± 0.40	42.0 ± 16.7	

IV, intravenous; PO, oral; ND, not determined.

Fig. 3. Interspecies comparison of the CL_r/GFR ratio in rat, dog, monkey, and human after intravenous (preclinical species) or oral (human) administration of PNU-288034. GFR values for preclinical species were determined experimentally by using creatinine or inulin clearance as described under *Materials and Methods*. Dose and numeric clearance values are shown in Table 2 with coefficients of variation of 12.6, 4.1, and 6.1% for rat, dog, and monkey, respectively. The coefficients of variation for human renal clearance measurements (Table 1) were 7 to 25% for the dose range of 100 to 1500 mg.

the disposition of PNU-288034 in monkeys coadministered probenecid and rats coadministered probenecid or cimetidine were also investigated. Probenecid, an Oat inhibitor, was used to chemically inhibit Oat-mediated renal transport in monkey and rat, whereas cimetidine was used to chemically inhibit Oct-mediated renal transport in rat. The administration of probenecid significantly decreased the renal clearance of PNU-288034 and increased the plasma exposure in monkey (Fig. 7). The total plasma $AUC_{0-\infty}$ was increased from 15.2 ± 5.5 to 26.1 ± 1.9 μg × h/ml ($p < 0.05$), whereas the plasma clearance was decreased from 11.8 ± 3.7 to 6.55 ± 0.40 ml/min/kg ($p < 0.05$), and the renal clearance was reduced by approximately 50%, from 5.63 ± 4.32 to 2.78 ± 1.18 ml/min/kg. The results from the rat transporter inhibition study are consistent with the observations in Oat3 gene knockout mice, because coadministration of probenecid was found to decrease the plasma clearance of PNU-288034 (Fig. 8). Unexpectedly, coadministration of cimetidine also decreased plasma and renal clearance of PNU-288034 to the same extent as probenecid. Given the results with HEK-hMATE1-transfected cells, this observation may reflect that inhibition of Mate1 can also dramatically reduce the renal secretion of PNU-288034. In total, the in vivo findings regarding these renal drug–drug interactions (DDIs) were found to correlate well with the in vitro assessment of transporter involvement described above.

Discussion

Urinary elimination is an important clearance pathway for several therapeutics (Lee and Kim, 2004; Wright and Dantzler, 2004). The extent of renal clearance is the net result of glomerular filtration, tubular secretion, and reabsorption. Clearance by glomerular filtration equals the product of GFR

(1.8 ml/min/kg in adult healthy men) and plasma free fraction. Renal secretion in proximal tubular cells is attributed mainly to renal transporters, and thus depends on the transporter kinetics, plasma free fraction, and blood flow rate (Li et al., 2006). In the present study the net secretion (CL_r/GFR) of PNU-288034 was evident because the renal clearance was larger than GFR × free fraction, too high for the compound to be considered for further clinical development. Although renal clearance in human has been predicted successfully by using physiologically based allometric procedures, the mechanism of clearance may complicate the interspecies scaling (Dedrick et al., 1970; Mahmood, 1998). In this study renal secretion in human was approximately 2-fold higher than that observed in rat (Fig. 3), indicating that the total and renal clearances predicted from rat data underestimated the values in human. Given that the high renal clearance limited the clinical development of PNU-288034, these results illustrate how transporter-mediated renal secretion can complicate extrapolation from preclinical to clinical studies and obscure the accurate prediction of human pharmacokinetics.

To elucidate the uptake transporters potentially governing the active renal secretion of PNU-288034 in human and preclinical species, we conducted in vitro uptake assays with transporter gene-transfected cell lines. The results demonstrate that PNU-288034 is a substrate of OAT3 and suggest that OAT3 is responsible for the uptake of the compound at the basolateral membrane of proximal tubule cells, thereby mediating the first step of active renal secretion. In addition, as a confirmed P-gp, BCRP, and MATE1 substrate in vitro, PNU-288034 could undergo efflux across the luminal membrane of proximal cells into the urine. This collaborative process ultimately would result in the vectorial transport of PNU-288034, leading to active urinary secretion. In total, the correlation between the transporters identified in the uptake assays and the increase in PNU-288034 plasma concentrations observed in Oat3(−/−) mice emphasize the utility of in vitro cellular uptake studies with respect to providing predictive information regarding the in vivo mechanism of active renal secretion.

The combined use of drugs that inhibit transporter function is a common approach for investigating the effects of transporters on drug disposition. In addition, the involvement of renal transporter-mediated DDI has attracted increasing interest (Feng et al., 2008; Yuan et al., 2009), because anions/cations that use the same secretory transport systems are prone to drug interactions with subsequent modification of pharmacokinetics/pharmacodynamics caused by impaired drug elimination. Therefore, identification of the specific renal transporters involved could provide support in understanding the molecular mechanisms of tubular secretion. For example, the inhibitory effect on OAT3-mediated

TABLE 3

Single-dose pharmacokinetic proportionality study of PNU-288034 in rats

Indicated doses were administered to rats by oral gavage. Plasma was collected to 24 h after dose and analyzed by LC-MS/MS. Pharmacokinetic parameters were calculated from concentration-time curves as described under *Materials and Methods*.

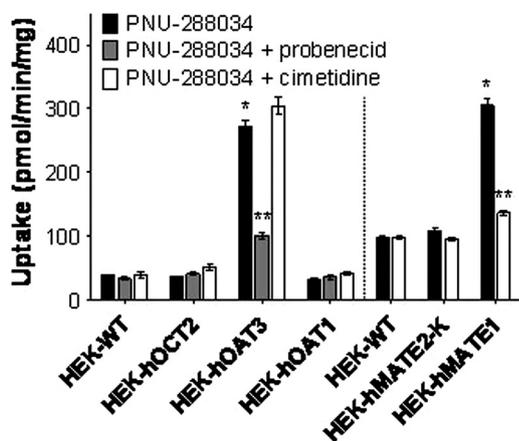| Dose	C_{max}	T_{max}	$t_{1/2}$	MRT	$AUC_{0-\infty}$	Unchanged in Urine	F
mg/kg	*μg/ml*	*h*	*h*	*h*	*μg × h/ml*	*%*	*%*
21.3	9.10 ± 2.0	0.5 ± 0.0	5.9 ± 1.4	2.63 ± 0.26	13.9 ± 0.8	73.2 ± 6.4	73.3 ± 9.3
30.6	10.9 ± 9.2	0.25 ± 0.00	9.6 ± 2.4	6.70 ± 4.5	24.1 ± 4.3	72.5 ± 0.8	88.0 ± 19
60	15.1 ± 1.6	0.75 ± 0.35	7.3 ± 3.4	3.78 ± 0.80	42.5 ± 3.9	63.9 ± 4.0	79.5 ± 6.0
107.9	20.7 ± 0.4	0.38 ± 0.18	5.9 ± 3.5	4.50 ± 1.1	55.8 ± 8.6	50.8 ± 2.4	53.2 ± 8.0

Fig. 4. Uptake of PNU-288034 by human renal transporters in vitro. PNU-288034 (10 μM) was incubated with control, HEK-hOAT1, HEK-hOAT3, HEK-hOCT2, HEK-hMATE1, and HEK-hMATE2-K cells (MATE-mediated transport was characterized in the presence of an oppositely directed H^+ gradient in control and transfected cells as described under *Materials and Methods*). Values are mean ± S.E., n = 3. PNU-288034 uptake in HEK-hOAT3 and HEK-hMATE1 cells was significantly increased over that in the corresponding control cells (∗, $p < 0.05$), and this increase was significantly inhibited by 10 μM probenecid or cimetidine, respectively (∗∗, $p < 0.05$).

transport is considered to be the mechanism for the probenecid–fexofenadine renal DDI (Tahara et al., 2006b). Probenecid has been reported to increase the half-life and AUC of the plasma concentration of methotrexate through inhibition of OAT-mediated renal secretion (Uwai et al., 2000). To further investigate the mechanism of basal and brush-border membrane transport as it relates to PNU-288034 elimination, the chemical inhibition of renal transporter function in rat and monkey was examined by using the coadministration of probenecid or cimetidine. Probenecid, which inhibited the OAT3-mediated uptake of PNU-288034 in vitro, caused a significant reduction in PNU-288034 renal clearance and the resultant increase in plasma exposure (170% AUC increase) in monkey. A similar trend in the level of inhibition was also observed for PNU-288034 plasma clearance in rats when probenecid was coadministered (Fig. 8). Overall, the results showed consistency between the in vitro and in vivo findings regarding probenecid inhibition of renal OATs. It is noteworthy that cimetidine was also observed to inhibit renal secretion of PNU-288034 in the rat inhibition study. Cimetidine is defined as a substrate and/or inhibitor for multiple renal transporters such as OCT2, OAT3, P-gp, and MATE (Lentz et al., 2000; Khamdang et al., 2004; Motohashi et al., 2004; Tsuda et al., 2009) and is known to cause DDIs involving the inhibition of the renal excretion of fexofenadine (Yasui-Furukori et al., 2005), varenicline (Feng et al., 2008), procainamide (Somogyi et al., 1983), dofetilide (Abel et al., 2000),

and metformin (Somogyi et al., 1987). However, it is thought that the inhibitory effect of cimetidine on P-gp and OAT3 should be negligible in the clinical situation, because cimetidine was not found to inhibit P-gp to any significant extent and the maximum plasma concentration of cimetidine (van Crugten et al., 1986) is low compared with the reported IC_{50} and K_m values for OAT3 [92 and 113 μM, respectively (Khamdang et al., 2004; Tahara et al., 2005)]. Therefore, the renal DDI caused by cimetidine may more accurately reflect an inhibitory effect on hMATE1, as the K_i value of cimetidine for hMATE1 is 1.1 μM (Matsushima et al., 2009; Tsuda et al., 2009). Indeed, our results showed that 10 μM cimetidine significantly inhibited the hMATE1-mediated PNU-288034 uptake in HEK gene-transfected cells. Although this inhibitory effect could be a large contributor to the in vivo findings regarding the reduction of plasma clearance in rat, the involvement of P-gp and BCRP were theoretically expected to compensate for the inhibition of MATE1, given the compensatory effects previously observed in renal drug transport systems (El-Sheikh et al., 2008). Surprisingly, compensatory effects were not evident because the inhibition by cimetidine was comparable with probenecid, suggesting that the exclusive collaboration of OAT3 and MATE1, but not P-gp or BCRP, govern the vectorial transport of PNU-288034. Although the failure of P-gp and BCRP to compensate for brush-border membrane efflux remains to be explained, the difference in pH between the intracellular and extracellular environments might alter the substrate specificity of P-gp or BCRP for PNU-288034 in an in vivo situation. In this regard, caution should still be exercised because the in vitro spectrum of transporter involvement may not necessarily translate directly to all in vivo situations.

Species differences in renal transporter expression (Buist and Klaassen, 2004; Terada et al., 2006) have long been recognized and can complicate pharmacokinetic scaling from preclinical species to humans. In the present studies, the renal secretion of PNU-288034 was examined across species, and extensive renal secretion was found in rat, monkey, and human, but not dog. Among the species tested, the monkey results were most similar to human and hence could provide the best pharmacokinetic prediction regarding the OAT3/MATE1-mediated renal secretion of PNU-288034 in humans (Fig. 3). These results are also consistent with previous findings that indicate monkey is a good predictor of renal organic anion uptake in humans (Tahara et al., 2005, 2006a). However, active renal secretion was not observed in dog ($CL_r/GFR < 1$), suggesting that significant interspecies differences in OAT3/MATE1-mediated PNU-288034 renal secretion exist. In the past decade, several laboratories have been focusing on elucidating
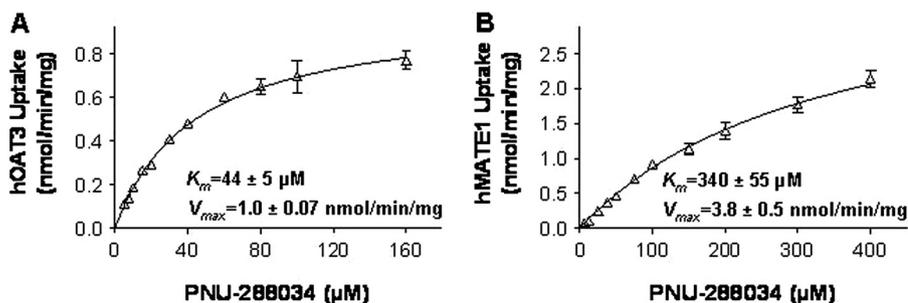

Fig. 5. Kinetic characterization of transporter-mediated uptake by human renal transporters in vitro. Concentration-dependent uptake of PNU-288034 in HEK-hOAT3 cells (A) and HEK-hMATE1 cells (B). Data points have been corrected for the corresponding uptake in control cells and represent the mean ± S.E., n = 4. Kinetic parameters were determined by nonlinear regression analysis (fit to the Michaelis-Menten equation).

TABLE 4
Permeability and transport of 2 μM PNU-288034 in Transwell monolayer efflux studies
Data are reported as the mean ± S.D., n = 3.

Cell Line	$P_{app (A \to B)}$	$P_{app (B \to A)}$	B → A/A → B Efflux Ratio	Transporter/WT Ratio of Ratios
	nm/s			
MDCK-WT	12.4 ± 0.55	19.2 ± 0.28	1.55 ± 0.07	—
MDCK-MDR1	13.8 ± 2.13	53.1 ± 1.70	3.86 ± 0.61	2.49 ± 0.41
MDCK-BCRP	9.87 ± 0.94	50.2 ± 2.77	3.33 ± 0.56	3.27 ± 0.39
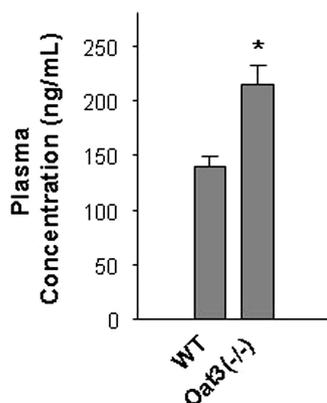
Fig. 6. Plasma concentration of PNU-288034 in Oat3(−/−) and WT mice. The mice were pump-infused with a 1 mg/ml dosing solution of PNU-288034 for 24 h (8 μl/h). Blood samples were collected at 24 h after infusion, and the plasma concentration of PNU-288034 was quantitated by LC-MS/MS. Values are mean ± S.E., n = 4 (∗, p < 0.05).
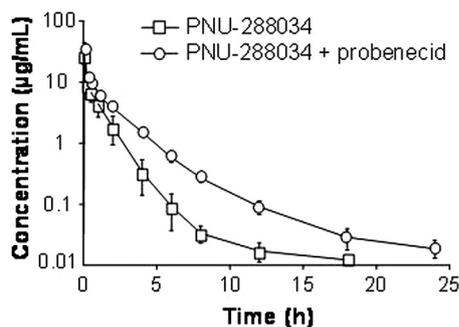

Fig. 7. Plasma elimination of PNU-288034 in monkey in the presence (○) or absence (□) of probenecid. The single intravenously dosed pharmacokinetics of PNU-288034 at 10 mg/kg was obtained from three monkeys. After a 2-week washout period, probenecid was orally administered to the monkeys every 6 h at 30 mg/kg starting 24 h before intravenously dosed PNU-288034 at 10 mg/kg. The samples were collected up to 24 h after dose, and plasma concentration of PNU-288034 was quantitated by LC-MS/MS. Values are mean ± S.E., n = 3.

the OAT3-mediated species differences between preclinical models and humans. It has been recognized, for example, that the dog is particularly susceptible to the effects of chlorinated phenoxyacetic acid herbicides including 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, and 4-chloro-2-methylphenoxyacetic acid (Arnold and Beasley, 1989). These organic acids have been widely used for decades in the control of weeds in both agriculture and aquaculture and rarely cause accumulative toxicity because of their efficient renal clearance in species other than dog (Gorzinski et al., 1987). It is noteworthy that the renal secretion of chlorinated phenoxyacetic acid is mediated by Oats (Sweet, 2005), further substantiating the notion that dogs
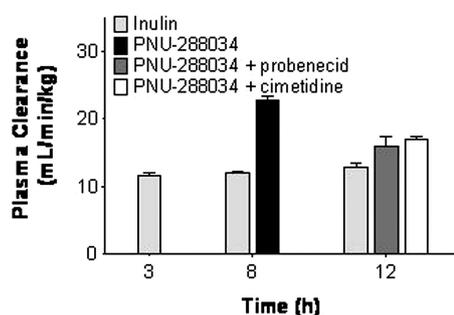

Fig. 8. Plasma elimination of PNU-288034 or inulin in rat in the presence or absence of probenecid or cimetidine. Radiolabeled [14C]inulin was infused to four rats at a rate of 0.2 μCi/9.6 μl/min from 0 to 4 h. At that time, a loading dose of 2.17 μCi/kg [3H]PNU-288034 was administered intravenously via the SVC, and the labeled inulin and PNU-288034 together were infused to rats at a rate of 0.2 μCi/9.6 μl/min from 4 to 12 h. Loading doses of probenecid or cimetidine at 70 or 50 mg/kg, respectively, were administered to two of the rats at 8 h via the SVC, and each was redosed five times at 40 mg/kg over the 8- to 12-h time period. Plasma samples were collected over 12 h for quantification of inulin and PNU-288034 by liquid scintillation. Plasma clearance was calculated at 3, 8, and 12 h. Inulin clearance is taken to be equal to GFR.

may have low renal Oat functions. Yet the mechanism underlying the inefficiency of active renal secretion in dog, whether caused by a difference in Oat3 or Mate1 renal expression, a species difference in Oat3 or Mate1 affinity for PNU-288034, or a combination thereof, remains to be investigated. Thus, caution should be exercised when extrapolating renal secretion mediated by Oat and Mate transporters from dog. Future work in this area will undoubtedly contribute to guidelines for the extrapolation of human pharmacokinetics from preclinical species.

In conclusion, the clinical and preclinical evidence demonstrates a strong in vitro–in vivo correlation for the active renal secretion of PNU-288034 mediated by the collaborative transport of the OAT3 and MATE1 transporters that could have limited the systemic exposure of PNU-288034 in humans. Species differences in active renal secretion of PNU-288034 were also observed and suggest that monkey is the best choice for predicting human pharmacokinetics. To the extent that transporters can alter plasma levels of their drug substrates and thus their efficacy and side effects, the current results emphasize that active renal secretion can limit the development opportunities of drug candidates in the clinic caused by inefficient systemic exposure and underscore the need to further recognize and characterize the contribution of renal mechanisms involved in drug elimination.

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