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**TITLE:** Prediction of Crizotinib-Midazolam Interaction using the Simcyp  
Population-based Simulator: Comparison of CYP3A Time-Dependent  
Inhibition between Human Liver Microsomes versus Hepatocytes

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#### ABBREVIATIONS:

AUC, area under the plasma concentration-time curve;  $C_{inlet,u}$ , unbound hepatic inlet concentration;  $C_{max}$ , maximum plasma concentration;  $C_{sys,u}$ , unbound systemic concentration;  $CL_{int}$ , hepatic intrinsic clearance; DDI, drug-drug interactions; EI, enzyme induction;  $f_m$ , fraction metabolized by an enzyme;  $K_i$ , inhibition constant;  $K_I$ , inactivation constant;  $k_{inact}$ , maximum inactivation rate constant;  $K_{I,app}$ , apparent inactivation constant;  $k_{deg}$ , degradation rate constant;  $k_{obs}$ , apparent inactivation rate constant;  $k_p$ , tissue to plasma partition coefficient;  $f_u$ , unbound fraction; HEP, human hepatocytes; HLM, human liver microsomes; HSP, human hepatocytes suspended in human plasma; PBPK, physiologically-based pharmacokinetics;  $P_{eff,man}$ , projected human jejunum permeability;  $R_{bp}$ , blood-to-plasma ratio; RI, reversible inhibition; TDI, time dependent inhibition;  $t_{max}$ , time to reach maximum plasma concentration;  $V_{ss}$ , volume of distribution at steady-state

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## ABSTRACT

Crizotinib (Xalkori<sup>®</sup>) is an orally available potent inhibitor of multiple tyrosine kinases including anaplastic lymphoma kinase and mesenchymal-epithelial transition factor. Objectives of the present study were to: 1) characterize crizotinib time-dependent inhibition (TDI) potency for CYP3A in human liver microsomes (HLM) and cryopreserved human hepatocytes suspended in plasma (HSP), 2) characterize crizotinib enzyme induction potency on CYP3A4 in cryopreserved human hepatocytes (HEP), 3) predict crizotinib steady-state plasma concentrations in patients (e.g., autoinhibition and autoinduction) using the mechanistic dynamic model, Simcyp population-based simulator, and 4) predict a clinical crizotinib-midazolam interaction using the dynamic model as well as the static mathematical model. Crizotinib inactivation constant ( $K_I$ ) and maximum inactivation rate constant ( $k_{inact}$ ) for TDI were estimated as, respectively, 0.37  $\mu\text{M}$  and 6.9  $\text{h}^{-1}$  in HLM and 0.89  $\mu\text{M}$  and 0.78  $\text{h}^{-1}$  in HSP. Thus, crizotinib inactivation efficiency ( $k_{inact} / K_I$ ) was approximately 20-fold lower in HSP relative to HLM. Crizotinib  $E_{max}$  and  $EC_{50}$  for CYP3A4 induction (measured as mRNA expression) were estimated as 6.4- to 29-fold and 0.47 to 3.1  $\mu\text{M}$ , respectively. Based on these in vitro parameters, the predicted crizotinib steady-state area under plasma concentration-time curve (AUC) with HLM-TDI was 2.1-fold higher than the observed AUC whereas that with HSP-TDI was consistent with the observed result ( $\leq 1.1$ -fold). The increase in midazolam AUC with co-administration of crizotinib (21-fold) was significantly over-predicted using HLM-TDI whereas the prediction using HSP-TDI (3.6-fold) was consistent with the observed result (3.7-fold). Collectively, the present study demonstrated the value of HSP to predict in vivo CYP3A-mediated drug-drug interaction.

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## INTRODUCTION

A clinically relevant drug-drug interaction (DDI) is generally considered a modification of pharmacological and/or toxicological effects of one drug (object) by another drug (precipitant). In many cases, DDI can be attributed to modulation of drug metabolizing enzymes, particularly CYP3A, as these are the most abundantly expressed in the liver and intestines (Shimada et al., 1994; Slaughter and Edwards, 1995). The enzyme inhibition mechanism can be categorized as reversible (competitive, uncompetitive or noncompetitive) inhibition (RI) or time-dependent (mechanism-based or metabolism-based) inhibition (TDI). For in vitro TDI evaluation, an inactivation constant ( $K_I$ ) and a maximum inactivation rate constant ( $k_{inact}$ ) are typically determined in human liver microsomes (HLM) (Grimm et al., 2009; Zimmerlin et al., 2011), and these kinetic parameters are used to predict potential in vivo DDI (Kanamitsu et al., 2000; Mayhew et al., 2000; Obach et al., 2007). In addition to HLM, TDI evaluation in human hepatocytes (HEP) has recently been reported for the known CYP3A time-dependent inhibitors such as diltiazem, erythromycin and ritonavir (Zhao et al., 2005; Xu et al., 2009; Chen et al., 2011; Kirby et al., 2011). HEP are also extensively used for the evaluation of enzyme induction (EI) for CYPs (Chu et al., 2009). Compared to HLM, HEP are intact cellular systems containing a full complement of phase I/II metabolizing enzymes and transporter proteins (Di et al., 2012). The presence of cell membranes in HEP is important in maintaining the effects of active uptake/efflux transporters and passive diffusion on intracellular drug concentration; therefore, these factors may substantially affect the determination of enzyme kinetic parameters such as  $K_I$  and  $k_{inact}$ . Generally, the known CYP3A inhibitors showed a less potent TDI in HEP relative to

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HLM, which have yielded an impact on the in vivo DDI prediction (Xu et al., 2009; Chen et al., 2011; Kirby et al., 2011). Furthermore, it has been reported that cryopreserved human hepatocytes suspended in plasma (HSP), compared to those in protein-free media, showed better DDI predictions for some of CYP inhibitors (Mao et al., 2011; Mao et al., 2012).

Recently, there has been a growing interest in physiologically-based pharmacokinetic (PBPK) models, where each physiological compartment (i.e., organ or tissue defined by a tissue volume) is connected with blood flow. The PBPK model provides disposition profiles to be predicted from physico- and bio-chemical properties of compounds (Jones et al., 2006; Lave et al., 2007; Nestorov, 2007). This dynamic approach is increasingly being employed in drug discovery and development setting to predict PK and DDI potential in the clinic. Traditionally, DDI predictions have been performed with static mathematical models using various inhibitor concentrations such as hepatic inlet or outlet concentrations in total (protein bound plus unbound) or unbound form (Mayhew et al., 2000; Obach et al., 2007; Fahmi et al., 2009; Boulenc and Barberan, 2011; Mao et al., 2012). In these reports, hepatic DDI magnitudes for RI were reasonably predicted using the projected unbound portal vein (or hepatic inlet) concentration ( $C_{inlet,u}$ ) whereas the use of unbound systemic (or hepatic outlet) concentration ( $C_{sys,u}$ ) yielded better prediction for TDI and EI compared to  $C_{inlet,u}$ . Based on advancements in the field, the Food and Drug Administration (FDA) has recently issued a revised draft DDI guidance for industry (FDA, 2012), which emphasizes the use of an integrated approach such as static mathematical model or mechanistic dynamic PBPK models to simultaneously utilize these DDI mechanisms (e.g., TDI, EI and RI) to

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predict potential clinical DDI of new chemical entities. In many cases, an inhibitor concentration used for DDI prediction with general PBPK models is considered to be comparable to  $C_{sys,u}$  at equilibrium since the tissue compartments are assumed to be perfusion-limited without any diffusion barrier.

Crizotinib (Xalkori®; PF02341066) was identified as an orally available potent inhibitor of multiple tyrosine kinases including anaplastic lymphoma kinase and mesenchymal-epithelial transition factor (Fig. 1). Crizotinib was recently approved by FDA for the treatment of patients with locally advanced or metastatic non-small cell lung cancer that is anaplastic lymphoma kinase-positive as detected by an FDA-approved test. It has been reported that crizotinib is a substrate of CYP3A (Johnson et al., 2011a). Objectives of the present study were to: 1) characterize crizotinib TDI potency on CYP3A in HLM and HSP, 2) characterize crizotinib EI potency in cryopreserved HEP, 3) predict crizotinib steady-state plasma concentrations in patients using the mechanistic dynamic model, and 4) predict a clinical crizotinib-midazolam interaction, utilizing the mechanistic dynamic model as well as the static mathematical model. The dynamic model used was a commercially available Simcyp population-based dynamic simulator (Jamei et al., 2009).

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## Materials and Methods

### Materials

Crizotinib ((R)-3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine), chemical purity >99%) was synthesized by Pfizer Worldwide Research and Development (San Diego, CA) (Cui et al., 2011). Pooled HLM from 50 individual donors (30 males and 20 females) were prepared and characterized at XenoTech LLC (Lenexa, KS, USA). Cryopreserved HEP suspension (3 males and 2 females) and In Vitro GRO<sup>TM</sup> HT Medium were obtained from Celsis In Vitro Technologies, Inc. (Baltimore, MD). Human plasma (heparin as anti-coagulant) was obtained from Lampire Biological Laboratories, Inc. (Pipersville, PA). HEP maintenance medium was obtained from Lonza, Inc. (Walkersville, MD). Midazolam, 1'-hydroxymidazolam (1'-OH midazolam) and [<sup>13</sup>C<sub>3</sub>]-1'-hydroxymidazolam (internal standard) were obtained from BD Gentest (Woburn, MA). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Sigma-Aldrich. All other commercially available reagents and solvents were of either analytical or high performance liquid chromatography (HPLC) grade.

### Crizotinib TDI Assay in HLM

A two-step incubation scheme was used to analyze the inhibition of midazolam hydroxylation by crizotinib in HLM. Crizotinib at final concentrations of 0.3 to 10  $\mu$ M (0.1% of final DMSO concentration) was preincubated with HLM (0.5 mg/mL) and NADPH (1 mM) at 37°C for 0, 5, 10 and 20 minutes. The incubation mixture was diluted (1:10) with buffer containing NADPH (1 mM), and midazolam (10  $\mu$ M) was added for an additional 3 minute incubation period to quantify 1'-hydroxymidazolam as the remaining



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CYP3A activity. The incubation was then terminated by the addition of 100  $\mu$ L of acetonitrile/methanol (3:1 v/v) containing 3% formic acid and the internal standard (0.1  $\mu$ M). The incubations were performed in duplicate. Samples were centrifuged at 2000g for 20 minutes and an aliquot of the supernatant was analyzed by liquid-chromatography tandem mass spectrometry (LC-MS/MS).

### **Crizotinib TDI Assay in HSP**

Crizotinib TDI potency was evaluated in HSP following a similar study design as reported previously (Mao et al., 2011). Briefly, the final crizotinib concentrations used in the incubation mixture were 0.13 to 100  $\mu$ M (0.5% of final methanol concentration). Incubations were performed in duplicate. A total of 25  $\mu$ l of stock cryopreserved HEP (prepared in human plasma as  $2 \times 10^6$  cells/ml) were added to 50  $\mu$ l of human plasma containing crizotinib and incubated for 0, 10 and 20 min (37°C, 5% CO<sub>2</sub>) before the addition of midazolam. Midazolam in human plasma (25  $\mu$ l) at final concentration of 30  $\mu$ M was added to the incubation mixture followed by a 35-min incubation to quantify 1'-hydroxymidazolam as the remaining CYP3A activity. The final concentration of HEP was  $0.5 \times 10^6$  cells/mL. Reactions were terminated by adding 200  $\mu$ L of acetonitrile/methanol (3:1 v/v) containing the internal standard (0.15  $\mu$ M). Samples were centrifuged at 2000g for 20 minutes and an aliquot of the supernatant was analyzed by LC-MS/MS.

### **Crizotinib EI Assay in Cryopreserved HEP**

Crizotinib CYP3A4 EI potency was evaluated in cryopreserved HEP from three donors (lot Hu4026, Hu8020 and HIE) using a procedure described previously (Fahmi et al., 2008). Briefly, cryopreserved HEP ( $3.5 \times 10^5$  viable cells per 0.5 mL of plating

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medium in each well of collagen I-precoated 24-well plates) were incubated for 24 hours (37°C, 95% relative humidity, 5% CO<sub>2</sub>) before the addition of crizotinib (0.1% of final DMSO concentration). The cells were treated with crizotinib at final concentrations of 0.25 to 7 μM daily for three consecutive days. Quantification of CYP3A4 mRNA was performed using the TaqMan two-step RT-PCR method, and the relative quantity of the target CYP3A4 gene compared with the endogenous control was determined by the  $\Delta\Delta\text{CT}$  method. An effect of crizotinib on cell viability was assessed using the WST-1 Cell Proliferation reagent.

### **LC-MS/MS Analysis for Midazolam Metabolite**

Concentrations of the CYP3A-mediated metabolite of midazolam, 1'-hydroxymidazolam, in the in vitro TDI assays were determined by an LC-MS/MS method after the protein precipitation. The LC-MS/MS system consisted of Shimadzu LC-10ADvp pumps (Shimadzu, Columbia, MD), a CTC PAL autosampler (Leap Technologies, Carrboro, NC), and a Sciex API 4000 mass spectrometer (Sciex, Foster City, CA) equipped with a turbo ion spray source. Chromatographic separation was achieved by a reverse-phase column (Agilent Zorbax SB-Phenyl, 5 μm, 50 × 2.1 mm column, Agilent, Santa Clara, CA) with a mobile phase consisting of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B) at a flow rate of 0.8 mL/min. The injection volume was 5 μL. Analytes were eluted using a step gradient from 1 to 40% mobile phase B over 3 minutes, and then increased to 90% in the next 0.01 minutes. Mobile phase B was held at 90% from 3.01 to 3.50 minutes, and the column was re-equilibrated to 1% over another 0.5 minutes. The MS was operated in the positive ionization mode using multiple reaction monitoring at specific parent ion →

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product ion transitions of  $m/z$  342→203 for 1'-hydroxymidazolam and 345 →171 for [ $^{13}\text{C}_3$ ]-1'-hydroxymidazolam (IS). The dynamic range of the assay ranged from 7.5 to 2540 nM. The back calculated calibration standard concentrations were within  $\pm 15\%$  of their nominal concentrations with coefficients of variation of less than 15%. No significant carryover and matrix effects were observed in the study.

### Estimation of Crizotinib TDI Kinetic Parameters

To determine TDI kinetic parameters (i.e.,  $K_I$  and  $k_{inact}$ ) in the HLM assay, the apparent inactivation rate constant ( $k_{obs}$ ) for each crizotinib concentration ( $[I]$ ) was first estimated from the slope of initial linear decline of CYP3A remaining activity on a natural logarithmic scale over the preincubation time. Apparent  $K_I$  ( $K_{I,app}$ ) and  $k_{inact}$  values were then determined by solving the following nonlinear equation (Mayhew et al., 2000):

$$k_{obs} = k_{inact} \cdot [I] / (K_{I,app} + [I]) \quad (1)$$

Subsequently, for prediction purpose, an estimate for  $K_I$  was calculated from  $K_{I,app}$  following the correction for in vitro non-specific binding in microsomes ( $f_{u,mic}$ ), which was measured by the equilibrium dialysis method (Yamazaki et al., 2011).

In the HSP assay, the previously reported mathematical equations incorporating reversible (eq. 2), irreversible (eq. 3), or both reversible and irreversible inhibitions (eq. 4) were employed to estimate the apparent reversible inhibition constant ( $K_{i,app}$ ) and/or TDI parameters ( $K_{I,app}$  and  $k_{inact}$ ) by simultaneously fitting three  $\text{IC}_{50}$  curves obtained from different incubation times using a weighted nonlinear regression analysis (Mao et al., 2011):

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$$\frac{Vi_t}{Vc_t} = \frac{[S] \cdot V_{max,t}}{(K_{m,MDZ} \cdot (1 + [I]/K_{i,app}) + [S]) \cdot Vc_t} \quad (2)$$

$$\frac{Vi_t}{Vc_t} = \frac{[S] \cdot V_{max,t} \cdot e^{-k_{inact} \cdot [I] \cdot t / (K_I \cdot app + [I])}}{(K_{m,MDZ} + [S]) \cdot Vc_t} \quad (3)$$

$$\frac{Vi_t}{Vc_t} = \frac{[S] \cdot V_{max,t} \cdot e^{-k_{inact} \cdot [I] \cdot t / (K_I \cdot app + [I])}}{(K_{m,MDZ} \cdot (1 + [I]/K_{i,app}) + [S]) \cdot Vc_t} \quad (4)$$

where,  $t$  is a total incubation time of a preincubation (0, 10 or 20 minutes) with an inhibitor followed by a 35-min incubation with midazolam,  $Vi_t$  represents the 1'-OH midazolam formation rate at a given inhibitor concentration during total incubation time,  $Vc_t$  represents the 1'-OH midazolam formation rate of vehicle control (no inhibitor) during total incubation time,  $K_{m,MDZ}$  is the Michaelis-Menten constant for 1'-OH midazolam formation in HSP (55  $\mu$ M total corresponding to 2  $\mu$ M free),  $V_{max,t}$  represents maximum 1'-OH midazolam formation rate,  $[I]$  is the nominal inhibitor concentration, and  $[S]$  is the final incubation concentration of midazolam (30  $\mu$ M).

The ratio of  $Vi_t$  and  $Vc_t$  normalized the baseline for the enzyme activity at the designated incubation time. Model selection was based on a number of criteria such as Akaike information criterion, estimates for each parameters and standard errors. Finally, an estimate for  $K_{I,app}$  was converted to  $K_I$  (as the input for Simcyp) following the correction for the unbound fraction in plasma ( $f_{u,plasma}$ ), which was measured by the equilibrium dialysis method (Yamazaki et al., 2011).

### **Prediction of Crizotinib and Midazolam Pharmacokinetics with Simcyp**

Physicochemical and in vitro pharmacokinetic parameters of crizotinib used for DDI prediction are summarized in Table 1. Based on these parameters, two compound files of crizotinib were created in Simcyp (version 11.1): one with the TDI parameters

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from HLM (henceforth referred to as CRZ-HLM) and the other with the TDI parameters from HSP (henceforth referred to as CRZ-HSP). The only difference in these two crizotinib Simcyp files was the TDI kinetic parameter value ( $K_I$  and  $k_{inact}$ ). Crizotinib in vitro inhibitory effect on CYP3A (i.e., RI) using midazolam as a probe substrate was negligible with  $IC_{50}$  value of  $>30 \mu\text{M}$  (FDA, 2011). Crizotinib EI parameters ( $E_{max}$  and  $EC_{50}$ ) estimated from three individual cryopreserved HEP were normalized to 2.4-fold and  $0.84 \mu\text{M}$ , respectively, with the rifampin data (mean  $E_{max}$  of 90-fold and  $EC_{50}$  of  $0.57 \mu\text{M}$ ) by the induction calibrator of prediction tool box implemented in Simcyp. These EI parameters were also incorporated into both CRZ-HLM and CRZ-HSP files.

Simcyp simulations of crizotinib plasma concentration-time profiles were performed by a full-PBPK model with a first-order absorption rate constant ( $k_a$ ) based upon the clinically observed plasma concentrations in healthy volunteers ( $n = 14$ ) after a single 50-mg intravenous 2-hour infusion or a single 250-mg oral administration. These single-dose clinical studies of crizotinib were conducted in a crossover design with a washout period of at least 14 days (FDA, 2011). In the Simcyp absorption model,  $k_a$  values were set to predict clinically observed time to reach maximum plasma concentration ( $t_{max}$ ), and an unbound fraction in the gut ( $f_{u,gut}$ ) was assumed to be equal to an unbound fraction in blood ( $f_{u,blood}$ ) calculated from  $f_{u,plasma}$  and blood-to-plasma ratio ( $R_{bp}$ ). The blood flow term ( $Q_{gut}$ ) of crizotinib in the “ $Q_{gut}$ ” model was estimated to be 4.0 L/h from crizotinib physicochemical properties. Using a retrograde model implemented in Simcyp, hepatic intrinsic clearance ( $CL_{int}$ ) was back-calculated from the in vivo intravenous plasma clearance (47 L/h) determined in the single intravenous infusion study as mentioned above (FDA, 2011). Crizotinib renal clearance was

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negligible in humans and the fraction metabolized by CYP3A4 ( $f_{m,CYP3A4}$ ) was suggested to be 0.8 based on the in vitro CYP phenotyping and the human mass-balance study with [ $^{14}$ C]crizotinib (Johnson et al., 2011a; Johnson et al., 2011b); therefore, 80% of the back-calculated  $CL_{int}$  was assigned to CYP3A4-mediated  $CL_{int}$  (194  $\mu$ L/min/mg protein) with the remaining 20% as additional  $CL_{int}$  (49  $\mu$ L/min/mg protein). The steady-state volume of distribution ( $V_{ss}$ ) of crizotinib was predicted using the mathematical model 2 (Rodgers et al., 2005) implemented in Simcyp. To improve goodness-of-fit between predicted and observed plasma concentration-time profiles, crizotinib  $pK_a$  values were adjusted from 5.4 and 8.9 (diprotic base) to 7.6 (monoprotic base) to predict the clinically observed  $V_{ss}$  of 25 L/kg from the single intravenous infusion study (FDA, 2011). Using the unadjusted  $pK_a$  values of 5.4 and 8.9, the predicted crizotinib  $V_{ss}$  was 7.0 L/kg, which resulted in poor fits to the clinically observed single-dose intravenous infusion and oral plasma concentration profiles. Although a minimal-PBPK model implemented Simcyp reasonably predicted the areas under the plasma concentration-time curve (AUC), the maximum plasma concentration ( $C_{max}$ ) was significantly under-predicted in both the single-dose intravenous infusion and oral plasma concentration profiles. Simulations for midazolam plasma concentration-time profiles were also performed by a full-PBPK model using a modified Simcyp-default midazolam file with the adjusted  $k_a$  of 2  $h^{-1}$  and  $pK_a$  values of 7.4 (ampholyte). Mathematical model 2 implemented in Simcyp was used to predict midazolam  $V_{ss}$ . These adjustments were required to simulate comparable midazolam plasma concentration-time profiles to the clinically observed results in patients ( $n = 14$ ) after a single oral administration of midazolam without co-administration of crizotinib (FDA, 2011). In addition to these adjustments, the predicted

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liver to plasma partition coefficients ( $k_p$ ) were set to unity from 22 and 1.3 for crizotinib and midazolam, respectively. These changes resulted in minimal effects on the predicted  $V_{ss}$  values for both crizotinib (from 25.3 to 24.8 L/kg) and midazolam (from 1.0 to 1.1 L/kg).

Clinical trial simulation in Simcyp was performed with a virtual population of healthy volunteers in 8 trials of 10 subjects, each aged 18 to 65 years with a female/male ratio of 0.34, whose CYP3A4 degradation rate constant ( $k_{deg}$ ) was  $0.019 \text{ h}^{-1}$  in the liver and  $0.030 \text{ h}^{-1}$  in the gastro-intestinal (GI) tract. The output sampling interval in Simcyp simulation tool box was set to 0.2 hours in all simulations. Trial designs used were as follows:

- Trial #1: A single oral dose of crizotinib 250 mg was administered on day 1; crizotinib plasma concentration was simulated for 7 days.
- Trial #2: Multiple doses of crizotinib, 250 mg twice daily with an interval of 12 hours, were orally administered for 28 days; crizotinib plasma concentration was simulated during crizotinib repeated administration.
- Trial #3: A single oral dose of midazolam 2 mg was co-administered on day 28 with repeated oral doses of crizotinib (250 mg twice daily with an interval of 12 hours) for 28 days; plasma concentrations of midazolam and crizotinib were simulated during crizotinib repeated administration.

In these simulations,  $C_{max}$ ,  $t_{max}$  and  $AUC_{0-\tau}$  were obtained from Simcyp output whereas  $AUC_{0-\infty}$  was calculated from simulated plasma concentrations using the linear trapezoidal rule:

$$AUC_{0-\infty} = AUC_{0-L} + C_L / \lambda \quad (5)$$

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where  $AUC_{0-L}$ ,  $C_L$  and  $\lambda$  represent the area under the plasma concentration-time curve from time zero to the last time point, the plasma concentration at the last time point and the elimination rate constant in the terminal phase of log plasma concentration-time curves determined by linear regression, respectively.

Geometric means of PK parameters in 8 simulation trials were compared to the clinically observed geometric mean in order to assess Simcyp prediction accuracy. In addition to the Simcyp outputs of 5<sup>th</sup> and 95<sup>th</sup> percentiles of all 80 subjects, the geometric mean values of 5<sup>th</sup> and 95<sup>th</sup> percentiles among 8 simulation trials were calculated by Microsoft Excel 2007 (Microsoft, Redmond, WA) to compare to the clinically observed 5<sup>th</sup> and 95<sup>th</sup> percentiles in the crizotinib-midazolam interaction study.

#### **Sensitivity Analysis for Crizotinib Hepatic $k_p$ and CYP3A4 $k_{deg}$ with Simcyp**

The sensitivity analyses for crizotinib hepatic  $k_p$  value ranging from 1 to 24 and hepatic CYP3A4  $k_{deg}$  from 0.693 to 0.00693 h<sup>-1</sup> (i.e.,  $t_{1/2}$  of 1 to 100 hours) were separately performed in the prediction of crizotinib-midazolam interaction with Simcyp using CRZ-HSP. Clinical trial simulation in Simcyp was the same as trial #3 as described above, and the PK parameters (e.g.,  $C_{max}$ ,  $t_{max}$ ,  $AUC_{0-\tau}$  and  $AUC_{0-\infty}$ ) and geometric mean values of 5<sup>th</sup> and 95<sup>th</sup> percentiles among 8 simulation trials were either obtained or calculated from Simcyp outputs as described above.

#### **Prediction of Crizotinib-Midazolam Interaction with a Static Mathematical Model**

A static mathematical model (Fahmi et al., 2008) was used to predict the fold-increase in midazolam  $AUC_{0-\infty}$  ( $AUC_R$ ) following co-administration with crizotinib:



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$$AUC_R = \left( \frac{I}{[A_h \times B_h \times C_h] \times f_m + (I - f_m)} \right) \times \left( \frac{I}{[A_g \times B_g \times C_g] \times (I - F_g) + F_g} \right) \quad (6)$$

$$A = \frac{k_{deg}}{k_{deg} + \frac{[I] \cdot k_{inact}}{[I] + K_i}} \quad (7)$$

$$B = I + \frac{d \cdot E_{max} \cdot [I]}{[I] + EC_{50}} \quad (8)$$

$$C = \frac{1}{1 + [I]/K_i} \quad (9)$$

where A, B and C represent TDI, EI and RI, respectively, and the subscripts *h* and *g* denote liver and GI tract, respectively.

Crizotinib input parameters and CYP3A4  $k_{deg}$  values in the liver and GI tract used for the static model were exactly the same as Simcyp input parameters in order to compare in vivo DDI predictions between the dynamic and static models. Therefore, the proposed empirical scaling factor for induction (i.e., *d*) in the static model was set to unity since the EI parameters were already normalized with rifampin data for the dynamic model.

The availability in the GI tract ( $F_g$ ) and  $f_{m,CYP3A4}$  for midazolam were set as 0.63 and 0.93, respectively (Ernest et al., 2005; Obach et al., 2006). Crizotinib steady-state unbound average plasma concentrations ( $C_{ave,u}$ ), that was calculated from  $AUC_{0-\tau}$  divided by the dosing interval of 12 hours followed by the correction for  $f_{u,plasma}$ , was used as the hepatic inhibitor concentrations ( $[I]_h$ ) in the static model (as consistent with a full-PBPK model with hepatic  $k_p$  of unity). The intestinal inhibitor concentrations ( $[I]_g$ ) were calculated from crizotinib parameters summarized in Table 1 using the following equation (Rostami-Hodjegan and Tucker, 2004):

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$$[I]_g = D \cdot k_a \cdot F_a \cdot f_{u,gut} / Q_{gut} \quad (10)$$

where  $D$ ,  $k_a$ ,  $F_a$ ,  $f_{u,gut}$  and  $Q_{gut}$  represent the dose amount per dose ( $\mu\text{mol}$ ), the first-order absorption rate constant ( $\text{h}^{-1}$ ), the fraction of dose absorbed, the unbound fraction in the GI tract and the enterocyte blood flow ( $\text{L/h}$ ), respectively.

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## RESULTS

### In vitro Effects of Crizotinib on CYP3A

Crizotinib TDI kinetic parameters for CYP3A were determined in HLM and HSP using midazolam as a CYP3A probe substrate. Estimated  $K_{I,app}$  and  $k_{inact}$  in HLM were 3.0  $\mu\text{M}$  and 6.9  $\text{h}^{-1}$ , respectively (Fig. 2). Crizotinib  $\text{IC}_{50}$  curves in HSP were adequately described by the irreversible model (eq. 3) with  $K_{I,app}$  of 9.6  $\mu\text{M}$  and  $k_{inact}$  of 0.78  $\text{h}^{-1}$  (Fig. 3). The estimates for  $K_I$  in HLM and HSP following the correction for in vitro non-specific binding (e.g.,  $f_{u,mic}$  and  $f_{u,plasma}$ , respectively) were 0.37 and 0.89  $\mu\text{M}$ , respectively. Thus, the  $K_I$  value in HSP was approximately 3-fold higher than that in HLM whereas the  $k_{inact}$  from HSP was approximately 9-fold lower than that from HLM, resulting in the 20-fold difference in the inactivation efficiency ( $k_{inact}/K_I$ ) between HLM and HSP (19 and 0.87  $\text{h}^{-1}\cdot\mu\text{M}^{-1}$ , respectively). These  $K_I$  and  $k_{inact}$  values were used for Simcyp simulation (Table 1).

In cryopreserved HEP from three donors, crizotinib concentration-dependently induced CYP3A4 mRNA expression with estimated  $E_{max}$  of 6.4 to 29-fold and  $EC_{50}$  of 0.47 to 3.1  $\mu\text{M}$ . It should be noted that relatively low cell viability (<0.7 ratio to vehicle control sample) was observed at higher crizotinib concentrations ( $\geq 5 \mu\text{M}$ ); thus, the accuracy of EI parameter estimation might have been impacted. Following the normalization with rifampin data (positive control), mean crizotinib  $E_{max}$  of 2.4-fold and  $EC_{50}$  of 0.84  $\mu\text{M}$  were used for Simcyp simulation (Table 1).

### Predicted Crizotinib Plasma Concentrations with Simcyp

Clinically observed crizotinib  $C_{max}$ ,  $t_{max}$  and  $\text{AUC}_{0-\infty}$  were 87 ng/mL, 4 hours and 1817 ng·h/mL, respectively, in cancer patients ( $n = 8$ ) after a single oral administration of

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crizotinib 250 mg (FDA, 2011). By the Simcyp simulation with CRZ-HLM, the predicted  $C_{\max}$  (89 ng/mL) was within 10% of the observed value whereas the  $AUC_{0-\infty}$  (3901 ng·h/mL) was over-predicted by 2.1-fold (Table 2). As shown in Fig. 4A, the predicted crizotinib plasma concentrations were slightly but consistently higher than the observed concentrations after  $t_{\max}$ . In contrast, the predicted  $AUC_{0-\infty}$  (1745 ng·h/mL) by Simcyp with CRZ-HSP was consistent with the observed value (<10%) whereas the predicted  $C_{\max}$  (42 ng/mL) was under-predicted by approximately 2-fold (Table 2). It remained unclear why the  $C_{\max}$  was under-predicted. One of the possible reasons could be the effect of multidrug-resistance transport protein, P-glycoprotein, on the absorption since crizotinib was reported to be a substrate of P-glycoprotein (FDA, 2011). The predicted crizotinib plasma concentrations with CRZ-HSP were in good agreement with the observed profiles after  $t_{\max}$  (Fig. 4B).

Clinically observed crizotinib steady-state  $C_{\max}$ ,  $t_{\max}$  and  $AUC_{0-\tau}$  were 328 ng/mL, 4 hours and 3054 ng·h/mL, respectively, in cancer patients (n =5) after 28-day repeated oral administration of crizotinib 250 mg twice daily (FDA, 2011). The predicted  $C_{\max}$  (543 ng/mL) and  $AUC_{0-\tau}$  (6494 ng·h/mL) by Simcyp with CRZ-HLM were over-predicted by 1.7- and 2.1-fold, respectively (Table 2 and Fig. 4C). In contrast, the Simcyp-predicted crizotinib steady-state plasma concentrations with CRZ-HSP were in good agreement with the observed concentrations (Fig. 4D). The predicted  $C_{\max}$  (273 ng/mL) and  $AUC_{0-\tau}$  (3258 ng·h/mL) were within 20% of the observed values (Table 2). Additionally, the effect of crizotinib TDI or EI on crizotinib steady-state plasma concentrations was examined in a virtual population of healthy volunteers by Simcyp using CRZ-HSP with/without TDI and/or EI parameters. The predicted  $C_{\max}$

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(288 ng/mL) and  $AUC_{0-\tau}$  (3432 ng·h/mL) with only TDI parameters were comparable to the predicted values (273 ng/mL and 3258 ng·h/mL, respectively) with both TDI and EI parameters (within 5%) whereas those with only EI parameters (128 ng/mL and 1519 ng·h/mL, respectively) were comparable to the predicted values (133 ng/mL and 1587 ng·h/mL, respectively) without TDI and EI parameters (within 5%). The predicted  $C_{max}$  and  $AUC_{0-\tau}$  with only TDI parameters were approximately 2-fold higher than the predicted values without TDI and EI parameters. Based on these comparisons, crizotinib steady-state plasma concentrations appeared to be accumulated by 2-fold due to TDI with a minimal EI effect.

### **Predicted Crizotinib-Midazolam Interaction with Simcyp**

Clinically observed midazolam  $C_{max}$ ,  $t_{max}$  and  $AUC_{0-\infty}$  in cancer patients ( $n = 14$ ) after a single oral administration of midazolam 2 mg were reported to be 13 ng/mL, 0.5 hours and 32 ng·h/mL, respectively, without co-administration of crizotinib (FDA, 2011). After 28-day oral administration of twice daily doses of crizotinib 250 mg in cancer patients ( $n = 8$ ), midazolam  $C_{max}$  and  $AUC_{0-\infty}$  increased to 26 ng/mL and 117 ng·h/mL, respectively. Thus, the fold-increase in midazolam  $C_{max}$  ( $C_{maxR}$ ) and  $AUC_R$  with co-administration of crizotinib were 2.0 and 3.7, respectively, suggesting crizotinib was a moderate CYP3A inhibitor (FDA, 2012). The 90% confidence intervals ( $CI$ ) were 1.4 to 2.9 for  $C_{maxR}$  and 2.6 to 5.1 for  $AUC_R$ . When the crizotinib-midazolam interaction was predicted by Simcyp with CRZ-HLM, the predicted midazolam plasma concentration-time profiles were much higher than the observed profiles (Fig. 5A). The midazolam  $C_{maxR}$  and  $AUC_R$  were 3.0 and 21, respectively (Table 3 and Fig. 6A), resulting in the 1.5- and 5.6-fold over-predictions of  $C_{maxR}$  and  $AUC_R$ , respectively. In

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contrast, the predicted midazolam plasma concentration-time profiles with CRZ-HSP were relatively consistent with the observed profiles (Fig. 5B). The midazolam  $C_{maxR}$  and  $AUC_R$  (1.9 and 3.6, respectively) were in good agreement with the clinically observed results (Table 3 and Fig. 6B). The 90%  $CI$  for predicted  $C_{max}$  (1.4 to 2.9) was relatively consistent with the observed  $CI$ , whereas that for predicted  $AUC_{0-\infty}$  (1.6 to 8.8) was slightly larger than the observed result.

### **Sensitivity Analysis for Crizotinib Hepatic $k_p$ and CYP3A4 $k_{deg}$ with Simcyp**

The sensitivity analysis for crizotinib hepatic  $k_p$  values of 1 to 24 revealed that the midazolam  $AUC_R$  with co-administration of crizotinib varied markedly from 3.6 to 13, while the predicted crizotinib  $AUC_{0-\tau}$  values were within 2-fold (3258 to 5434 ng·h/mL) (see Supplemental Data, Figure S1). Therefore, DDI prediction with a full-PBPK model in Simcyp was highly sensitive to crizotinib hepatic  $k_p$  value even though the PBPK model was basically constructed with perfusion-limited compartments in the absence of any diffusion barrier.

In the sensitivity analysis for hepatic CYP3A4  $k_{deg}$  values of 0.693 to 0.00693  $h^{-1}$  (i.e.,  $t_{1/2}$  of 1 to 100 hours), the predicted crizotinib steady-state  $C_{max}$  and  $C_{ave}$  varied from 141 to 385 ng/mL and 140 to 383 ng/mL, respectively, indicating that the CYP3A4  $t_{1/2}$  of 25 to 35 hours yielded the best prediction for crizotinib  $C_{ave}$  (Supplemental Data, Figure S2). The midazolam  $C_{maxR}$  and  $AUC_R$  varied from 1.3 to 2.4 and 1.3 to 7.7, respectively. Thus, the  $AUC_R$  was more sensitive to the hepatic  $k_{deg}$  values relative to  $C_{maxR}$ . The  $t_{1/2}$  of 30 to 40 hours yielded the best prediction of the midazolam  $AUC_R$ . Therefore, the hepatic  $k_{deg}$  value of 0.019  $h^{-1}$  ( $t_{1/2}$  of 36 hours) used in this study was

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within a range of reasonable predictions on both the crizotinib steady-state plasma concentrations and crizotinib-midazolam interaction.

### **Predicted Crizotinib-Midazolam Interaction by Static Models**

The predicted midazolam  $AUC_R$  values with the static mathematical model using either clinically observed or Simcyp-predicted crizotinib  $C_{ave,u}$  are summarized in Table 4. Using the clinically observed crizotinib  $C_{ave,u}$ , the predicted midazolam  $AUC_R$  values with HLM-TDI and HSP-TDI were 17 and 3.2, respectively. Thus, the midazolam  $AUC_R$  with HLM-TDI was over-predicted by approximately 5-fold whereas the prediction with HSP-TDI was consistent with the observed  $AUC_R$ . In the static model, the midazolam  $AUC_R$  in the liver (11 to 12) with HLM-TDI was much higher than that with HSP-TDI (2.5 to 2.6), whereas the  $AUC_R$  in the intestines was relatively comparable between HLM-TDI and HSP-TDI (1.6 and 1.3, respectively). This comparable intestinal  $AUC_R$  between HLM-TDI and HSP-TDI appeared to be largely due to midazolam  $F_g$  of 0.63, which resulted in the maximal effect of 1.6-fold increase. Using the Simcyp-predicted crizotinib  $C_{ave,u}$  by HLM-TDI and HSP-TDI, the predicted midazolam  $AUC_R$  values were 19 and 3.3, respectively. Thus, the prediction results of crizotinib-midazolam interaction with the static model using the Simcyp-predicted crizotinib  $C_{ave,u}$  were relatively consistent with those with the Simcyp full-PBPK models with  $k_p$  of unity.

In addition, the predicted midazolam  $AUC_R$  values with Simcyp static model ( $R_{ss}$ ) were 13 and 2.3 with HLM-TDI and HSP-TDI, respectively. These  $AUC_R$  values were approximately 1.5-fold lower than the predicted values with the Simcyp full-PBPK models (i.e., 21 and 3.6, respectively). The difference in the predicted ratios between the  $R_{ss}$  and full-PBPK predictions appeared to be largely due to the crizotinib TDI effects on

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the accumulation of crizotinib steady-state plasma concentrations, which was not taken into account for the Simcyp- $R_{ss}$  prediction, where the inhibitor concentration was calculated from the input PK parameters such as  $D$  and  $CL$ .



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## DISCUSSION

In the present study, the estimated in vitro inactivation efficiency ( $k_{inact}/K_I$ ) for crizotinib TDI potency was approximately 20-fold lower in HSP relative to HLM. Consistent with the present results, the known CYP3A inhibitors (e.g., diltiazem, erythromycin and verapamil) were also reported to be less potent in HEP compared to HLM, and the difference in TDI potency have yielded an impact on in vivo DDI prediction (Xu et al., 2009; Chen et al., 2011). The advantages in utilization of HEP over HLM are considered to be that HEP are intact cellular systems containing not only a full complement of phase I/II metabolizing enzymes but also functional cell membranes. The estimated  $k_{inact}$  in HEP is generally considered an apparent hybrid kinetic parameter consisting of 1) the rate of diffusion through the cell membrane, 2) the total metabolic consumption rate (including sequential metabolism), and 3) the intrinsic enzyme inactivation rate. Therefore, these factors could be important for some drugs showing substantially different intra- versus extracellular concentrations and/or multiple metabolic pathways, particularly those associated with non-CYPs (Zhao, 2008). Crizotinib was primarily metabolized by CYP3A (Johnson et al., 2011a), and its passive permeability was relatively low (approximately  $1 \times 10^{-6}$  cm/sec) in the recently reported permeability assay system using low-efflux Madin-Darby canine kidney cells (Di et al., 2011). Crizotinib was also reported to be a substrate of P-glycoprotein as mentioned above. Thus, these factors could potentially lead to differences in intra- versus extracellular concentrations, and, correspondingly, to differences in the estimated TDI potency between HLM versus HSP.

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Mathematical models used in the Simcyp-PBPK model are the well-stirred and “ $Q_{gut}$ ” models in the liver and GI tract, respectively (Yang et al., 2007; Jamei et al., 2009). The well-stirred model assumes that drug distribution into liver is perfusion-limited without any diffusion delay; therefore, unbound drug concentration within liver is in equilibrium with unbound drug concentration in liver outlet, e.g.,  $C_{sys,u}$ . Accordingly, we intended to use crizotinib  $C_{sys,u}$  as the inhibitor concentration for DDI prediction by adjusting hepatic  $k_p$  value (i.e., unity) as described in Materials and Methods. Consistent with our findings, other investigators (Obach et al., 2007; Fahmi et al., 2009; Boulenc and Barberan, 2011) reported that the use of  $C_{sys,u}$  (e.g.,  $C_{max,u}$  or  $C_{ave,u}$ ) as the inhibitor concentrations yielded the most accurate DDI predictions for TDI and EI by the static models. When the projected/calculated  $C_{inlet,u}$  was used, the DDI predictions for TDI and EI were generally over-predicted while those for RI were more accurately predicted. These findings may suggest that RI mainly occurs during the first-pass metabolism process, whereas TDI and EI continue to take place beyond the first-pass metabolism. Consistently, the clinically observed crizotinib-midazolam interaction was more significant on midazolam  $AUC_R$  than  $C_{maxR}$ , and the DDI prediction using crizotinib  $C_{sys,u}$  yielded a reasonable prediction for both midazolam  $AUC_R$  and  $C_{maxR}$ . Thus, crizotinib  $C_{sys,u}$  appears to be a more appropriate inhibitor concentration than  $C_{inlet,u}$  for the prediction of crizotinib-midazolam interaction.

The  $Q_{gut}$  model in the GI tract is basically constructed by the well-stirred model, where the blood flow term (i.e.,  $Q_{gut}$ ) is a hybrid parameter consisting of a compound-dependent permeability through enterocyte membrane and a physiological villous blood flow. Crizotinib  $Q_{gut}$  value was estimated to be 4 L/h based on a calculated  $P_{eff,man}$  value

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of  $0.58 \times 10^{-4}$  cm/s derived from its physicochemical properties. This  $P_{eff,man}$  value was within a moderate range among seventeen compounds reported (Yang et al., 2007). Since crizotinib  $F_g$  was estimated to be approximately 0.9 by the  $Q_{gut}$  model, its moderate  $P_{eff,man}$  did not appear to be a limiting factor for the first-pass metabolism in the GI tract. In contrast, the experimentally measured crizotinib in vitro permeability ( $1 \times 10^{-6}$  cm/sec) seemed to be lower than the value expected from its physicochemical properties, suggesting that the calculated  $P_{eff,man}$  value might be over-predicted. However, crizotinib permeability can be taken into account to estimate TDI kinetic parameters in HSP because this system contains intact cell membranes. We therefore believe that a perfusion-limited model is appropriate for PK and/or DDI prediction when the kinetic parameters were determined in an in vitro system containing cell membranes. On the other hand, a diffusion rate through the cell membrane would likely be an important factor for PBPK model of low permeable compounds when their kinetic parameters were determined in assay systems without functional cell membranes (e.g., HLM). In such a case, a diffusion-limited model would be likely required for their PK and/or DDI prediction. Moreover, when compounds are metabolized by multiple enzymes such as microsomal and cytosolic enzymes, HEP would be more appropriate to determine TDI kinetic parameters by taking into account of an overall in vivo metabolic pathway. It has been reported that multiple enzymes (e.g., CYP3A and aldehyde oxidase) were involved in the crizotinib metabolism (Johnson et al., 2011a); therefore, this might be another factor that contributed to the difference in the TDI kinetics between HLM and HSP. These considerations are graphically summarized in Fig. 7. For PBPK model-based DDI prediction, the selection of either a perfusion- or diffusion-limited model should be

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carefully considered based upon each inhibitor's property and in vitro assay system used. It should also be important to select/simulate appropriate surrogate concentrations of both object and precipitant drugs as the unbound concentrations at target sites. In vivo DDI predictions are expected to be more accurate based on plasma concentration-profiles predicted by the PBPK model compared to a fixed inhibitor concentration (e.g.,  $C_{max,u}$  and  $C_{ave,u}$ ) used for the static model. In the prediction of crizotinib-midazolam interaction, it would be worth noting that the Simcyp minimal- and full-PBPK models (with hepatic  $k_p$  of unity) yielded comparable DDI predictions when the predicted crizotinib concentrations in plasma were comparable between these models (Supplemental Data, Table S1). Moreover, these DDI predictions were roughly comparable to the predictions from the static models such as a Simcyp-static model (i.e.,  $R_{ss}$ ) and a static mathematical model (Supplemental Data, Table S1). Therefore, there might not be significant differences in DDI prediction between dynamic and static models if the factors related to DDI were appropriately incorporated into these models. However, these comparable predictions for crizotinib-midazolam interaction could be largely due to the relatively flat crizotinib plasma concentration time-profiles (i.e.,  $C_{max} \approx C_{ave}$ ) during dosing interval as shown in Fig 4.

Several other factors related to TDI mechanism should also be considered for the in vivo DDI prediction. Quantitative measurement of in vivo CYP3A4  $k_{deg}$  in humans remains a challenge as  $k_{deg}$  is often associated with an uncertainty in the DDI prediction (Obach et al., 2007; Rowland Yeo et al., 2011). Not surprisingly, a wide range of hepatic  $k_{deg}$  values has been reported for CYP3A4 as turnover half-lives ( $t_{1/2}$ ) of 10 to 140 h (Yang et al., 2008; Grimm et al., 2009; Xu et al., 2009; Chen et al., 2011). We used the

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Simcyp default  $k_{deg}$  values of  $0.019 \text{ h}^{-1}$  ( $t_{1/2} = 36$  hours) in the liver and  $0.030 \text{ h}^{-1}$  ( $t_{1/2} = 23$  hours) in the intestines. Based on the sensitivity analysis for hepatic CYP3A4  $k_{deg}$  values ranging from  $0.693$  to  $0.00693 \text{ h}^{-1}$ , i.e.,  $t_{1/2}$  of 1 to 100 hours, the hepatic  $k_{deg}$  value of  $0.019 \text{ h}^{-1}$  ( $t_{1/2}$  of 36 hours) used in this study was within a range of reasonable predictions on both the crizotinib steady-state plasma concentrations and crizotinib-midazolam interaction (Supplemental Data, Figure S2). Another important parameter is the fraction metabolized by a drug-metabolizing enzyme, e.g.,  $f_{m,CYP3A4}$ . The estimated crizotinib  $f_{m,CYP3A4}$  was approximately 0.8 based on the in vitro CYP phenotyping study and the human mass-balance study with [ $^{14}\text{C}$ ]crizotinib (Johnson et al., 2011a; Johnson et al., 2011b); the  $f_{m,CYP3A4}$  of 0.8 used in the present study yielded reasonable PK/DDI predictions.

Despite these reasonable DDI predictions, the method used in the present study included potential limitations that should be addressed. First, neither in vitro non-specific binding nor any decrease in crizotinib concentration during incubation was considered for the correction of in vitro crizotinib EI parameters (e.g.,  $E_{max}$  and  $EC_{50}$ ), as previously reported (Fahmi et al., 2008). In contrast, the obtained TDI parameters in HLM and HSP were corrected for the in vitro non-specific binding. Secondly, and possibly more importantly, the physiological CYP3A4 kinetic (or systems) parameters (i.e.,  $k_{syn}$  and  $k_{deg}$ ) for the TDI prediction were assumed to remain constant in the presence of inhibitor, and an additional enzyme degradation pathway was introduced to reach new steady-state concentration as  $k_{obs}$  (eq. 1). This assumption may not be valid when EI occurs in parallel to TDI. In fact, there appears to be limited understanding of underlying simultaneous in vitro-to-in vivo extrapolation for TDI and EI (and its possible interplay).

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Moreover, although TDI and EI of CYP3A are known to occur in both liver and GI tract, their magnitudes may be different in these organs. Despite these limitations, a PBPK modeling approach for DDI prediction affords an opportunity to gain greater insight into the underlying mechanisms mediating these complex interactions as a function of time with the effects of intrinsic factors such as organ dysfunction, age and genetics.

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### **Authorship Contribution**

*Participated in research design:* Mao and Yamazaki

*Conduct experiments:* Mao, Shen and Yamazaki

*Performed data analysis:* Mao and Yamazaki

*Wrote or contribute to the writing of the manuscript:* Mao, Shen, Johnson and Yamazaki



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## Footnotes

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## Legends for Figures

### Fig. 1. Chemical structure of crizotinib

**Fig. 2. Time-dependent inhibition by crizotinib for CYP3A in HLM.** The x-axis represents crizotinib concentration in micromolars and the y-axis represents the observed apparent inactivation rate constant,  $k_{obs}$ , (●) and its fitting curve (—) in reciprocal hours. Data are the mean of duplicate determination.

**Fig. 3. Time-dependent inhibition by crizotinib for CYP3A in HSP.** The x-axis represents crizotinib concentration in micromolars on a logarithmic scale and the y-axis represents the ratio of remaining CYP3A enzyme activity to the vehicle control at the end of 35-minute incubation with midazolam following 0 (●), 10 (◆) or 20 (■)-minute preincubation with crizotinib alone. The dashed lines represent the best fitting curves for each incubation period by the irreversible model. Data are the mean of duplicate determination.

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**Fig. 4. Observed and Simcyp-predicted plasma concentrations of crizotinib in patients after a single or 28-day repeated oral administration of crizotinib.**

Crizotinib was orally administered at a single dose of 250 mg (n = 8) or multiple doses of 250 mg twice daily (n = 5) to cancer patients. The x-axis represents the time after a single (A and B) or 28-day repeated (C and D) oral administration of crizotinib in hours and the y-axis represents the observed (○) and Simcyp-predicted (—) plasma concentrations of crizotinib in nanograms per milliliter on a logarithmic scale using TDI parameters from HLM (A and C) or HSP (B and D). The observed and predicted plasma concentrations are expressed as mean ± SD and mean with 90% confidence interval (dashed line), respectively.

**Fig. 5. Observed and Simcyp-predicted plasma concentrations of midazolam in patients before and after 28-day repeated oral administration of crizotinib.**

Midazolam (2 mg) was orally administered to cancer patients before (n = 14) and after (n = 8) twice daily doses of crizotinib 250 mg for 28 days. The x-axis represents the time after a single oral administration of midazolam in hours and the y-axis represents the observed plasma concentrations of midazolam before (○) and after (●) oral administration of crizotinib in nanograms per milliliter on a logarithmic scale along with the predicted plasma concentrations before (---) and after (—) crizotinib administration by Simcyp with TDI parameters from HLM (A) or HSP (B). The observed and predicted plasma concentrations are expressed as mean ± SD and mean, respectively.

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**Fig. 6. Clinically observed and Simcyp-predicted fold-increase in midazolam**

**$AUC_{0-\infty}$  with co-administration of crizotinib.** Midazolam (2 mg) was orally administered to cancer patients before ( $n = 14$ ) and after ( $n = 8$ ) twice daily doses of crizotinib 250 mg for 28 days. The x-axis represents the observed (Obs) and predicted 8 clinical trials (#1 to 8) of 10 individuals by Simcyp with TDI parameters from HLM (A) or HSP (B) and the y-axis represents the fold-increase in midazolam  $AUC_{0-\infty}$  ( $AUC_R$ ) with co-administration of crizotinib. The observed and predicted  $AUC_R$  values in each trial are expressed as geometric mean with 90% confidence interval. The solid and dashed lines represent geometric mean and 90% confidence interval, respectively, of all trials.

**Fig.7. Proposed general scheme of PBPK model-based in vivo TDI prediction.**

TABLE 1

## Physicochemical and pharmacokinetic parameters of crizotinib used for DDI prediction

Parameters (units)		Parameters (units)	
Molecular weight	450.34	$CL_{int}$ ( $\mu\text{L}/\text{min}/\text{mg protein}$ )	
$\log P$	4.29	$CL_{int,CYP3A4}$	194
$pK_a^a$	5.4 & 8.9	$CL_{int,others}$	49
$PSA$ ( $\text{\AA}^2$ )	78	Time-dependent inhibition on CYP3A in HLM	
$f_{u,plasma}$	0.093	$K_I$ ( $\mu\text{M}$ )	0.37
$R_{bp}$	1.1	$k_{inact}$ ( $h^{-1}$ )	6.9
$f_{u,blood}^b$	0.085	Time-dependent inhibition on CYP3A in HSP	
$f_{u,mic}$	0.12	$K_I$ ( $\mu\text{M}$ )	0.89
$F_a$	0.6	$k_{inact}$ ( $h^{-1}$ )	0.78
$k_a$ ( $h^{-1}$ ) <sup>c</sup>	0.01 - 0.18	CYP3A4 induction in HEP <sup>d</sup>	
$Q_{gut}$ ( $L/h$ )	4.0	$E_{max}$ (fold)	2.4
$f_{u,gut}$	0.085	$EC_{50}$ ( $\mu\text{M}$ )	0.84
$V_{ss}$ ( $L/kg$ )	25	Reversible Inhibition on CYP3A4 in HLM	
		$IC_{50}$ ( $\mu\text{M}$ )	>30

<sup>a</sup> The value of  $pK_a$  was adjusted from 5.4 and 8.9 (diprotic base) to 7.6 (monprotic base) to predict clinically observed  $V_{ss}$  of 25 L/kg.

<sup>b</sup> The value of  $f_{u,blood}$  was calculated by  $f_{u,plasma}$  and  $R_{bp}$ .

<sup>c</sup> The values of  $k_a$  were adjusted to simulate clinically observed  $t_{max}$  of approximately 4 hours in each simulation.

<sup>d</sup> The values of  $E_{max}$  and  $EC_{50}$  were determined in cryopreserved human hepatocytes from three donors (6.4 to 29-fold and 0.47 to 3.1  $\mu\text{M}$ , respectively) followed by normalization with rifampin data (positive control)



TABLE 2

**Clinically observed and Simcyp-predicted pharmacokinetic parameter estimates of crizotinib in humans after a single or 28-day repeated oral administration of crizotinib.**

	Single Dose (250 mg)		Repeated Doses (250 mg bid)	
	$C_{max}$ <i>ng/mL</i>	$AUC_{0-\infty}$ <i>ng·h/mL</i>	$C_{max}$ <i>ng/mL</i>	$AUC_{0-\tau}$ <i>ng·h/mL</i>
Observed	87 (34)	1817(33)	328 (25)	3054 (32)
Predicted				
HLM <sup>a</sup>	89 (43)	3901 (57)	543 (41)	6494 (41)
P/O ratio <sup>b</sup>	1.0	2.1	1.7	2.1
HSP <sup>a</sup>	42 (52)	1745 (72)	273 (64)	3258 (65)
P/O ratio <sup>b</sup>	0.48	0.96	0.83	1.1

Data are expressed as geometric mean (% coefficient of variation) for single dose (n = 8 patients) and 28-day repeated doses (n = 5 patients).

<sup>a</sup> Simcyp simulation was performed using TDI parameters from either HLM or HSP.

<sup>b</sup> Calculated mean ratio of predicted to observed pharmacokinetic parameters.

TABLE 3

**Clinically observed and Simcyp-predicted pharmacokinetic parameter estimates of midazolam in humans before and after 28-day repeated oral administration of crizotinib twice daily doses of 250 mg**

	Crizotinib Administration	$C_{max}$ <i>ng/mL</i>	$AUC_{0-\infty}$ <i>ng·h/mL</i>	Fold-increase in midazolam oral exposure <sup>a</sup>			
				$C_{maxR}$	90% CI	$AUC_R$	90% CI <sup>a</sup>
Observed	Pre	13 (39)	32 (41)				
	Post	26 (44)	117 (61)	2.0	1.4 - 2.9	3.7	2.6 - 5.1
Predicted	Pre	10 (58)	27 (90)				
	P/O ratio <sup>b</sup>	0.77	0.83				
HLM <sup>c</sup>	Post	30 (39)	547 (70)	3.0	1.8 – 6.9	21	4.7 – 72
	P/O ratio <sup>b</sup>	1.2	4.7	1.5		5.6	
HSP <sup>c</sup>	Post	19 (53)	97 (109)	1.9	1.4 - 2.7	3.6	1.6 – 8.8
	P/O ratio <sup>b</sup>	0.72	0.83	0.94		1.0	

Data are expressed as geometric mean (% coefficient of variation) for pre- and post-doses of crizotinib (n = 14 and 8 patients, respectively).

<sup>a</sup> Fold-increase in  $C_{max}$  ( $C_{maxR}$ ) and  $AUC_{0-\infty}$  ( $AUC_R$ ) with co-administration of crizotinib with 90% confidence interval (CI).

<sup>b</sup> Calculated predicted to observed pharmacokinetic parameter ratio.

<sup>c</sup> Simcyp simulation was performed using TDI parameters from either HLM or HSP.

**TABLE 4****Predicted fold-increase in midazolam AUC<sub>0-∞</sub> by the static models using crizotinib steady-state average plasma concentration**

TDI <sup>a</sup>	Crizotinib $C_{ave}$ <sup>b</sup>		Predicted fold-increase in midazolam AUC <sub>0-∞</sub>			
			Static mathematical model			Simcyp $R_{ss}$
	ng/mL total	nM free	Liver	Gut	Liver + Gut	
HLM	255 <sup>c</sup>	53 <sup>c</sup>	11	1.6	17	–
	541 <sup>d</sup>	112 <sup>d</sup>	12	1.6	19	13
HSP	255 <sup>c</sup>	53 <sup>c</sup>	2.5	1.3	3.2	–
	271 <sup>d</sup>	56 <sup>d</sup>	2.6	1.3	3.3	2.3

–, not applicable

<sup>a</sup> Static mathematical models utilized TDI parameters from either HLM or HSP.<sup>b</sup> Average steady-state plasma concentrations ( $C_{ave}$ ) in total (ng/mL) and unbound (nM) forms were calculated from either the observed or Simcyp-predicted AUC<sub>0-τ</sub> (listed in Table 2) divided by the dosing interval of 12 hours.<sup>c</sup> Clinically observed crizotinib  $C_{ave}$  was used for the prediction of crizotinib-midazolam interaction with the static model.<sup>d</sup> Simcyp-predicted crizotinib  $C_{ave}$  was used for the prediction of crizotinib-midazolam interaction with the static model.

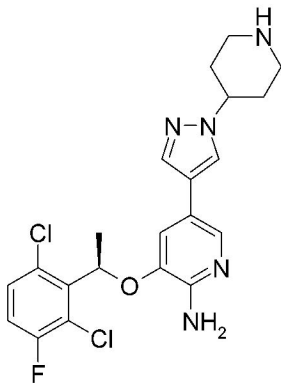


Figure 1

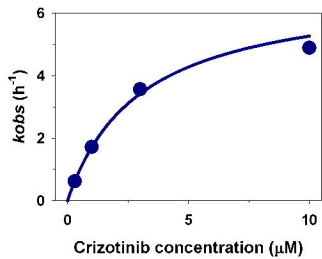


Figure 2

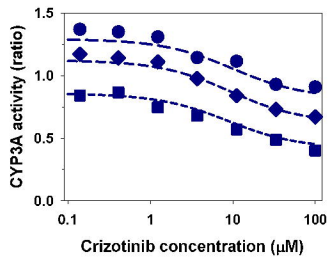


Figure 3

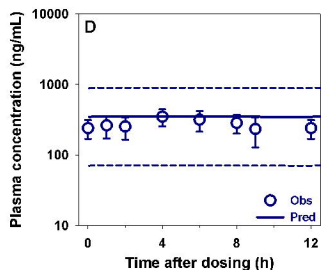
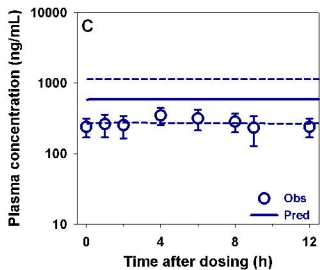
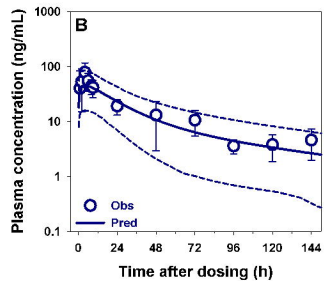
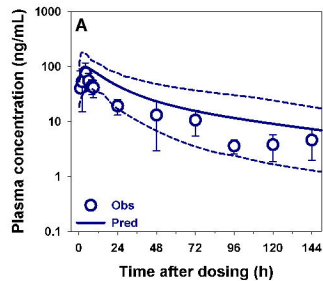


Figure 4

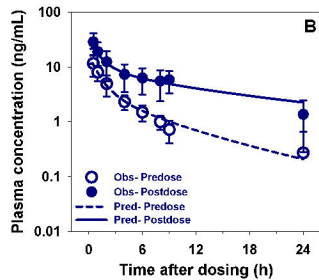
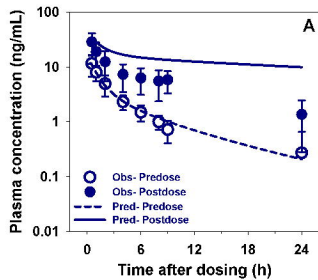


Figure 5



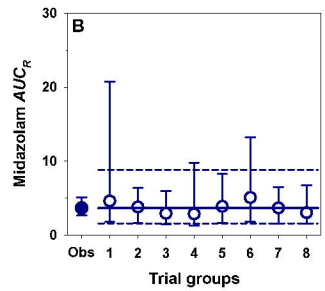
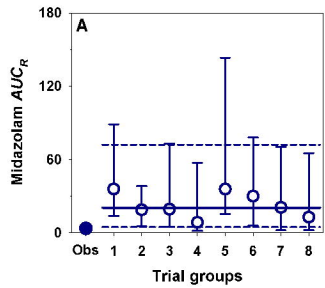


Figure 6

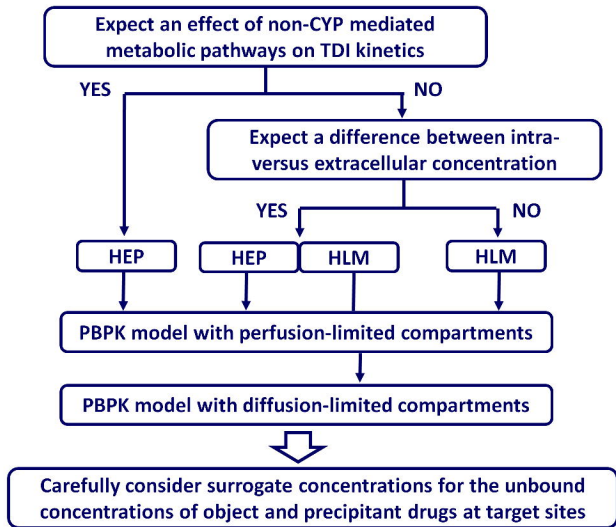


Figure 7