Preclinical Species and Human Disposition of PF-04971729, a Selective Inhibitor of the Sodium-Dependent Glucose Cotransporter 2 and Clinical Candidate for the Treatment of Type 2 Diabetes Mellitus

Amit S. Kalgutkar, Meera Tugnait, Tong Zhu, Emi Kimoto, Zhuang Miao, Vincent Mascitti, Xin Yang, Beijing Tan, Robert L. Walsky, Jonathan Chupka, Bo Feng, and Ralph P. Robinson


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ABSTRACT:

PF-04971729, (1S,2S,3S,4R,5S)-5-[4-Chloro-3-(4-ethoxybenzyl)phenyl]-1-hydroxymethyl-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol (PF-04971729), a potent and selective inhibitor of the sodium-dependent glucose cotransporter 2, is currently in phase 2 trials for the treatment of diabetes mellitus. This article describes the preclinical species and in vitro human disposition characteristics of PF-04971729 that were used in experiments performed to support the first-in-human study. Plasma clearance was low in rats (4.04 ml · min⁻¹ · kg⁻¹) and dogs (1.64 ml · min⁻¹ · kg⁻¹), resulting in half-lives of 4.10 and 7.63 h, respectively. Moderate to good bioavailability in rats (69%) and dogs (94%) was observed after oral dosing. The intermediate biotransformation profile of PF-04971729 in liver microsomes and cryopreserved hepatocytes from rat, dog, and human was qualitatively similar; prominent metabolites include monoxygenation, O-deethylation, and glucuronidation. No human-specific metabolites of PF-04971729 were detected in in vitro studies. Reaction phenotyping studies using recombinant enzymes indicated a role of CYP3A4/3A5, CYP2D6, and UGT1A9/2B7 in the metabolism of PF-04971729. No competitive or time-dependent inhibition of the major human cytochrome P450 enzymes was discerned with PF-04971729. Inhibitory effects against the organic cation transporter 2-mediated uptake of [³⁵S]metformin by PF-04971729 also were very weak (IC₅₀ = 900 μM). Single-species allometric scaling of rat pharmacokinetics of PF-04971729 was used to predict human clearance, distribution volume, and oral bioavailability. Human pharmacokinetic predictions were consistent with the potential for a low daily dose. First-in-human studies after oral administration indicated that the human pharmacokinetics/dose predictions for PF-04971729 were in the range that is likely to yield a favorable pharmacodynamic response.

Introduction

Type 2 diabetes mellitus (T2DM), a chronic disease prevalent worldwide (King et al., 1998), is characterized by insulin resistance in muscle and liver, resulting in higher blood glucose levels. As blood glucose levels escalate, more insulin is required over time, and with increasing severity of insulin resistance, hyperinsulinemia develops into insulin deficiency as a result of progressive β-cell failure in the pancreas (DeFronzo, 1988, 2009). Approximately 85% of patients with T2DM are obese or overweight, a key factor underlying the development and maintenance of insulin resistance (Mokdad et al., 2003). Individuals with T2DM have an increased risk of developing both microvascular (nephropathy, neuropathy, and retinopathy) and macrovascular complications and have an increased mortality rate from cardiovascular disease compared with that of adults who are not diabetic (Stratton et al., 2000).

In normal individuals, glucose filtered from the blood in the glomerulus is almost completely reabsorbed, such that <1% of glucose is excreted in urine. The low-affinity, high-capacity sodium-dependent glucose cotransporter (SGLT2), expressed in the proximal tubule of the nephron, is largely responsible for the reabsorption process (Kanai et al., 1994; Wallner et al., 2001). Although the high-affinity SGLT1 is expressed to some extent in the kidney and contributes to glucose reabsorption, it is mainly expressed in the small intestine, where it plays a role in glucose absorption (Pajor and Wright, 1992; Wright, 2001).

It is now well established that suppressing the activity of SGLT2 inhibits renal glucose reabsorption, thereby increasing the excretion of excess glucose from the body and assisting in the reduction of hyper-
glycemia in T2DM (Oku et al., 1999; Adachi et al., 2000; Zhang et al., 2005, 2006; Boldys and Okopień, 2009; Neumiller et al., 2010; Robinson et al., 2010). Because of their insulin-independent mechanism of action, SGLT2 inhibitors are associated with a low risk for hypoglycemia (Katsuno et al., 2007; Fujimori et al., 2008; Wilding et al., 2009). Furthermore, there is the potential for clinically significant weight loss and reduced hepatic glucose production and amelioration of glucotoxicity (Abdul-Ghani and DeFronzo, 2008; Nair and Wilding, 2010).

Several SGLT2-selective inhibitors are in various stages of clinical trials (Aires and Calado, 2010; Nair and Wilding, 2010; Sha et al., 2011); proof-of-concept has been reported with dapagliflozin (Meng et al., 2008) in phase 2b studies in patients with type 2 diabetes (List et al., 2009; Kasichayanula et al., 2011). Results from a 24-week phase 3 clinical study with dapagliflozin have also demonstrated significant mean reductions in the primary endpoint, glycosylated hemoglobin levels and in the secondary endpoint, fasting plasma glucose, when added to metformin in people with T2DM inadequately controlled with metformin alone (Bailey et al., 2010). The study also evaluated the potential impact of dapagliflozin on weight loss and demonstrated that individuals receiving dapagliflozin had statistically greater mean reductions in body weight than individuals taking placebo.

We recently disclosed a novel class of SGLT2 inhibitors bearing a unique dioxa-bicyclo[3.2.1]octane (bridged ketal), from which 4-methyl-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol (PF-04971729) (Fig. 1) emerged as a candidate for further advancement (Mascitti et al., 2011). PF-04971729 demonstrated >2000-fold selectivity for SGLT2 inhibition (relative to SGLT1) in vitro and revealed a concentration-dependent glucosuria after oral administration to rats. To evaluate the potential for further clinical development and toxicological evaluations, preclinical pharmacokinetics and disposition of PF-04971729 were characterized in rats and dogs and in vitro human hepatic tissue. Results from these studies were used to predict human pharmacokinetic parameters via the allometric scaling approach. Preliminary assessments of PF-04971729 in the clinic suggest a fairly precise prediction of human pharmacokinetics, which should translate into a favorable pharmacodynamic response.

Materials and Methods

Materials. PF-04971729 and its O-deethylated phenol metabolite M1 (chemical and isometric purity >99% by HPLC and NMR), N-(3,4-dimethoxyphenethyl)-4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2-[H]-yl)-1-hydroxy-methyl-6,8-dioxabicyclo[3.2.1]octane-2,3,4-triol (PF-04971729) (Fig. 1) was noted. PF-04971729 was added to each well. Each time point was transferred to 96-well blocks and acetonitrile (200 μl) was added. Each matrix was normalized to the other by the addition of equal volumes of the opposite matrix, (i.e., plasma to buffer and buffer to plasma). Samples were then centrifuged at 3000 g for 10 min; the supernatants were analyzed by liquid chromatography (LC)-tandem mass spectrometry (MS/MS). Nonspecific binding of PF-04971729 to the equilibrium dialysis device was minimal. The percentage of PF-04971729 bound to plasma proteins was calculated as 100 − [(concentration of PF-04971729 in buffer/concentration of PF-04971729 in plasma) × 100].

Blood Cell Partitioning. Blood to plasma partitioning of PF-04971729 was determined in fresh rat, dog, and human whole blood and plasma to which was added PF-04971729 to yield an final concentration of 2.3 μM (n = 3). Samples were incubated on a shaking water bath for 120 min at 37°C. Postincubation, whole blood samples were centrifuged at 3000 g to obtain plasma. Aliquots (50 μl) of plasma sample incubations and plasma isolated from whole blood were mixed with acetonitrile (200 μl) containing an internal standard (molecular weight = 390, 250 ng/ml) and centrifuged. Supernatants were analyzed using LC-MS/MS. A blood/plasma partitioning ratio for PF-04971729 was calculated from the concentrations in blood and plasma.

P450 Inhibition Studies. Competitive inhibition. Standard marker substrates of P450 isoforms were incubated with pooled human liver microsomes in the presence of NADPH (1.3 mM) in 100 mM KH2PO4, pH 7.4, containing 3.3 mM MgCl2, at 37°C open to air. The incubation volume was 0.2 ml. Microsomal protein concentrations, substrate concentrations, incubation times and reaction termination solvents for each activity have been described in detail previously (Walsky and Obach, 2004). Probe substrate concentrations used were near Km values that had been determined previously, and incubation times were selected on the basis of previous determinations of reaction velocity linearity (Walsky and Obach, 2004). Incubation mixtures contained PF-04971729 at concentrations of 0, 0.0952, 0.301, 0.951, 3.00, 9.49, and 30 μM. Stock solutions of PF-04971729 were prepared in 95:5 acetonitrile-water. Incubations were started with the addition of NADPH.

Time-dependent inhibition. Pooled human liver microsomes (protein concentration = 0.1–1.0 mg/ml, depending on which isozyme was assayed) were preincubated with PF-04971729 at a concentration of 300 μM, in 100 mM KH2PO4, pH 7.4, containing 3.3 mM MgCl2, at 37°C open to air in the presence and absence of NADPH (1.3 mM). Preincubations were performed for 30 min at 37°C. After preincubation, a portion of the incubation mixture (20 μl) was added to a mixture containing a probe P450 isozyme substrate (concentration of approximately the Km value) (Walsky and Obach, 2004) in 100 mM KH2PO4, pH 7.4, containing 3.3 mM MgCl2 and NADPH (1.3 mM) at 37°C. The final incubation volume was 0.2 ml. At the end of the incubation period, acetonitrile containing internal standard was added, and the mixture was filtered using a Millipore Multiscreen HA filter plate to remove microbial protein. Filtered solutions were analyzed by LC-MS/MS using a Micromass Ultima tandem quadrupole mass spectrometer (Waters, Milford, MA) fitted with an electrospray interface. The HPLC system consisted of a Shimadzu SCL-10Avp controller (Shimadzu Scientific Instruments, Columbia, MD) with LC-10Amp pumps and CTC Analytics Leap autosampler (LEAP Technologies, Carrboro, NC). Metabolites of probe P450 substrates were analyzed using validated bioanalytical conditions established previously (Walsky and Obach, 2004). IC50 values for inhibition of P450 isoforms were estimated using the Sigma Plot curve-fitting software (SYSTAT Inc., San Jose, CA).

Fig. 1. Structure of PF-04971729.
hOCT2 Inhibition Studies. Human embryonic kidney 293 cells transfected with hOCT2 were obtained from Professor Kathleen M. Giacomini (University of California, San Francisco, San Francisco, CA). hOCT2 was subcloned into the mammalian expression vector pcDNA3/FRT and transfected into HEK293 flip-in cells (Invitrogen). Nontransfected human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle’s medium, 10% v/v heat-inactivated fetal bovine serum, 1% v/v penicillin/streptomycin, and 100 μg/ml Zeocin. Stably transfected hOCT2 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1% gentamicin, and 50 μg/ml Hygromycin B. hOCT2 assays were performed in 24-well poly-l-lysine-coated plates (Biocoat, Horsham, PA). Cells were seeded to reach confluence (80–100%) 48 to 72 h before each experiment. Immediately before the experiment, cells were placed on a 37°C heat block and were washed twice with 1 ml of Hank’s balanced salt solution, pH 7.4, prewarmed to 37°C. After a 5-min acclimation period, the cells were incubated with 150 μl of Hank’s balanced salt solution buffer containing [14C]metformin (5 μM). To increase assay sensitivity, the metformin concentration chosen for the inhibition study was below its reported K_m value (Bachmakov et al., 2009). Thus, the uptake of metformin at the concentration of 5 μM in the HEK293 cells transfected with hOCT2 is 10-fold greater than the corresponding uptake in wild-type HEK293 cells, which provides a large dynamic window to assess inhibitory potency against the cation transporter. After 5 min at 37°C, the cellular uptake was terminated by washing the cells three times with 1 ml of ice-cold Hank’s balanced salt solution. The cells were lysed in 400 μl of 1% SDS in Dulbecco’s phosphate-buffered saline. The cells were covered and shaken for 10 to 15 min at room temperature. Accumulated radioactivity was determined by combining all of the cell lysate solution with 6.6 ml of scintillation fluid and mixed until homogeneous. Radioactivity in each sample was determined by liquid scintillation counting on a Packard Tri-Carb 2900TR (Thermo Fisher Scientific) scintillation counter and recorded as disintegrations per minute. The IC50 value for hOCT2 inhibition was determined in incubations with seven different concentrations of PF-04971729 ranging from 1.4 to 1000 μM (n = 3). Stock solutions of PF-04971729 were prepared in dimethyl sulfoxide (DMSO). The hOCT2 inhibitor quinidine (1 mM) was used as a positive control (Zolk et al., 2009). Incubations were performed in 24-well culture plates using [14C]metformin (5 μM) spiked with PF-04971729 and applied simultaneously to the two cell lines. The mean and S.D. of the substrate uptake rate were calculated for each sample. These values were then converted to percentage of uptake relative to the control (substrate uptake without inhibitor), with the control representing 100% uptake. The IC50 value for PF-04971729 was estimated from a semi-logarithmic plot of concentrations versus percentage of net uptake relative to the control.

Caco-2 Cell Permeability. Caco-2 cells, obtained from the American Type Culture Collection (Manassas, VA), were seeded in 24-well Falcon Multwell plates (polyethylene terephthalate membranes, pore size 1.0 μm) at 4.0 × 10^4 cells/well. The permeability studies on PF-04971729 at a concentration of 1 μM were conducted using a protocol published previously (Frederick et al., 2009). Studies were conducted in the presence or absence of the dual P-glycoprotein (P-gp)/breast cancer resistance protein (BCRP) inhibitor CP-100,356 (10 μM) to both the apical and the basolateral compartments in the bidirectional permeability determination (Kalugkat et al., 2009).

Animal Pharmacokinetic Studies. All animal care and in vivo procedures conducted were in accordance with guidelines of the Pfizer Animal Care and Use Committee. Male Sprague-Dawley rats (0.31–0.36 kg) and male beagle dogs (9.4–11.6 kg) were used as animal models for allometric scaling of human pharmacokinetics. For oral pharmacokinetic studies, animals were fasted overnight before dosing, whereas access to water was provided ad libitum. PF-04971729 was administered intravenously via the jugular vein of rats (n = 2) or the cephalic vein for dogs (n = 2). For oral studies, PF-04971729 was administered as the l-propramide cocaine salt by oral gavage to rats (n = 3) and dogs (n = 3). PF-04971729 was administered at 2.0 mg/kg i.v. and 5.0 mg/kg p.o. doses in rats and at a 2.0 mg/kg dose for the intravenous and oral studies in dogs. Animals were fed after collection of the 4-h blood samples. PF-04971729 was formulated as a solution in dimethyl sulfoxide-polyethylene glycol 400–30% solubitylther-β-cyclodextrin (10:30:60, v/v/v) and 0.5% (w/v) methylcellulose with 10% (v/v) polyethylene glycol 400 for rat intravenous and oral studies, respectively. For assessment of dog intravenous and oral pharmacokinetics, PF-04971729 was formulated as a solution in 5% polyethylene glycol 400 in 23% hydroxypropyl-β-cyclodextrin and 0.5% (w/v) methylcellulose with 10% (v/v) polyethylene glycol 400, respectively. After dosing, serial plasma samples were collected at appropriate times and kept frozen at −20°C until LC/MS/MS analysis. Urine samples (0–7.0 and 7.0–24 h) were also collected after intravenous administration to rats and dogs.

Pharmacokinetic Parameter Generation in Preclinical Species. A plasma concentration versus time profile was generated for each animal. Standard noncompartmental pharmacokinetic analysis was performed using Watson Bioanalytical LIMS (version 72003; Thermo Fisher Scientific) to determine plasma clearance (CLp) and volume of distribution at steady state (Vdss). CLp was calculated as the intravenous dose divided by the area under the plasma concentration-time curve from zero to infinity (AUC0→∞). AUC0→∞ was calculated by the linear trapezoid rule. The terminal slope of the ln (concentration) versus time plot was calculated by linear least-squares regression, and the half-life was calculated as 0.693 divided by the absolute value of the slope. The maximum plasma concentration (Cmax) observed after oral dosing and the time at which it was observed (Tmax) were determined directly from the individual plasma concentration-time profiles. The bioavailability (F) of the oral doses was calculated using the following equation: 

\[ F = \frac{\text{AUC}_{0→\infty}^{\text{i.v.}}}{\text{AUC}_{0→\infty}^{\text{p.o.}}} \times \frac{\text{dose}_{\text{i.v.}}}{\text{dose}_{\text{p.o.}}} \]

Bioanalytical Methodology. Mass spectrometry was performed on a Sciex API 4000 system equipped with a TurboIonSpray source (Applied Biosystems, Foster City, CA) operated in negative ion mode. HPLC analysis was conducted on a Shimadzu 10ADvp Binary HPLC system (Shimadzu Scientific Instruments) with a CTC-PAL (Thermo Fisher Scientific) autosampler. Chromatographic separations were performed on a Synergi Max-RP 2 5 mm × 50 μm HPLC column (Phenomenex, Torrance, CA) using mobile phase A (10 mM ammonium acetate and 1% isopropyl alcohol in water) and mobile phase B (acetonitrile). A linear gradient from 5% B to 95% B over 1.5 min with the flow rate of 0.3 ml/min was performed to elute PF-04971729 and the internal standard. Analyst (version 1.4.1; Applied Biosystems) was used to control the instrument operation and acquire data in multiple reaction monitoring mode. The ion transition for PF-04971729 was 435 → 303, and for the internal standard was 391 → 313. The dynamic range of the assay ranged from 5 to 5000 ng/ml using linear regression with a weighting of 1/x^2.

Pharmacokinetic Projections to Human. The single-species allometric method was used to predict human pharmacokinetic parameters of PF-04971729. CLp and Vdss observed in rats were corrected for species differences in protein binding and then scaled to humans according to eqs. 1 and 2 below (Frederick et al., 2009). This approach uses a fixed allometric exponent of 0.75 and 1.0 for clearance and volume, respectively, BW is the body weight (kilograms) [assumed to be 70 for human; for rat actual body weight of animals (0.314, 0.361) in the study was used]:

\[ \text{CL}_{\text{human}} = \text{CL}_{\text{animal}} \times \left( \frac{\text{BW}_{\text{human}}}{\text{BW}_{\text{animal}}} \right)^{0.75} \]  
\[ \text{Vdss}_{\text{human}} = \text{Vdss}_{\text{animal}} \times \left( \frac{\text{BW}_{\text{human}}}{\text{BW}_{\text{animal}}} \right)^{1.0} \]

Human pharmacokinetic predictions assumed a simple one-compartment pharmacokinetic model with first-order absorption. Thus, human half-life was predicted according to eq. 3:

\[ t_{1/2,\text{human}} = \frac{\ln(2)}{\text{Vdss}_{\text{human}}} \times \text{CL}_{\text{human}} \]

Metabolite Identification. Liver microsomes and recombinant P450 enzymes. Stock solutions of PF-04971729 were prepared in methanol. The final concentration of methanol in the incubation medium was 0.2% (v/v). Incubations were performed at 37°C for 60 min in a shaking water bath. The incubation volume was 1 ml and consisted of the following: 0.1 M potassium phosphate buffer (pH 7.4), liver microsomes from rat, dog, and human (P450 concentration = 0.5 μM) or recombinant P450 isoforms (50 pmol of CYP1A2, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5, NADPH (1.0 mM), and PF-04971729 (10–20 μM). The reaction mixture was prewarmed at 37°C for 2 min before addition of NADPH. Incubations that lacked NADPH served as negative controls, and reactions were terminated by the addition of ice-cold acetonitrile.
collision-induced dissociation (CID) spectra of the molecular ions (MH+
liams’ E medium supplemented with 24 mM NaHCO3 and 10% fetal bovine
one female and two males) hepatocytes were thawed and suspended in Wil-
of 15–20 livers), dog (pool of 4 – 8 male livers), or human (pool of three livers,
for metabolite formation by LC-MS/MS.
autopilot was programmed to inject 50
with a electrospray source and coupled to a Thermo Fisher Scientific Separa-
LTQ or LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) operated
C-18 250
mobile phase was introduced into the mass spectrometer via an ion spray
for 6 min before next analytical run. Postcolumn flow was split such that the
from 45 to 52 min where it was held for 3 min and then returned to 95:5 (v/v)
(4 ml). The solutions were centrifuged (3000
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1612 KALGUTKAR ET AL.
lood and human plasma . The mean percentages ± S.D. of plasma protein
binding of PF-04971729 at concentrations of 2.3 and 23 µM
were 96.0 ± 0.4 and 96.4 ± 0.5% (rat), 96.8 ± 0.02 and 96.8 ± 0.1% (dog), and 93.6 ± 0.1 and 94.7 ± 0.3% (human). These results show that
PF-04971729 is highly bound to plasma proteins among the species examined, and binding is independent of concentration. PF-
04971729 was stable in rat, dog, and human plasma for >6 h at 37°C
at the concentrations used in the plasma free fraction determination.
Blood Cell Partitioning. The blood/plasma ratios for PF-04971729
were estimated to be 0.66 ± 0.01, 0.58 ± 0.02, and 0.66 ± 0.07 in rat, dog,
and human, respectively, suggesting that PF-04971729 preferentially
distributed into plasma over red blood cells.
Human P450 Isozyme Inhibition. PF-04971729 exhibited no relevant
reversible and time-dependent inhibitory effects against CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, or CYP3A4/5 (felodipine oxidase, midazolam-1-hydroxylase, and
testosterone-6β-hydroxylase) activities. IC50 values could not be calculated
because PF-04971729 did not inhibit any P450 activity more than 40%
at the highest concentration tested. Under the present experimental conditions, known P450 isozyme-specific inhibitors
furafylline, clopidogrel, montelukast, sulfaphenazole, (+)-N-3-benzyliminoro
quinidine, and ketoconazole demonstrated potent competitive
inhibition of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 activity, respectively, in human liver microsomes (data not shown).
Inhibition of hOCT2 by PF-04971729. PF-04971729, at a concentration range of 1.4 to 1000 µM, was evaluated as an inhibitor of the
hOCT2 transporter. The calculated IC50 for inhibition of hOCT2-
mediated uptake of [14C]metformin by PF-04971729 was 917 µM
(Fig. 2). Under these experimental conditions, the positive control
quinidine (1 mM) (Zolk et al., 2009) inhibited
activity, respectively, in human liver microsomes (data not shown).
Caco-2 Permeability. The permeability coefficient of PF-
04971729 across Caco-2 cells in the apical (A) to basolateral (B)
direction was 4.1 × 10−6 cm/s, a value that is comparable to those of
drugs that exhibit moderate to good oral absorption in humans (Elshby
et al., 2008). Under identical experimental conditions, the B to A
permeability coefficient of PF-04971729 was 8.6 × 10−6 cm/s
(BA/AB ratio of 2.1). The presence of the dual P-gp/BCRP inhibitor

Results

Plasma Protein Binding. The extent of in vitro binding of PF-
04971729 to plasma proteins was evaluated by equilibrium dialysis in
rat, dog, and human plasma . The mean percentages ± S.D. of plasma
protein binding of PF-04971729 at concentrations of 2.3 and 23 µM
was 96.0 ± 0.4 and 96.4 ± 0.5% (rat), 96.8 ± 0.02 and 96.8 ± 0.1% (dog), and 93.6 ± 0.1 and 94.7 ± 0.3% (human). These results show that
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quinidine (1 mM) (Zolk et al., 2009) inhibited >80% [14C]metformin
uptake mediated by hOCT2 (data not shown).

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direction was 4.1 × 10−6 cm/s, a value that is comparable to those of
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(BA/AB ratio of 2.1). The presence of the dual P-gp/BCRP inhibitor

Fig. 2. Inhibition of hOCT2-mediated uptake of [14C]metformin by PF-04971729. The net uptake values were obtained by subtracting the uptake in vector-transfected cells (control) from those in OCT2-expressing cells. The net uptake without inhibitor was defined as 100%. The IC50 value for PF-04971729 was estimated from a semilogarithmic plot of concentrations versus percentage of net uptake relative to the control. Each value represents the mean ± S.D. (n = 3).
CP-100,356 (Kalgutkar et al., 2009) did not greatly increase the absorptive permeability (4.7 × 10⁻⁶ cm/s) of oral absorption of PF-04971729. Thus, P-gp and BCRP do not appear to be limiting factors for oral absorption of PF-04971729.

**Pharmacokinetics of PF-04971729 after Single Doses in Rats and Dogs.** The pharmacokinetic parameters describing the disposition of PF-04971729 after intravenous and oral administration in preclinical species is shown in Table 1, and mean plasma concentration versus time profiles are depicted in Fig. 3. After an intravenous dose of 2.0 mg/kg, PF-04971729 exhibited a low blood clearance (CLp) of 6.12 ml/minute/kg⁻¹ (obtained by dividing the CLp, of 4.94 ml/minute/kg⁻¹ by the blood/plasma ratio of 0.66), which is ~6% of rat hepatic blood flow of 70 ml/minute/kg⁻¹. The Vdss was moderate (1.13 l/kg) compared with 0.6 l/kg for total body water in the rat, indicating extravascular distribution. The mean elimination half-life (t₁/₂) was estimated to be 4.10 h. The percentage of unchanged PF-04971729 excreted over a 24-h period in the urine was 27.5%. After oral administration of PF-04971729 as aL-pyroglutamic acid cocrystal to dogs at 2.0 mg/kg, systemic exposure of PF-04971729 as ascertained from Cmax and AUC₀⁻₂₄ h was 2500 ± 215 ng/ml and 19,100 ± 3650 ng-h/ml, respectively. The time to reach Cmax was 0.83 ± 0.29 h. The mean F was 94%.

**In Vitro Metabolic Profile.** Liver microsomes. Figure 4 depicts the extracted ion chromatograms of incubation mixtures of PF-04971729 in NADPH-supplemented liver microsomes from rat, dog, and human. PF-04971729 was the major peak in all microsomal incubations, and there were no human-unique metabolites. Four metabolites (labeled M1, M2, M3, and M4) of PF-04971729 were detected in liver microsomes from rat, dog, and human at the retention times (tR) noted in Table 2. M7 was a rat-specific metabolite. M1 to M4 and M7 were not detected when NADPH was omitted from the microsomal incubations, suggesting that P450 isozymes catalyzed the rate-limiting step in their formation. PF-04971729 exhibited an ammonium adduct [M + NH₄⁺], which possessed an exact mass of 454.1632 (Table 2; Fig. 5). Theoretical exact masses for the proposed fragment ion structures for m/z 329, 273, 207, and 135 in the CID spectrum of PF-04971729 (Fig. 5) were consistent with the observed accurate masses (<2 ppm difference). MS/MS data, proposed fragmentation patterns and structures for the metabolites of PF-04971729 are shown in Table 2 and Fig. 6, respectively. M1 possessed an exact mass of 470.1580 [M + NH₄⁺], an addition of 16 Da to the molecular mass of PF-04971729, which suggested that it was a monohydroxylated derivative of PF-04971729. The CID spectra showed diagnostic fragment ions at m/z 435, 407, 299, and 243. The ion at m/z 435 was due to the sequential loss of NH₃ and H₂O. The most abundant fragment ion, which occurred at m/z 407 (loss of 28 Da from m/z 435) was derived from the loss of an ethylene unit. The absence of fragment ions derived from benzylic cleavage (e.g., m/z 207 and 135 seen in the mass spectrum of PF-04971729) in the CID spectrum of M1 suggested the benzylic position as the most likely site of hydroxylation. A proposed structure for M1 that is consistent with the observed
fragment ions is depicted in Fig. 6. Metabolite M2 possessed a molecular ion at 426.1320 \([\text{M} + \text{NH}_3]^+\), a loss of 28 Da from the molecular mass of PF-04971729; the CID spectrum and \(t_R\) were identical to those of the synthetic standard of the O-deethylated phenol metabolite of PF-04971729 (Fig. 6). Like M1, metabolites M3 and M4 (\([\text{M} + \text{NH}_3]^+ = 470.15\)) were derived from monohydroxylation of PF-04971729. A common fragment ion at \(m/z\) 207 in the mass spectrum of M3 and PF-04971729 suggested that the chlorobenzyl group was unaltered. The ions at \(m/z\) 123 and 151, which are 16 Da higher than the characteristic ions at \(m/z\) 107 and 135 in PF-04971729, suggested that the hydroxylation in M3 occurred on the ethoxyphenyl ring; a proposed structure that is consistent with the fragmentation pattern as depicted in Fig. 6. For M4, the CID spectrum showed fragment ions at \(m/z\) 435, 417, 399, 329, 207, and 135. The ion at \(m/z\) 435 resulted from sequential loss of \(\text{NH}_3\) and \(\text{H}_2\text{O}\), whereas the ion at \(m/z\) 207 indicated that the chlorophenyl moiety was unchanged. On the basis of these data, a tentative structure for M4 is shown in Fig. 6. Rat-specific metabolite M7 showed an ammonia adduct ion \([\text{M} + \text{NH}_3]^+\) at \(m/z\) 442.12, which is 12 Da lower than parent compound and 16 Da higher than M2. This observation suggested that M7 was a monohydroxylated derivative of M2. The fragment ion at \(m/z\) 407 in the CID spectrum of M7 resulted from sequential loss of \(\text{NH}_3\) and \(\text{H}_2\text{O}\). Lack of ions that resulted from the characteristic benzylic cleavage seen in PF-04971729 suggested that the site of oxidation was the benzylic position in M2. A proposed structure for M7 is shown in Fig. 6.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>(t_R) (min)(a)</th>
<th>Observed ([\text{M} + \text{NH}_3]^+)(b)</th>
<th>LC-MS(c) fragments, (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6a</td>
<td>11.6</td>
<td>602.16404</td>
<td>MS(^2) on (m/z) 602.16404: 409.1049, 301.0626, 207.0207; MS(^3) on (m/z) 602 → 409: 301.0626, 207.0207, 107.0491</td>
</tr>
<tr>
<td>M7</td>
<td>11.9</td>
<td>442.12</td>
<td>MS(^2) on (m/z) 442.12: 424/37, 397.19, 299.08, 271.20</td>
</tr>
<tr>
<td>M6b</td>
<td>12.2</td>
<td>602.16401</td>
<td>MS(^2) on (m/z) 602.16401: 409.1049, 301.0626, 207.0207; MS(^3) on (m/z) 602 → 409: 301.0626, 207.0207, 107.0491</td>
</tr>
<tr>
<td>M6c</td>
<td>12.3</td>
<td>602.16402</td>
<td>MS(^2) on (m/z) 602.16402: 409.1049, 301.0626, 207.0207; MS(^3) on (m/z) 602 → 409: 301.0626, 207.0207, 107.0491</td>
</tr>
<tr>
<td>M8</td>
<td>13.9</td>
<td>646.19</td>
<td>MS(^2) on (m/z) 646.19: 470.06, 453.12, 345.04, 207.14; MS(^3) on (m/z) 426 → 453: 345, 297; MS(^4) on (m/z) 453 → 345: 207, 151, 139, 123</td>
</tr>
<tr>
<td>M2</td>
<td>15.9</td>
<td>426.1320</td>
<td>MS(^2) on (m/z) 426.1320: 409.1050, 301.0626; MS(^3) on (m/z) 426 → 409: 301.0628; MS(^4) on (m/z) 426 → 409 → 301: 207.0208, 107.4914</td>
</tr>
<tr>
<td>M1</td>
<td>17.3</td>
<td>470.1580</td>
<td>MS(^2) on (m/z) 470.1580: 435.1204; MS(^3) on (m/z) 470 → 435: 407.0891, 299.0470, 243.0209</td>
</tr>
<tr>
<td>M3</td>
<td>18.1</td>
<td>470.1583</td>
<td>MS(^2) on (m/z) 470.1583: 453.1311, 345.0889, 207.0206, 151.0755; MS(^3) on (m/z) 470 → 435: 301.0626, 151.0754, 139.0754, 123.0440</td>
</tr>
<tr>
<td>M5a</td>
<td>18.7</td>
<td>630.1952</td>
<td>MS(^2) on (m/z) 630.1952: 437.1362, 329.0940, 135.082</td>
</tr>
<tr>
<td>M5b</td>
<td>19.1</td>
<td>630.1954</td>
<td>MS(^2) on (m/z) 630.1954: 437.1362, 329.0940, 135.0802</td>
</tr>
<tr>
<td>M4</td>
<td>20.1</td>
<td>470.15</td>
<td>MS(^2) on (m/z) 470.15: 435.0; MS(^3) on (m/z) 470 → 435: 417.1, 369.1, 329.0, 207.1, 135.1</td>
</tr>
<tr>
<td>M5c</td>
<td>20.6</td>
<td>630.1953</td>
<td>MS(^2) on (m/z) 630.1953: 437.1362, 329.0940, 135.0802</td>
</tr>
<tr>
<td>PF-04971729</td>
<td>31.0</td>
<td>454.1632</td>
<td>MS(^2) on (m/z) 454.1632: 437.1359; MS(^3) on (m/z) 454 → 329: 273.0677, 207.0206, 135.0802</td>
</tr>
</tbody>
</table>

\(a\) Under HPLC conditions listed under Materials and Methods.

\(b\) Accurate mass data for PF-04971729 and its metabolites was generated using Orbitrap MS.
Cryopreserved hepatocytes. Figure 7 depicts the extracted ion chromatograms of incubation mixtures of PF-04971729 in cryopreserved hepatocytes from rat, dog, and human. PF-04971729 was the major peak in all hepatocyte incubations, and there were no human-unique metabolites. Apart from the formation of oxidative metabolites M1 to M4 and M7, metabolites (M5a, M5b, M5c, M6a, M6b, M6c, and M8) derived from the glucuronidation of PF-04971729 and its oxidative metabolites were also observed in hepatocyte incubations. Metabolites M5a, M5b, and M5c were regioisomers that displayed identical ammonia adduct ions [M + NH₄⁺] at m/z 630, 176 Da higher than PF-04971729, suggesting that they were obtained from glucuronidation of the glycoside hydroxyl groups (Fig. 6). Metabolites M6a, M6b, and M6c also were regioisomers that displayed identical ammonia adduct ions [M + NH₄⁺] at m/z 602, suggesting that they were obtained from glucuronidation of M2. The CID spectra showed fragment ions at m/z 459, 409, 391, 301, 283, 207, and 107. The ion at m/z 409 resulted from loss of glucuronic acid and NH₃ components, whereas the ion m/z 207 indicated the integrity of the chlorophenyl moiety. Proposed structures of M6a, M6b, and M6c, which are consistent with the fragmentation pattern, are shown in Fig. 6. M8 showed an ammonia adduct ion [M + NH₄⁺] at m/z 646, 192 Da higher than the molecular mass of PF-04971729, suggesting that M8 was a glucuronide conjugate of a monohydroxylated metabolite of PF-04971729. The MS² and MS³ spectra of M8 showed fragment ions at m/z 470, 453, 345, 207, 151, 139, and 123. The ions at m/z 470 and m/z 453 resulted from loss of the components of glucaric acid (176 Da) and NH₃, respectively. The diagnostic ion at m/z 207, also observed in PF-04971729, suggested that the chlorophenyl moiety was unaltered in M8. The MS³ spectrum of m/z 345 was identical to the MS³ spectrum of m/z 345 of M3, suggesting that M8 was a glucuronide of M3. The proposed structure of M8 is shown in Fig. 6.

Oxidative Metabolism of PF-04971729 by Recombinant Human P450 Enzymes. To identify the human P450 isozyme(s) responsible for the metabolism of PF-04971729, PF-04971729 (20 μM) was incubated in NADPH-supplemented cDNA-expressed P450 isozymes CYP1A2, CYP2D6, CYP2C9, CYP2C19, CYP2E1, CYP3A4 and CYP3A5 at 37°C for 1 h. LC-MS/MS analysis indicated that CYP3A4/3A5 were the principle enzymes responsible for the biotransformation of PF-04971729 to metabolites M1 to M4 (Fig. 8). Trace amounts of M2 were also seen in recombinant CYP2D6 incubations of PF-04971729.
Glucuronidation of PF-04971729 by Recombinant Human UGT Enzymes. To identify the human UGT isozyme(s) that were responsible for the glucuronidation of PF-04971729, PF-04971729 (20 μM) was incubated in UDP-glucuronic acid-supplemented recombinant UGT isozymes (UGT1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10, 2B4, 2B7, and 2B15) at 37°C for 40 min. Among the panel of UGT
enzymes evaluated, only UGT1A9 and UGT2B7 catalyzed the glucuronidation of PF-04971729 to M5 regioisomers (Fig. 8).

**Human Pharmacokinetic Projections.** A single-species allometric scaling approach (Frederick et al., 2009; Hosea et al., 2009) of rat CL\(_p\) and V\(_d\)\(_{ss}\) values (normalized for protein binding) was used to project human clearance and volume. This exercise led to a predicted low CL\(_p\) (intravenous clearance = 1.7 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\) and oral clearance = 2.6 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\)) and moderate V\(_d\)\(_{ss}\) value of 1.8 l/kg for PF-04971729 in humans. The half-life of PF-04971729 in humans as derived from the equation \(t_{1/2} = 0.693 \times V_d/CL_p\) was estimated to be \(\sim 12\) h. Extrapolation of oral bioavailability from rat to human, using single species allometry, provided an estimate of \(\sim 65\%\).

**Single-Dose Pharmacokinetics of PF-04971729 in Humans.** After single oral dose administration to healthy volunteers, PF-04971729 was rapidly absorbed with mean plasma concentrations occurring at 0.5 to 1.5 h postdose. Systemic exposure as ascertained from C\(_{\text{max}}\) and AUC\(_{0-\infty}\) appeared to be dose-proportional over the dose range of PF-04971729 evaluated (0.5–300 mg) in healthy human volunteers (data not shown). The terminal half-life (\(t_{1/2}\)) of PF-04971729 was 11 to 17 h and oral clearance (CL\(_{p}\)) was within 1.5-fold of that predicted from single-species allometric scaling.

**Discussion**

The pharmacokinetics of the potent and selective SGLT2 inhibitor, PF-04971729, in rats and dogs was characterized by low clearance (<7 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\)) and a moderate steady-state distribution volume (0.8–1.1 l/kg). After oral administration as a methylcellulose/polyethylene glycol 400 suspension, PF-04971729 was rapidly absorbed in preclinical species with \(T_{\text{max}}\) values of \(\sim 0.5\) to 1 h. The fraction of the oral dose absorbed (\(F_a\)) in rat and dog was estimated using the equation \(F_a = F/\left(1 - CL_{\text{int}}/Q\right)\). Using rat and dog hepatic blood flows of 70 and 35 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\), \(F_a\) was estimated to be \(\sim 75\%\) and \(\sim 100\%\) for rats and dogs, respectively. Human oral \(F\) was predicted using rat \(F_a\), assuming linear correlation between the oral fraction absorbed in rat and human (Chiou and Barve, 1998). Thus, using the rat \(F_a\) value of 0.75 and predicted human clearance of 1.7 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\), the oral \(F\) of PF-04971729 in humans is anticipated to be \(\sim 65\%\) (Chiou and Barve, 1998). Thus, the oral \(F\) prediction for PF-04971729 is in agreement with the moderate absorptive permeability discerned in the Caco-2 assay. The BA/AB efflux ratio of 2.1, discerned in the Caco-2 permeability assay, suggests that PF-04971729 is potentially a substrate for efflux. However, the presence of the dual P-gp/BCRP inhibitor CP-100,356 did not greatly increase the absorptive permeability of PF-04971729. Although the overall impact of efflux transport on oral absorption of PF-04971729 remains unclear, high oral \(F\) was observed in rats and dogs. Furthermore, because of the linear pharmacokinetic response in oral preclinical species toxicity evaluation and first-in-human studies (data not shown), it is unlikely that efflux plays an important role in the oral absorption of PF-04971729.

Consistent with the observed low in vivo CL\(_{\text{int}}\), stability studies in liver microsomes and hepatocytes from preclinical species and humans did not lead to significant compound turnover. The inability to measure turnover of PF-04971729 in standard in vitro systems meant that traditional approaches of scaling in vitro intrinsic clearance data from human liver microsomes and hepatocytes to predict hepatic clearance in vivo could not be pursued for this compound. Thus, a single-species scaling approach that previously had been shown to be successful for a large data set of proprietary compounds (Frederick et al., 2009; Hosea et al., 2009) was used to predict human clearance. The predicted human CL\(_{\text{int}}\) (intravenous = 1.7 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\) and oral = 2.6 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\)) and V\(_d\)\(_{\text{ss}}\) (1.8 l/kg) for PF-04971729 obtained from single-species allometric scaling of rat pharmacokinetic data led to a predicted half-life of \(\sim 12\) h in human. The observed CL\(_{\text{int}}\)/F of PF-04971729 in humans over the dose range examined was within 1.5-fold of the predicted value of 2.4 ml \(\cdot\) min\(^{-1}\) \(\cdot\) kg\(^{-1}\). Likewise, the terminal half-life of 11 to 17 h in humans is in the vicinity of the predicted half-life of \(\sim 12\) h. Overall, the oral pharma-
cokinetics of PF-04971729 in humans was generally favorable as judged from the C_{max} and AUC, which increased in a dose-dependent fashion over the dose range examined.

Preliminary in vitro metabolite identification studies using a combination of liver microsomes and hepatocytes indicated that both phase I (P450-mediated oxidation) and phase II (UGT-mediated conjugation) metabolic pathways contributed to the biotransformation of PF-04971729. All metabolites detected in human in vitro systems were also observed in microsomes and hepatocytes from rat and dog, the preclinical species for toxicological evaluation. Studies in human hepatocytes indicated that glucuronidation of the glycosidic OH group(s) to the regioisomers M5b and M5c constituted the major metabolic fate of PF-04971729. Because PF-04971729 demonstrated no turnover in NADPH-supplemented human liver microsomes, preliminary reaction phenotyping was conducted using recombinant P450 enzymes. Results from this analysis revealed that oxidative metabolites of PF-04971729 were formed principally through the catalytic action of CYP3A4/3A5 enzymes. Similar studies examining the role of UGT isoforms in the glucuronidation of PF-04971729 revealed that UGT1A9 and UGT2B7 isoforms played a role. The likelihood that PF-04971729 will undergo significant biliary excretion in humans appears to be low because studies addressing this clearance mechanism in bile duct-exenterated rats revealed very little biliary excretion (<5% of the administered dose) after intravenous administration of PF-04971729. Furthermore, analysis of human urine in the clinical study revealed that unchanged PF-04971729 in urine was >72 h accounted for ~1% of the oral dose (data not shown). This finding contrasts with the observation in rats, wherein ~27% of the intravenous dose of PF-04971729 was excreted in the urine in the parent form. Whether the differences in renal excretion profile in rats and humans are due to differences in substrate affinity of PF-04971729 toward organic anion transporters in the respective species needs to be investigated further.

From a drug-drug interaction standpoint, the finding that PF-04971729 was devoid of competitive or time-dependent inhibition of the catalytic activities of the major human P450 enzymes suggests that there is low potential for pharmacokinetic interaction of PF-04971729 with coadministered drugs that are metabolized by these P450 enzymes. Likewise, inhibitory effects against the human OCT2 transporter by PF-04971729 were weak (IC_{50} > 900 μM), and at the unbound systemic exposures (free drug) of PF-04971729 (1.1–13 mg) are unlikely to cause pharmacokinetic interactions with the OCT2 substrate, metformin, a treatment of choice for T2DM (Setter et al., 2003). In the case of metformin, the principal clearance mechanism involves active renal excretion in the unchanged form, which is mediated by OCT2 (Kimura et al., 2005); modulation of this elimination mechanism through inhibition and/or genetic polymorphisms is known to result in drug-drug interactions (Bachmakov et al., 2009; Chen et al., 2009). Finally, with the involvement of multiple elimination mechanisms (e.g., CYP3A4/3A5 and CYP2D6 oxidation and UGT1A9/2B7 glucuronidation) for PF-04971729, the fraction of drug metabolized via a single clearance pathway is reduced and so is the potential for pharmacokinetic interactions involving that pathway. From a drug safety perspective, PF-04971729 did not form glutathione conjugates (consistent with reactive metabolite formation) in NADPH- and glutathione-fortified human liver microsomes; therefore, PF-04971729 should not present a risk for immune-mediated toxicity due to reactive metabolite formation.

To conclude, this study provides important information pertaining to the disposition profile of PF-04971729, a novel and selective SGLT2 inhibitor, in preclinical species and humans. A key highlight of this work was the ability to predict human pharmacokinetics with reasonable confidence using allometric scaling of in vivo data from a single animal species. The favorable absorption, distribution, metabolism, and excretion characteristics and lack of drug-drug interaction concerns with PF-04971729 supported its progression into clinical trials.

**Authorship Contributions**

**Participated in research design:** Kalgutkar, Tugnit, Zhu, Miao, Mascitti, Feng, and Robinson.

**Conducted experiments:** Kimito, Miao, Yang, Tan, Walsky, and Chupka.

**Contributed new reagents or analytic tools:** Mascitti and Robinson.

**Performed data analysis:** Kimito, Miao, Yang, Walsky, and Chupka.

**Wrote or contributed to the writing of the manuscript:** Kalgutkar, Tugnit, and Zhu.

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Address correspondence to: Dr. Amit S. Kalgutkar, Pharmacokinetics, Dynamics, and Metabolism Department, Pfizer Global Research and Development, Groton, CT 06340. E-mail: amit.kalgutkar@pfizer.com