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

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ABSTRACT:

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 (SGLT) 2, expressed mainly in the S1/S2 segment of 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-
gycemia 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 mecha-
nism of action, SGLT2 inhibitors are associated with a low risk for hy-
poglycemia (Katsuno et al., 2007; Fujimori et al., 2008; Wilding et
al., 2009). Furthermore, there is the potential for clinically signif-
icient weight loss and reduced hepatic glucose production and amelioration of gluctotoxicity (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 insulin. Furthermore, there is the potential for clinically signif-
icant weight loss and reduced hepatic glucose production and amelioration of glucotoxicity (Abdul-Ghani and DeFronzo, 2008; Nair and Wilding, 2010).

We recently disclosed a novel class of SGLT2 inhibitors bearing a unique dioxa-bicyclo[3.2.1]octane (bridged ketal), from which (1S,2S,3R,5S)-5-[4-chloro-3-(4-ethoxybenzyl)phenyl]-1-hydroxy-
methyl-6,8-dioxbicyclo[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 pharmacokinetic 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 isomeric purity >99% by HPLC and NMR), N-(3,4-dimethoxyphenyl)-4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2-[1H]-yl)-6,7-dimethoxyquino-
zolin-2-amine (CP-100,356) and the selective cytochrome P450 P450 (P450) 2C19 inhibitor (++)-N-benzylindanol were synthesized at Pfizer Global Research and Development (Groton, CT). NADPH was purchased from Sigma-Aldrich (St. Louis, MO). Probe P450 substrates (phenacetin, bupropion, amodiaquine, diclofe-
nac, S-mephentoin, dextromethorphan, felodipine, testosterone, and midazolam) for individual P450 enzymes and recombinant human UDP-glucuronosyltransferase (UGT) isoforms were purchased from BD Gentest (Woburn, MA). Recom-
binant human P450 isoforms were purchased from Invitrogen (Carlsbad, CA). Cryopreserved rats, dog, and human hepatocytes were obtained from Celsis (Chi-

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 polystyrene-coated plates (Biorad, 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 Hanks’ balanced salt solution, pH 7.4, prewarmed to 37°C. After a 5-min acclimation period, the cells were incubated with 150 μl of Hanks’ balanced salt solution buffer containing [3H]metformin (5 μM). To increase assay sensitivity, the metformin concentration chosen for the inhibition study was below its reported Kᵢₐ 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 cotransporter. After 5 min at 37°C, the cellular uptake was terminated by washing the cells three times with 1 ml of ice-cold Hanks’ 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 [3H]metformin (5 μM) spiked with PF-04971729 and applied simultaneously to the cells. 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 Multiblock plates (polyethylene terephthalate membranes, pore size 1.0 μm) at 4.0 × 10⁴ 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 (Kalugtik 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 through 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-γ-prolylglutamate cocrystal 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. in 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% sulfobutylether-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 trapezoidal 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 absolute bioavailability (F) of the oral doses was calculated by using the following equation:

\[ F = \frac{AUC_{p.o.}}{AUC_{i.v.}} \times \frac{dose_{i.v.}}{dose_{p.o.}} \]

Bioanalytical Methodology. Mass spectrometry was performed on a Sciex API 4000 system equipped with a TurbolonSpray 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 250 × 5 mm 4 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/x².

Pharmacokinetic Projections to Human. The single-species allometric method was used to predict human pharmacokinetic parameters of PF-04971729. CLp and Vdss were 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]:

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

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

\[ t_{1/2,\text{human}} = \frac{\ln(2)}{\text{Vdss}_{\text{human}}/\text{CL}_{\text{human}}} \]
collision-induced dissociation (CID) spectra of the molecular ions (MH+ with synthetic standard(s), and structural information was generated from the LC-MS/MS of 15–20 livers), dog (pool of 4–8 male livers), or human (pool of three livers, one female and two males) hepatocytes were thawed and suspended in Williams’ E medium supplemented with 24 mM NaHCO3 and 10% fetal bovine serum at 2 × 106 viable cells/ml. PF-04971729 (10 µM) was incubated with hepatocytes at 37°C for 4 h with gentle agitation. A gas mixture of O2:CO2 (95:5) maintained at −2.5 kPa for −5 s was passed through this mixture at every hour of incubation. Flasks were crinkled immediately after gassing. Reactions were stopped by addition of ice-cold acetonitrile (10 ml) and centrifuged (3000 × g; 15 min). The supernatants were dried under a steady nitrogen stream, reconstituted with 25% aqueous acetonitrile (250 µl), and analyzed by LC-MS/MS for metabolite formation.

Cryopreserved hepatocytes. Cryopreserved rat (male Sprague-Dawley, pool of 15–20 livers), dog (pool of 4–8 male livers), or human (pool of three livers, one female and two males) hepatocytes were thawed and suspended in Williams’ E medium supplemented with 24 mM NaHCO3 and 10% fetal bovine serum at 2 × 106 viable cells/ml. PF-04971729 (10 µM) was incubated with hepatocytes at 37°C for 4 h with gentle agitation. A gas mixture of O2:CO2 (95:5) maintained at −2.5 kPa for −5 s was passed through this mixture at every hour of incubation. Flasks were crinkled immediately after gassing. Reactions were stopped by addition of ice-cold acetonitrile (10 ml) and centrifuged (3000 × g, 15 min). The supernatants were dried under a steady nitrogen stream, reconstituted with 25% aqueous acetonitrile (250 µl), and analyzed by LC-MS/MS for metabolite formation.

Bioanalytical Methodology for Metabolite Identification. Qualitative assessment of the metabolism of PF-04971729 was conducted using a Thermo LTQ or LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) operated with a electrospray source and coupled to a Thermo Fisher Scientific Separation SpectroMonitor 3200 UV. The monitoring wavelength was 254 nm. Analyses were chromatographically separated using an HPLC system, which was composed of an HP-1050 solvent delivery system, HP-1050 membrane degasser, and HP-1050 autoinjector (Hewlett Packard, Palo Alto, CA). The autoinjector was programmed to inject 50 µl of sample onto a Synergi Fusion C-18 250 × 4.6 mm 5-µm column (Phenomenex) using a gradient consisting of a mixture of 5 mM ammonium formate (pH 3.0) (solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml/min. The binary gradient was as follows: solvent A to solvent B ratio was held at 95:5 (v/v) for 3 min and then adjusted to 55:45 (v/v) from 0 to 35 min, 30:70 (v/v) from 35 to 45 min, and 5:95 (v/v) from 45 to 52 min where it was held for 3 min and then returned to 95:5 (v/v) for 6 min before next analytical run. Postcolumn flow was split such that the mobile phase was introduced into the mass spectrometer via an ion spray interface at a rate of 50 µl/min. The remaining flow was diverted to the UV detector positioned in line to provide simultaneous UV detection (λ = 254 nm) and a total ion chromatogram. The mass spectrometer was operated in the positive ion mode, and the ion was selected at a spray voltage 4.5 kV. The scan event cycle used a full-scan mass spectrum at unit resolution (LTQ) or at 30,000 resolution (Fourier transform). The exact mass measurement was based on external calibration performed on the same day. Initial full scans were performed between m/z 50 and 1000. PF-04971729 and its identifiable metabolites eluted in the first 30 min. Metabolites were identified in the full-scan mode (from m/z 100 to 850) by comparing r = 0 samples with r = 40 to 60 min (liver microsomes)/r = 240 min (hepatocytes) samples or through comparison with synthetic standard(s), and structural information was generated from the collision-induced dissociation (CID) spectra of the molecular ions (MH+). Because of the lack of nitrogen atoms and/or other positively charged sites in the structural architecture of PF-04971729, CID spectra of parent and metabolites were obtained from the corresponding ammonium adducts, and structure elucidation was based on these adducts.

Clinical Pharmacokinetic Data. The pharmacokinetics of PF-04971729 were evaluated in healthy volunteer subjects in a randomized, placebo-controlled, ascending single oral dose, crossover study over the dose range of 0.5 to 300 mg. The protocol was reviewed and approved by an independent ethics committee, and all subjects gave written informed consent. There were two cohorts in the study with 12 healthy volunteers per cohort. Subjects in cohort 1 received two single doses of PF-04971729 and one placebo dose in random order; in addition, one dose of PF-04971729 was administered in the fed state. Subjects in cohort 2 received two single doses of PF-04971729 and one dose of placebo in a random order. Subjects were healthy volunteers aged 18 to 55 years, and females participating in this study were of non-childbearing potential.

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 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, CYP2C19, 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, clotidigrel, montelukast, sulfaphenazole, (+)-N-3-benzylirvalno, 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 >80% [14C]Metformin uptake mediated by hOCT2 (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

![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).](Link to image)
In the dog, after a intravenous dose of 2.0 mg/kg, PF-04971729 exhibited a low blood clearance (CL\textsubscript{b}) of 2.0 mg/kg, PF-04971729 ascertained from C\textsubscript{max} and AUC\textsubscript{0–\infty} was 2500 ± 215 ng/ml and 19,100 ± 3650 ng-h/ml, respectively. The time to reach C\textsubscript{max} 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 (t\textsubscript{R}) 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 isoforms catalyze the rate-limiting step in their formation. PF-04971729 exhibited an ammonium adduct ([M + NH\textsubscript{4}]\textsuperscript{+}, 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 + NH\textsubscript{4}]\textsuperscript{+}, 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 fragments at m/z 435, 407, 299, and 243. The ion at m/z 435 was due to the sequential loss of NH\textsubscript{3} and H\textsubscript{2}O. 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 structures for M1 to M4 is shown in Table 1, and mean plasma concentration versus time profiles are depicted in Fig. 3. An intravenous dose of 2.0 mg/kg, PF-04971729 exhibited a low blood clearance (CL\textsubscript{p}) of 4.04 ml \cdot min\textsuperscript{-1} \cdot kg\textsuperscript{-1} (obtained by dividing the CL\textsubscript{p} of 4.04 ml \cdot min\textsuperscript{-1} \cdot kg\textsuperscript{-1} by the blood/plasma ratio of 0.66), which ~6% of rat hepatic blood flow of 70 ml \cdot min\textsuperscript{-1} \cdot kg\textsuperscript{-1}. The V\textsubscript{dss} 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 (t\textsubscript{1/2}) was estimated to be 4.10 h. The percentage of unchanged PF-04971729 excreted over a 24-h period in the urine was 7.48 ± 0.13 h.

### 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 (CL\textsubscript{p}) of 6.12 ml \cdot min\textsuperscript{-1} \cdot kg\textsuperscript{-1} (obtained by dividing the CL\textsubscript{p} of 4.04 ml \cdot min\textsuperscript{-1} \cdot kg\textsuperscript{-1} by the blood/plasma ratio of 0.66), which ~6% of rat hepatic blood flow of 70 ml \cdot min\textsuperscript{-1} \cdot kg\textsuperscript{-1}. The V\textsubscript{dss} 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 (t\textsubscript{1/2}) was estimated to be 4.10 h. The percentage of unchanged PF-04971729 excreted over a 24-h period in the urine was 7.48 ± 0.13 h.

### Table 1

<table>
<thead>
<tr>
<th>Species (strain)</th>
<th>Gender/No.</th>
<th>Dose\textsuperscript{a}</th>
<th>Route</th>
<th>C\textsubscript{max}</th>
<th>T\textsubscript{max}</th>
<th>CL\textsubscript{p}</th>
<th>V\textsubscript{dss}</th>
<th>AUC\textsubscript{0–\infty}</th>
<th>t\textsubscript{1/2}</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (Sprague-Dawley)</td>
<td>M/2</td>
<td>2.0 i.v.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>4.04 (3.39, 4.68)</td>
<td>1.13 (1.18, 1.08)</td>
<td>8480 (9830, 7040)</td>
<td>4.08 (4.85, 3.31)</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>Dog (beagle)</td>
<td>M/3</td>
<td>5.0 p.o.</td>
<td>1940 ± 185</td>
<td>1.0 ± 0.0</td>
<td>N.A.</td>
<td>14,700 ± 4320</td>
<td>N.A.</td>
<td>7.63 (7.42, 7.84)</td>
<td>N.A.</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>M/3</td>
<td>2.0 i.v.</td>
<td>1940 ± 185</td>
<td>1.0 ± 0.0</td>
<td>N.A.</td>
<td>20,400 (19,400, 21,400)</td>
<td>N.A.</td>
<td>7.48 ± 0.13</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M/3</td>
<td>2.0 i.v.</td>
<td>2500 ± 215</td>
<td>0.83 ± 0.29</td>
<td>N.A.</td>
<td>19,100 ± 365</td>
<td>N.A.</td>
<td>7.48 ± 0.13</td>
<td>94</td>
<td></td>
</tr>
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</table>

\textsuperscript{a} The dosing vehicle used in intravenous and oral dosing to rats was DMSO-polyethylene glycol 400–30% sulfobutylether-β-cyclodextrin (10:30:60, v/v/v) and 0.5% (w/v) methyl cellulose with 10% (v/v) polyethylene glycol 400. The dosing vehicle used in intravenous and oral dosing to dogs was 5% polyethylene glycol 400 in 23% hydroxypropyl-β-cyclodextrin and 0.5% methyl cellulose (w/v) with 10% (v/v) polyethylene glycol 400, respectively.

\textsuperscript{b} AUC\textsubscript{0–\infty}.

CP-100,356 (Kalgutkar et al., 2009) did not greatly increase the absorptive permeability (4.7 ± 10 \times 10\textsuperscript{-6} cm/s) of fosaprepitant. Thus, P-Gp and BCRP do not appear to be limiting factors for oral absorption of PF-04971729.

**Fig. 3.** Concentrations of PF-04971729 in plasma of rats (A) and dogs (B) after intravenous and oral administration. Each point represents the mean of two or three determinations.
fragment ions is depicted in Fig. 6. Metabolite M2 possessed a molecular ion at 426.1320 ([M + NH₄]⁺, a loss of 28 Da from the molecular mass of PF-04971729); the CID spectrum and tᵱ 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 ([M + NH₄]⁺ = 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 chloro-benzyl 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 NH₃ and H₂O, 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 [M + NH₄]⁺ 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 NH₃ and H₂O. 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>tᵱ (min)ᵃ</th>
<th>Observed [M + NH₄]⁺ᵇ</th>
<th>LC-MS⁶ fragments, m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₆a</td>
<td>11.6</td>
<td>602.1640</td>
<td>MS² on m/z 602.16404: 409.1049, 301.0626, 207.0207; MS³ 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>MS² 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>MS² on m/z 602.16401: 409.1049, 301.0626, 207.0207; MS³ 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>MS² on m/z 602.16402: 409.1049, 301.0626, 207.0207; MS³ 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>MS² on m/z 646.19: 470.06, 453.12, 345.04, 207.14; MS³ on m/z 646.19 → 453: 345, 297; MS⁴ on m/z 646.19 → 435: 345 → 207, 151, 139, 123</td>
</tr>
<tr>
<td>M₂</td>
<td>15.9</td>
<td>426.1320</td>
<td>MS² on m/z 426.1320: 409.1050, 301.0626; MS³ on m/z 426 → 409: 301.0628; MS⁴ 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>MS² on m/z 470.1580: 435.1204; MS³ 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>MS² on m/z 470.1583: 453.1311, 345.0889, 207.0206, 151.0755; MS³ on m/z 470 → 435: 207.0208, 151.0754, 139.0754, 123.0440</td>
</tr>
<tr>
<td>M₅a</td>
<td>18.7</td>
<td>630.1952</td>
<td>MS² 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>MS² 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>MS² on m/z 470.15: 435.1204; MS³ 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>MS² 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>MS² on m/z 454.1632: 437.1359; MS³ 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.

Fig. 4. Extracted ion chromatograms of incubation mixtures of PF-04971729 (10 µM) in NADPH-supplemented liver microsomes from rat (A), dog (B), and human (C).
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 \([M + NH_4]^+\) 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 \([M + NH_4]^+\) 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 NH$_3$ 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 \([M + NH_4]^+\) 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 MS$^2$ and MS$^3$ 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 glucuronic acid (176 Da) and NH$_3$, respectively. The diagnostic ion at \(m/z\) 207, also observed in PF-04971729, suggested that the chlorophenyl moiety was unaltered in M8. The MS$^3$ spectrum of \(m/z\) 345 was identical to the MS$^3$ 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 \(\mu\)M) 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.

*Fig. 5. CID spectrum of PF-04971729. Top, MS$^2$ spectrum of \((M + NH_4)^+ = 454\). Bottom, MS$^3$ spectrum of \((m/z + H)^+ = 329.\)*
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 isozymes tested, UGT1A1, 1A4, and 1A9 were found to be responsible for the glucuronidation of PF-04971729.

FIG. 6. Proposed structures and assignments of the MS fragmentation pattern for the metabolites of PF-04971729 in liver microsomes and hepatocytes from rat, dog, and human.

FIG. 7. Extracted ion chromatograms of incubation mixtures of PF-04971729 (10 μM) in cryopreserved hepatocytes from rat (A), dog (B), and human (C).
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 Vd\(_\text{ss}\) 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·min\(^{-1}\)·kg\(^{-1}\) and oral clearance = 2.6 ml·min\(^{-1}\)·kg\(^{-1}\)) and moderate Vd\(_\text{ss}\) value of 1.8 l/kg for PF-04971729 in humans. The half-life of PF-04971729 in humans as derived from the equation \(t_{1/2} = 0.693 \times \text{Vd}/\text{CL}_p\) was estimated to be \(~12\) h. Extrapolation of oral bioavailability from rat to human, using single species allometry, provided an estimate of \(~65\%\).

**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\(_\text{0-}\infty\) 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/F) 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·min\(^{-1}\)·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 ~0.5 to 1 h. The fraction of the oral dose absorbed (\(F_A\)) in rat and dog was estimated using the equation \(F_A = F(1 - \text{CL}_p/Q)\). Using rat and dog hepatic blood flows of 70 and 35 ml·min\(^{-1}\)·kg\(^{-1}\), \(F_A\) was estimated to be ~75 and ~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·min\(^{-1}\)·kg\(^{-1}\), the oral \(F\) of PF-04971729 in humans is anticipated to be ~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\(_p\), 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\(_p\) (intravenous = 1.7 ml·min\(^{-1}\)·kg\(^{-1}\) and oral = 2.6 ml·min\(^{-1}\)·kg\(^{-1}\)) and Vd\(_\text{ss}\) (1.8 l/kg) for PF-04971729 obtained from single-species allometric scaling of rat pharmacokinetic data led to a predicted half-life of ~12 h in human. The observed CL/F of PF-04971729 in humans over the dose range examined was within 1.5-fold of the predicted value of 2.4 ml·min\(^{-1}\)·kg\(^{-1}\). Likewise, the terminal half-life of 11 to 17 h in humans is in the vicinity of the predicted half-life of ~12 h. Overall, the oral pharma-
cokinetics of PF-04971729 in humans was generally favorable as judged from the $C_{\text{max}}$ and AUC, which increased in a dose-dependent fashion over the dose range examined.

Preliminary in vitro metabolism 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 recombiant 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-extirpated 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 over 72 h accounted for ~1% of the oral dose (data not shown). This finding contrasts with the observations 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 ($IC_{50} > 900 \; \mu M$), and at the unbound systemic exposures (free $C_{\text{max}} = 1.0–11 \; nM$) associated with efficacious daily doses 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 needs to be investigated further.

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, Tugnait, 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:** Kalgutkar, Miao, Yang, Walsky, and Chupka.

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

References


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