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Examination of the Human Cytochrome P4503A4 Induction Potential of PF-06282999, an Irreversible Myeloperoxidase Inactivator: Integration of Preclinical, *In Silico*, and Biomarker Methodologies in the Prediction of the Clinical Outcome

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Abbreviations used are: CYP, cytochrome P450; mRNA, messenger RNA; DDI, Drug-drug interaction; PF-06282999, 2-(6-(5-chloro-2-methoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamide; MPO, myeloperoxidase; FIH, first-in-human; PK, pharmacokinetics; MAD, multiple ascending dose; AUC, area under the plasma concentration time curve; PBPK, physiologic-based pharmacokinetic modeling; DMSO, dimethyl sulfoxide; TID, three times daily; BID, twice daily; LC–MS/MS, liquid chromatography tandem mass spectrometry; C_{\max} , peak plasma concentration, t_{\max} , time to reach C_{\max} ; C_{\min} , minimum or trough concentration; C_{av} , average concentration; $t_{1/2}$, half-life; m/z , mass-to-charge; CI, confidence interval.

Abstract:

The propensity for cytochrome P450 (CYP)3A4 induction by 2-(6-(5-chloro-2-methoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamide (PF-06282999), an irreversible inactivator of myeloperoxidase, was examined in the present work. Studies using human hepatocytes revealed moderate increases in CYP3A4 messenger RNA (mRNA) and midazolam-1'-hydroxylase activity in a PF-06282999 dose-dependent fashion. At the highest tested concentration of 300 μ M, PF-06282999 caused maximal induction in CYP3A4 mRNA and enzyme activity ranging from 56%–86% and 47%–72%, respectively, of rifampicin response across the three hepatocyte donor pools. In a clinical drug-drug interaction (DDI) study, mean midazolam C_{\max} and AUC following 14-day treatment with PF-06282999 decreased in a dose-dependent fashion with a maximum decrease in midazolam $AUC_{0-\infty}$ and C_{\max} of ~57.2% and 41.1% observed at the 500 mg BID dose. The moderate impact on midazolam pharmacokinetics at the 500 mg BID dose of PF-06282999 was also reflected in statistically significant changes in plasma 4 β -hydroxycholesterol/cholesterol and urinary 6 β -hydroxycortisol/cortisol ratios. Changes in plasma and urinary CYP3A4 biomarkers did not reach statistical significance at the 125 mg TID dose of PF-06282999, despite a modest decrease in midazolam systemic exposure. Predicted DDI magnitude based on the in vitro induction parameters and simulated pharmacokinetics of perpetrator (PF-06282999) and victim (midazolam) using Simcyp population-based simulator were in reasonable agreement with the observed clinical data. As the magnitude of the 4 β -hydroxycholesterol or 6 β -hydroxycortisol ratio change was generally smaller than the magnitude of midazolam AUC change with PF-06282999, a pharmacokinetic interaction study with midazolam ultimately proved important for assessment of drug-drug interaction via CYP3A4 induction.

Introduction

The important role of CYP enzymes in drug metabolism is highlighted by the fact that the CYP1, 2, and 3 families combined are responsible for the biotransformation of most xenobiotics including 70–80% of all medicinal products in clinical use (Nelson, 2006; Guengerich, 2008; Zanger et al., 2008). Within the human CYP superfamily of drug-metabolizing enzymes, constitutively expressed CYP3A4 has the highest liver expression and plays a major role in the metabolism of ~55% of drugs from almost all therapeutic categories (Wienkers and Heath, 2005; Bu, 2006; Liu et al., 2007). DDIs caused by inhibition and/or induction of drug-metabolizing enzymes, particularly CYP3A4, can impact the efficacy and safety of co-administered drugs (Lin and Lu, 1998; Isoherranen et al., 2012). In vitro characterization of CYP inhibitory/induction potential of new investigational drug substances and interpretation of the generated results are critical steps in the drug development process, particularly in the design of relevant clinical DDI studies, and is also required for regulatory filing

(<http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf>;
http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf).

2-(6-(5-Chloro-2-methoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2*H*)-yl)acetamide (PF-06282999, Figure 1) is an irreversible inactivator of the human myeloperoxidase (MPO) enzyme (Ruggeri et al., 2015) and is a clinical candidate for the treatment of acute coronary syndrome and related cardiovascular disorders. PF-06282999 has been evaluated in first-in-human (FIH) clinical studies for assessment of safety and tolerability (Dong et al., 2016). PF-06282999 is virtually resistant to metabolic turnover in human liver microsomes and human

hepatocytes, and is principally eliminated in unchanged form via renal excretion in healthy adult volunteers (Dong et al., 2016). Although PF-06282999 was devoid of inhibitory (reversible and/or time-dependent) effects against major human CYP enzymes including CYP3A4, subsequent studies in cryopreserved human hepatocytes revealed a propensity for CYP3A4 induction upon pretreatment with PF-06282999. Because the degree of MPO inhibition that leads to a clinical benefit in cardiovascular disease remain uncharacterized at the present time, there was much uncertainty around the clinically efficacious dose (and relevant systemic exposure) of PF-06282999 in relation to DDI potential with “victim” drugs (e.g., calcium channel blockers, lipid-lowering agents, etc.) that are subject to metabolism by CYP3A4 in a cardiovascular disease patient population (Niemi et al., 2003; Neuvonen et al., 2006).

Assessment of clinical DDIs arising from inhibition and/or induction of CYP3A4 by investigational drug substances usually relies on the use of midazolam as a victim drug and an exogenous marker of CYP3A4 metabolic activity (Sinz et al., 2008; Fahmi et al., 2009; Kirwan et al., 2010; Jones et al., 2016). Apart from pharmacokinetics (PK) interaction studies with probe CYP3A4 substrates, changes in plasma and urinary ratios of 4 β -hydroxycholesterol to cholesterol and 6 β -hydroxycortisol to cortisol, respectively, can be used as endogenous biomarkers of CYP3A4 activity (Bodin et al., 2001; Nowak et al., 2002; Galteau and Shamsa, 2003; Konishi et al., 2004; Diczfalusy et al., 2011; Marde Arrhen et al., 2013). The selective CYP3A4-mediated metabolic conversion of cholesterol to 4 β -hydroxycholesterol and/or cortisol to 6 β -hydroxycortisol, respectively, is utilized increasingly in clinical studies as non-invasive phenotypic markers of CYP3A4 induction, and are often implemented in phase 1 multiple ascending dose (MAD) clinical studies (Bjorkhem-Bergman et al., 2013; Dutreix et al., 2014). In our present work, a MAD study in healthy volunteers was conducted with PF-06282999 to

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assess the safety, tolerability and PK upon repeated oral dosing, with a particular emphasis on the measurement of the endogenous plasma (4 β -hydroxycholesterol/cholesterol) and urine (6 β -hydroxycortisol/cortisol) biomarkers as surrogates for CYP3A4 induction. A DDI protocol was then integrated into the study design and included the co-administration of PF-06282999 with midazolam to estimate the magnitude of area under the plasma concentration time curve (AUC) change for midazolam as a result of CYP3A4 induction by PF-06282999. Finally, *in vitro* CYP3A4 induction data in human hepatocyte cultures and the available clinical PK data were used to rationalize the clinical observations via dynamic physiologically-based pharmacokinetic (PBPK) modeling using the Simcyp population-based simulator. The collective findings are summarized, herein.

Materials and Methods

Materials. PF-06282999 (chemical purity > 99% by high-performance liquid chromatography and nuclear magnetic resonance) was synthesized at Pfizer Worldwide Research and Development (Groton, CT). Hepatocyte thawing, plating, and incubation media as well as the Torpedo antibiotic mix for the media were purchased from Bioreclamation IVT Celsis (Baltimore, MD) and CellzDirect (Pittsboro, NC). Rifampin, midazolam, 1'-hydroxymidazolam, Dulbecco's phosphate-buffered saline, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Cryopreserved human hepatocytes lot # HH1089 (Caucasian female, 57 years), HC7-4 (Caucasian male, 7 years), and FOS (Arabic male, 34 years) were purchased from In Vitro ADMET Laboratories (Columbia, MD), XenoTech (Lenexa, KS), and Bioreclamation IVT Celsis, (Baltimore, MD), respectively.

CYP3A4 Induction in Human Hepatocytes. Cryopreserved human hepatocytes were used to examine CYP3A4 induction potential of PF-06282999 using established protocols (Fahmi et al., 2008b). Briefly, primary cultures of human hepatocytes from three donors (HH1089, HC7-4, and FOS) were seeded in collagen I-precoated 24-well plates, and each well had a cell density of $\sim 2 \times 10^5$ viable cells. Hepatocyte cultures were treated for 3 consecutive days with medium containing solvent (0.1% DMSO) control, PF-0628999 (0.3, 2, 5, 10, 20, 30, 40, 50, 75, 100, 150, 200, and 300 μM), or prototypical CYP3A4 inducer rifampicin (0.01, 0.1, 0.5, 1, 5 and 10 μM). Following the incubation phase of the experiment, the medium containing test compounds or vehicle was removed and the cells were washed with phosphate buffered saline and fresh medium was added to all wells for a 30 minute wash. Following removal of the wash media, midazolam (30 μM) in 0.1% DMSO was added to each well and the plate was returned to the incubator for 30 minutes. After incubation, 200 μl of the medium from each well was removed and analyzed for 1-hydroxymidazolam formation using established liquid chromatography tandem mass spectrometry (LC-MS/MS) protocols (Walsky and Obach, 2004). Quantification of CYP3A4 mRNA was performed using the TaqMan[®] as previously described (Fahmi et al., 2008b).

In Vitro Data Analysis. Data for mRNA and activity (normalized to DMSO vehicle control) were plotted versus the concentration of the PF-06282999 or rifampin. Individual fitting was carried out on each hepatocyte donor using GraphPad Prism (version 6), and was fit to either a four-parameter sigmoidal model (equation 1, fitting E_{max} , Hill slope γ , and EC_{50} , with baseline fixed at 1) or a three-parameter sigmoidal model (equation 2, fitting E_{max} and EC_{50} , with Hill slope and baseline each fixed at 1). The four parameter model was preferred if adding the extra

parameter produced a fit with a p value < 0.05 compared to the three parameter model by the built-in GraphPad extra sum-of-squares F test.

$$\text{Fold Induction} = 1 + \frac{(E_{\text{max}}-1) \cdot \text{Conc}^{\gamma}}{EC_{50}^{\gamma} + \text{Conc}^{\gamma}} \quad \text{equation 1}$$

$$\text{Fold Induction} = 1 + \frac{(E_{\text{max}}-1) \cdot \text{Conc}}{EC_{50} + \text{Conc}} \quad \text{equation 2}$$

Clinical DDI Study Design. The DDI potential of PF-06282999 was characterized in a clinical study (clinical trial identifier NCT01707082) comprising of healthy adult volunteers. This study was conducted according to the ethical principles of the Declaration of Helsinki and all protocols and informed consent documents were approved by the Ethics Committee for the study center. All subjects signed an approved written informed consent form before any study-related activities. There were two parts to this clinical study. Part A was a blinded, randomized, placebo-controlled, parallel group, escalating multiple oral dose study in healthy overweight adult subjects to assess the safety, tolerability and PK of PF-06282999 following 14 days of oral administration. A total of 48 male subjects and 2 female subjects (86% Caucasian, 4% Black and 10% Other) aged 22 to 55 years old, with a body weight range of 62.2-116.5 kg and a BMI range of 27.0–34.8 kg/m², were enrolled in 5 cohorts (10 per cohort, 8 active and 2 placebo). The doses tested were 10 mg three times daily (TID), 30 mg TID, 100 mg TID, 250 mg TID and 500 mg twice daily (BID), all administered with food. PF-06282999 was administered as an immediate-release tablet during both periods of the study. The safety and tolerability of PF 06282999 was assessed by adverse events, electrocardiograms, vital signs, and safety laboratory measurements. In addition to collection of safety and PK data, concentrations of plasma (4 β -hydroxycholesterol/cholesterol) and urinary (6 β -hydroxycortisol/cortisol) biomarkers associated with CYP3A4 induction were also assessed at the various PF-06282999 dose groups.

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Part B of the study was an open label, fixed sequence DDI study in healthy adult volunteers evaluating the impact of PF-06282999 on the single dose PK of oral midazolam. A total of 18 male subjects (94% Caucasian and 6% Other) aged 20 to 48 years old, with a body weight range of 57.3–86.0 kg and a body mass index range of 19–27.3 kg/m², were enrolled in 2 cohorts (9 per cohort) in order to evaluate the effect of 2 dose levels of PF-06282999 on the PK of oral midazolam. The two dose levels of PF-06282999 (125 mg TID and 500 mg BID) were selected based on review of emerging data from Part A of the study, including the review of the available urinary and plasma biomarker data. This DDI part of the study was conducted in 2 periods. On day 1 of period 1 after an overnight fast, subjects received a single 7.5 mg tablet oral dose of midazolam followed by collection of blood samples for up to 16 hours post dose for evaluation of midazolam PK. On the next day, period 2 day 1 commenced when subjects received their initial dose of PF-06282999. All doses were provided at approximately the same time of day throughout the study for 13 days. All doses of PF-06282999 were administered following completion of a standard meal. On day 14 of period 2 after an overnight fast, subjects received another single 7.5 mg oral dose of midazolam. Two hours later, PF-06282999 was administered following completion of a standard meal (the only dose to be administered on day 14). Midazolam was administered at a similar time on day 1 period 1 and day 14 period 2.

Evaluation of PF-06282999 PK and Endogenous Biomarkers of CYP3A4 Induction. For part A, serial blood samples were collected prior to dosing and up to 48 hours after the first and the last dose for quantitation of circulating levels of PF-06282999. Sparse sampling was also performed on subsequent dosing days to confirm steady-state status of systemic exposure. For part B, only pre-dose blood samples were collected on selected days to confirm adequate exposures to PF-06282999. Blood samples were centrifuged to generate plasma and

concentrations of PF-06282999 were determined using a validated LC-MS/MS method (Dong et al., 2016). PK parameters were calculated using non-compartmental analysis (eNCA, a Pfizer internally validated system of non-compartmental analysis). The steady state plasma PK parameters area under the concentration-time curve over dosing interval ($AUC_{0-\tau}$), peak plasma concentration (C_{max}), time to peak concentration (t_{max}), minimum or trough concentration (C_{min}), and average concentration (C_{av}) over a dosing interval, half-life ($t_{1/2}$), and the observed accumulation ratio for $AUC_{0-\tau}$ and C_{max} were summarized descriptively by dose.

Measurement of plasma (cholesterol and 4 β -hydroxycholesterol) and urine (cortisol and 6 β -hydroxycortisol) biomarkers was performed at SGS Cephac Europe (Saint Benoît Cedex, France). Blood samples were also collected in both part A and part B segments of the study for analysis of plasma cholesterol and 4 β -hydroxycholesterol concentrations. Cholesterol levels were measured following chemical derivatization of plasma cholesterol to the corresponding dansylated derivative (Tang and Guengerich, 2010). Plasma samples were diluted (100-fold) with a solution of bovine serum albumin in phosphate buffered saline (40 g/l) containing 1% butyl-4-hydroxytoluene (22.69 mM), followed by the addition of internal standard [2H_6]-cholesterol (50 μ g/ml) to ~ 100 μ l portions of the diluted plasma samples. The samples were then treated with ethanolic sodium hydroxide solution (9 M) in water and incubated for 5-15 minutes at 37 °C followed by the addition of 200 μ l of derivatization solution (dansyl chloride (150 mg) and dimethylaminopyridine (150 mg) in 15 ml of dichloromethane) in the presence of 2% diisopropylethylamine solution and heated to approximately 65 °C for 1 hour as previously described (Tang and Guengerich, 2010). The samples were centrifuged and the organic phase was evaporated to dryness, and the residue was reconstituted with 200 μ l of mobile phase (5 mM ammonium acetate in acetonitrile) prior to LC-MS/MS analysis. Samples (5-10 μ l) were

analyzed on a API-4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) with a Phenomenex, Kinetex C18 column (2.6 μm , 150 x 2.1 mm, 100 \AA) at 40-55 $^{\circ}\text{C}$. For separation of dansyl cholesterol from endogenous plasma components, liquid chromatography was performed under isocratic conditions using a mobile phase comprising of 5 mM ammonium acetate in acetonitrile at a flow rate of 0.5 ml/min. Under these conditions, the retention time for dansyl cholesterol (and its stable-labeled internal standard) was 1.8 minutes. Dansylated derivatives of cholesterol and the internal standard [$^2\text{H}_6$]-cholesterol were detected using electrospray ionization (positive ion mode) in the multiple reaction monitoring mode, monitoring for mass-to-charge (m/z) transitions 620 \rightarrow 252 (cholesterol) and 626 \rightarrow 252 ([$^2\text{H}_6$]-cholesterol). The lower limit of quantification was 1.0 $\mu\text{g/ml}$ with an overall assay precision of $\leq 8.0\%$, and % relative error from -8.26% to 2.07%.

For measurement of 4 β -hydroxycholesterol plasma levels, plasma samples (200 μl) were diluted with methanol (20 μl) containing the internal standard ([$^2\text{H}_7$]-4 β -hydroxycholesterol (1250 ng/ml). The samples were centrifuged (3000 x g) for 10 min and the supernatant was dried under a steady stream of nitrogen. The residue was reconstituted with hexane containing 0.5% isopropanol, loaded onto solid phase extraction cartridges (Biotage, Isolute Si 100 mg, 3cc), and eluted with 3 ml of hexane/isopropanol (70/30; v/v). The eluate was evaporated and reconstituted with HPLC mobile phase prior to analysis. The liquid chromatography method consisted of a 6 minute gradient with first two minutes at 20% solvent A (5 mM ammonium acetate in methanol/water (80/20; v/v)) and 80% solvent B (5 mM ammonium acetate in methanol) followed by gradual increase (1.5 minutes) to 100% solvent B. The flow rate was 0.3 ml/minute and the retention time for 4 β -hydroxycholesterol and its stable-labeled internal standard were 3.0 and 2.9 minutes, respectively. 4 β -Hydroxycholesterol and [$^2\text{H}_7$]-4 β -

hydroxycholesterol were detected using electrospray ionization (positive ion mode) in the multiple reaction monitoring mode, monitoring for m/z transitions 402→385 (4β-hydroxycholesterol) and 409→392 ($[^2\text{H}_7]$ -4β-hydroxycholesterol). The lower limit of quantification was 3.0 ng/ml with overall assay precision of <5.0%, and % relative error from -5.20% to 4.59%.

Twenty four-hour urine collections both before and after the 14-days oral administration of PF-06282999 were analyzed for the amount of urinary 6β-hydroxycortisol and cortisol. Urine samples (500 μl) diluted with 500 μl phosphate buffer (pH 8.0) were treated with 25 μl of the internal standard (30000/1000 ng/mL ($[^2\text{H}_3]$ -6β-hydroxycortisol/ $[^2\text{H}_4]$ -cortisol in 50/50 methanol/water; v:v) and loaded onto preconditioned (with 0.5 ml methanol and 0.5 ml water) Oasis HLB cartridges and eluted with 300 μl of methanol. The samples were centrifuged (3500 x g, 1 minute at 5 °C), evaporated to dryness under a steady stream of nitrogen, and the residue was reconstituted with 0.3 ml of a methanol/water (50/50) mixture containing 0.1% formic acid. The samples (20 μl) were analyzed on an API-4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) with a Merck Chromolith RP-18e column (100 x 4.6 mm) at 30 °C. Analytes were eluted using a binary gradient comprising of water/acetonitrile (90/10; v/v) with 0.5% formic acid (mobile phase A) and water/acetonitrile (80/20; v/v) with 0.5 % formic acid (mobile phase B). Initial conditions (100% A) were held at 4 minutes at a flow rate of 2.0 ml/minute and then changed to 100% B over 7.5 minutes (flow rate = 3.0 ml/min) and held for 2 minutes. The flow rate was split into the mass spectrometer at a flow rate of 0.5 ml/minute. The retention times for 6β-hydroxycortisol and cortisol were at 3.0 minutes. The retention times of the internal standards were at 9.8 minutes. The mass spectrometer was operated in positive electrospray mode and the m/z transitions used were 363→121 (cortisol), 379→343 (6β-

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hydroxycortisol), 367→121 ($[^2\text{H}_4]$ -cortisol) and 382→346 ($[^2\text{H}_3]$ -6 β -hydroxycortisol). The lower limit of quantification of cortisol was 1.00 ng/ml with assay precision of $\leq 6.0\%$, and % relative error from -4.40% to 0.33%. The lower limit of quantification of 6 β -hydroxycortisol was 10.0 ng/ml with assay precision of $\leq 5.0\%$, and % relative error from -0.89% to 6.56%.

The 4 β -hydroxycholesterol/cholesterol ratio and 6 β -hydroxycortisol/cortisol ratio were calculated for individual subjects at pre- and post-treatment. For part A, the fold induction was derived as the ratio of post-/pre-treatment 4 β -hydroxycholesterol ratio or 6 β -hydroxycortisol ratio for each of the PF-06282999 dose group or placebo. Pairwise comparison between each PF-06282999 dose group versus placebo was performed using *t*-test to examine the dose-dependent changes in the biomarkers of CYP3A4 induction. For Part B, 4 β -hydroxycholesterol ratio and 6 β -hydroxycortisol ratio data were log-transformed then back transformed to derive the fold induction with a 90% confidence interval (CI). *P*-values were from *t*-test and a 90% CI excluding 1 would indicate statistically significant change from baseline in the markers of CYP3A4 induction.

Assessment of Clinical DDI with Midazolam. In the DDI part of the study, serial blood samples were collected for midazolam PK analysis (16 hour PK profile) on day 1 of period 1 for midazolam given alone and day 14/period 2 for midazolam and PF-06282999 co-administration. Plasma samples were analyzed for midazolam concentrations at Covance Bioanalytical Services (Shanghai) using a validated, sensitive and specific LC-MS/MS method (Covance data on file). Calibration standard responses were linear over the range of 0.05–50 ng/ml, using a weighted ($1/\text{concentration}^2$) linear least squares regression. The lower limit of quantification for midazolam was 0.05 ng/ml. The overall assay precision was $\leq 7.9\%$, with % relative error of -

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5.6% to 0.0%. PK parameters for midazolam were calculated using non-compartmental analysis (eNCA). Plasma midazolam PK parameters C_{max} , $AUC_{0-16hour}$, and AUC_{0-inf} were summarized descriptively by dose and period. The interactive effect on C_{max} , $AUC_{0-16hour}$, and AUC_{0-inf} of midazolam were determined by construction 90% CI's around the estimate difference between the test and reference treatments using a mixed-effects model based on the log transformed data. The mixed effect models with treatment as fixed effects and subject as a random effect was implemented using SAS Proc Mixed, with REML estimation method and Kenward-Roger degrees of freedom algorithm. Estimates of the adjusted mean differences (test/reference) and corresponding 90% CI's for the differences were exponentiated to provide estimates of the ratio of adjusted geometric means (test/reference) and 90% CIs for the ratios. Midazolam alone was the reference treatment, while the midazolam co-administered with PF-06282999 was the test treatment.

Simcyp Model. PBPK modeling was used to simulate the effect of PF-06282999 on midazolam metabolism. Simulations were carried out using the Simcyp population-based absorption, distribution, metabolism, and elimination (ADME) simulator (Simcyp Ltd, Sheffield, UK; version 15.1). The PF-06282999 Simcyp perpetrator file was built using the clinical PK information to ensure the models reasonably recapitulated day 14 plasma-concentration time profiles reported in part A. Input parameters for PF-06282999 are shown in Table 1; data for absorption and renal clearance parameters of PF-06282999 at the dose used in the clinical study were obtained from previous clinical studies (Dong et al., 2016). For midazolam, the Simcyp default midazolam model was used. Individual in vitro CYP3A4 mRNA induction data (EC_{50} , E_{max} and the Hill coefficient, γ) for PF-06282999 and rifampin from the three hepatocyte donors (Table 2) were integrated into the Simcyp induction calibrator to allow for correction of PF-

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06282999 induction parameters in reference to the known inducer rifampin (Table 3). E_{\max} and EC_{50} values were directly inputted as Ind_{\max} and Ind_{C50} ; media binding was calculated to be negligible for PF-06282999 (Kalvass and Maurer, 2002).

Simulations were performed for 100 healthy subjects of North American Caucasian origin (i.e., 10 different subjects were chosen randomly for each of the 10 trial simulations); simulated subjects had an average body weight of 74 kg, were aged between 20 and 50 years, and included both genders. The AUC_T of the test (co-administration of midazolam and PF-06282999) and that of the reference (midazolam alone) treatment were determined by Simcyp algorithms. Dose, dose interval, and duration of administration of PF-06282999 and midazolam were set according to the clinical protocol, such that PF-06282999 was administered 125 mg TID or 500 mg BID for 15 days with co-administration of midazolam (7.5 mg single dose) with the first dose of PF-06282999 on Day 14.

Results

In Vitro Induction Assessment. The potential for induction of CYP3A4 mRNA and enzyme activity was examined in cryopreserved human hepatocytes treated with PF-06282999 at several concentrations (0.3-300 μ M). PF-06282999 demonstrated moderate induction of CYP3A4 mRNA levels and midazolam-1'-hydroxylase activity in a dose-dependent fashion in all three donor hepatocytes (Figure 1). In contrast, treatment with positive control rifampicin led to a marked induction of CYP3A4 as measured from increases in mRNA and midazolam-1'-hydroxylase activity in cryopreserved human hepatocytes from the three donors. The estimated EC_{50} and E_{\max} values for fold increases in CYP3A4 mRNA levels and enzyme activity in the three lots of human hepatocytes for PF-06282999 and rifampicin are shown in Table 2. A

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cytotoxicity assay using MTS Cell Proliferation reagent (Promega, WI) and visual inspection showed no apparent reduction in hepatocyte viability after treatment with PF-06282999 at the tested concentrations.

Safety and Clinical Pharmacokinetics. PF-06282999 was safe and well tolerated after multiple oral doses up to 500 mg BID for 14 days in healthy adult volunteers. The corresponding C_{\max} of PF 06282999 was achieved with a median t_{\max} of 2.5-3.5 hours on day 1 and 2.0-3.0 hours on day 14 (Table 4). Steady state exposures were attained by day 2 with minimal to modest accumulation (<2-fold) of PF 06282999 in plasma observed after BID and TID dosing, which was consistent with $t_{1/2}$ values in the range of 4.4 to 7.3 hours in this study as well as an earlier single ascending dose study in healthy volunteers (Dong et al., 2016). Both C_{\max} and $AUC_{0-\tau}$ increased proportionally with increasing dose from 10 mg to 250 mg TID and 500 mg BID, with maximum C_{\max} and $AUC_{0-\tau}$ of 5.14 $\mu\text{g/ml}$ and 35.6 h $\mu\text{g/ml}$ respectively, achieved at 500 mg BID (Table 4). For the midazolam DDI part of the study (part B), mean C_{\min} PF-06282999 concentrations were 1.57 $\mu\text{g/ml}$ on Day 7 and 1.90 $\mu\text{g/ml}$ on Day 14 for 500 mg BID, and 1.11 $\mu\text{g/ml}$ on Day 7 and 1.09 $\mu\text{g/ml}$ on Day 14 for 125 mg TID, respectively. These C_{\min} values were comparable to those achieved in part A at similar doses (Table 4).

Effects on Biomarkers of CYP3A4 Induction. Because PF-06282999 induced CYP3A4 activity in human hepatocytes, endogenous clinical biomarkers of CYP3A4 activity were monitored in the MAD study with PF-06282999. Dose-dependent increases in both plasma 4 β -hydroxycholesterol ratio and 24-hour urinary 6 β -hydroxycortisol ratio were observed following daily administrations of PF-06282999 for 14 days, with an apparent induction effect starting at the 100 mg TID dose level (Part A of study) (Figure 2). Comparison between placebo and PF-06282999 dose groups was performed using the *t*-test. For 4 β -hydroxycholesterol ratio,

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significant differences from placebo were detected in the 250 mg TID and 500 mg BID dose groups ($p=0.0044$ and $p=0.0003$, respectively). For the 6β -hydroxycortisol ratio, significant differences from placebo were found in the 100 mg TID, 250 mg TID and 500 mg BID dose groups ($p=0.0206$, $p=0.0029$ and $p=0.0018$, respectively).

Midazolam DDI Study. The observed changes in the biomarkers of CYP3A4 induction during part A of the study formed the basis of dose selection for the DDI portion of the study (part B), i.e., to confirm the extent of CYP3A4 induction following 125 mg TID and 500 mg BID PF-06282999 administrations for 15 days using oral midazolam as a probe. Median midazolam plasma concentrations over the 16 hours sampling interval were lower in the presence of multiple PF-06282999 administrations than when midazolam was administered alone. Midazolam was rapidly absorbed following a single 7.5 mg oral dose administered either alone or with multiple-dose PF 06282999 (500 mg BID or 125 mg TID), with similar median t_{\max} of 0.5 to 1 hour. Mean terminal $t_{1/2}$ for midazolam was similar when administered alone and with multiple-dose PF 06282999 ranging from 3.3 to 4.6 hours. Following the co-administration of midazolam and PF-06282999, midazolam AUC and C_{\max} values were lower than those when midazolam was administered alone (Table 5). Results of the statistical comparison indicate that multiple-dose administration of PF-06282999 significantly decreased both peak and total midazolam exposures in a dose-dependent manner (Table 5). Following co-administration of 500 mg BID PF-06282999, the mean (90% CI) midazolam $AUC_{0-\text{inf}}$ and C_{\max} were decreased by 57.2% (52.1%, 61.7%) and 41.1% (24.6%, 54.06%), respectively, compared to when midazolam was administered alone. The exposures were decreased by 34.0% (26.2%, 41.0%) for $AUC_{0-\text{inf}}$ and 30.8% (11.4%, 46.0%) for C_{\max} , respectively, for midazolam with PF-06282999 125 mg TID.

Comparison of CYP3A4 Induction Biomarkers. In the midazolam DDI part of the study, the CYP3A4 induction after 2 weeks of daily 125 mg TID or 500 mg BID of PF-06282999 treatment were also assessed by measuring plasma 4 β -hydroxycholesterol/cholesterol ratio and urinary 6 β -hydroxycortisol/cortisol ratio. There appeared to be comparable levels of baseline CYP3A4 enzyme activities (based on plasma 4 β -hydroxycholesterol/cholesterol ratio, urinary 6 β -hydroxycortisol/cortisol ratio and midazolam pharmacokinetics) before treatment with PF-06282999 within the two dose cohorts (Figure 3). Statistical analysis comparing the after PF-06282999 treatment values with baseline values revealed significant changes in the 4 β -hydroxycholesterol/cholesterol ratio and 6 β -hydroxycortisol/cortisol ratio following 500 mg PF-06282999 BID dosing regimen (Table 6). This was associated with moderate impact on midazolam pharmacokinetics (ratio to baseline of 0.44 for midazolam AUC_{0-16hours} and 0.59 for midazolam C_{max}, respectively, Table 6). Dosing of PF-06282999 at 125 mg TID did not demonstrate significant changes in the 4 β -hydroxycholesterol/cholesterol ratio or 6 β -hydroxycortisol/cortisol ratio, albeit a modest impact on midazolam exposure was observed (Table 6). The magnitude of fold induction was the largest for the midazolam AUC changes, followed by the 6 β -hydroxycortisol/cortisol ratio, and with plasma 4 β -hydroxycholesterol/cholesterol ratio showing the minimal change (Table 6). Whereas both 4 β -hydroxycholesterol/cholesterol ratio and 6 β -hydroxycortisol/cortisol ratio performed similarly in detecting CYP3A4 induction, the 6 β -hydroxycortisol/cortisol ratio in urine showed more inter-individual variability (Figure 3).

Simcyp Simulation. Simcyp was used to simulate the effect of co-administration of PF-06282999 on midazolam PK. Model simulations for PF-06282999 successfully captured the day 14 plasma-concentration time profiles from the part A (Figure 4). Incorporation of the most

sensitive estimate of rifampin calibrated mRNA CYP3A4 induction parameters for PF-06282999 (donor FOS) reasonably characterized the magnitude of CYP3A4 enzyme induction on the PK of midazolam (Figure 5). The Simcyp model predicted an AUCR of 0.62 (90% CI: 0.59-0.65), for midazolam with 500 mg BID PF-06282999, compared to the observed interaction (AUCR = 0.43) in part B (Table 7). At the lower dose of 125mg TID PF-06282999 the model predicted an AUCR 0.91 (90% CI: 0.90-0.92) for midazolam compared to the observed interaction (AUCR = 0.66) (Table 7).

Discussion

PF-06282999 is devoid of reversible and/or time-dependent inhibitory effects against major human CYP enzymes including CYP3A4, which largely mitigates the likelihood of clinical DDI's arising from CYP inhibition by PF-06282999 (Dong et al., 2016). Concentration-dependent increases in CYP3A4 mRNA and midazolam-1'-hydroxylase activity, however, were noted in human hepatocytes treated with PF-06282999 indicating that PF-06282999 is a CYP3A4 inducer in vitro. At the highest tested concentration of 300 μ M, PF-06282999 caused maximal induction in CYP3A4 mRNA and enzyme activity ranging from 56% to 86% and 47% to 72%, respectively, of the positive control rifampicin response in the three individual hepatocyte lots. Consistent with previous reports (Fahmi et al., 2008a; 2008b), significant variability in the EC_{50} (43–105 μ M) and E_{max} (12.7–24.5) values for induction of CYP3A4 mRNA was noted amongst the 3 different lots of human hepatocytes. Resistance of PF-06282999 towards metabolic turnover in human hepatocytes (Ruggeri et al., 2015; Dong et al., 2016) suggests that CYP3A4 induction is solely mediated by the parent compound with little to no contribution of metabolites. Clinical PK and safety studies in healthy adult volunteers revealed that PF-06282999 was safe and well tolerated after administration of multiple oral doses

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(10 mg TID-500 mg BID) for 14 days. Systemic exposures as reflected by the C_{\max} and $AUC_{0-\tau}$ increased in a dose-proportional manner; maximum C_{\max} and $AUC_{0-\tau}$ values of 5.14 $\mu\text{g/ml}$ and 35.6 h $\mu\text{g/ml}$ respectively, were achieved at the top 500 mg BID dose group. The corresponding unbound C_{\max} (5.94 μM , PF-06282999 molecular weight = 325 and human plasma unbound fraction = 0.376) and C_{avg} (1.71 μM) concentrations of PF-06282999 at the 500 mg BID dose were approximately 2.2–7.8-fold in excess over the IC_{50} value (0.76 μM) for inhibition of MPO activity in human whole blood (Ruggeri et al., 2015), and this dose was potentially considered to be a therapeutic dose to test proof-of-pharmacology in phase 2 efficacy studies.

Because PF-06282999 induced CYP3A4 activity in human hepatocytes, measurement of plasma 4 β -hydroxycholesterol/cholesterol and urinary 6 β -hydroxycortisol/cortisol ratios was incorporated in the MAD study. Daily administration of PF-06282999 for 14 days led to dose-dependent increases in both plasma 4 β -hydroxycholesterol/cholesterol ratio and 24-hour urinary 6 β -hydroxycortisol/cortisol ratios. The urinary biomarker 6 β -hydroxycortisol/cortisol ratio was more variable than the plasma 4 β -hydroxycholesterol/cholesterol ratio, although in the present scenario, the 6 β -hydroxycortisol/cortisol ratio appeared to be more sensitive in detecting mean fold induction upon repeated dosing with PF-06282999. High inter- and intrasubject variabilities in urinary 6 β -hydroxycortisol/cortisol ratio, previously noted across several clinical studies in healthy volunteers, have been attributed to interindividual variability in CYP3A4 expression in human liver and/or variability in cortisol secretion into the circulation that is influenced by several factors such as stress, infections and circadian rhythm (Forrester et al., 1992; Galteau and Shamsa, 2003; Dutreix et al., 2014). Consistent with the dose-dependent increases in endogenous CYP3A4 biomarker ratios in the MAD study, mean midazolam C_{\max} and AUC following 14-day treatment with PF-06282999 decreased in a dose-dependent fashion with a

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maximum decrease in midazolam AUC_{0-inf} and C_{max} of 57.2% and 41.1% observed at the 500 mg BID PF-06282999 dose group. The moderate impact on midazolam PK at the 500 mg BID dose of PF-06282999 was also reflected in statistically significant changes in plasma 4 β -hydroxycholesterol/cholesterol and urinary 6 β -hydroxycortisol/cortisol ratio. Interestingly, changes in plasma and urinary CYP3A4 biomarkers did not reach statistical significance at the 125 mg TID dose of PF-06282999, despite a modest impact on midazolam systemic exposure.

In retrospect, both plasma and urinary biomarkers performed relatively well in the MAD study as early indicators of the CYP3A4 induction potential of PF-06282999. However, the magnitude of CYP3A4 induction determined by oral midazolam systemic exposure was larger than the fold induction ascertained from plasma and urinary 4 β -hydroxycholesterol/cholesterol and 6 β -hydroxycortisol/cortisol ratios, respectively. Similar findings have also been recently reported in a clinical 14-day rifampin/midazolam DDI study (Björkhem-Bergman et al., 2013). Across several rifampin dose groups (10, 20 and 100 mg), the median fold induction from baseline was 2.0, 2.6, and 4.0 for the estimated midazolam clearance; 1.7, 2.9, and 3.1 for the 6 β -hydroxycortisol/cortisol ratio; and 1.3, 1.6, and 2.5 for the 4 β -cholesterol/cholesterol ratio. It is possible that the plasma 4 β -hydroxycholesterol/cholesterol ratio is not at steady state in the 2-week MAD/DDI studies because of the slow turnover of 4 β -hydroxycholesterol ($t_{1/2}$ = 17 days), and therefore does not fully reflect the induced CYP3A4 levels. In contrast, the 6 β -hydroxycortisol/cortisol ratio reaches steady state relatively faster because of the shorter $t_{1/2}$ (~ 1 hour) of cortisol and its 6 β -hydroxyl metabolite, with little delay or lag time for changes in CYP3A4 activity in vivo (Weitzman et al., 1971; Dutreix et al., 2014). In addition, oral midazolam pharmacokinetics takes into account intestinal and hepatic metabolism by CYP3A4. Therefore, the greater fold induction of midazolam AUC changes may be due to induction of

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both intestinal and hepatic CYP3A4 activity by the perpetrator agents (e.g., PF-06282999 and/or rifampin). As such, simcyp simulation of the steady state pharmacokinetics of PF-06282999 at the 125 mg TID and 500 mg BID dose groups using the calibrated induction data predict approximately similar increases in active CYP3A4 enzyme of ~1.1- and 1.5-fold respectively, further strengthening the hypothesis around DDI arising from induction of both intestinal and liver CYP3A4 activity. However, in accordance with previous literature reports (Dresser et al., 2003; Imai et al., 2008; Davis et al., 2012), lack of significant changes in the terminal $t_{1/2}$ for midazolam in the 14-day DDI study with both dose groups of PF-06282999 suggests that PF-06282999 mainly induces intestinal CYP3A4. Additional clinical studies between PF-06282999 and midazolam (e.g., measurement of intravenous and oral AUC and/or clearance of midazolam in the presence of PF-06282999) will be required in order to delineate DDIs arising from CYP3A4 induction in the gut wall or the liver or both. Overall, the clinical studies conducted to investigate the potential of PF-06282999 as DDI perpetrator confirmed that this compound was a moderate inducer of CYP3A4 according to the Food and Drug Administration USA classification system

(<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf>)

Simcyp, a population-based ADME simulator, has been successfully utilized by various laboratories to quantitatively predict metabolism-based DDIs (Almond et al., 2016; Xu et al., 2011; Fahmi et al., 2009). Relying on the in vitro CYP3A4 induction data, the Simcyp simulator can predict the extent of in vivo induction in a virtual population using a concentration-dependent dynamic induction model, as was recently demonstrated with rifampin, carbamazepine, and phenobarbital (Xu et al., 2011). Consequently, a retrospective analysis of

the clinical PF-06282999/midazolam DDI data was performed using Simcyp. The predicted steady-state PK of PF-06282999 (125 mg TID and 500 mg BID) and the PK (C_{\max} and AUC) of midazolam after a single 7.5 mg dose correlated reasonably well with the data obtained from our clinical study. Using PF-06282999 and midazolam kinetics and the in vitro induction parameters (EC_{50} and E_{\max} for the three individual hepatocyte lots) for CYP3A4 mRNA increase, the induction DDIs were simulated. The predicted magnitudes of DDIs were shown to be in reasonable agreement particularly at the high PF-06282999 dose (500 mg BID). PF-06282999 exhibited dose- and dosing duration-dependent effects on the magnitude of midazolam victim DDI. Consistent with the conclusions noted in previous studies (Almond et al. 2016), Simcyp could be a useful tool for prospectively predicting clinical DDI risks for drug candidates that induce CYP3A4 enzyme levels.

Induction of expression of drug metabolizing enzymes (and hepatobiliary transport proteins) by small molecule xenobiotics, including clinical drugs, often occurs through activation of nuclear receptors, resulting in transcriptional upregulation of the receptor's target genes. Nuclear receptors commonly involved in regulation of CYP enzymes include the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR). Activation of AhR results in upregulation of the CYP1A genes, while activation of PXR and/or CAR results in upregulation of CYP3A, CYP2B and CYP2C genes (Raucy et al., 2002; Raucy, 2003; Hewitt et al., 2007; Tirona and Kim, 2005; Sahi et al., 2009). Induction of CYP3A4 expression by xenobiotics (including drugs) primarily occurs via PXR activation, although there is overlap with CAR both in terms of ligand activators and target genes (Moore et al., 2000; Wilson and Kliewer, 2002; Wang et al., 2003; Wang et al., 2004; Faucette et al., 2006; Faucette et al., 2007). Induction of CYP3A4 expression by PF-06282999, which ultimately manifested in

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a clinical DDI with midazolam, has been clearly demonstrated in this work; however, the propensity for DDI's resulting from the potential induction of CYP2B6, CYP2C9, and/or CYP2C19 enzymes by PF-06282999 remain to be examined. As such, PF-06282999 (2-300 μ M) does not induce CYP1A2-mediated ethoxyresorufin-*O*-deethylase activity in human hepatocytes, suggesting that PF-06282999 does not activate AhR (Pfizer data on file). In vitro studies using PXR and CAR reporter assays (Luo et al., 2002) are currently in progress to ascertain whether the biochemical basis of CYP3A4 induction by PF-06282999 arise through activation of PXR and/or CAR. The knowledge gained from these ongoing studies can be potentially utilized to design the next generation of selective MPO inactivators that are devoid of the CYP3A4 induction liability in the clinic. Likewise, the clinical development plan for PF-06282999 has been modified to include additional in vitro studies in human hepatocytes to examine induction of CYP2C9 and/or CYP2C19 mRNA and activity levels, information that can be potentially used in designing appropriate clinical DDI studies with relevant isozyme-selective substrates of the CYP2C family.

Our studies clearly demonstrate the informative value of endogenous biomarker measurement in FIH studies to examine CYP3A4 induction in vivo; However, as the magnitude of the 4 β -hydroxycholesterol or 6 β -hydroxycortisol ratio change was generally smaller than the magnitude of an oral midazolam AUC change with PF-06282999, an oral midazolam DDI study was ultimately needed to gauge DDI via CYP3A4 induction, and is likely to be the case for weak to moderate CYP3A4 inducers or inducers that have a significant intestinal CYP3A4 induction component.

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Authorship Contributions

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Declaration of interest

All authors are employees of, and/or hold stock in Pfizer Inc. when this work was carried out.

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Figure Legends

Figure 1. Fold induction in mRNA expression (A) and enzyme activity (B) of CYP3A4 (A) in cultured human hepatocyte cultures treated with PF-06282999 (0.3, 2, 5, 10, 20, 30, 40, 50, 75, 100, 150, 200, 300 μ M) from three human donors (■ HH1089, ■ HC7-4, ■ FOS). For purposes of comparison, increases in CYP3A mRNA and enzyme activity with rifampicin (10 μ M) are also depicted. The chemical structure of PF-06282999 is also shown.

Figure 2. Dose-dependent fold-induction of urinary and plasma biomarkers of CYP3A4 induction, 4 β -hydroxycholesterol/cholesterol ratio (4 β -OHcholesterol Ratio) and 6 β -hydroxycortisol/cortisol ratio (6 β -OHCortisol Ratio), following daily administrations of placebo or PF-06282999 for 14 days.

The numbers at the top designate the median fold induction in each treatment group. Pairwise comparison was performed between each treatment group versus placebo using t-test.

Figure 3. Comparison of three different markers for CYP3A4 activities: 4 β -hydroxycholesterol/cholesterol ratio (4 β -OHcholesterol Ratio), 6 β -hydroxycortisol/cortisol ratio (4 β -OHCortisol Ratio), midazolam AUC_{0-16h}, and midazolam C_{max} before and after 14 days of PF-06282999 administrations with graphical illustration of the treatment effect.

Figure 4. Simcyp-simulated versus observed plasma profile of PF-06282999 following 500 mg BID oral administration for 14 days.

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Figure 5. Simcyp-simulated versus observed plasma profiles following a single oral dose of 7.5 mg midazolam without and with 14 days co-administration of PF-06282999 500 mg BID (upper panel) and 125 mg TID (lower panel).

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Tables

TABLE 1
PF-06282999 Simcyp model input parameters

Input Parameters			
Mol. wt (g/mol)	325.77	F_a	1.0
cLog P	0.49	k_a (h^{-1})	0.3
pK _a (Monoprotic base)	7.82	Vd _{ss} (L/kg)	0.45
Blood/plasma (R_{bp})	0.94		
$f_{u,plasma}$	0.376	CL _{po} (L/hr)	14
$f_{u,gut}$	equal to $f_{u,plasma}$	CL _{renal} (L/h)	7.8
Q _{gut} (l /hour)	5.0		
CYP3A4			
$k_{deg,CYP3A4}$ ($hour^{-1}$)	0.0289		

cLogP—calculated partition coefficient (Dong et al., 2016), pK_a—acid dissociation constant (Dong et al., 2016), R_{bp} —blood to plasma ratio (Dong et al., 2016), $f_{u,plasma}$ —unbound fraction in plasma (Dong et al., 2016), $f_{u,gut}$ —unbound fraction in gut, Q_{gut}—gut blood flow, $k_{deg,CYP3A4}$ —degradation rate constant for CYP3A4, F_a —fraction absorbed, k_a —absorption rate constant (Dong et al., 2016), Vd_{ss}—steady-state volume of distribution (Dong et al., 2016), CL_{po}—oral clearance (Dong et al., 2016), CL_{renal}—renal clearance (Dong et al., 2016).

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TABLE 2

EC_{50} , E_{max} , and γ (Hill slope) (\pm standard error (SE)^a) for induction of CYP3A4 mRNA and midazolam-1'-hydroxylase activity by PF-06282999 and Rifampin

PF-06282999							
Donor	CYP3A4 mRNA ^b			CYP3A4 Activity ^b			n^c
	$EC_{50} \pm$ SE (μ M)	$E_{max} \pm$ SE	$\gamma \pm$ SE ^d	$EC_{50} \pm$ SE (μ M)	$E_{max} \pm$ SE	$\gamma \pm$ SE ^d	
HH1089	105 \pm 11	24.5 \pm	1.25 \pm	92.1 \pm	3.76 \pm 0.34	1.0	3
		1.27	0.07	25.6			
HC7-4	56 \pm 4.5	27.1 \pm	2.20 \pm	62.3 \pm	3.70 \pm 0.14	1.6 \pm	3
		1.28	0.31	6.2		0.2	
FOS	43 \pm 2.9	12.7 \pm	1.99 \pm	75.5 \pm	4.64 \pm 0.23	1.0	3
		0.45	0.24	11.5			

Rifampin							
Donor	CYP3A4 mRNA ^b			CYP3A4 Activity ^b			n^c
	EC_{50}	EC_{50}	$E_{max} \pm$	EC_{50}	EC_{50}	$E_{max} \pm$	
	Geo.	Range ^e	SE	Geo.	Range	SE	
	Mean (μ M)	(μ M)		Mean (μ M)	(μ M)		
HH1089	0.60	0.53 –	45.2 \pm	0.57	0.30 –	6.1 \pm	2
		0.68	16.7		1.09	0.9	
HC7-4	1.0	0.82 –	53.6 \pm	0.57	0.28 –	11.1 \pm	4
		1.22	10.4		1.13	1.8	
FOS	1.16	0.54 –	37.1 \pm	0.36	0.22 –	5.0 \pm	3
		2.47	10.2		0.60	1.2	

^aSE for PF-06282999 parameters reflects fit parameter uncertainty; SE for rifampin parameters determined by variability in fit parameters obtained for each independent run. ^bfold induction in CYP3A4 mRNA or enzyme activity (midazolam hydroxylation) expressed as pmol/min/10⁶ cells. ^cn = number of replicates (for PF-06282999) or number of independent runs (for

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rifampin).^d where tabulated $\gamma \neq 1.0$, CYP3A4 mRNA and activity data was fit using a four-parameter sigmoidal model; otherwise data was fit using a three-parameter sigmoidal model.^e EC_{50} range for rifampin reflects ± 1 geometric standard error.

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TABLE 3

Simcyp calibrated in vitro CYP3A4 induction parameters for PF-06282999

PF-06282999			
CYP3A4 mRNA			
Donor	Ind _{max} (μ M)	Ind _{C50} (μ M)	γ
HH1089	8.98	56.0	1.25
HC7-4	8.44	17.9	2.20
FOS	5.86	12.0	1.99

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TABLE 4

Summary of PF-06282999 pharmacokinetic parameter following daily PF-06282999 administrations for 14 days

Parameter	10 mg TID	30 mg TID	100 mg TID	250 mg TID	500 mg BID
<i>N</i>	8	7	8	8	8
AUC_{0-τ}, hours μg/ml					
Geometric mean	1.05	3.37	8.26	19.7	35.6
% CV	23	19	10	13	6
C_{max}, μg/ml					
Geometric mean	0.198	0.604	1.52	3.54	5.14
% CV	22	18	17	15	5
t_{max}, hours					
Median	2.02	2.00	3.00	2.00	3.01
Range	2.00-3.00	1.00-3.98	2.00-4.02	2.00-4.00	3.00-4.00
C_{av}, μg/ml					
Geometric mean	0.131	0.422	1.03	2.47	2.97
% CV	23	19	10	13	6
C_{min}, μg/ml					
Geometric mean	0.0714	0.231	0.584	1.36	1.15
% CV	29	27	11	22	15
t_{1/2}, hours					
Arithmetic mean	4.43	4.90	4.75	7.35	6.08
SD	0.88	0.62	0.20	3.21	1.14
R_{ac,AUC}					
Geometric mean	1.62	1.77	1.46	1.52	1.15
% CV	9	18	12	11	5
R_{ac,Cmax}					
Geometric mean	1.62	1.56	1.33	1.36	1.07
% CV	11	16	8	15	7

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$AUC_{0-\tau}$ area under the plasma concentration-time curve over a dosing interval τ , BID twice daily, C_{av} average concentration, C_{max} peak concentration, C_{min} trough concentration, CV coefficient of variation, $R_{ac,AUC}$ accumulation ratio over 14 days for AUC, $R_{ac,Cmax}$ accumulation ratio over 14 days for C_{max} , SD standard deviation, $t_{1/2}$ half-life, t_{max} time to peak concentration, TID three times daily, τ the dosing interval is 12 hours for BID dosing and 8 hours for TID dosing.

TABLE 5

Statistical summary of pharmacokinetic parameters after an oral dose of 7.5 mg midazolam without and with co-administration of PF-06282999

Midazolam parameter	Interaction with 125 mg TID PF-06282999 ^a			Interaction with 500 mg BID PF-06282999 ^a		
	With PF-06282999 (Test)	Without PF-06282999 (Reference)	Ratio of Test/Reference (90% CI) ^b	With PF-06282999 (Test)	Without PF-06282999 (Reference)	Ratio of Test/Reference (90% CI) ^b
AUC _{0-inf} , hour ng/ml						
Adjusted geometric mean	72.3	110	66.0 (59.0, 73.8)	45.4	106	42.9 (38.3, 47.9)
AUC _{0-16hours} , hour ng/ml						
Adjusted geometric mean	70.0	104	67.4 (60.5, 75.2)	44.6	102	43.7 (39.2, 48.7)
C _{max} , ng/ml						
Adjusted geometric mean	29.8	43.1	69.2 (54.0, 88.6)	22.1	37.5	58.9 (46.0, 75.4)

AUC_{0-inf} area under the plasma concentration-time curve from zero to infinity, AUC_{0-16hours} area under the plasma concentration-time curve from zero to 16 hours, BID twice daily, C_{max} peak concentration, CI confidence interval, TID three times daily.

^a n= 9 subjects per PF-06282999 dose.

^b The ratios (and 90% CI's) are expressed as percentages.

TABLE 6

Comparison of three different markers for CYP3A4 activities: 4 β -hydroxycholesterol/cholesterol ratio (4 β -OHcholesterol Ratio), 6 β -hydroxycortisol/cortisol ratio (6 β -OHCortisol Ratio), midazolam AUC_{0-16hours}, and midazolam C_{max} before and after 14 days of PF-06282999 administrations with statistical analysis of the treatment effect

Markers for CYP3A4	125 mg BID PF-06282999 ^a		500 mg BID PF-06282999 ^a	
	Ratio to baseline ^b (90% CI)	p-value ^c	Ratio to baseline ^b (90% CI)	p-value ^c
4 β -OHcholesterol Ratio	1.03 (0.95, 1.12)	0.4657	1.14 (1.06, 1.24)	0.0145
6 β -OHCortisol Ratio	1.10 (0.88, 1.39)	0.4497	1.53 (1.35, 1.73)	0.0002
Midazolam AUC _{0-16hours}	0.67 (0.63, 0.73)	<0.0001	0.44 (0.38, 0.51)	<0.0001
Midazolam C _{max}	0.69 (0.55, 0.87)	0.0158	0.59 (0.44, 0.79)	0.0007

^a n= 9 subjects per PF-06282999 dose.

^b Values have been back-transformed from the log scale.

^c p-values from *t*-test.

TABLE 7

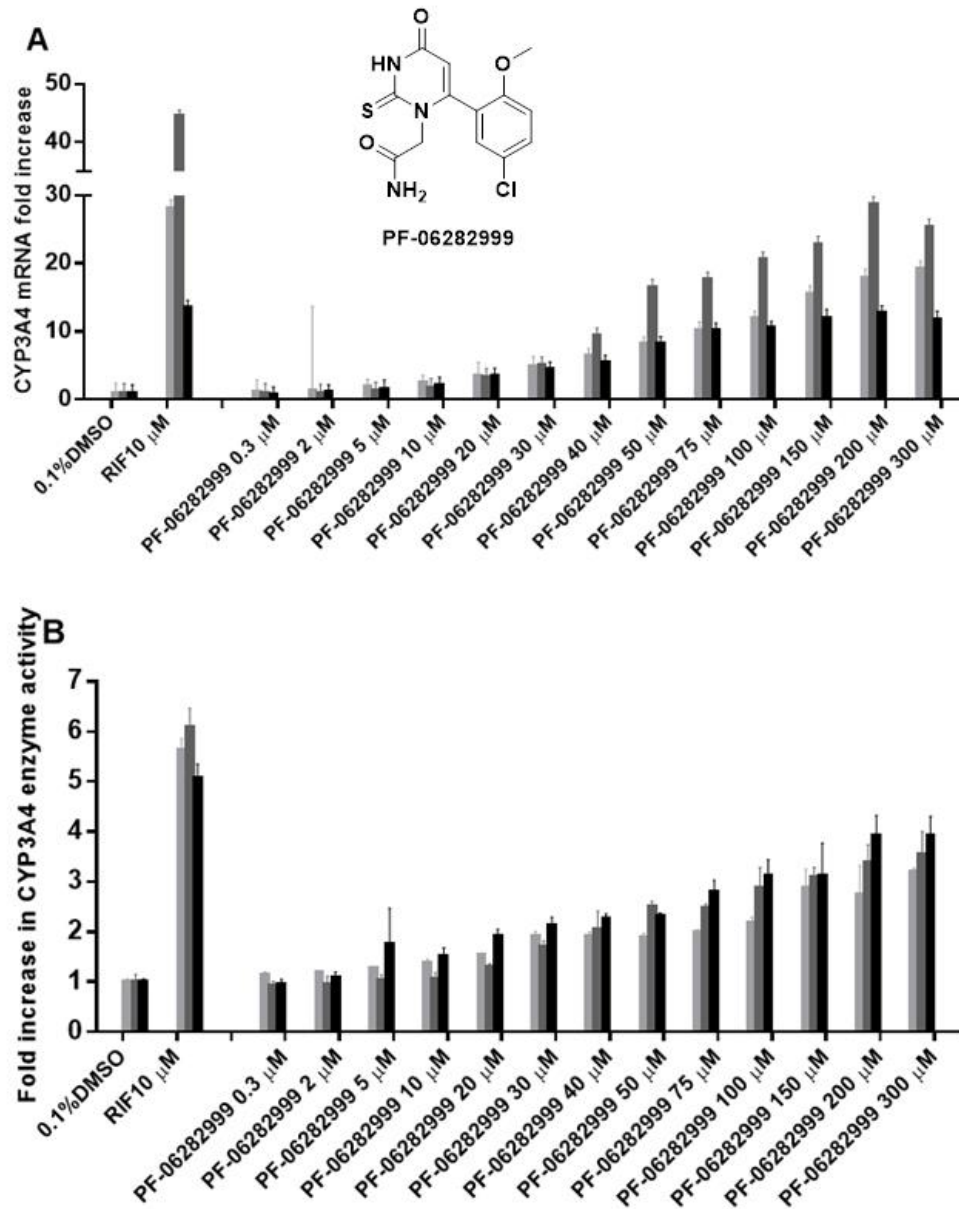
Summary of predicted vs observed DDI using Simcyp

Perpetrator drug	Victim drug	Predicted DDI AUCR Geometric Mean (90% CI)	Observed DDI AUCR
PF-6282999 500 mg BID	Midazolam 7.5 mg	0.62 (0.59-0.65)	0.43 (0.38, 0.48)
PF-6282999 125 mg TID		0.91 (0.90–0.92)	0.66 (0.59, 0.74)

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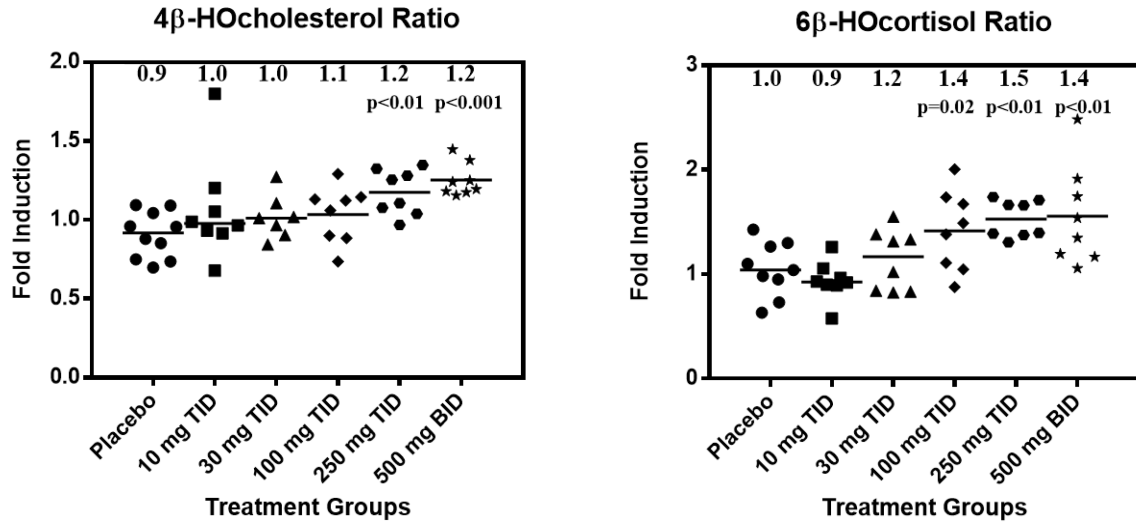
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Figure 1



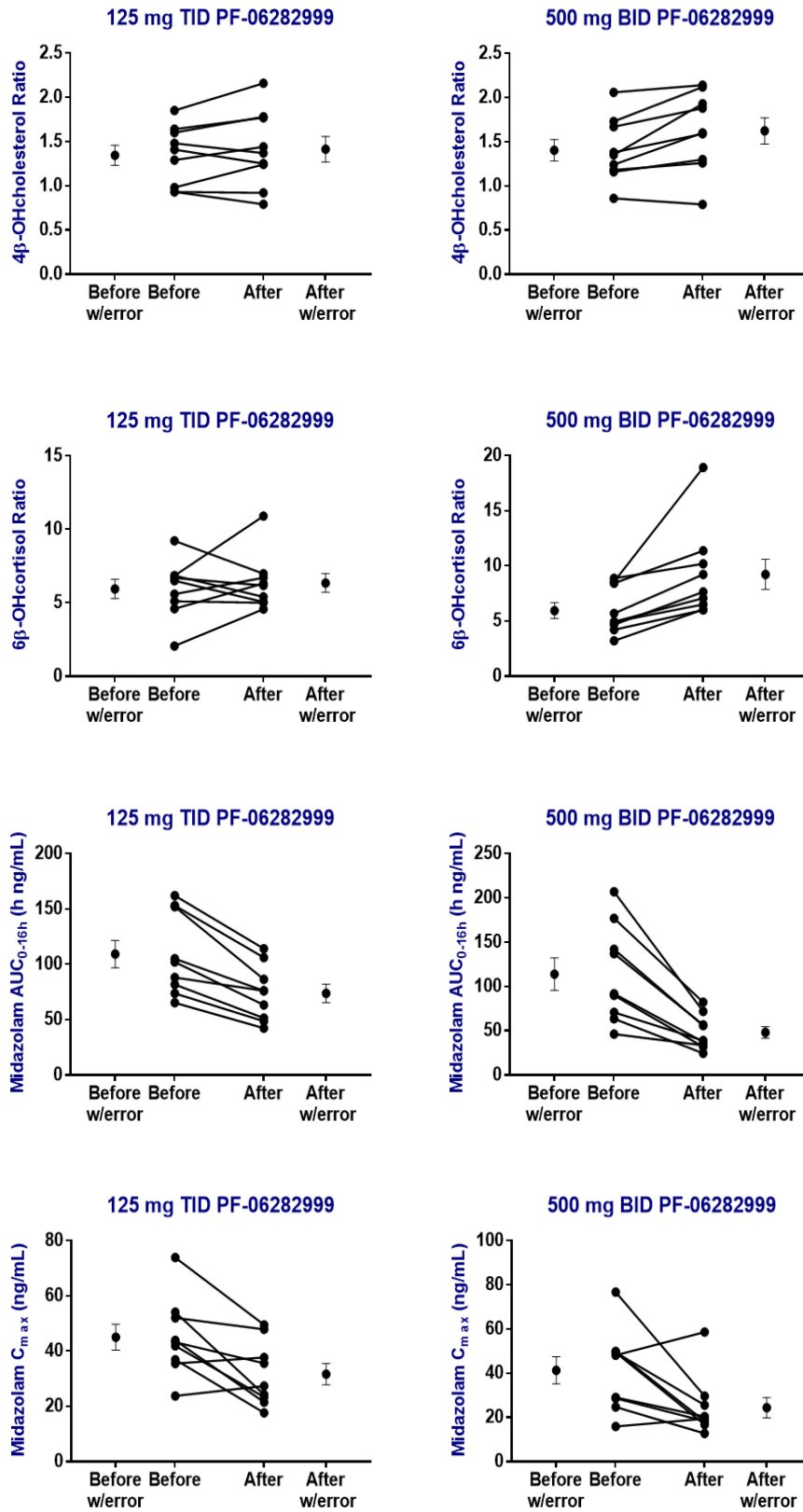
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Figure 2



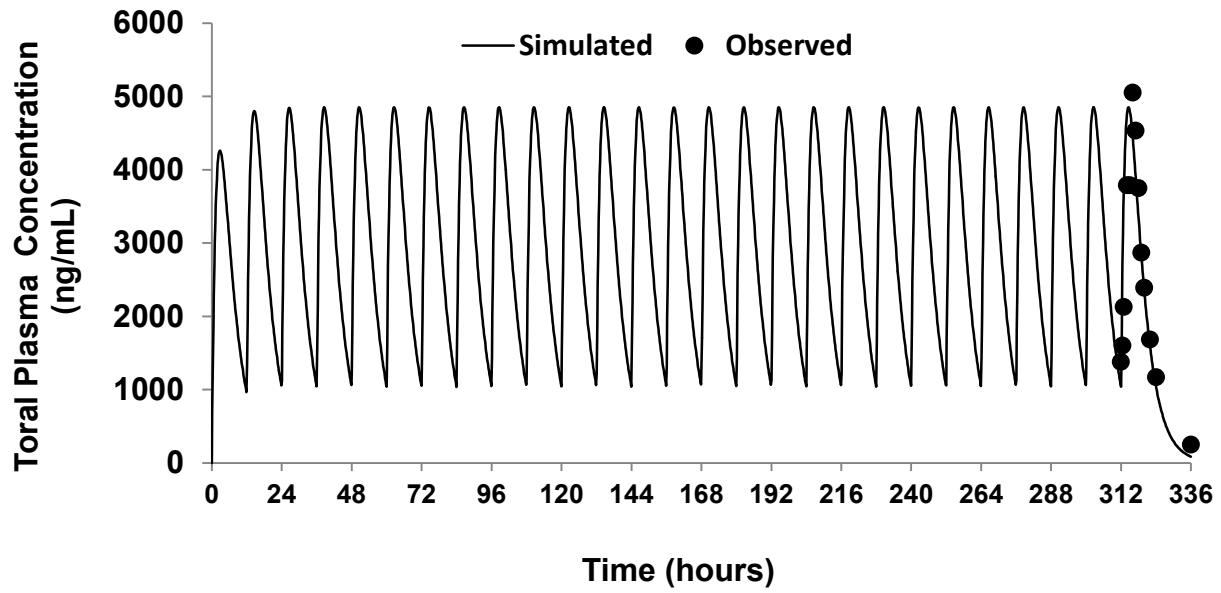
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Figure 3



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Figure 4



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Figure 5

