Translational Pharmacokinetic-Pharmacodynamic Modeling for an Orally Available Novel Inhibitor of Anaplastic Lymphoma Kinase and c-Ros Oncogene 1

Shinji Yamazaki, Justine L. Lam, Helen Y. Zou, Hui Wang, Tod Smeal, and Paolo Vicini

Pharmacokinetics, Dynamics and Metabolism (S.Y., J.L.L., P.V.) and Oncology Research Unit (H.Y.Z., H.W., T.S.), Pfizer Worldwide Research & Development, San Diego, California

Received May 30, 2014; accepted July 25, 2014

ABSTRACT
An orally available macrocyclic small molecule, PF06463922 ([10R]-7-amino-12-fluoro-2,10,16-trimethyl-15-oxo-10,15,16,17-tetrahydro-2H-8,4-(metheno)pyrazolo[4,3-h][2,5,1]benzoxadiazacyclotetradecine-3-carbonitrile), is a selective inhibitor of anaplastic lymphoma kinase (ALK) and c-Ros oncogene 1 (ROS1). The objectives of the present study were to characterize the pharmacokinetic-pharmacodynamic relationships of PF06463922 between its systemic exposures, pharmacodynamic biomarker (target modulation), and pharmacologic response (antitumor efficacy) in athymic mice implanted with H3122 non–small cell lung carcinomas expressing echinoderm microtubule-associated protein-like 4 (EML4)-ALK mutation (EML4-ALK L1196M) and with NIH3T3 cells expressing CD74-ROS1. In these nonclinical tumor models, PF06463922 was orally administered to animals with EML4-ALK L1196M and CD74-ROS1 at twice daily doses of 0.3–20 and 0.01–3 mg/kg per dose, respectively. Plasma concentration-time profiles of PF06463922 were adequately described by a one-compartment pharmacokinetic model. Using the model-simulated plasma concentrations, a pharmacodynamic indirect response model with a modulator sufficiently fit the time courses of target modulation (i.e., ALK phosphorylation) in tumors of EML4-ALK L1196M–driven models with EC50, in vivo of 36 nM free. A drug-disease model based on an indirect response model reasonably fit individual tumor growth curves in both EML4-ALK L1196M and CD74-ROS1–driven models with the estimated tumor stasis concentrations of 51 and 6.2 nM free, respectively. Thus, the EC50, in vivo (52 nM free) for ALK inhibition roughly corresponded to the tumor stasis concentration in an EML4-ALK L1196M–driven model, suggesting that 60% ALK inhibition would be required for tumor stasis. Accordingly, we proposed that the EC50, in vivo for ALK inhibition corresponding to the tumor stasis could be considered a minimum target efficacious concentration of PF06463922 for cancer patients in a phase I trial.

Introduction
Lung cancer has long been one of the most common and lethal malignancies worldwide, with 1.8 million new cases and 1.6 million deaths in 2012, representing 13% of new cancers and 19% of cancer mortality, as estimated by the International Agency for Research on Cancer of the World Health Organization, GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012 (http://globoCAN.iarc.fr/Default.aspx). Most lung cancers (~90%) are non–small cell lung cancers (NSCLCs), which consist of a number of subtypes driven by various activated oncogenes (Ettinger et al., 2010; Larsen et al., 2011). Recent advances in molecular profiling technologies have significantly enhanced the development of personalized, molecularly targeted therapies based on individual genetic or protein profiles (Meric-Bernstam et al., 2013; Arnedos et al., 2014). Consequently, molecularly targeted agents for NSCLC patients have become one of the successful personalized cancer therapies (Moreira and Thornton, 2012; Cardarella and Johnson, 2013; Li et al., 2013). For example, the identification of activating epidermal growth factor receptor (EGFR) mutation in NSCLC patients led to personalized cancer therapy with the first-generation small molecule inhibitors gefitinib and erlotinib. Unfortunately, clinical responses to these EGFR inhibitors have not been durable in most cancer patients, as tumor cells can readily acquire drug resistance by multiple mechanisms, such as the secondary mutations in EGFR (e.g., T790M), the amplification of mesenchymal-epithelial transition factor (MET), and the increased activation of the receptor tyrosine kinase AXL (Chong and Janne, 2013; Remon et al., 2014). Accordingly, the identification of these drug resistance mechanisms led to the development of second-generation inhibitors (Robinson and Sandler, 2013; Yu and Riely, 2013). These principles and practices on personalized cancer therapy have significantly influenced the accelerated approval of the first-generation anaplastic lymphoma

ABBREVIATIONS: ALK, anaplastic lymphoma kinase; CL/F, oral clearance; PF06463922, (10R)-7-amino-12-fluoro-2,10,16-trimethyl-15-oxo-10,15,16,17-tetrahydro-2H-8,4-(metheno)pyrazolo[4,3-h][2,5,1]benzoxadiazacyclotetradecine-3-carbonitrile; EGFR, epidermal growth factor receptor; EML4, echinoderm microtubule-associated protein-like 4; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MET, mesenchymal-epithelial transition factor; NSCLC, non–small cell lung cancer; OFV, objective function value; PKPD, pharmacokinetic-pharmacodynamic; ROS1, c-Ros oncogene 1; V/F, oral volume of distribution.
kinase (ALK) inhibitor, crizotinib (Xalkori; PF02341066; Pfizer, New York, NY), by the Food and Drug Administration (FDA) (Gerber and Minna, 2010; Ou, 2012). The FDA’s approval of crizotinib in 2011 was less than 4 years after ALK rearrangements [e.g., echinoderm microtubule-associated protein-like 4 (EML4)-ALK] in NSCLC patients were first reported (Rikova et al., 2007; Soda et al., 2007). As an example of personalized cancer medicine, the labeling language states that “Xalkori is a kinase inhibitor indicated for the treatment of patients with locally advanced or metastatic NSCLC that is ALK-positive as detected by an FDA-approved test” (http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/202570s000lbl.pdf). Unfortunately, as seen with other target therapies, such as the first-generation EGFR inhibitors, resistance to crizotinib attributable to EML4-ALK mutations has been reported even before its approval, leading to the rapid development of second-generation ALK inhibitors for crizotinib-resistant NSCLC patients (Choi et al., 2010; Casaluce et al., 2013; Gridelli et al., 2014).

PF06463922 ((10R)-7-amino-12-fluoro-2,10,16-trimethyl-15-oxo-10,15,16,17-tetrahydro-2H,8,4-(metheno)pyrazolo[4,3-h][2,5,11]benzoxadiazacyclotetradecine-3-carbonitrile) has been identified as an orally available ATP-competitive selective inhibitor of ALK (including mutations) and c-Ros oncogene (ROS1) (Johnson et al., 2014). PF06463922 is highly potent against ALK phosphorylation in the cell-based assay (H3122 NSCLC cells expressing the EML4-ALK fusion protein) with an IC50 of ∼2 nM against the wild-type EML4-ALK (without ALK mutations) and ∼20 nM against one of the most frequently detected crizotinib-resistant EML4-ALK mutations, EML4-ALKL1196M. Thus, PF06463922 is ∼10-fold potent against the wild-type EML4-ALK relative to its most frequently detected mutation (EML4-ALKL1196M) and ∼40-fold potent against EML4-ALKL1196M compared with crizotinib, with an approximate IC50 of ∼800 nM. Moreover, PF06463922 showed significant in vivo inhibition of ALK phosphorylation and antitumor efficacy in an H3122 NSCLC xenograft model with EML4-ALKL1196M, whereas crizotinib failed to exhibit a significant antitumor efficacy in the xenograft model at twice-daily oral doses of 75 mg/kg per dose, which yielded unbound plasma concentrations higher in mice than in patients at clinically recommended twice daily oral doses of 250 mg (Zou et al., manuscript in preparation).

We previously reported the pharmacokinetic-pharmacodynamic (PKPD) modeling of crizotinib to target modulation and antitumor efficacy driven by the inhibition of ALK (wild-type) and MET phosphorylation in nonclinical tumor models (Yamazaki et al., 2008, 2012; Yamazaki, 2013). PKPD modeling is a powerful mathematical approach linking drug exposures to pharmacodynamic biomarkers and/or pharmacologic responses as a function of time, providing a quantitative assessment of in vivo drug potency together with mechanistic insights in drug action (Derendorf et al., 2000; Chien et al., 2005). The objectives of the present study were to characterize PKPD relationships of plasma concentrations of PF06463922 to inhibition of ALK phosphorylation in tumors (target modulation) and antitumor efficacy (pharmacologic response) in athymic mice implanted with H3122 NSCLC cells expressing the EML4-ALKL1196M and together with the PKPD relationship to antitumor efficacy in athymic mice implanted with NIH3T3 cells expressing CD74-ROS1. The present PKPD results will be helpful in understanding the PKPD relationships of PF06463922 and also in guiding dose escalation or de-escalation to maintain efficacious exposure in the clinic.

### Materials and Methods

#### Chemicals

PF06463922 (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) (Johnson et al., 2014). 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 (Zou et al., 2013; Zou et al., manuscript in preparation). Briefly, three separate repeated oral-dose PKPD studies were conducted with PF06463922 in female athymic nu/nu mice implanted subcutaneously with H3122 NSCLC cells expressing the EML4-ALKL1196M (studies 1 and 2) or NIH3T3 cells expressing CD74-ROS1 (study 3). These nonclonal tumor models with H3122 NSCLC-EML4-ALKL1196M and NIH3T3-CD74-ROS1 are henceforth referred to as ALK- and ROS1-tumor models, respectively. PF06463922 was orally administered to animals twice daily, 7 hours apart, at doses of 0.3, 1, 3, and 10 mg/kg per dose for 4 days (study 1); 0.5, 1, 3, 10, and 20 mg/kg per dose for 13 days (study 2); and 0.01, 0.03, 0.1, 0.3, 1, and 3 mg/kg per dose for 9 days (study 3). On the last dosing day, a subset of mice was humanely euthanized at 1, 3, 7, 8, 24, and 36 hours after the first daily dose in study 1; at 1, 3, 7, 8, 24 hours after the first daily dose in study 2; and at 1, 3, 7, and 24 hours after the first daily dose in study 3. Blood samples (n = 3/time point) were collected by exsanguinations via cardiac puncture to determine plasma concentrations of PF06463922. Resected tumors (n = 3/time point) were snap-frozen and pulverized using liquid nitrogen–cooled cryomortar. Protein lysates were generated, and the level of total phosphorylated ALK protein (ALK phosphorylation) was determined using a capture enzyme-linked immunosorbent assay method (studies 1 and 2). Tumor volume was measured during the treatment period by electronic Vernier calipers and calculated as the product of its length × width2 × 0.4 (studies 2 and 3). Tumor growth inhibition in each treatment group of PF06463922 was calculated as 100 × (1 – [ΔT/Cu]), where ΔT and Cu are the differences in the median tumor volumes between the first and last dosing days in the treatment and vehicle control groups, respectively. Tumor regression was calculated as 100 × (ΔT/T_initial), where T_initial is the median tumor volume on the first dosing day. All 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.

#### In Vivo Protein Binding

The free fraction of PF06463922 was determined in mouse plasma at 2 μM (0.8 μg/ml) using the equilibrium dialysis technique as described previously (Yamazaki et al., 2008). Briefly, the study was conducted in a 96-well Teflon dialysis chamber (HTDialysis LLC, Gales Ferry, CT) using a semipermeable membrane (SpectraPor; Spectrum, Laguna Hills, CA) with a 12,000–14,000 Da molecular mass cutoff. After incubation at 37°C for 6 hours, aliquots of plasma and buffer samples were extracted with aliquots of acetonitrile:methanol mixture (1:1, v/v) containing the internal standard and analyzed by a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method as described subsequently herein. The free fraction in plasma (f0) was calculated by eq. 1:

$$f_0 = C_{\text{buffer}} / C_{\text{plasma}}.$$  

where $C_{\text{buffer}}$ and $C_{\text{plasma}}$ denote the concentrations of PF06463922 in buffer and plasma, respectively, after the incubation.
PF06463922 Analysis

Plasma concentrations of PF06463922 were determined by an LC-MS/MS method after protein precipitation of plasma samples. The LC-MS/MS system consisted of Waters Acquity UPLC system (Waters, Milford, MA) and an API 5500 triple-stage quadrupole mass spectrometer (Applied Biosystems, Grand Island, NY). Both instruments were controlled by Analyst 1.5.2 software (Applied Biosystems). Chromatographic separation of the analytes was achieved using a reverse-phase column (Phenomenex Kinetex phenyl-hexyl, 50 × 2 mm, 1.7 μm; Torrance, CA) at a flow rate of 0.5 ml/min. A binary mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient started at 5% B for 0.2 minutes, increased to 95% B over 1.3 minutes, and then held at 95% B for 0.5 minutes. The gradient was returned to the initial condition of 5% B in 0.1 minutes and equilibrated at 5% B for 0.5 minutes before the next injection. The mass spectrometer was operated in the positive ionization mode using multiple-reaction monitoring at specific precursor ion—product ion transition, m/z 407.3→228.0 for PF06463922 and m/z 472.3→432.6 for the internal standard. The standard calibration curve was constructed using weighted (1/x^2) linear regression. The calibration curve range was 0.5 to 5000 ng/ml. The back-calculated calibration standard concentrations were within 15% of their theoretical concentrations, with coefficients of variation of less than 15%. The precision and accuracy of the quality control samples were within 15%.

Pharmacokinetic Analysis

A naive-pooled pharmacokinetic analysis was used to determine pharmacokinetic parameters of PF06463922 in mice. That is, all individual data at each dose were pooled together for pharmacokinetic analysis as if they came from a single individual since plasma concentrations of PF06463922 were available from only a subset of mice (n = 3/time point) (Sheiner, 1984). Pharmacokinetic analysis was performed with a standard one-compartment model as implemented in NONMEM version 7.1.2 (University of California at San Francisco, San Francisco, CA) (Beal and Sheiner, 1992). This model (subroutine ADVAN2 with TRANS2) was parameterized using a reverse-phase column (Phenomenex Kinetex phenyl-hexyl, 50 × 2 mm, 1.7 μm; Torrance, CA) at a flow rate of 0.5 ml/min. A binary mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient started at 5% B for 0.2 minutes, increased to 95% B over 1.3 minutes, and then held at 95% B for 0.5 minutes. The gradient was returned to the initial condition of 5% B in 0.1 minutes and equilibrated at 5% B for 0.5 minutes before the next injection. The mass spectrometer was operated in the positive ionization mode using multiple-reaction monitoring at specific precursor ion—product ion transition, m/z 407.3→228.0 for PF06463922 and m/z 472.3→432.6 for the internal standard. The standard calibration curve was constructed using weighted (1/x^2) linear regression. The calibration curve range was 0.5 to 5000 ng/ml. The back-calculated calibration standard concentrations were within 15% of their theoretical concentrations, with coefficients of variation of less than 15%. The precision and accuracy of the quality control samples were within 15%.

PKPD Modeling

Target Modulation. The response of ALK phosphorylation in tumor (expressed as the ratio to vehicle control animal data) to plasma concentrations of PF06463922 was first modeled by an indirect response model. The indirect response model assumed that ALK phosphorylation at baseline was maintained by the balance of formation and degradation rates (Dayneka et al., 1993; Jusko and Ko, 1994). The addition of PF06463922 was considered to inhibit the formation rate, since PF06463922 was a competitive ATP-binding ALK inhibitor. Therefore, the following differential equation (eq. 2) was used to determine the EC50 required for the inhibition of ALK phosphorylation (R):

$$\frac{dR}{dt} = k_{in} \left( 1 - \frac{E_{\text{max}} \times C_p}{EC_{50} + C_p} \right) - k_{out} \cdot R$$

(2)

where $k_{in}$ is the zero-order formation rate constant (h^-1), $E_{\text{max}}$ is maximum effect, $C_p$ is the plasma concentration of PF06463922 (ng/ml), $EC_{50}$ is the plasma concentration of PF06463922 (ng/ml) causing one-half $E_{\text{max}}$, $h$ is the Hill coefficient, and $k_{out}$ is the first-order degradation rate constant (h^-1) of ALK phosphorylation.

Since a rebound of ALK phosphorylation was observed at 24 to 36 hours postdose (i.e., the ALK phosphorylation ratio of greater than unity in the treatment groups relative to the control group), a modulator was incorporated into the indirect response model as a precursor to take account of the observed rebound phenomena, as has been reported previously (Sharma et al., 1998). The precursor PKPD model assumed that a modulator ($M$) was synthesized at a zero-order rate ($k_{in}$) and degraded at a first-order rate ($k_{out}$), and an ALK phosphorylation level was maintained by the balance of the first-order formation rate provided by the modulator degradation rate (i.e., $k_{out}$) and the ALK degradation rate ($k_{out}$). PF06463922 was considered to inhibit the ALK formation rate (i.e., $k_{out}$). Accordingly, the following differential equations (eqs. 3 and 4) were used to estimate EC50 required for PF06463922-mediated ALK inhibition:

$$\frac{dM}{dt} = k_{in} - k_{out} \cdot \left( 1 - \frac{E_{\text{max}} \times C_p}{EC_{50} + C_p} \right) \cdot M$$

(3)

$$\frac{dR}{dt} = k_{out} \cdot \left( 1 - \frac{E_{\text{max}} \times C_p}{EC_{50} + C_p} \right) \cdot M - k_{out} \cdot R$$

(4)

Antitumor Efficacy. Drug-disease modeling for antitumor efficacy to plasma concentration of PF06463922 was performed based on absorption rate constant ($k_{in}$, h^-1), oral clearance (CL/F, l/h per kilogram), and oral volume of distribution (V/F, l/kg), that were separately determined in each study. Residual variability was characterized by a proportional error model.

TABLE 1
Pharmacokinetic parameter estimates of PF06463922 in nonclinical tumor models after twice daily oral administration.

<table>
<thead>
<tr>
<th>Study</th>
<th>Dose (mg/kg)</th>
<th>CL/F (l/h per kg)</th>
<th>V/F (l/kg)</th>
<th>$k_{in}$ (h^-1)</th>
<th>OFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3–10</td>
<td>1.2 (0.1)</td>
<td>5.3 (0.8)</td>
<td>2.0 (0.6)</td>
<td>543</td>
</tr>
<tr>
<td>2</td>
<td>0.3–20</td>
<td>1.1 (0.2)</td>
<td>7.0 (0.9)</td>
<td>1.3 (0.1)</td>
<td>802</td>
</tr>
<tr>
<td>3</td>
<td>0.01–1</td>
<td>1.7 (0.1)</td>
<td>11 (1)</td>
<td>4.0 (2.2)</td>
<td>275</td>
</tr>
</tbody>
</table>

Fig. 1. Observed and model-fitted plasma concentrations of PF06463922 in athymic mice implanted with H3122 NSCLC cells expressing the EML4-ALKL1196M after repeated oral administration. PF06463922 was orally administered to animals (n = 3 per time point) at doses of 0.3–10 mg/kg per dose twice daily, 7 hours apart, for 4 days. The x-axis represents the time after dosing in hours, and the y-axis represents the observed plasma concentrations of PF06463922 (OBS) with the model-fitted individual (IPRED) and typical (PRD) profiles in nanograms per milliliter on a logarithmic scale in study 1.
a modified indirect response model (Yamazaki et al., 2008, 2011; Wonget al., 2009). In our approach, a tumor growth model was first established to characterize tumor growth curves in the vehicle control group. Typical tumor growth curves in nonclinical tumor models are known to follow an exponential growth in the early phases followed by a linear growth, and then eventually reach a plateau phase (Bissery et al., 1996; Bernard et al., 2012). Accordingly, the individual tumor growth curves in the vehicle control group were first modeled by using a first-order growth rate with and without a logistic function that constrains the maximum tumor volume. An exponential tumor growth model without a logistic function is defined as in eq. 5:

\[
\frac{dT}{dt} = k_{bg} \cdot T,
\]

where \(T\) is tumor volume (mm\(^3\)) and \(k_{bg}\) is the first-order net growth rate constant (h\(^{-1}\)).

In contrast to the exponential tumor growth model, the tumor growth model with a logistic function (logistic tumor growth model) is defined as in eq. 6:

\[
\frac{dT}{dt} = k_{bg} \cdot T \cdot (1 - T/T_{\text{ss}}),
\]

where \(T_{\text{ss}}\) represents the maximum sustainable tumor volume (mm\(^3\)), which is assumed to be constant whereas \(T\) changes over time.

When \(T\) is relatively small in the early stage of tumor growth, the net tumor growth rate is roughly first-order (i.e., exponential growth) since the ratio of \(T/T_{\text{ss}}\) approximates zero. Thereafter, the net tumor growth rate approaches zero when the \(T/T_{\text{ss}}\) ratio becomes unity. Thus, the logistic model is applicable if tumor growth starts to slow down in the later stage of tumor growth. In the present study, the logistic model was used in study 2 as the basic tumor growth model 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). The difference in tumor growth function between these studies could simply reflect tumor growth dynamics which might differ among nonclinical tumor models and individual studies.

Subsequently, the response of tumor volume (\(T\)) to plasma concentration of PF06463922 (\(C_p\)) was modeled based on the assumption that PF06463922 ultimately stimulated the tumor killing rate, thus inhibiting tumor growth rate characterized by either the exponential or logistic tumor growth model (eq. 7):

\[
\frac{dT}{dt} = g(T) - \left( \frac{K_{\text{max}} \times C_p}{K_{\text{C50}^*} + C_p} \right) \cdot T.
\]

where \(g(T)\) is tumor growth function, \(K_{\text{max}}\) is the maximal tumor killing rate (h\(^{-1}\)) caused by PF06463922, and \(K_{\text{C50}^*}\) is the plasma concentration of PF06463922 (nanograms per milliliter) causing one-half \(K_{\text{max}}\).

Tumor stasis concentration (\(T_{\text{st}}\), defined as the plasma concentration of PF06463922 required to maintain tumor burden constant at steady-state (i.e., 100% tumor growth inhibition meaning zero net tumor growth rate), was calculated using eq. 7 with the obtained pharmacodynamic parameter estimates, assuming zero tumor growth rate (i.e., \(dT/dt = 0\) as \(C_p = T_{\text{st}}\)).

Data Analysis. All PKPD modeling analyses were performed with NONMEM version 7.1.2 with the subroutine ADVAN. The initial conditions at time zero for the gastrointestinal tract compartment, ALK phosphorylation ratio, and tumor volume were the dose amount (milligrams per kilogram), 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 drug-disease model, interanimal variability on \(k_{bg}\) was estimated by mixed-effect modeling using an exponential variance model, and Hill coefficients (\(\gamma\)) were fixed to be unity. Model selection was based on a number of criteria, such as the NONMEM objective function values (OFVs), estimates, standard errors, and scientific plausibility, as well as exploratory analysis of standard goodness-of-fit plots. The difference in the OFVs 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 (Wahby et al., 2001).

### Results

Pharmacokinetic Analysis for PF06463922. The increase in plasma concentrations of PF06463922 was roughly dose-proportional at the dose ranges tested in studies 1, 2, and 3. Therefore, pharmacokinetic parameters were estimated together at all doses of each study. Typical pharmacokinetic parameter estimates for \(CL/F, V/F, \) and \(k_{\text{out}}\) were, respectively, 1.2 l/h per kilogram, 5.3 l/kg, and 2.0 h\(^{-1}\) in study 1; 1.1 l/h per kilogram, 7.0 l/kg, and 1.3 h\(^{-1}\) in study 2; and 1.7 l/h per kilogram, 11 l/kg, and 4.0 h\(^{-1}\) in study 3 (Table 1). These

<table>
<thead>
<tr>
<th>Model</th>
<th>(E_{\text{EC50}})</th>
<th>(E_0)</th>
<th>(E_{\text{max}})</th>
<th>(k_{\text{out}})</th>
<th>(k_{\text{out}})</th>
<th>(\gamma)</th>
<th>OFV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDR</td>
<td>137</td>
<td>1</td>
<td>1</td>
<td>4.8</td>
<td>na</td>
<td>0.95</td>
<td>262</td>
</tr>
<tr>
<td>PCS</td>
<td>58</td>
<td>(fixed)</td>
<td>(fixed)</td>
<td>(1.7)</td>
<td>(0.08)</td>
<td>(0.8)</td>
<td>(0.03)</td>
</tr>
</tbody>
</table>

\(E_0\), ALK phosphorylation baseline; IDR, indirect response model; \(k_{\text{out}}\), first-order degradation rate constant for a modulator; \(k_{\text{out}}\), first-order degradation rate constant; na, not applicable; PCS, precursor model.

Fig. 2. Indirect-response model-fitted and observed ALK inhibition by PF06463922 in athymic mice implanted with H3122 NSCLC cells expressing the EML4-ALKL1196M after repeated oral administration. PF06463922 was orally administered to animals (\(n = 3\) per time point) twice daily, 7 hours apart, at doses of 0.3–10 mg/kg per dose for 4 days in study 1 (S1) and 0.3–20 mg/kg for 13 days in study 2 (S2). The y-axis represents the time after the last dosing in hours, the left side of the y-axis represents the observed (ALK OBS) and model-fitted (ALK PRED) ALK inhibition in the ratio to the mean value of control animal data, and the right side of the y-axis represents the model-fitted plasma concentrations of PF06463922 (CP PRED) in nanograms per milliliter on a logarithmic scale.
pharmacokinetic parameter estimates with twice daily doses were comparable to those determined in other studies where the same cumulative dose amounts were delivered with once daily doses. The standard errors of most of the estimated pharmacokinetic parameters in studies 1 to 3 were small (CV < 30%), with residual variability of 12–24%. OFVs were 543, 802, and 275 in studies 1, 2, and 3, respectively. A representative example of the observed and one-compartment pharmacokinetic model-fitted plasma concentrations of PF06463922 in study 1 is shown in Fig. 1. Overall, the plasma concentration-time courses of PF06463922 in studies 1, 2, and 3 were adequately described by the one-compartment model. The typical pharmacokinetic parameters thus obtained (i.e., $CL/F$, $V/F$ and $k_{a}$) were used to simulate plasma concentrations as a function of time after oral administration to drive the pharmacodynamic models.

**PKPD Modeling for Target Modulation.** The inhibition of ALK phosphorylation was sustained after the first and second daily doses and thereafter returned to near or above the baseline at 24 to 36 hours postdose in studies 1 and 2. In contrast, the plasma concentrations of PF06463922 reached the maximal levels at 1 hour postdose and then rapidly declined. The observed and indirect-response model-fitted ALK phosphorylation–time courses along with the simulated plasma concentrations of PF06463922 are graphically presented in Fig. 2. The indirect response model did not fit the ALK inhibition well at 24 to 36 hours postdose in studies 1 and 2 because the model was unable to account for the ALK rebound. The $EC_{50,\text{in vivo}}$ was estimated at 137 ng/ml (Table 2). In contrast to the indirect response model, the precursor model fit the time courses of ALK inhibition sufficiently well, particularly 24 to 36 hours postdose (Fig. 3), yielding the OFV value of $-284$ smaller (i.e., statistically better) than that ($-262$) from the indirect response model. The estimated $EC_{50,\text{in vivo}}$ was 58 ng/ml, which was approximately 2-fold lower than that from the indirect response model. The estimated $k_{\text{out}}$ and $k_{\text{md}}$ were 1.8 and 0.021 h$^{-1}$, respectively.

**Drug-Disease Modeling for Antitumor Efficacy.** In an ALK-tumor model, the observed tumor growth inhibition by PF06463922 was 57, 87, 101, 121 (63% regression), and 120% (66% regression) at the doses of 0.3, 1, 3, 10, and 20 mg/kg per dose, respectively, on the last dosing day (study 2). The model-fitted individual and typical tumor growth inhibition curves with the observed tumor volumes are presented in Fig. 4. The drug-disease model reasonably fit the observed individual tumor growth curves in all groups, with an estimated $KC_{50}$ of 33 ng/ml (Table 3). The estimated $K_{\max}$ (0.011 h$^{-1}$) was 1.1-fold higher than the estimated $k_{\text{ng}}$ (0.0094 h$^{-1}$), indicating...
that the model-predicted maximal antitumor efficacy was greater than tumor stasis (i.e., tumor regression). The tumor stasis concentration \( (T_{sc}) \), which was the plasma concentration of PF06463922 required to maintain tumor stasis at steady state, was calculated at 83 ng/ml.

In a ROS1-tumor model, the observed tumor growth inhibition by PF06463922 was 26, 38, 84, 104 (20% regression), 116 (73% regression), and 120% (85% regression) at doses of 0.01, 0.03, 0.1, 0.3, 1, and 3 mg/kg per dose, respectively, on the last dosing day (study 3). The model-fitted individual and typical tumor growth inhibition curves with the observed tumor volumes are presented in Fig. 5. The drug-disease dynamic parameters (e.g., EC50, in vivo and T sc) of the second-generation ALK inhibitor for target modulation (including rebounds) and antitumor efficacy in an ALK-tumor model was approximately 10-fold lower than that (51 nM free) in an ALK-tumor model, demonstrating that PF06463922-mediated antitumor efficacy was more potent in a ROS1-tumor models than an ALK-tumor model as consistent with the difference in the EC50, in vitro values of 0.2 and 15 nM for ROS1 and ALK inhibition, respectively.

**Quantitative Comparison of PKPD Relationships.** The concentration-response curves of ALK inhibition and tumor growth inhibition based on the obtained pharmacodynamic parameters (e.g., EC50, in vivo and EC60) from ALK- and ROS1-tumor models are graphically presented in Fig. 6. It may be worth noting that the tumor growth inhibition ranges from 0 to 120%, whereas the range of ALK inhibition is 0 to 100% in Fig. 6. Based on the calculation method for tumor growth inhibition, (EC50), as indicated in Materials and Methods, the maximum tumor growth inhibition was near 110%, even though the estimated \( K_{max} \) was 2,3-fold greater than the estimated \( k_{net} \) in a ROS1-tumor model. In an ALK-tumor model, the EC50, in vivo (58 ng/ml) for ALK inhibition was 1.4-fold lower than the estimated \( T_{sc} \) (83 ng/ml). Thus, the \( T_{sc} \) was roughly comparable to the EC60, in vivo (85 mg/ml) for ALK inhibition. The EC50, in vivo estimate for ALK inhibition and \( T_{sc} \) as total plasma concentrations (bound plus unbound) corresponded to 52 and 51 nM free, respectively, with the correction for an unbound fraction of 0.25 in mouse plasma (Table 4). The estimated \( T_{sc} \) (10 ng/ml corresponding to 6.2 nM free) in a ROS1-tumor model was approximately 10-fold lower than that (51 nM free) in an ALK-tumor model, demonstrating that PF06463922-mediated antitumor efficacy was more potent in a ROS1-tumor models than an ALK-tumor model as consistent with the difference in the EC50, in vitro values of 0.2 and 15 nM for ROS1 and ALK inhibition, respectively.

**Discussion**

In the present study, we quantified the relationships of plasma concentrations of PF06463922 to inhibition of ALK phosphorylation and tumor growth inhibition in a crizotinib-resistant ALK-tumor model (i.e., H3122 NSCLC with EML4-ALKL1196M) using a mathematical modeling approach. This is the first report to quantitatively characterize PKPD relationships of a second-generation ALK inhibitor for target modulation (including rebounds) and antitumor efficacy in an ALK-tumor model. Unexpectedly, the rebounds of ALK phosphorylation in vivo were observed at 24–36 hours after repeated oral administration of PF06463922. By that time, the plasma concentrations of PF06463922 declined to less than 5 ng/ml (3 nM free), which was >10-fold lower than the EC50, in vivo (36 nM free). The observations that ALK responses

![Fig. 5.](image-url)

**Fig. 5.** Observed tumor volumes and model-fitted tumor growth inhibition curves in athymic mice implanted with NIH3T3 cells expressing the CD74-ROS1 after repeated oral administration of PF06463922. Animals \( n = 12 \) per group orally received PF06463922 twice daily, 7 hours apart, at doses of 0.01–3 mg/kg for 9 days (study 3). 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 (IPRED) (A) and typical (PRED) (B) tumor growth curves in cubic millimeters.
It was critical to incorporate a modulator into an indirect response model to take account of the time-dependent ALK responses, including rebound phenomena. Since the biologic mechanism for ALK rebounds in an ALK-tumor model is still unclear, we have applied several previously proposed feedback and precursor PKPD models to the present results in addition to an indirect response model (Gabrielsson and Weiner, 2000). None of these PKPD models, except for a precursor model, could acceptably fit the time course of ALK responses. The EC_{50,in vivo} (58 ng/ml) estimated by the precursor model was >2-fold lower than that (137 ng/ml) with an indirect response model, suggesting that accounting for the ALK rebounds would be important to estimate pharmacodynamic parameters.

It has been known that adaptive resistance to molecularly target agents (e.g., tyrosine kinase inhibitors) can occur immediately in cancer patients through a rapid wiring of cancer cell signaling (Soria et al., 2012; Rosell et al., 2013). There are also limited data suggesting that a signaling rebound may occur after the cessation of cancer therapy with tyrosine kinase inhibitors in patients who develop drug resistance (Riely et al., 2007). Thus, the network signals can possibly and quickly undergo adaptive changes during cancer therapy with tyrosine kinase inhibitors. It has also been reported that changes in total receptor abundance could affect tyrosine kinase inhibitor–mediated target modulation, when it was measured as amount of phosphorylated protein corrected for total protein (Kirouac et al., 2013). Some ATP-competitive tyrosine kinase inhibitors (such as crizotinib) could affect an interaction between the heat shock protein 90 charperone and its client kinases, resulting in changes of kinase stabilization (Taipale et al., 2013). The biologic feedback mechanisms, including the observed ALK rebounds in vivo, might be one of the potential reasons for the 2-fold difference in the estimated EC_{50} between in vivo (36 nM free) and in vitro (15 nM free) (Table 4), although the 2-fold difference could be within the expected variability derived from in vitro and in vivo experiments. It would be worth noting that significant antitumor efficacy of PF06463922 was consistently observed throughout the treatment period in an ALK-tumor model we studied, despite the ALK responses being partially back to near or above the baseline before each dose. For example, the ALK phosphorylation ratios at 24 hours postdose were near baseline on day 4 and approximately 2 on day 13 at the dose of 3 mg/kg per dose twice daily, whereas tumor growth inhibition at this dose was consistently near-tumor stasis during the treatment period with 101% tumor growth inhibition on day 13. Therefore, the ALK rebounds in nonclinical models may not be related to antitumor resistance to ALK inhibitors.

**TABLE 4**

Summary of pharmacodynamic parameter estimates for ALK inhibition and antitumor efficacy by PF06463922 in two different nonclinical tumor models

<table>
<thead>
<tr>
<th>Nonclinical Model</th>
<th>Target Modulation</th>
<th>Antitumor Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC_{50,in vivo}^{nM free}</td>
<td>EC_{50,in vivo}^{nM free}</td>
</tr>
<tr>
<td>H3122-EML4-ALK\textsuperscript{L1196M}</td>
<td>15</td>
<td>36</td>
</tr>
<tr>
<td>NIH3T3-CD74-ROS1</td>
<td>0.2</td>
<td>—</td>
</tr>
</tbody>
</table>

\textsuperscript{nM free} — not available; T_{sc}, tumor stasis concentration.

\textsuperscript{nM free} Values are cited from previous reports (Zou et al., 2013).
Regarding the PKPD relationship of target modulation to antitumor efficacy, the EC\textsubscript{60, in vivo} (52 nM free) for ALK inhibition was comparable to the estimated T\textsubscript{50} (51 nM free) in an ALK-tumor model (Table 4). This relationship suggested that >60% ALK inhibition would be required for tumor stasis. We previously developed an integrated PKPD model to comprehensively characterize relationships between inhibitor concentrations, biomarker responses, and antitumor efficacy (Yamazaki et al., 2011; Yamazaki, 2013). In the integrated model, pharmacodynamic parameters obtained by the PKPD model were used to simulate biomarker responses as a function of time to model antitumor efficacy using a proposed “inhibition index” (1/E-1) as the variable driving the effect. However, in the present study, the inhibition index became negative when ALK rebounds were observed (i.e., E > 1), resulting in a predicted increase in model-simulated tumor growth rate that was not supported by our observations. Therefore, we did not apply the integrated model to the present study. In a previous nonclinical study (Yamazaki et al., 2012), the PKPD relationship of crizotinib-mediated ALK inhibition to antitumor efficacy in an H3122 NSCLC model with wild-type EML4-ALK (without ALK mutations) was characterized by a similar PKPD modeling approach, where a link model was applied to estimate crizotinib-mediated ALK inhibition. One of the potential reasons for the difference in PKPD models applied between crizotinib and PF06463922 could be their difference in tumor distribution. That is, crizotinib extensively distributed into tumors of nonclinical models with an approximate tumor/plasma area under the concentration-time curve ratio of 4 at steady state, whereas the distribution of PF06463922 into tumor was less extensive with the approximate tumor/plasma ratio being close to unity. Differently from the present study with PF06463922, 50% ALK inhibition by crizotinib (EC\textsubscript{50, in vivo} = 19 nM free) was associated with 50% tumor growth inhibition (EC\textsubscript{50} = 20 nM free) in the previous studies (Yamazaki et al., 2012). Thus, PF06463922-mediated ALK inhibition in an ALK-tumor model (with ALK mutation) appeared to lead to more pronounced antitumor efficacy than crizotinib-mediated ALK inhibition in an H3122 NSCLC model with wild-type EML4-ALK. In a previously reported crizotinib PKPD simulation in patients using clinically observed pharmacokinetic parameters (Yamazaki, 2013), the predicted crizotinib-mediated ALK inhibition could reach 75% at steady state in patients at the clinically recommended doses of 250 mg twice daily. Collectively, the EC\textsubscript{75, in vivo} (100 nM free) for PF06463922-mediated ALK inhibition could be considered a target plasma concentration for crizotinib-resistant NSCLC patients to achieve crizotinib-equivalent antitumor efficacy, as observed in crizotinib-sensitive NSCLC patients with wild-type ALK rearrangements (Kwak et al., 2010; Camidge et al., 2012). Furthermore, the EC\textsubscript{50, in vivo} (52 nM free) could be considered a minimum target efficacious concentration in NSCLC patients with EML4-ALK mutations since tumor stasis was achieved with 60% ALK inhibition in an ALK-tumor model.

In a ROS1-tumor model, we could not determine the EC\textsubscript{50, in vivo} for ROS1 inhibition because of a lack of specificity of the ROS1 antibody, whereas the decrease in the numbers of Ki67 positive cells in tumor cells was confirmed with an antiproliferation marker with an immune-histochemistry analysis (Zou et al., 2013). The EC\textsubscript{50, in vitro} estimate (0.2 nM free) for ROS1 inhibition was >50-fold lower than that for

---

**Fig. 7.** PKPD modeling summary of target modulation and antitumor efficacy by PF06463922 in nonclinical tumor models. D, dose; k\textsubscript{a}, absorption rate constant (h\textsuperscript{-1}); C\textsubscript{p}, plasma concentration (nanograms per milliliter); V, volume of distribution (l/kg); k\textsubscript{e}, elimination rate constant (h\textsuperscript{-1}); M, modulator; k\textsubscript{f0}, zero-order formation rate constant for a modulator (h\textsuperscript{-1}); k\textsubscript{md}, first-order degradation rate constant for a modulator and formation rate constant for ALK (h\textsuperscript{-1}); E\textsubscript{max}, maximum effect; EC\textsubscript{50}, plasma concentration causing one-half E\textsubscript{max} (nanograms per milliliter); \gamma, Hill coefficient; E, ALK response (ratio to baseline); k\textsubscript{out}, first-order degradation rate constant for ALK (h\textsuperscript{-1}); T\textsubscript{max}, maximum sustainable tumor volume (mm\textsuperscript{3}).

\[
\frac{dE}{dt} = k_{md} \left( 1 - \frac{E_{max} \cdot C_p^\gamma}{EC_{50}^\gamma + C_p^\gamma} \right) \cdot M - k_{out} \cdot E
\]

\[
\frac{dT}{dt} = g(T) = \frac{K_{max} \cdot C_p^\gamma}{KC_{50}^\gamma + C_p^\gamma} \cdot T
\]

\[
g(T) = k_{eg} \cdot T \cdot (1 - T/T_{ss})
\]
ALK inhibition, and the $T_{sc}$ estimate in a ROS1-tumor model was approximately 10-fold lower than that in an ALK-tumor model (Table 4). One might expect more pronounced antitumor efficacy by PF06463922 in a ROS1-tumor model, but antitumor potency could result from its target modulation through complex biologic mechanisms. For example, previous crizotinib reports suggested that >50% ALK inhibition corresponded to >50% tumor growth inhibition in an H3122 NSCLC model, as discussed already, whereas >90% MET inhibition was required for the same degree of tumor growth inhibition in a GTL16 gastric cancer model (Yamazaki et al., 2008, 2012; Yamazaki, 2013). Recent emerging systems pharmacology approaches also suggest 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; Kirouac et al., 2013). When the EC$_{50\text{in vitro}}$ (0.2 nM) for ROS1 inhibition was compared with the observed plasma concentration of PF06463922 in an ROS1-tumor model, the average plasma concentration (0.5 nM free) at the lowest dose of 0.01 mg/kg was approximately 3-fold greater than the EC$_{50\text{in vitro}}$. However, the observed antitumor efficacy at this dose level was minimal with tumor growth inhibition of 26% on the last dosing day, suggesting that the EC$_{50\text{in vivo}}$ for ROS1 inhibition could be greater than the EC$_{50\text{in vitro}}$ to achieve an antitumor efficacy comparable to that observed in an ALK-tumor model. Nevertheless, the comparison of PF06463922-mediated antitumor efficacy between ROS1 and ALK suggested that antitumor efficacy in cancer patients with ROS1 rearrangements would be achieved more readily. Therefore, we could expect a significant antitumor efficacy of PF06463922 in patients with ROS1 rearrangements when its systemic exposure reached a proposed targeted efficacious concentration based on ALK inhibition.

In conclusion, the PKPD relationships among systemic exposures of PF06463922, target modulation, and antitumor efficacy in nonclinical tumoral models were well characterized in a quantitative manner using a mathematical modeling approach (Fig. 7). The present modeling efforts suggest that >60% ALK inhibition would be required for tumor stasis in an ALK-tumor model. Accordingly, we proposed that the EC$_{50\text{in vivo}}$ for ALK inhibition (∼50 nM free) could be considered a minimum target efficacious concentration of PF06463922 in NSCLC patients with EML4-ALK rearrangements (with and without ALK mutations). The proposed minimum efficacious concentration could also be enough to lead to a significant antitumor efficacy in patients with ROS1 rearrangements. Overall, we believe the present PKPD results will be helpful in understanding clinical PKPD relationships of PF06463922 and also in guiding dose escalation or de-escalation to maintain efficacious exposure to PF06463922 in the clinic.

Acknowledgments
The authors thank the members of Pharmacokinetics, Dynamics and Metabolism Department (Pfizer Worldwide Research and Development, Groton, CT), especially, Amanda King-Ahmad, Andre Negahban, Jian Lin, Beiyan Tu, and Yishong Zhang for their in vitro studies and bioanalytical analyses, and Duc Dinh, Lars Engstrom, Hieu Lam, Nathan Lee, Qiuhua Li, Ruth W Tang, Sergei Timofeevski, Konstantinos Tsaparakos, and Melissa West (Oncology Research Unit, Pfizer Worldwide Research and Development, San Diego, CA) and Hovhannes Gukasyan (La Jolla Research Support, Pfizer Worldwide Research and Development, San Diego, CA) for the in vivo studies and pharmacodynamic analyses, as well as Bhaskar Shetty and Bill Smith (Pharmacokinetics, Dynamics and Metabolism, Pfizer Worldwide Research & Development, San Diego, CA) for excellent input on the draft manuscript.

Authorship Contributions
Participated in research design: Lam, Wang, Yamazaki, Zou. Conducted experiments: Wang, Zou. Performed data analysis: Lam, Vicini, Yamazaki, Zou. Wrote or contributed to the writing of the manuscript: Lam, Smeal, Vicini, Wang, Yamazaki, Zou.

References
Beal S and Sheiner L (1992) NONMEM user guides. NONMEM project group, University of California at San Francisco, San Francisco, CA.
Beal S and Sheiner L (1992) NONMEM user guides. NONMEM project group, University of California at San Francisco, San Francisco, CA.


Address correspondence to: Dr. Shinji Yamazaki, Pharmacokinetics, Dynamics and Metabolism, La Jolla Laboratories, Pfizer Worldwide Research and Development, 10177 Science Center Drive, San Diego, CA 92121. E-mail: shinji.yamazaki@pfizer.com