TITLE: Pharmacokinetic-Pharmacodynamic Modeling of Crizotinib for Anaplastic Lymphoma Kinase Inhibition and Anti-tumor Efficacy in Human Tumor Xenograft Mouse Models

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ABBREVIATIONS:

ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; \( C_e \), effect-site concentration of crizotinib; \( CL/F \), oral clearance; \( C_p \), plasma concentration of crizotinib; \( EC_{50} \), drug concentration causing 50% of maximum effect; crizotinib (PF02341066), \((R)-3-[1-(2,6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine; E_0 \), ALK phosphorylation baseline; \( E_{max} \), maximum effect; EML4, echinoderm microtubule-associated protein-like 4; \( \gamma \), Hill coefficient; \( k_a \), absorption rate constant; \( k_{e0} \), rate constant for equilibration with the effect site; \( k_{id} \), first-order tumor death rate constant; \( k_{tg} \), first-order tumor growth rate constant; MET, hepatocyte growth factor receptor; NPM, nucleophosmin; NSCLC, non–small-cell lung cancer; \( OFV \), objective function value; PKPD, pharmacokinetic-pharmacodynamic; \( T \), tumor volume; TGI, tumor growth inhibition; \( T_{sw} \), maximum sustainable tumor volume; \( V_d/F \), oral volume of distribution.
ABSTRACT

Crizotinib (Xalkori®, PF02341066) is an orally available dual inhibitor of anaplastic lymphoma kinase (ALK) and hepatocyte growth factor receptor. The objectives of the present studies were to characterize: 1) the pharmacokinetic-pharmacodynamic relationship of crizotinib plasma concentrations to inhibition of ALK phosphorylation in tumor, and 2) the relationship of ALK inhibition to anti-tumor efficacy in human tumor xenograft models. Crizotinib was orally administered to athymic nu/nu mice implanted with H3122 non-small cell lung carcinomas or immunodeficient SCID/beige mice implanted with Karpas299 anaplastic large cell lymphomas. Plasma concentration-time courses of crizotinib were adequately described by a one-compartment pharmacokinetic model. A pharmacodynamic link model reasonably fit the time-courses of ALK inhibition in both H3122 and Karpas299 models with EC₅₀ of 233 and 666 ng/mL, respectively. A tumor growth inhibition model also reasonably fit the time-course of individual tumor growth curves with EC₅₀ of 255 and 875 ng/mL, respectively. Thus, the EC₅₀ for ALK inhibition roughly corresponded to the EC₅₀ for tumor growth inhibition in both xenograft models, suggesting that >50% ALK inhibition would be required for significant anti-tumor efficacy (>50%). Furthermore, based on the observed clinical pharmacokinetic data coupled with the pharmacodynamic parameters obtained from the present non-clinical xenograft mouse model, >70% ALK inhibition was projected in NSCLC patients administered the clinically recommended dosage of crizotinib, twice daily doses of 250 mg (500 mg/day). The result suggests that crizotinib could sufficiently inhibit ALK phosphorylation for significant anti-tumor efficacy in patients.
INTRODUCTION

Recent advances in a number of molecular profiling technologies, the so-called “-omics” sciences, such as genomics, transcriptomics, proteomics and metabolomics, have significantly enhanced the potential and actual development of personalized medicine, which targets individualized treatment and care based on genetic and individual variations. Some examples of successful approaches to personalized medicine have been reported in cancer therapy, e.g., small-molecule epidermal growth factor receptor (EGFR) inhibitors, gefitinib and erlotinib, for non–small-cell lung cancer (NSCLC) patients with activating EGFR mutations (Lynch et al., 2004; Pao et al., 2004) and an anti-Her2 monoclonal antibody, trastuzumab, for breast cancer patients with HER2 gene over-expression (Slamon et al., 2001; Spector, 2008). Such accomplishments illustrate the ever-increasing evidence that a personalized medicine approach can yield promising clinical responses in a subset of cancer patients. On the other hand, rapidly acquired drug resistance still remains an important limitation to long-term successful cancer therapy (Engelman and Settleman 2008). For example, in patients with NSCLC who demonstrated a primary response to EGFR inhibitors, acquired resistance against EGFR inhibitor monotherapy typically develops after 6 to 12 months (Sharma et al., 2007). One of the mechanisms for this acquired resistance appears to be genomic amplification of the hepatocyte growth factor receptor, MET (also named cMet or HGFR), since a combination of MET and EGFR inhibitors can potentially improve efficacy (Engelman et al., 2007; McDermott et al., 2010). Thus, a clinical outcome improvement in some cancer patients is likely to be achieved by identification of the molecular events that underlie their specific pathogenesis.
In recent years, the identification of activating mutations or translocations of the anaplastic lymphoma kinase (ALK) gene has been reported in various types of cancer such as anaplastic large cell lymphoma (ALCL) (Kutok and Aster, 2002), inflammatory myofibroblastic tumor (Pulford et al., 2004), neuroblastoma (George et al., 2008; Mosse et al., 2008) and NSCLC (Soda et al., 2007; Mano, 2008). Nucleophosmin (NPM) is the most common fusion partner of ALK (80% of translocations) in ALCL. Echinoderm microtubule-associated protein-like 4 (EML4)-ALK fusion protein has been identified in ~7% of NSCLC patients whereas other rare fusion partners for ALK (e.g., LIF5B, TFG, etc) have also been detected in NSCLC patients (Takeuchi et al., 2009). The genetic ALK rearrangements rarely coexist with KRAS or EGFR mutations, and the patients with ALK rearrangement tend to be younger than those without the rearrangements and most of the patients have little or no exposure to tobacco (Soda et al., 2007; Mano, 2008; Perner et al., 2008; Wong et al., 2009, Kwak et al., 2010). These findings suggest that the ALK rearrangement is a promising therapeutic target as well as a diagnostic molecular marker in NSCLC patients.

Crizotinib (Xalkori®, PF02341066, (R)-3-[1-(2, 6-dichloro-3-fluoro-phenyl)-ethoxy]-5-(1-piperidin-4-yl-1H-pyrazol-4-yl)-pyridin-2-ylamine) was identified as an orally available, ATP-competitive dual inhibitor for ALK and MET. Crizotinib has recently been approved by the Food and Drug Administration (FDA) for the treatment of locally advanced or metastatic NSCLC patients who are positive for ALK rearrangement as detected by an FDA-approved test. We previously reported the pharmacokinetic-pharmacodynamic (PKPD) modeling of crizotinib for the inhibition of MET phosphorylation and anti-tumor efficacy in athymic mice implanted with GTL16 gastric...
carcinomas or U87MG glioblastomas (Yamazaki et al., 2008). PKPD modeling is a useful mathematical approach linking drug exposure to pharmacologic responses as a function of time, providing a quantitative assessment of in vivo drug potency with mechanistic insight of drug action (Derendorf et al., 2000; Chien et al., 2005; Workman et al., 2005). The previous PKPD modeling results of crizotinib suggested that >90% inhibition of MET phosphorylation would be required for significant anti-tumor efficacy (>50%).

The objectives of the present study were to characterize: 1) the PKPD relationship of crizotinib plasma concentrations to the inhibition of ALK phosphorylation in tumors (biomarker), and 2) the relationship of ALK inhibition to anti-tumor efficacy (pharmacological response) in athymic mice implanted with H3122 NSCLC cells harboring the EML4-ALK fusion protein or Karpas299 ALCL cells carrying the NPM-ALK fusion protein. Subsequently, we compared the present PKPD relationships in the ALK-driven xenograft mouse models with the previous PKPD results in the MET-driven xenograft models. Furthermore, based on these non-clinical PKPD modeling results, the biomarker inhibitions of ALK and MET phosphorylation were projected by PKPD simulation using the observed crizotinib clinical PK results in NSCLC patients. The present PKPD results will be helpful in understanding PKPD relationships of crizotinib and also in guiding dose escalation or de-escalation to achieve or maintain efficacious exposure in patients with ALK or MET-positive tumors.
MATERIALS AND METHODS

Chemicals

Crizotinib (chemical purity >99%) and a structurally-related in house compound (internal standard for analysis) were synthesized by Pfizer Worldwide Research and Development (San Diego, CA) (Cui et al., 2011). All other reagents and solvents were commercially available and were of either analytical or high performance liquid chromatography grade.

In Vivo PKPD Study

The experimental designs and methods of the in vitro and in vivo PKPD studies were previously reported in part by Zou et al. (2011). Briefly, three separate repeated oral-dose PKPD studies were conducted with crizotinib in athymic nu/nu mice implanted with H3122 NSCLC xenografts (studies 1 and 2) or in immunodeficient SCID/beige mice implanted with Karpas299 ALCL xenografts (study 3). Mice were treated with crizotinib at the doses of 25, 50, 100, and 200 mg/kg once daily in studies 1 and 2 (14 and 18 days of administration, respectively), or at the doses of 25, 50 and 100 mg/kg once daily in study 3 (13 days of administration). A subset of mice was humanely euthanized at 1, 4, 7 and 24 hours after the last dose. Blood samples (n= 3/time point) were collected by exsanguination via cardiac puncture to determine plasma concentrations of crizotinib. Resected tumors (n= 3/time point) were snap frozen and pulverized using liquid nitrogen-cooled cryomortar. Protein lysates were generated, and then the level of total phosphorylated ALK protein (ALK phosphorylation) was determined using a capture ELISA method (studies 1 and 3). Tumor volume was measured during the treatment period by electronic Vernier calipers and was calculated as the product of its length ×
width$^2 \times 0.4$ (studies 2 and 3). All of the procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and with Pfizer Animal Care and Use Committee guidelines. More detailed information about the in vitro and in vivo PKPD studies will be published elsewhere (Zou et al., manuscript in preparation).

**Crizotinib Plasma Concentration Analysis**

A quantitative assay method to determine crizotinib plasma concentrations was previously reported (Yamazaki et al., 2008). Briefly, mouse plasma samples were prepared by protein precipitation with a methanol: acetonitrile mixture (25:75, v/v). After the centrifugation, an appropriate volume of the resulting supernatant was analyzed by liquid-chromatography tandem mass spectrometry. The chromatography was performed with a Shimadzu high performance liquid chromatography system, equipped with a binary solvent delivery system LC-10 ADvp and a controller SLC-10Avp (Shimadzu Scientific Instrument, Columbia, MD) using a reverse phase column (Agilent XDB-C18, 2.1 × 50 mm, 5 μm). Mass spectrometric analysis was performed on an API 4000 triple quadrupole mass spectrometer (AB Sciex, Foster city, CA) using turbo-ion spray ionization. Sample analysis was performed in the positive ionization, multiple reaction monitoring mode with unit resolution for the transitions of m/z 450 to 260 for crizotinib and m/z 377 to 348 for the internal standard. The calibration curve range was 1 to 1000 ng/mL. The back-calculated calibration standard concentrations were within ±15% of their nominal concentrations with coefficients of variation (CV) of less than 15%. The precision and accuracy of the quality control samples were within ±15%.
Pharmacokinetic Analysis

A naïve-pooled pharmacokinetic analysis was used to determine crizotinib pharmacokinetic parameters in mice since a subset of mice (n=3/time points) was humanely euthanized at each time point to collect blood samples. Therefore, all individual data at each dose were pooled together for the pharmacokinetic analysis as if they came from a single individual (Sheiner, 1984). This approach provided a better fitting than non-linear pharmacokinetic model with Michaelis-Menten elimination (data not shown). Pharmacokinetic analysis was performed with a standard one-compartment model as implemented in NONMEM® version VI (University of California at San Francisco, San Francisco, CA) (Beal and Sheiner, 1992). This model (subroutine ADVAN2 with TRANS2) was parameterized using absorption rate constant ($k_a$, h$^{-1}$), oral clearance ($CL/F$, L/h/kg) and oral volume of distribution ($V_d/F$, L/kg). Residual variability was characterized by a proportional error model. The pharmacokinetic parameters thus obtained were used to simulate plasma concentrations as a function of time following oral administration to drive the time-dependent pharmacodynamic models.

PKPD Modeling

PKPD modeling for the response of ALK phosphorylation in tumor to plasma concentration of crizotinib in studies 1 and 3 was performed by a link model (an effect-compartment model) (Sheiner et al., 1979). Briefly, the effect site concentration of crizotinib ($C_e$, ng/mL) was expressed by the following differential equation:

$$\frac{dC_e}{dt} = k_{e0} \cdot (C_p - C_e)$$

(1)

where $k_{e0}$ is the rate constant for equilibration with the effect site (h$^{-1}$) and $C_p$ is the plasma concentration of crizotinib (ng/mL).
In the link model, the following equation was used to determine \( EC_{50} \) (the concentration causing one-half maximum effect, \( E_{\text{max}} \)) for the inhibition of ALK phosphorylation \( (E) \):

\[
E = E_0 \times \left( 1 - \frac{E_{\text{max}} \times C_p^\gamma}{EC_{50}^\gamma + C_p^\gamma} \right)
\]

where \( E_0 \) is the baseline of ALK phosphorylation (ratio to control animals) and \( \gamma \) is the Hill coefficient.

An alternative model, the indirect response model, assumes that ALK phosphorylation at baseline is maintained by the balance of formation and degradation rates (Jusko and Ko, 1994). The addition of crizotinib was considered to inhibit the model’s formation rate, since crizotinib was a competitive ATP-binding ALK inhibitor. Therefore the following differential equation was used to determine the \( EC_{50} \) required for the inhibition of ALK phosphorylation \( (R) \):

\[
\frac{dR}{dt} = k_{in} \cdot (1 - \frac{E_{\text{max}} \times C_p^\gamma}{EC_{50}^\gamma + C_p^\gamma}) - k_{out} \cdot R
\]

where \( k_{in} \) is the zero-order formation rate constant (h\(^{-1}\)) and \( k_{out} \) is the first-order degradation rate constant (h\(^{-1}\)).

PKPD modeling for the tumor growth inhibition (TGI) to crizotinib plasma concentration in studies 2 and 3 was performed by a modified indirect response model as previously reported (Yamazaki et al., 2008). Briefly, crizotinib plasma concentration inhibited tumor growth assuming the effect of crizotinib ultimately decreased the tumor growth rate:

\[
\frac{dT}{dt} = k_{tg} \cdot \left( 1 - \frac{T}{T_{ss}} \right) \cdot (1 - \frac{E_{\text{max}} \times C_p^\gamma}{EC_{50}^\gamma + C_p^\gamma}) \cdot T - k_{t\text{d}} \cdot T
\]
where \( T \) is tumor volume, \( k_{tg} \) is the first-order tumor growth rate constant (\( h^{-1} \)), \( k_{td} \) is the first-order tumor death rate constant (\( h^{-1} \)) and \( T_{ss} \) represents the maximum sustainable tumor volume (carrying capacity).

The ratio of \( T/T_{ss} \) approximates zero when \( T \) is relatively small, meaning the net growth rate is roughly first-order (i.e., exponential growth). The tumor growth thereafter approaches zero when the ratios of \( T/T_{ss} \) reaches unity. Thus, the logistic model is applicable when tumor growth starts to slow down at later growth stages of the study period. When the estimate of \( T_{ss} \) was much greater than the observed maximum tumor volume, tumor growth simply followed exponential growth curve in a study period. Therefore, the above TGI model could be simplified to the following differential equation:

\[
\frac{dT}{dt} = k_{tg} \cdot (1 - \frac{E_{max} \cdot C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}) \cdot T - k_{td} \cdot T
\]

In the present study, the logistic model was used in study 2, whereas the exponential growth model was used in study 3, since each respective model provided a better fit to the individual tumor growth curves (data not shown). This difference may simply reflect tumor growth dynamics which differ among xenograft models. Hill coefficients (\( \gamma \)) were fixed to be unity in both studies.

All PKPD modeling analyses were performed with NONMEM version VI and S-Plus 6.2 (Insightful Corporation, Seattle, WA). The NONMEM subroutine ADVAN6 was used for the link model whereas the ADVAN8 was used for the indirect response and TGI models. The initial conditions at time zero for the GI tract compartment, ALK phosphorylation ratio and tumor volume were the dose amount (mg/kg), the ALK
baseline ratio (i.e., unity) and the measured initial individual tumor volume (mm$^3$), respectively. Residual variability was characterized by a proportional error model. In the TGI model, an inter-animal variability on $k_{tg}$ or $k_{td}$ was estimated using an exponential variance model. Model selection was based on a number of criteria such as the NONMEM objective function values (OFV), estimates, standard errors, and scientific plausibility, as well as exploratory analysis of standard goodness-of-fit plots. The difference in the OFV between two nested models was compared with a $\chi^2$ distribution in which a difference of 6.63 was considered significant at the 1% level (Wahlby et al., 2001). The final model parameter estimates were evaluated by running a bootstrap procedure with five thousand datasets (Efron and Tibshirani, 1993). The parametric statistics of the parameters (median, 10$^{th}$ and 90$^{th}$ percentiles) thus generated were compared with the final parameter estimates generated by the NONMEM analysis.

**PKPD Simulation in Patients**

Crizotinib plasma concentrations were first simulated as a function of time in patients following clinically recommended dosage of crizotinib, twice daily doses of 250 mg (500 mg/day), for 14 days, using a one-compartment pharmacokinetic model with $CL/F$ of 70 L/h, $V_d/F$ of 1500 L and $k_a$ of 0.75 h$^{-1}$. These pharmacokinetic parameters were adjusted from the clinically observed single-dose pharmacokinetic parameters to simulate comparable steady-state plasma concentrations to the clinically observed results previously reported (Tan et al., 2010): the differences in steady-state maximum plasma concentrations and area under the plasma concentration-time curves between the simulated (342 ng/mL and 3570 ng·h/mL, respectively) and observed values (368 ng/mL and 3641 ng·h/mL, respectively) were within 10%. Based on the simulated
crizotinib plasma concentrations, the crizotinib-mediated inhibitions of ALK and MET phosphorylation in tumors were simulated using the pharmacodynamic parameters determined in the present study for ALK inhibition and the previous study for MET inhibition (Yamazaki et al., 2008). Since the crizotinib clinical studies were mainly conducted in advanced NSCLC patients who were positive for EML4-ALK fusion (Kwak et al., 2010), the pharmacodynamic parameters estimated from an EML4-ALK positive H3122 NSCLC xenograft model were used for the PKPD simulations in patients. The $EC_{50}$ values from the non-clinical xenograft models were corrected for the difference in plasma protein binding between humans and mice, thus assuming that the unbound $EC_{50}$ values were comparable between patients and xenograft mouse model. The PKPD simulation was performed by NONMEM version VI with the subroutine ADVAN8. The initial condition at time zero for the GI tract compartment was the dose amount (i.e., 250 mg), while those for the ALK and MET phosphorylation were their baselines (i.e., unity).
RESULTS

Crizotinib Pharmacokinetics

Plasma concentration-time courses of crizotinib in both H3122 and Karpas299 xenograft mouse models after repeated oral administration of crizotinib were adequately described by a one-compartment model. The observed and model-fitted plasma concentrations of crizotinib in both xenograft models are shown in Fig. 1. Pharmacokinetic parameter estimates for $k_a$, $CL/F$ and $V_d/F$ were 0.076 to 1.8 h$^{-1}$, 0.76 to 5.3 L/h/kg and 0.52 to 17 L/kg, respectively (Table 1). The $CL/F$ values tended to be higher at the lower doses than at the higher doses, suggesting non-linear pharmacokinetics at the dose range of 25 to 200 mg/kg. The observed dose-dependent pharmacokinetics could be, in part, due to an inhibition of crizotinib hepatic/intestinal clearance at higher doses since crizotinib was reported to be a substrate and inhibitor of CYP3A isozymes (Johnson et al., 2011; Tan et al., 2011). The standard errors of the majority of pharmacokinetic parameter were relatively small (CV<40%). Residual variability was estimated to be 28%, 17% and 8% in the studies 1, 2 and 3, respectively, and $OFVs$ were 682, 566 and 475, respectively. Final parameter estimates (median values) from the bootstrap procedure were 0.078 to 1.8 h$^{-1}$, 0.77 to 5.5 L/h/kg and 0.54 to 17 L/kg for $k_a$, $CL/F$ and $V_d/F$, respectively. Thus, the final parameter estimates (50th percentile) for the bootstrap validation were in good agreement with the estimates of the final pharmacokinetic model (< ±3%).

PKPD Relationships for ALK and TGI

PKPD modeling for ALK inhibition: Crizotinib plasma concentrations slowly declined in both H3122 and Karpas299 xenograft models after reaching the maximum
concentrations at 1 to 4 hours post-dose whereas the inhibition of ALK phosphorylation was sustained throughout most of the dosing interval of 24 hours, especially at higher doses. Thus it was important to incorporate a time-delay (hysteresis) factor between the crizotinib plasma concentrations and the ALK inhibition into PKPD modeling. The observed and link model-fitted ALK phosphorylation-time profiles, along with the predicted crizotinib concentrations in plasma and effect compartment, are graphically presented in Fig. 2. The link model reasonably fit the time-courses of ALK inhibition with \( EC_{50} \) of 233 and 666 ng/mL in studies 1 and 3, respectively (Table 2). The standard errors of \( EC_{50} \), \( k_{e0} \) and \( \gamma \) were, respectively, 66, 42 and 20% of the estimates in study 1 and 12, 30 and 8% in study 3. \( OFV \) values were -153 in the study 1 and -178 in the study 3. In contrast to the link model, an indirect response model did not fit the time-courses of ALK inhibition well in both studies 1 and 3, providing higher \( OFV \) values of -132 and -162, respectively. Final parameter estimates of \( EC_{50} \) from the bootstrap procedure were 233 and 667 ng/mL in studies 1 and 3, respectively, and other final PKPD parameter estimates were also in good agreement with the estimates of the final model (< ±1%).

**PKPD modeling for TGI:** The TGI model fit the individual tumor growth curves well during crizotinib repeated-dose treatment in both H3122 and Karpas299 xenograft models (studies 2 and 3, respectively) (Fig. 3). The \( EC_{50} \) values were estimated to be 255 and 875 ng/mL in studies 2 and 3, respectively (Table 3). \( OFVs \) were 4011 in the study 2 and 2377 in the study 3. Final parameter estimates of \( EC_{50} \) for the bootstrap procedure were 257 and 881 ng/mL in the studies 2 and 3, respectively, and other final parameter estimates of the PKPD parameters were also in good agreement with the estimates of the
final model (< ±1%). The concentration-response curves for ALK and TGI using a sigmoidal $E_{\text{max}}$ model with the estimated pharmacodynamic parameters ($EC_{50}$, $E_{\text{max}}$ and $\gamma$) from H3122 and Karpas299 xenograft mouse models are graphically presented in Fig. 4A and 4B, respectively. The concentration-response curves for MET and TGI in a GTL16 xenograft model from the previous study (Yamazaki et al., 2008) are also shown in Fig. 4C for the comparison. The $EC_{50}$ values for ALK inhibition were comparable to those for TGI in both H3122 and Karpas299 xenograft models (233 vs 255 and 666 vs 875 ng/mL, respectively) (Table 4). On the other hand, the $EC_{50}$ for MET inhibition (19 ng/mL) in a GTL16 xenograft model was approximately 10-fold lower than that for TGI (213 ng/mL), which was roughly comparable to the $EC_{90}$ for MET inhibition (167 ng/mL) (Table 4).

**PKPD Simulation for ALK and MET Inhibition in Patients**

The crizotinib-mediated inhibitions of ALK and MET phosphorylation in tumors were simulated in a population of patients following the recommended clinical dosage of crizotinib, twice daily doses of 250 mg (500 mg/day), for 14 days (Fig. 5). The simulated MET inhibition rapidly reached near-complete inhibition (~95%) whereas the simulated ALK inhibition was approximately 75% at steady-state. The simulated ALK and MET inhibitions in patient tumors were relatively sustained throughout the dosing interval, which seemed to be largely due to the relatively small $k_e$ values (0.030 and 0.14 h$^{-1}$ for ALK and MET, respectively) combined with the twice daily dosing regimen. Overall the simulation results indicated that crizotinib could significantly inhibit the phosphorylation of ALK (>70%) and MET (>90%) in patient tumors during the treatment of recommended clinical dosage of 250 mg twice daily.
DISCUSSION

The present non-clinical PKPD study demonstrated that crizotinib was associated with the inhibition of ALK phosphorylation in H3122 NSCLC and Karpas299 ALCL xenograft mouse models. The estimated in vivo EC\textsubscript{50} in a H3122 NSCLC xenograft model was 19 nM free (Table 4), which was 3-fold lower than in vitro EC\textsubscript{50} of 56 nM (Zou et al., 2011). The EC\textsubscript{50} in a Karpas299 ALCL xenograft model was 53 nM free, which was 1.5-fold higher than in vitro EC\textsubscript{50} of 35 nM (Christensen et al., 2007; Zou et al., 2011). Thus, the estimated EC\textsubscript{50} values for ALK inhibition in these cancer cell lines were relatively consistent between in vitro and in vivo studies. In the previous study using a GTL16 gastric carcinoma xenograft model (Yamazaki et al., 2008), the in vivo EC\textsubscript{50} for MET inhibition was 1.5 nM free, which was 7-fold lower than in vitro EC\textsubscript{50} of 11 nM (Zou et al., 2007). Crizotinib showed relatively high non-specific binding (approximately 90%) in hepatic microsomes and hepatocytes, along with plasma protein binding of 91 to 96% across species (Yamazaki et al., 2011). Therefore, the correction of non-specific binding in the cell-based assay systems might be required to further evaluate the EC\textsubscript{50} difference between in vitro and in vivo. In addition, it has been reported that the expression levels of drug-metabolizing enzymes and transporters could be altered after inoculation of tumor cells into athymic mice, which might cause the discrepancies between in vivo and in vitro activities of antitumor drugs (Sugawara et al., 2010).

Crizotinib is a substrate of CYP3A isozymes and multidrug-resistance transport protein, P-glycoprotein (Johnson et al., 2011). Therefore, the in vivo and in vitro EC\textsubscript{50} difference of crizotinib might be, in part, due to the changes of the expression levels of drug-
metabolizing enzymes and transporters in human tumor xenografts implanted subcutaneously into athymic or immunodeficient mice.

Regarding the PKPD relationships of biomarker inhibition to anti-tumor efficacy, the \( EC_{50} \) for ALK inhibition was consistent with the \( EC_{50} \) for the TGI in both H3122 NSCLC and Karpas299 ALCL xenograft models (19 vs 20 nM free and 53 vs 70 nM free, respectively). This relationship suggests that >50% inhibition of ALK phosphorylation would be required for significant anti-tumor efficacy (>50%). Therefore, the \( EC_{50} \) value for ALK inhibition could be considered as a minimum target efficacious concentration in the clinic. This is, to our knowledge, the first report describing the PKPD relationships of drug concentration to ALK inhibition and TGI. In our previous study (Yamazaki et al., 2008), PKPD relationship of MET inhibition to anti-tumor efficacy in a GTL16 xenograft model was characterized with a similar PKPD modeling approach. Differently from the present study, it was the \( EC_{90} \) (13 nM free) for MET inhibition which was comparable to the \( EC_{50} \) (17 nM free) for TGI (Table 4), suggesting that near-complete inhibition of MET phosphorylation (>90%) would be required for significant anti-tumor efficacy (>50%). A similar crizotinib PKPD relationship with MET inhibition to TGI was also suggested in a U87MG glioblastoma xenograft mouse model (Yamazaki et al., 2008). Therefore, the crizotinib PKPD relationships of target modulation to anti-tumor efficacy in the xenograft models appear to be different between ALK and MET inhibition. These considerations are graphically summarized in Fig. 4. The concentration-response curves for ALK and TGI were similar in magnitude and had comparable \( EC_{50} \) values. In contrast, the concentration-response curve for TGI was shifted to right compared with the MET response curve, resulting in approximately 10-fold difference in the \( EC_{50} \) values.
between MET inhibition and TGI. The difference in these response curves between ALK-TGI and MET-TGI in the non-clinical xenograft models appears to suggest that targeting ALK may be more effective than MET to achieve similar levels of anti-tumor efficacy in cancer patients. Such an extrapolation, of course, assumes similar PKPD relationships from non-clinical xenograft models to patients. Although xenograft mouse models are extensively used as the most common non-clinical anti-tumor efficacy model (Kelland, 2004; Burchill, 2006; Hollingshead, 2008), there are obviously several assumptions associated with such an extrapolation of PKPD relationship, which should be addressed.

One of the main assumptions is that the tumor environment in subcutaneous tumor xenograft models is similar to that in human tumors, which also presumes a similar drug distribution between xenograft mouse models and human tumors. In other words, the plasma concentrations of crizotinib (and other inhibitors) required for biomarker response (or target modulation) and anti-tumor efficacy is assumed to be equivalent between the xenograft models and patient tumors. In this context, we assumed that unbound plasma concentrations of crizotinib were efficaciously equivalent between xenograft mouse models and patients. Therefore, in addition to accounting for species-related difference in pharmacokinetics, only the difference in crizotinib plasma protein binding between mice and humans was accounted for in the simulation of crizotinib-mediated ALK and MET inhibition in patients, (Fig. 5). Crizotinib extensively distributed into tumors of the xenograft mouse models with an approximate tumor/plasma area under the concentration-time curve ratio of 4 at steady-state (in-house data), despite its being a substrate of P-glycoprotein. This observation seems to be consistent with the
significant biomarker inhibition and TGI observed in the xenograft models. Marked differences in tumor growth rate between xenograft models and patients may also have a significant impact on an evaluation of anti-tumor efficacy (Komlodi-Pasztor et al., 2011). Up to this point, the promising clinical responses by crizotinib as a single agent has been reported in EML4-ALK-positive NSCLC patients (Kwak et al., 2010): an overall response rate of 57% (confirmed partial and complete responses) and a rate of stable disease of 33% (stable disease plus unconfirmed partial responses) in 85 patients, the majority of whom had received multiple previous therapies. These clinical responses of crizotinib in the ALK-positive NSCLC patients appear to derive from the crizotinib ALK inhibition activity, since tumors from 33 patients with available tissues were negative for MET amplification, (Kwak et al., 2010). These clinical results therefore appear to be consistent with the present PKPD simulation in patients, where ALK inhibition by crizotinib at steady-state was greater than 70% (Fig. 5). We could also expect that crizotinib would show promising clinical responses in MET-positive patients since the simulated MET inhibition by crizotinib at steady-state was greater than 90% in patients (Fig. 5). A recent report indicated that an NSCLC patient with MET amplification, but no ALK rearrangement, achieved a rapid and durable response to crizotinib (Ou et al., 2011); however, extensive clinical results have not been reported yet.

Furthermore, the comparison between ALK and MET response curves in Fig. 4 might hint that a combination approach of a MET inhibitor with another tyrosine kinase inhibitor, e.g., EGFR inhibitor, would be a promising therapeutic strategy for cancer patients, although several assumptions would be required for the extrapolation of the PKPD relationships, as mentioned above. Recent emerging data (e.g., systems
biology/pharmacology) support the view of an extensive and intricate signaling cross-talk and scaffold networks within cancer cells during tumorigenesis and tumor progression (Rikova et al., 2007; Guo et al., 2008). These networks can also possibly undergo adaptive changes during long-term therapy where sustained inhibition is maintained such as an acquired resistance against EGFR inhibitor due to MET amplification (Engelman et al., 2007; McDermott et al., 2010). This suggests a multi-targeted approach may be better for certain cancer therapies including overcoming acquired treatment resistance. Interestingly, some clinical MET inhibitor candidates are considered to be highly selective while others are multi-targeted inhibitors (Faoro et al., 2009; Tirgan et al., 2009). It remains to be seen whether highly selective MET inhibitors or multi-targeted inhibitors (or a combination of selective MET inhibitor with other tyrosine kinase inhibitor) will provide a better success in the clinic.

In conclusion, the PKPD relationships among crizotinib systemic concentration, ALK or MET inhibition, and TGI in human tumor xenograft models were well characterized in a quantitative manner using PKPD modeling (Fig. 6). The present modeling efforts suggests that >50% ALK inhibition would be required for significant anti-tumor efficacy (>50%). Accordingly, we proposed that the $EC_{50}$ value for ALK inhibition could be considered a minimum target efficacious concentration in the clinic. PKPD simulation based on the results from a NSCLC xenograft model suggests that crizotinib could inhibit ALK phosphorylation by greater than 70% in cancer patients following clinically recommended dosing regimen (250 mg twice a day), which appears to be consistent with the reported clinical response results (Kwak et al., 2010). Overall the present PKPD results will be helpful in understanding PKPD relationships of
crizotinib and also in guiding dose escalation or de-escalation to achieve or maintain efficacious exposure to crizotinib in cancer patients. It would be interesting to compare clinical biomarker responses between ALK and MET in crizotinib-treated patients, which has not been reported to date.
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AUTHORSHIP CONTRIBUTION

Participated in research design: Christensen, Yamazaki and Zou

Conduct experiments: Lee, Li, Shen and Zou

Contributed new reagents or analytic tools: Lee, Li, Shen and Zou

Performed data analysis: Vicini and Yamazaki

Wrote or contribute to the writing of the manuscript: Christensen, Shen, Shetty, Smith, Vicini and Yamazaki
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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Observed and model-fitted plasma concentrations of crizotinib in H3122 NSCLC or Karpas299 ALCL xenograft mouse models following repeated oral administration. The x-axis represents the time after dosing in hours and the y-axis represents the observed crizotinib plasma concentrations (OBS) with the model-fitted typical profiles (PRED) in nanograms per milliliter on a logarithmic scale in studies 1, 2 and 3 (S1, S2 and S3, respectively).

Fig. 2. Observed and model-fitted ALK inhibition by crizotinib in H3122 NSCLC or Karpas299 ALCL xenograft mouse models following repeated oral administration. The x-axis represents the time after dosing in hours, the left side of the y-axis represents the model-fitted crizotinib concentrations in plasma (CP PRED) and the effect compartment (CE PRED) in nanograms per milliliter on a logarithmic scale and the right side of y-axis represents the observed (ALK OBS) and model-fitted (ALK PRED) ALK responses in the ratio to the mean value of control animal data in studies 1 and 3 (S1 and S3, respectively).

Fig. 3. Observed and model-fitted tumor growth inhibition curves by crizotinib in H3122 NSCLC or Karpas299 ALCL xenograft mouse models during repeated oral administration. The x-axis represents the treatment period in days and the y-axis represents the observed individual tumor volumes (OBS) with the model-fitted individual tumor growth curves (IPRE) in cubic millimeters in studies 2 and 3 (S2 and S3, respectively).
Fig. 4. Comparison of crizotinib concentration-response curves for biomarkers and tumor growth inhibition in ALK- and MET-driven human tumor xenograft mouse models. Concentration-response curves for ALK, MET and TGI were simulated at the concentration range of 1 to 1000 ng/mL with sigmoidal $E_{\text{max}}$ model using the pharmacodynamic parameters ($EC_{50}$, $E_{\text{max}}$ and $\gamma$) estimated from the mouse xenograft models of H3122 non-small cell lung cancer cells (A), Karpas299 anaplastic large cell lymphoma (B), and GTL16 gastric carcinoma (C). The x-axis represents the plasma concentrations of crizotinib in nanograms per milliliter on a logarithmic scale and the y-axis represents the simulated responses for ALK, MET and TGI in the baseline ratio. Results for MET and TGI are cited from the previous report (Yamazaki et al., 2008).

Fig. 5. Simulated MET and ALK phosphorylation in tumors of cancer patients during 14-day repeated oral administration of crizotinib. The x-axis represents the treatment period in days, the left side of the y-axis represents the model-simulated crizotinib plasma concentrations (Cp) in nanograms per milliliter on a logarithmic scale and the right side of y-axis represents the model-simulated ALK and MET responses in the baseline ratio.

Fig. 6. PKPD modeling summary of ALK and MET inhibition and anti-tumor efficacy by crizotinib in human tumor xenograft models.
### TABLE 1

Pharmacokinetic parameter estimates of crizotinib in H3122 NSCLC or Karpas299 ALCL xenograft mouse models following once daily oral administration

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose mg/kg</th>
<th>$k_a$ $h^{-1}$</th>
<th>CL/F $L/h/kg$</th>
<th>V$_d$/F $L/kg$</th>
<th>OFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>0.25 (0.01)</td>
<td>4.4 (0.7)</td>
<td>3.1 (1.3)</td>
<td>682</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.11 (0.02)</td>
<td>2.6 (0.3)</td>
<td>5.2 (3.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.096 (0.013)</td>
<td>1.9 (0.2)</td>
<td>1.0 (0.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.8 (0.7)</td>
<td>1.9 (0.3)</td>
<td>17 (4)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0.33 (0.01)</td>
<td>5.3 (1.0)</td>
<td>2.2 (0.7)</td>
<td>566</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.29 (0.01)</td>
<td>3.8 (0.3)</td>
<td>1.7 (0.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.17 (0.01)</td>
<td>3.8 (0.3)</td>
<td>1.9 (0.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.094 (0.012)</td>
<td>3.5 (0.3)</td>
<td>1.7 (0.4)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>0.22 (0.03)</td>
<td>1.0 (0.1)</td>
<td>2.4 (0.8)</td>
<td>475</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.21 (0.02)</td>
<td>1.1 (0.1)</td>
<td>1.6 (0.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.076 (0.013)</td>
<td>0.76 (0.05)</td>
<td>0.52 (0.19)</td>
<td></td>
</tr>
</tbody>
</table>

Precision of the estimates is expressed as S.E. in parentheses.
**TABLE 2**

Pharmacodynamic parameter estimates of crizotinib for ALK phosphorylation in H3122 NSCLC or Karpas299 ALCL xenograft mouse models following once daily oral administration

<table>
<thead>
<tr>
<th>Study</th>
<th>EC50 (ng/mL)</th>
<th>E0</th>
<th>Emax</th>
<th>ke0 (h^-1)</th>
<th>γ</th>
<th>OFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>233</td>
<td>1</td>
<td>1</td>
<td>0.030</td>
<td>0.56</td>
<td>-153</td>
</tr>
<tr>
<td></td>
<td>(153)</td>
<td>(fixed)</td>
<td>(fixed)</td>
<td>(0.013)</td>
<td>(0.11)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>666</td>
<td>1</td>
<td>1</td>
<td>0.055</td>
<td>1.8</td>
<td>-178</td>
</tr>
<tr>
<td></td>
<td>(83)</td>
<td>(fixed)</td>
<td>(fixed)</td>
<td>(0.017)</td>
<td>(0.2)</td>
<td></td>
</tr>
</tbody>
</table>

Precision of the estimates is expressed as S.E. in parentheses.
TABLE 3
Pharmacodynamic parameter estimates of crizotinib for tumor growth inhibition in H3122 NSCLC or Karpas299 ALCL xenograft mouse models following once daily oral administration

<table>
<thead>
<tr>
<th>Study</th>
<th>EC50 (ng/mL)</th>
<th>E_max</th>
<th>k_{lg} (h^{-1})</th>
<th>k_{id} (h^{-1})</th>
<th>T_{ss}</th>
<th>OFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>255</td>
<td>1</td>
<td>0.0126</td>
<td>0.00115</td>
<td>1410</td>
<td>4011</td>
</tr>
<tr>
<td></td>
<td>(22) (fixed)</td>
<td></td>
<td>(0.0008)</td>
<td>(0.000003)</td>
<td>(155)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>875</td>
<td>1</td>
<td>0.0087</td>
<td>0.00016</td>
<td>-</td>
<td>2377</td>
</tr>
<tr>
<td></td>
<td>(198) (fixed)</td>
<td></td>
<td>(0.0002)</td>
<td>(0.00010)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Precision of the estimates is expressed as S.E. in parentheses.

--, not available.
TABLE 4
Summary of pharmacodynamic parameter estimates for biomarker inhibition and anti-tumor efficacy by crizotinib in ALK- and MET-driven human tumor xenograft mouse models

<table>
<thead>
<tr>
<th>Xenograft Model</th>
<th>PD Parameter</th>
<th>EC_{50} \text{ ng/mL total}</th>
<th>nM \text{ free}</th>
<th>EC_{90} \text{ ng/mL total}</th>
<th>nM \text{ free}</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3122 ALK</td>
<td></td>
<td>233</td>
<td>19</td>
<td>2097</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>TGI</td>
<td>255</td>
<td>20</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Karpas299 ALK</td>
<td></td>
<td>666</td>
<td>53</td>
<td>2198</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>TGI</td>
<td>875</td>
<td>70</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GTL16 MET</td>
<td></td>
<td>19</td>
<td>1.5</td>
<td>167</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>TGI</td>
<td>213</td>
<td>17</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Pharmacodynamic parameters were obtained from mouse xenograft models with H3122 NSCLC cells or Karpas299 ALCI cells in the present study and GTL16 gastric carcinoma cells in a previously reported study (Yamazaki et al., 2008).

–, not calculated.
Figure 1

The figure illustrates the plasma concentration (ng/mL) over time (h) following dosing at different concentrations for S1, S2, and S3, with lines representing observed (OBS) and predicted (PRED) values.
Figure 2

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Figure 2: Concentration-time profiles of ALK in different dosing groups. The graphs show the concentration (ng/mL) on the y-axis and time after dosing (h) on the x-axis. Different colored lines and markers represent different predicted and observed concentrations for each dose level (S1: 25 mg/kg, S1: 50 mg/kg, S1: 100 mg/kg, S3: 25 mg/kg, S3: 50 mg/kg, S3: 100 mg/kg). The y-axis is a logarithmic scale for concentration.
Figure 3
Figure 4B

Response (ratio)

Plasma concentration (ng/mL)

10^0
10^1
10^2
10^3
10^4

0.0
0.5
1.0

ALK
TGI

B
Figure 4C

Plasma concentration (ng/mL) vs. Response (ratio) for MET and TGI.
Figure 5
**Pharmacokinetics**

ALK & MET Dual inhibitor
Crizotinib (PF02341066)

**Pharmacodynamics**

ALK & MET Dual inhibitor
Crizotinib (PF02341066)

Dose (PO)

ALK or MET-Driven
Human Tumor
Xenograft Models

PK in Plasma

PK Simulation in Tumor

ALK Inhibition

Tumor Growth Inhibition

Biomarker (E)

Biomarker (E)

Biomarker (E)

Biomarker (E)

Tumor (T)

Tumor (T)

Tumor (T)

Tumor (T)