Impact of Hydrolysis Mediated Clearance on the Pharmacokinetics of Novel Anaplastic Lymphoma Kinase Inhibitors

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Abstract

Compound 1, a new, potent, selective anaplastic lymphoma kinase (ALK) inhibitor with potential application for the treatment of cancer, was selected as candidate to advance into efficacy studies in mice. However, the compound underwent mouse specific enzymatic hydrolysis in plasma to a primary amine product (M1). Subsequent intravenous (IV) PK studies in mice showed that compound 1 had high CL and a short half-life. Oral dose escalation studies in mice indicated that elimination of compound 1 was saturable, with higher doses achieving sufficient exposures above in vitro IC50. Chemistry efforts to minimize hydrolysis resulted in the discovery of several analogs that were stable in mouse plasma. Three were taken in vivo into mice and showed decreased CL corresponding to increased in vitro stability in plasma. However, the more stable compounds also showed reduced potency against ALK. Kinetic studies in NADPH fortified and un-fortified microsomes and plasma produced sub-micromolar Km values and could help explain the saturation of elimination observed in vivo. Predictions of CL based on kinetics from hydrolysis and NADPH-dependent pathways produced predicted hepatic CL values of 3.8, 3.0, 1.6, and 1.2 L/(h*kg) for compounds 1, 2, 3, and 4, respectively. The in vivo observed CL for compounds 1, 2, 3, and 4 were 5.52, 3.51, 2.14, and 2.66 L/hr*kg, respectively. These results indicate that in vitro metabolism kinetic data, incorporating contributions from both hydrolysis and NADPH-dependent metabolism, could be used to predict the systemic CL of compounds cleared via hydrolytic pathways provided that the in vitro assays thoroughly investigate the processes, including the contribution of other metabolic pathways and the possibility of saturation kinetics.
Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase member of the insulin receptor superfamily. Although ALK is expressed at high levels prenatally, its expression is dramatically decreased in healthy adult tissues. Aberrant expression of full-length ALK occurs in neuroblastoma (George et al., 2008). In addition, novel fusion-ALK proteins that possess constitutive kinase activity, and contribute to oncogenic processes are generated by chromosomal translocation or inversion of the ALK gene. Two of these fusion ALK proteins nucleophosmin (NPM-ALK) and echinoderm microtubule-associated protein-like 4 (EML4-ALK) have been validated as driving oncogenes in a subset of anaplastic large cell lymphomas and non-small cell lung carcinomas, respectively (Morris et al., 1994; Soda et al., 2007). The clinical validation of EML4-ALK as a therapeutic target by crizotinib has provided compelling evidence for the importance of ALK in oncology (Shaw et al., 2011).

Compound 1 ((E)-4-fluoro-N-(6-((4-(2-hydroxypropan-2-yl)piperidin-1-yl)methyl)-1-((1s,4s)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzo[d]imidazol-2(3H)-ylidene)benzamide), is a member of a new class of potent and selective anaplastic lymphoma kinase inhibitor with potential application in the treatment of cancer (Lewis et al., 2012). In vitro screening of compound 1 resulted in excellent inhibitory activity against the ALK enzyme (IC50 = 1 nM) and ALK expressing Karpas-299 cells (4 nM) (Lewis et al., 2012). Rat in vivo studies showed acceptable PK with a CL of 0.8 L/hr/kg, a t1/2 of 5.6 hr, and MRT of 8 hrs (Lewis et al., 2012). Based on potency and preliminary rodent pharmacokinetics, compound 1 was selected as a candidate to advance into efficacy studies in mice. However, during bioanalysis it was found that the compound was subject to extensive hydrolysis to a resultant primary amine product (M1). Enzyme mediated hydrolytic metabolism of compound 1 was examined to determine whether or not it would be a liability in the further development of this molecule for potential use in humans.
Although not as common as cytochrome P450 mediated oxidations, hydrolysis reactions play an important role in the metabolism of xenobiotics (Testa and Mayer, 2003). A variety of hydrolytic enzymes have been implicated in the hydrolysis of esters, thioesters, amides, and epoxides. These hydrolytic enzymes include carboxylesterases, cholinesterases, organophosphatases, and amidases/peptidases. Cholinesterases and aminopeptidases are the most commonly associated with the hydrolysis of amide bonds (Uetrecht and Trager, 2007). Species difference in amide hydrolysis is well precedent. For example, considerable species differences in extent of hydrolysis was observed for lidocaine forty years ago (Keengham and Boyes, 1972). With regards to compounds containing biaryl amide links, species differences in extent of hydrolysis have been reported for ameltoide (Potts et al., 1989), the anticonvulsant agent D2624 (Martin et al., 1997), pranulkast, (Luan et al., 1997), piroxicam (Hobbs and Twomey, 1981), and more recently a bile acid Takeda G-protein coupled receptor 5 (TGR5) agonist (Eng et al., 2010) and the Bruton’s Tyrosine Kinase Inhibitor GDC-0834 (Liu et al., 2011).

Species specific enzyme mediated amide hydrolysis reactions in drug discovery often present multiple problems, including high CL in PK, instability in in vitro assays that may contain the hydrolytic enzymes, and instability in bioanalytical matrices such as blood or plasma. Instability in in vitro and ex vivo assays may lead to erroneous results and potentially causes the subsequent termination of a promising candidate. Therefore, early identification and examination of the hydrolytic process(es) can be used to design experiments that deliver more accurate results, provide rationale for the progression (or early termination) of a lead molecule, or support the design of hydrolytically stable analogs that might offer better success in development.

In early development, knowledge of the hydrolysis mediated metabolism of a lead molecule may influence the approach taken to predict human pharmacokinetics prior to advancement of the compound to the clinic. For example, although they performed kinetic
studies to determine Vmax and Km through formation of the hydrolysis metabolite in multiple
species, Liu and co-authors (2011) used the substrate depletion method at a single arbitrary
concentration to arrive at the predicted CL. They reported substantial uncertainty in the IVIVE
techniques used, given the large species differences observed in the hydrolysis pathway.
Unfortunately, they did not appear to address issues such as plasma protein binding, binding to
microsomal proteins, saturation kinetics, or kinetics of hydrolysis versus other metabolic
pathways such as P450 dependent metabolism. Further study on the impact of hydrolysis on
IVIVE predictive techniques is warranted.

In a discovery setting, extrahepatic metabolism such as hydrolysis in plasma, is
occasionally proposed as a possible explanation for poor predictivity of in vitro-in vivo
extrapolation (IVIVE) based upon data obtained from liver microsomes or hepatocytes. To our
knowledge, there are no reports in the literature that deal with an IVIVE method for hydrolysis
which takes into account plasma hydrolysis, saturation kinetics, and contribution of NADPH
dependent metabolism. In this paper, we report our experiences with compound 1, a novel
potent ALK inhibitor that is rapidly enzymatically hydrolyzed in mouse plasma and liver
microsomes. We report our rationale for the successful guidance of compound progression
and the use of IVIVE of the hydrolysis reaction to drive an informed decision. In addition, the
contributions of hydrolysis in plasma and liver versus NADPH-dependent metabolism in vitro are
considered in this context.
Materials and Methods

Materials. Liver microsomes isolated from male Sprague-Dawley rats (n=50), male CD-1 mice (n=200), male beagle dogs (n=5), and human (n=50) were purchased from BD Biosciences (San Jose, CA). Plasma from male Sprague-Dawley rats, CD-1 mice, beagle dogs and human was purchased from Bioreclamation, Inc. (Westbury, CT). All fresh plasma and blood was obtained from the in vivo group at Amgen. All proprietary ALK inhibitors were synthesized by the Department of Medicinal Chemistry at Amgen (Cambridge, MA) and all synthetic routes have already been published elsewhere (Lewis et al., 2012). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless otherwise specified.

Determination of stability or CL_{int} in plasma. Compound 1 and analogs were incubated in mouse, rat, dog, or human plasma at 37 C for up to 3 hrs. For initial stability screening in mouse plasma, test compounds were incubated at a concentration of 0.83 µM. For determination of metabolism kinetics in mouse plasma selected compounds (compound 1, compound 2, compound 3, and compound 4) were incubated at a compound concentration range of 0.0023 µM to 5 µM. At various time points, samples were collected from the incubations into acetonitrile containing internal standard (tolbutamide), centrifuged at 3500 g for 15 min, and analyzed by LC-MS/MS. For each compound/concentration, peak areas at each time point were converted to the natural log of the percent remaining relative to the 0-minute samples. The resulting initial linear portion of the slope (k) of these values relative to time was converted to in vitro t_{1/2} where t_{1/2} = -0.693/k. In determination of metabolism kinetics, observed CL_{int} was calculated using the relationship (Obach, 2002):

\[
\text{CL}_{\text{int,obs}} = \frac{0.693}{t_{1/2}} \times (1/M)
\]

Eq. 1

where, in this case, M is the concentration of plasma in the incubation (e.g. 1 mL plasma per mL of incubation).
**Determination of CL_{int} in liver microsome.** Initial experiments with compound 1 were conducted in mouse, rat, dog, and human liver microsomes (0.25 mg/mL protein) in phosphate buffer (0.1 M, pH 7.4). Compound 1 (0.1 and 1 µM) was incubated with NADPH (1 mM). Samples were collected from the incubations at 0, 10, 20, 30 and 40 min into an equal volume of acetonitrile containing internal standard (tolbutamide), centrifuged at 3500 g for 15 min, and analyzed by LC-MS/MS. Subsequent metabolism kinetics (V_{max} and K_{m}) for compounds 1, 2, 3 and 4 in mouse liver microsomes were determined as above in the presence and absence of NADPH at a compound concentration range of 0.0023 µM to 5 µM. For each compound/concentration, CL_{int,obs} was calculated using Eq. 1, where, in this case, M is the concentration of microsomes in the incubation.

**Plasma protein binding and microsomal binding.** Fraction unbound in mouse plasma (f_{up}) was determined in triplicate by equilibrium dialysis, using the Rapid Equilibrium Dialysis device (Thermo Scientific, Rockford, IL). Three hundred microliter aliquots of plasma containing 0.1 µM, 0.5 µM or 2 µM test compound were dialyzed against 500 µL of phosphate buffered saline for 7 h at 37 °C in an incubator maintained at 5% CO₂. Preliminary experiments with selected compounds indicated that a 7 h incubation time was sufficient to achieve equilibrium. Since some test compounds were rapidly hydrolyzed in plasma, plasma was pre-incubated for 20 min in the presence of 100 µM diisopropyl fluorophosphate (DFP) to inactivate hydrolytic enzymes. Recovery of test compounds in the presence of DFP was >80%, indicating DFP did an adequate job of preventing hydrolysis. Aliquots of the post-dialysis buffer were transferred into an equal volume of blank plasma. Samples of the dialyzed plasma were transferred to an equal volume of buffer. Samples were extracted with acetonitrile containing internal standard (tolbutamide), then centrifuged at 3500 g for 15 min and analyzed by LC-MS/MS as described below. The fraction unbound in plasma was calculated by dividing the amount of drug found in the post dialysis buffer by the amount found in the dialyzed plasma.
The binding of test compounds to liver microsomes was conducted as described above for plasma, except substituting plasma with 0.25 mg/mL liver microsomes solution in phosphate buffer.

**Blood to plasma ratio (BP).** BP was determined in vitro after incubation of compound with and without DFP in fresh whole blood. Blood was warmed to 37°C and compound was spiked to a concentration of 1 µM. After incubation for 1 h the blood samples were processed for plasma. Reference plasma was also spiked with compound to a concentration of 1 µM. The same incubations were performed after pre-incubation for 20 min with 100 µM of DFP to inhibit amide hydrolysis. Recovery of test compounds in the presence of DFP was >80%. Samples were extracted with 6 volumes of acetonitrile containing internal standard, centrifuged, and analyzed on the LC-MS/MS system described below. BP was calculated by dividing the peak area observed in the reference plasma (representing nominal blood concentration) by the peak area observed in the treated plasma (representing plasma concentration).

**Pharmacokinetic studies in mice.** All animal procedures were conducted under protocols approved by the Amgen (Cambridge, MA) Institutional Animal Care and Use Committee. Male CD-1 mice were purchased from Taconic (Germantown, NY). Mice (60) were housed in a humidity- and temperature-controlled environment subject to a 12 h:12 h light:dark cycle and had access to water and a standard laboratory diet ad libitum. The animals designated for oral administration were fasted overnight prior to dosing and food was returned 2 h post-dose.

Following a 5 day acclimation period, the mice were administered a single dose of test material by bolus intravenous injection through the lateral tail vein (2 mg/kg) or by oral gavage (20, 50, 100 mg/kg). Mice were euthanized by CO₂ asphyxiation at 0.083 (IV only), 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hours after dose administration. Blood samples (0.3 mL) were obtained by cardiac puncture and then transferred to heparinized tubes on ice. Plasma was separated by centrifugation at 4 °C and fifty microliters aliquots were immediately transferred to tubes containing six volumes of a mixture of 0.1% formic acid and IS in acetonitrile. The remaining
plasma was flash frozen on dry ice and stored at -80 °C until required for follow-up analysis. Plasma samples were analyzed using the LC-MS/MS method described below.

Analysis of pharmacokinetic samples. The LC-MS/MS system consisted of a Waters Acquity UPLC system (Waters) and an API4000 mass spectrometer (Applied Biosystems Group, Foster City, CA) equipped with either an atmospheric pressure chemical ionization (APCI) source or electrospray ionization (ESI) source. For each analyte, the mass spectrometer electronics were tuned to the most intense mass transition. The following transitions were used for data acquisition: compound 1, m/z 578 → m/z 435.3; compound 2, m/z 596.4 → m/z 453.3; compound 3, m/z 612.3 → m/z 469.2; compound 4, m/z 628.4 → m/z 485.3; M1, m/z 456.4 → m/z 313.2.

Calibration curve standards and QC samples were prepared in mouse plasma at concentrations ranging from 0.3 to 10000 ng/mL for each compound. Fifty microliters of each plasma sample, calibration standard, and QC sample were extracted by adding 6 volumes of a mixture containing 0.1% formic acid in water and an internal standard. The samples were mixed by vortexing and centrifuged. The resulting supernatants were diluted with an equal volume of water.

Samples were injected onto an Acquity UPLC Phenyl 1.7µm BEH column (2.1×50 mm Waters) with a 0.5 µm PEEK guard filter. Analytes were separated using a gradient solvent system consisting of two components: solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The percentage of solvent B was increased in a linear fashion from 10% to 95% over 2 min, then maintained at 95% for an additional 0.5 min; flow rate was 0.6 mL/min.

Raw data was collected using Sciex Analyst 1.5.1 (Applied Biosystems, Foster City, CA) residing on a computer running a Windows XP operating system. Following peak area integration, the data was exported to the Small Molecules Discovery Assay (SMDA) Watson® software package (version 7.0.0.01, Thermo Fisher Scientific, Waltham, MA) where concentrations were
determined by a weighted \((1/x^2)\) linear regression of peak area ratios versus the peak area ratios of known calibration standards.

**Noncompartment PK Analysis.** The non-compartmental pharmacokinetics parameters were calculated using Small Molecules Discovery Assay (SMDA) Watson® software (version 7.0.0.01; Thermo Fisher Scientific, Waltham, MA). The area under the plasma concentration-time curve from time 0 to the last time point \(t\) with a quantifiable concentration \(C_p\), \(AUC_{0–t}\), was calculated using the linear trapezoidal rule. The area under the plasma concentration-time curve was extrapolated to infinity time \(AUC_{0–\text{inf}}\) by the equation \(AUC_{0–\text{inf}} = AUC_{0–t} + C_p/k\), where \(k\) is the elimination rate constant determined by linear regression of the last two to four quantifiable data points in the log plasma concentration-time curves. Plasma clearance (CL), \(t_{1/2}\), and \(V_{ss}\) were calculated by the equations, \(CL = \text{Dose}/AUC_{0–\text{inf}}\), \(t_{1/2} = 0.693/k\), and \(V_{ss} = CL * AUMC_{0–\text{inf}}/AUC_{0–\text{inf}}\), where \(AUMC_{0–\text{inf}}\) is the area under the first moment of the plasma concentration-time curve from time 0 to infinity. MRT was calculated by the equation \(MRT = V_{ss}/CL\). Bioavailability (F) was calculated using the equation \(F = (AUC_{p.o.}/AUC_{i.v.}) * (\text{Dose}_{i.v.}/\text{Dose}_{p.o.})\), where \(AUC_{i.v.}\) and \(AUC_{p.o.}\) are AUC estimates after the intravenous and oral administration, respectively, and \(\text{Dose}_{i.v.}\) and \(\text{Dose}_{p.o.}\) are the administered doses for intravenous and oral administration, respectively.

**Analysis of samples from in vitro metabolism, blood to plasma ratio, and plasma protein binding experiments.** The LC-MS/MS system consisted of two LC-10AD HPLC pumps and a DGU-14A degasser (Shimadzu, Columbia, MD), a CTC PAL autoinjector (Leap Technologies, Carrboro, NC), and either an API3000 or API4000 mass spectrometer (Applied Biosystems, Foster City, CA), according to the requirements of the compound. For each analyte, the mass spectrometer electronics were tuned to the most intense mass transition. The following transitions were used for data acquisition: compound 1, \(m/z\) 578 → \(m/z\) 435.3; compound 2, \(m/z\) 596.4 → \(m/z\) 453.3; compound 3, \(m/z\) 612.3 → \(m/z\) 469.2; compound 4, \(m/z\) 628.4 → \(m/z\) 485.3; M1, \(m/z\) 456.4 → \(m/z\) 313.2. Samples from in vitro experiments were injected onto a Sprite
Armor C18 analytical column (20 mm x 2.1 mm, 5 µm pore size; Analytical Sales and Products, Pompton Plains, NJ) with a 0.5 µm PEEK guard filter. Analytes were separated using a gradient solvent system consisting of 2 components, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The percentage of solvent B was increased in a linear fashion from 2 to 95% over 1 min; flow rate was 0.4 mL/min.

**Metabolite Identification.** The LC-MS/MS system consisted of two Shimadzu LC-20AD HPLC pumps, a DGU-14A degasser and an autosampler (Shimadzu, Columbia, MD), and a Therm LTQ-Orbitrap (ThermoElectron Corp., San Jose, CA). Electrospray ionization with positive ion detection was used. The source temperature was set at 250° C, and the ion spray voltage was held at 5 kV.

**Calculation of kinetic constants.** Kinetic constants apparent V<sub>max</sub> and K<sub>m</sub> were determined for compound 1, compound 2, compound 3, and compound 4 from depletion experiments with mouse plasma and liver microsomes by assuming that Michaelis-Menten kinetics were sufficient to describe the metabolic processes. Kinetic constants for the hydrolysis pathways in plasma or liver microsomes in the absence of NADPH were determined using the relationship:

\[
CL_{int, \text{hydrolysis}} = \frac{V_{\text{max, hydrolysis}}}{K_{\text{m, hydrolysis}} + C}
\]  

Eq. 2

where CL<sub>int,hydrolysis</sub> is the observed CL<sub>int</sub> due to hydrolysis in plasma or in liver microsomes in the absence of NADPH, and C is the concentration of test compound in the incubation.

CL<sub>int</sub> observed in incubations with mouse liver microsomes in the presence of NADPH (CL<sub>int,NADPH</sub>) reflects the contribution of hydrolysis together with NADPH-dependent processes. Therefore, the apparent kinetic constants for NADPH-dependent metabolism were estimated while considering the contribution of the kinetics of the hydrolysis pathway, as measured in incubations with mouse liver microsomes in the absence of NADPH. In other words, the
apparent $V_{\text{max}}$ and $K_m$ of the hydrolysis pathway were solved first from incubations in the absence of NADPH. The hydrolysis $\text{CL}_{\text{int}}$ term was calculated for specific concentrations and this information was fed into equation 3 in order to estimate the apparent $V_{\text{max}}$ and $K_m$ for NADPH-dependent metabolism in the presence of NADPH, as shown in the relationship:

$$\text{CL}_{\text{int}, \text{NADPH}} = \text{CL}_{\text{int}, \text{hydrolysis}} + V_{\text{max}, \text{NADPH}}/(K_m, \text{NADPH} + C) \quad \text{Eq. 3}$$

where $\text{CL}_{\text{int}, \text{hydrolysis}}$ is also concentration dependent and determined by fitting Eq. 2 to results from incubations in the absence of NADPH. Eqs. 2 and 3 were fit by nonlinear regression using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Metabolite formation velocity ($v$) was also simulated using the estimated apparent $V_{\text{max}}$ and $K_m$ values for hydrolysis or NADPH-dependent metabolism in plasma and liver microsomes using the relationship:

$$v = V_{\text{max}} * C/(K_m + C) \quad \text{Eq. 4}$$

**Prediction of CL from in vitro data.** CL caused by metabolism in the plasma and liver were predicted separately using the corresponding apparent kinetic constants. Maximum $\text{CL}_{\text{int}}$ ($\text{CL}_{\text{int}, \text{max}}$) for hydrolysis in plasma, hydrolysis in liver or NADPH-dependent metabolism in liver was calculated by:

$$\text{CL}_{\text{int}, \text{max}} = V_{\text{max}}/K_m \quad \text{Eq. 5}$$

CL caused by hydrolysis in plasma ($\text{CL}_{\text{plasma}}$) was considered independent of blood-flow and was predicted by the relationship
\[ CL_{\text{plasma}} = CL_{\text{int, max}} \times SF \]  \hspace{1cm} \text{Eq. 6}

where SF is a scaling factor with a value of 48.8 mL plasma per kg body weight in mice (Davies and Morris, 1993).

CL caused by combined hydrolysis and NADPH-dependent metabolism in the liver (CL_{\text{hepatic}}) was predicted using the well-stirred model:

\[
CL_{\text{hepatic}} = \frac{CL_{\text{int, hepatic}} \times SF_1 \times SF_2}{fu_{\text{mic}}} \times f_{ub} \times Q_h \times SF_{\text{hepatic}} CL_{\text{int, hepatic}} \times SF_1 \times SF_2}{fu_{\text{mic}}} \times f_{ub} + Q_h
\]  \hspace{1cm} \text{Eq. 7}

where CL_{\text{int, hepatic}} is the sum of the CL_{\text{int, max}} values from the hydrolysis and NADPH-dependent metabolic pathways in liver microsomes; SF1 and SF2 are scaling factors with values of 45 mg microsomal protein per g liver and 87.5 g liver per kg body weight in mice, respectively; f_{u,\text{mic}} is the fraction unbound in microsomes; f_{ub} is the fraction unbound in blood, equal to the fraction unbound in plasma (f_{up}) divided by the blood-to-plasma concentration ratio (Rb); and Q_h is the hepatic blood flow, or 5.4 L/(h*kg) in mice. Since a value for SF1 is not available for mouse liver, the value for SF1 used was that determined for rat liver (Houston and Galetin, 2008), and was assumed to reasonably represent mouse liver.
Results

**Compound 1 Studies.** As a prelude to conducting murine efficacy studies, we investigated the PK disposition of compound 1 in mice. During analysis, it was discovered that compound 1 rapidly degraded in plasma. Figure 1 shows the chromatogram of mouse plasma incubated with 5 μM compound 1 at 37 °C for 20 min. The major degradant was identified as resulting from hydrolysis of the benzamide moiety to release the corresponding primary amine with MH⁺ ion at m/z 456.3324 and the same chromatographic retention time as the chemically synthesized authentic standard. The product ion of the MH⁺ ion of compound 1 shows fragments of mass 435.2179 and 268.0879 together with the fluorobenzacyl cleavage fragments of the MH⁺ ion 313.2025 and 146.0711 (Figure 2). Furthermore, the product ion spectra of M1 and the authentic standard were identical.

Since the hydrolytic reaction was facilitated by increased temperature (data not shown), a method was developed that minimized the time from sample collection to extraction and the exposure of the matrix to temperatures greater than 4 °C during storage and processing. Quality control samples processed under similar conditions (see experimental) were within 20 % of nominal concentrations across the standard curve (relative standard deviations 5%-20%). In addition, no hydrolysis products were detected in the processed QC samples or standards. This method was used for analysis of compound 1 in mouse plasma and a mouse IV PK study with compound 1 was conducted successfully. As shown in Table 1, compound 1 was rapidly cleared from plasma with a half life of 1 hr and CL of 5.5 L/hr/kg. Compound 1 was stable to incubation with rat, dog, and human plasma and liver microsomes, indicating that the observed hydrolysis phenomenon was mouse specific. Table 2 shows the CL\textsubscript{int} values of compound 1 in liver microsomes from dog, human, rat, and mouse. The mouse is clearly unique as shown by the NADPH independent CL. To further investigate whether in vivo exposure in mice would permit achievement of free drug concentrations approaching the *in vitro* ALK IC50, we
proceeded to dose mice orally at 20, 50, and 100 mpk. Figure 3 shows the plasma levels of compound 1 and its hydrolysis product after PO dosing with 20, 50, 100 mpk. At a dose of 100 mpk, significant target coverage appeared to be achievable. It can be observed that the exposures were not dose proportional, with a decrease in CL/F increasing with dose (Table 3). In addition, the proportion of the exposure of hydrolysis product to compound 1 was significantly higher at the lower doses. The data suggested saturation kinetics, and additional results and discussion are given below. In response to the results above, the medicinal chemistry efforts continued toward the design of compounds with attenuated hydrolysis in mouse plasma.

Minimizing Hydrolysis. Discovery efforts focused on minimizing hydrolysis in mouse plasma showed that the biggest impact on mouse plasma stability was achieved by altering the nature of the substituents on the benzamide ring (Figure 4). Bulky electron withdrawing groups appear to decrease the rate while no substitution or substitution with electron donating groups appears to increase the rate. Figure 5 shows the PK profiles of three selected compounds (compounds 2, 3, and 4) with half-lives of 4 to 8 hrs. Compound 1 was included for comparison. Table 1 shows the non-compartmental PK parameters of the 4 compounds. A decrease in CL associated with increased plasma stability was observed.

Kinetics of hydrolysis in mouse plasma. Figure 6 shows the kinetics of metabolism of selected ALK inhibitors versus concentration in mouse plasma. The $V_{\text{max}}$ and $K_m$ values for the hydrolysis of these compounds in mouse plasma are shown in Table 4. The $V_{\text{max}}$ for compound 1 hydrolysis in plasma was significantly higher than the other compounds while the $K_m$s for all were comparable and lower than 1 μM.

Kinetics of metabolism in mouse liver microsomes. Considering that both hydrolysis and NADPH-dependent metabolic pathways could contribute to the elimination of these compounds, the concentration-dependent metabolism kinetics were also determined in liver microsomes in the absence (hydrolysis only) and presence (hydrolysis + NADPH-dependent metabolism) of
NADPH (Figure 7). The $V_{\text{max}}$ and $K_m$ values for the hydrolysis in the absence of NADPH and the NADPH-dependent metabolism of ALK inhibitors in mouse liver microsomes are shown in Table 4. The $V_{\text{max}}$ for the hydrolysis of compound 1 is significantly higher than the other compounds while the $K_m$ is comparable. As seen from Figure 7a, the total $CL_{\text{int}}$ (Hydrolysis+NADPH-dependent) and the hydrolytic $CL_{\text{int}}$ for compound 1 are similar, indicating very little contribution of oxidative CL pathways at the concentrations measured. For compounds 2, 3, and 4, the hydrolytic CL is a progressively smaller fraction as compared to the contribution of the NADPH-dependent pathways. The $K_m$ values for NADPH-dependent metabolism are larger than those for the hydrolysis pathway ($\sim 6 - 30x$ larger), indicating that the saturation of the hydrolysis pathway begins at lower concentrations than that of the NADPH-dependent pathways. It should be noted that uncertainty is evident in the estimates of $V_{\text{max}}$ and $K_m$ for the NADPH-dependent pathway for compound 1. This is because NADPH-dependent metabolism was estimated by subtracting out the contribution of the hydrolytic pathway. Since hydrolysis accounted for the vast majority of the metabolism ($>90\%$) of compound 1 in NADPH-fortified liver microsomes, only a small amount of residual activity remained from which to estimate the contribution of NADPH-dependent metabolism. Although there is likely uncertainty in the reported data, there is still some value in reporting them. For example, it is unlikely that the structural modification of compound 1 to give analogs 2, 3 and 4 resulted in meaningful changes to the extent of NADPH-dependent metabolism, as seen in the calculated $CL_{\text{int,max}}$ values (Table 4). Therefore, the primary effect of chemical modification was to reduce extent of hydrolysis among these ALK inhibitor analogs.

**IVIVE.** Plasma protein binding, microsomal binding, and blood to plasma ratio were measured after inactivation of the hydrolytic enzymes with diisopropylfluorophosphate. Recovery of drug in the presence of DFP was $>80\%$, indicating inhibition of hydrolytic activity. Blood to plasma concentration ratio for each compound was determined to be close to 1 (0.90-1.02 with SD 0.02-0.19), therefore $f_{\text{ub}}$ was taken to be equivalent to $f_{\text{up}}$. Table 5 shows the
results of the predictions from in vitro data and the comparison to observed in vivo CL. Maximal compound 1 CL$_{int}$ values in plasma and microsomes via hydrolysis were 71.4 µL/(min*mL) and 1876 µL/(min*mg), respectively. The maximal NADPH-dependent CL$_{int}$ for compound 1 was estimated to be 173 µL/(min*mg). The predicted CL attributed to hydrolysis in plasma, which was calculated by scaling for the total plasma volume, was 0.21 L/(h*kg). The predicted hepatic CL was 3.8 L/(h*kg). The observed CL of compound 1 was 5.52 L/(h*kg). This indicates that hydrolysis in the liver is likely a substantially more important contributor to CL than hydrolysis in the plasma following an IV dose in mice, despite the short t$_{1/2}$ in plasma incubations in vitro.

At low drug concentrations, compound 2 has similar CL$_{int}$ via hydrolysis and NADPH-dependent metabolism 175 and 153 µL/(min*mg), respectively. The maximal CL$_{int}$ in plasma was 10.7 µL/(min*mL). The predicted hepatic CL was 3.0 L/(h*kg), which was very close to the observed CL of 3.51 L/(h*kg). The predicted CL attributed to hydrolysis in plasma was 0.031 L/hr*kg, suggesting hydrolysis in plasma contributes very little to the systemic CL.

Compounds 3 and 4 had predicted hepatic CL of 1.6 and 1.2 L/(h*kg), respectively. The observed CL for compound 3 and 4 (2.14 and 2.16), were within approximately 2-fold of the predicted CL. Clearance for these analogs appeared to be primarily driven by NADPH-dependent metabolic pathways.
Discussions

*Compound 1 Hydrolysis.* The observation of a susceptibility to plasma hydrolysis of compound 1 early in the drug discovery phase could have presented significant obstacles in the progression of this class of ALK inhibitors. However, the sequence of experiments described here quickly quantified the obstacles and allowed a rational decision regarding the progression of compounds to the efficacy testing phase. The first problem was the stability of the compound in plasma. This led to inability to accurately quantify the plasma levels. Modification of the bioanalytical method to prevent hydrolysis post sample-collection enabled us to measure the plasma levels and obtain PK parameters that correctly reflected the properties of the compound in-vivo. The second obstacle to the progression was the possible presence or absence of the hydrolytic cleavage pathway in other species. Experiments to address the species selectivity showed that the enzymatic hydrolytic pathway was highly specific to mouse with no hydrolysis observed *in vitro* (liver microsomes and plasma) in rat, dog, and human. Furthermore, *in vivo*, the hydrolysis pathway was not detectable in the plasma of PK samples from rats and dogs. Species specificity with regard to enzyme mediated hydrolytic cleavage is not uncommon and has been observed for related amides (Eng et al., 2010; Hobbs and Twomey, 1981; Luan et al., 1997; Liu et al., 2011; Martin et al. 1997; Potts et al., 1989). High CL due to hydrolysis in mouse was problematic because of the desire to use readily available immune-compromised animals for the evaluation of in vivo efficacy in xenograft models of disease. Examining the PO PK closely showed a saturation of elimination as exemplified by the disproportional increase in exposure and the increase in bioavailability between the two lowest dose groups. Furthermore, the ratio of M1 exposure relative to compound 1 decreased with increased dose suggesting that the hydrolytic pathway was saturation limited. Metabolite M1 was also tested for activity in our ALK inhibition assays and showed no potency. Exposures levels achieved in the mice used in disease efficacy models were similar to those observed in the PK studies and full data will be
presented in a communication describing the evaluation of compound 1 in mouse xenograft models.

Minimizing Hydrolysis. The medicinal chemistry efforts attempted to design potent analogs with reduced hydrolytic potential. Minimizing enzyme mediated hydrolytic cleavage via changes to the substitution patterns of a benzamide moiety has been described by Douch and Gahagan in the metabolism of niclosamine (Douch and Gahagan, 1977). Their work showed that substituents ortho to the benzamide bond substantially decreased hydrolytic potential. Although this decrease in hydrolysis was attributed to steric hindrance, electronic effects may also be implicated since substantial decrease in hydrolysis may also be achieved by para substitution (Testa and Mayer, 2003). In the case of the ALK inhibitors described here, ortho substitution was not tolerated, most probably due to the distortion of the co-planar conformation which was required for an optimum interaction with the ALK protein (Lewis et al., 2012). Studies of meta or para substitution on the benzamide moiety demonstrated clear SAR. Di-substitutions and bulkier electronegative groups decreased the rate of hydrolysis. Unfortunately, the ALK-cell potencies of the most hydrolytically stable compounds decreased significantly relative to the less stable analogs. For example, the cell potencies of the compounds 1, 2, 3 and 4 were 4 nM, 6 nM, 333 nM, and 90 nM, respectively. The in vitro plasma t_{1/2} for these same compounds (from Fig 3), compound 1, 2, 3, and 4 were 0.2 hr, 2.9 hr, 8.2 hr, and 8.5 hr respectively. The IV PK of the compounds with minimized hydrolysis showed decreased CL and better exposures. The decrease in systemic CL exhibited by hydrolytically stable compounds suggested that the in vitro stability in plasma qualitatively related to the in vivo exposure. This led us to investigate the IVIVE relationship to learn if in vitro experimental results could quantitatively predict CL when hydrolysis contributes to elimination.

IVIVE. In our IVIVE experiments several features were notable. First, in initial experiments, it was found that the mouse microsomal CL_{int} of compound 1 at 0.01 µM was significantly higher than that at 1 µM; 898 µL/(min*mg) versus 110 µL/(min*mg). This suggested
that the hydrolytic pathway possessed a very low \( K_m \) and therefore experiments at 1 \( \mu M \) could dramatically under predict the \textit{in vivo} CL observed following a low IV dose. Indeed, more detailed experiments revealed that the \( K_m \)s of the hydrolytic reaction for all the ALK inhibitors cited in this work (obtained using the substrate depletion method at concentrations starting at single nanomolar levels) were far below 1 \( \mu M \), a default concentration often used in depletion experiments reported in the literature (e.g. Hallifax et al., 2010; Liu et al., 2011). Therefore it can be concluded that arbitrarily choosing a single substrate concentration might not always produce \( \text{CL}_{\text{int}} \) values that can be used for reasonable \textit{in vivo} prediction. Further investigation of saturation kinetics is recommended.

Secondly, since these ALK inhibitors were also hydrolyzed in mouse plasma, \textit{in vitro} plasma \( \text{CL}_{\text{int}} \) values were also experimentally obtained. The \( \text{CL}_{\text{int}} \) values in plasma were calculated using a similar approach to the microsomal work. The results show that the contribution of plasma hydrolysis to the overall CL is likely minor even for the compounds with the shortest plasma half-lives. This illustrates the potential pitfalls of following only rates of plasma hydrolysis to screen compounds for stability. Rowland and Riegleman (Rowland and Riegleman, 1968) have also elegantly demonstrated the relatively minor contribution of hydrolysis in blood to the total CL of acetylsalicylic acid via hydrolysis to salicylic acid. It seems that half-life determinations in plasma (or blood) can potentially be used help detect the presence of a hydrolysis mechanism but it should not be automatically inferred that plasma (or blood) is the main tissue responsible for elimination, even for compounds with short half-lives in plasma (or blood) in vitro. Subsequent scaling exercises, however, using in vitro half-life data and appropriate scaling factors can help estimate the contribution of hydrolysis in plasma to CL relative to the contribution of elimination or metabolism by other organs.

Thirdly, for microsomal \( \text{CL}_{\text{int}} \) experiments in mouse, both hydrolysis and NADPH-dependent metabolic pathways were considered. Metabolite identification clearly showed that the only metabolite formed in microsomes in the absence of NADPH was the hydrolysis product.
Obach and Reed-Hagen (2002) have shown that measurement of $K_m$ and $V_{max}$ by the substrate depletion method gives similar results as those obtained by conventional formation kinetics. In our work, the $CL_{int}$ from the incubation in the absence of NADPH was considered to be due to hydrolytic CL and the difference between the +NADPH and -NADPH CL values was attributed to NADPH-dependent (e.g. P450 mediated) reactions. There was good agreement between the predicted and the actual CL values for the compounds (~2-fold or less), with a slight trend toward underprediction of CL. Underprediction of systemic CL has been widely reported by others investigating IVIVE with liver microsomes or hepatocytes (e.g. Hallifax et al., 2010).

Explanations for the phenomenon have been proposed, including reduced (or a lack of) enzyme activity in various in vitro preparations compared to in vivo, study designs that do not consider saturation kinetics, metabolism by organs other than the liver, passive renal or hepatic elimination, impact of hepatic uptake transporters, and elimination by active transport. Conclusive explanations for lack of predictive IVIVE are rarely provided. For compounds that are hydrolyzed by esterases, metabolism in extrahepatic tissues could likely be a major reason for under prediction, since hydrolytic enzymes are present throughout the body. In the present example, contrary to expectations, hydrolysis in plasma appeared to contribute little to systemic CL, and consideration of hepatic metabolism alone was sufficient to achieve a reasonable prediction of CL. However, the possibility of some relatively minor contribution to CL by other tissues cannot be ruled out.

In summary, *in vitro* kinetic and *in vivo* PK studies indicated that the primary CL pathway of compound 1 in mouse, amide hydrolysis, was saturation limited, and that plasma levels of free drug exceeding in-vitro IC50 were achievable at higher doses. Reducing the extent of hydrolysis was possible through chemical modification of the compound 1 structure leading to compound 2 with better exposure at lower doses. Compound 2 has substantial efficacy in a murine xenograft model of ALK driven cancer (Lewis et al, 2012). Rendering this chemotype more stable to hydrolysis was associated with significant loss in potency against ALK *in vitro*. 

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Finally, from the prediction results it can be concluded that IVIVE methods can be used to predict hydrolytically mediated CL, provided that the *in vitro* assays thoroughly investigate the processes, including the contribution of other metabolic pathways and the possibility of saturation kinetics.
Acknowledgments

We thank Adria Colletti, Meghan Canfield and John Roberts for conducting the *in vivo* procedures.

Authorship Contributions

Participated in research design: Teffera, Lewis, Brake, Saffran, and Zhao

Conducted experiments: Moore, Liu, and Berry

Performed data analysis: Teffera, Berry, Moore, and Liu

Wrote or contributed to the writing of the manuscript: Teffera, Lewis, Berry, and Zhao
References


Figure Legends

Figure 1. Compound 1 in mouse plasma A) t=0 min, B) t =20 min, C) Hydrolysis Standard

Figure 2. Product ion of A) compound 1, B) Hydrolysis product from plasma, and C) Authentic hydrolysis product

Figure 3. Plasma concentrations of compound 1 (A) and metabolite M1 (B) following PO administration of compound 1 to mice.

Figure 4. Structure activity relationship (SAR) of Hydrolysis for Selected ALK Inhibitors (0.83 μM)

Figure 5. Plasma concentrations of ALK inhibitor analogs following 2 mg/kg IV administration to mice.

Figure 6. Intrinsic clearance (CL$_{int}$) and hydrolysis metabolite formation rate (v), respectively, for compound 1 (A and B), compound 2 (C and D), compound 3 (E and F), and compound 4 (G and H) at various concentrations in mouse plasma. Symbols are observed data, mean of two replicates. Lines represent the simulations using the best fit for apparent Vmax and Km values.

Figure 7. Intrinsic clearance (CL$_{int}$) and metabolite formation rate (v), respectively, for compound 1 (A and B), compound 2 (C and D), compound 3 (E and F), and compound 4 (G and H) at various concentrations in mouse liver microsomes in the presence and absence of NADPH. Symbols are observed data, mean of two replicates. Diamonds are total CL$_{int}$ or v in the presence of NADPH (hydrolysis + NADPH-dependent metabolism). Squares are CL$_{int}$ and v due to hydrolysis. Circles are metabolite formation and v due to NADPH-dependent metabolism. Lines represent the simulations using the best fit for apparent Vmax and Km values for hydrolysis and NADPH-dependent metabolism.
Table 1. Mean noncompartmental pharmacokinetics parameters for ALK inhibitors following 2 mg/kg IV administration to mice. A total of 27 animals (3 per time point) were used to generate the data for each compound.

<table>
<thead>
<tr>
<th></th>
<th>(\text{AUC}_{0-\infty})</th>
<th>CL</th>
<th>MRT</th>
<th>(T_{1/2})</th>
<th>Vss</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM*h</td>
<td>L/(h*kg)</td>
<td>h</td>
<td>h</td>
<td>L/kg</td>
<td></td>
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<tr>
<td>Compound 1</td>
<td>0.627</td>
<td>5.52</td>
<td>0.474</td>
<td>1.05</td>
<td>2.62</td>
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<tr>
<td>Compound 2</td>
<td>0.957</td>
<td>3.51</td>
<td>1.28</td>
<td>1.01</td>
<td>4.49</td>
</tr>
<tr>
<td>Compound 3</td>
<td>1.53</td>
<td>2.14</td>
<td>2.56</td>
<td>2.08</td>
<td>5.47</td>
</tr>
<tr>
<td>Compound 4</td>
<td>1.20</td>
<td>2.66</td>
<td>2.92</td>
<td>2.20</td>
<td>7.78</td>
</tr>
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</table>
Table 2. Intrinsic liver microsomal clearance ($CL_{int}$, $\mu$L/(min*mg)) of compound 1 in liver microsomes.

<table>
<thead>
<tr>
<th>Concentration $\mu$M</th>
<th>Mouse +NADPH</th>
<th>Rat +NADPH</th>
<th>Dog +NADPH</th>
<th>Human +NADPH</th>
<th>Mouse -NADPH</th>
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<tbody>
<tr>
<td>0.1</td>
<td>891</td>
<td>14</td>
<td>51</td>
<td>93</td>
<td>805</td>
</tr>
<tr>
<td>1</td>
<td>110</td>
<td>13</td>
<td>33</td>
<td>67</td>
<td>50</td>
</tr>
</tbody>
</table>

$CL_{int}$ in Rat, dog, and human -NADPH was <5 $\mu$L/(min*mg).
Table 3. Mean noncompartmental pharmacokinetics parameters for compound 1 and M1 following PO administration of compound 1 to mice. A total of 24 animals (3 per time point) were used to generate the data for each dose group.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-inf&lt;/sub&gt; (µM*h)</th>
<th>CL/F (L/(h*kg))</th>
<th>F&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.782</td>
<td>0.25</td>
<td>1.23</td>
<td>1.31</td>
<td>26.2</td>
<td>21.1</td>
</tr>
<tr>
<td>50</td>
<td>10.7</td>
<td>1.0</td>
<td>2.30</td>
<td>23.7</td>
<td>3.65</td>
<td>151</td>
</tr>
<tr>
<td>100</td>
<td>15.2</td>
<td>1.0</td>
<td>2.17</td>
<td>37.4</td>
<td>4.63</td>
<td>120</td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.881</td>
<td>1.0</td>
<td>ND</td>
<td>2.99</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50</td>
<td>2.75</td>
<td>2.0</td>
<td>ND</td>
<td>10.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>100</td>
<td>3.27</td>
<td>4.0</td>
<td>ND</td>
<td>23.3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND indicates not determined. T<sub>max</sub> is defined as the time at which the maximum concentration of drug in plasma (C<sub>max</sub>) is observed. <sup>a</sup> Relative to 2 mg/kg IV dose.
Table 4. Apparent $V_{\text{max}}$ and $K_{\text{m}}$ values ± S.D. for hydrolysis or NADPH-dependent metabolism of the test compounds in mouse plasma or mouse liver microsomes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plasma Hydrolysis</th>
<th>Liver Microsomes Hydrolysis</th>
<th>NADPH-dependent metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_{\text{m}}$</td>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td></td>
<td>pmol/(min*ml)</td>
<td>µM</td>
<td>pmol/(min*mg)</td>
</tr>
<tr>
<td>Compound 1</td>
<td>19.7 ± 5.6</td>
<td>0.276 ± 0.088</td>
<td>82.0 ± 6.0</td>
</tr>
<tr>
<td>Compound 2</td>
<td>3.81 ± 0.85</td>
<td>0.357 ± 0.088</td>
<td>5.12 ± 0.43</td>
</tr>
<tr>
<td>Compound 3</td>
<td>0.305 ± 0.133</td>
<td>0.146 ± 0.072</td>
<td>2.49 ± 0.63</td>
</tr>
<tr>
<td>Compound 4</td>
<td>1.14 ± 0.16</td>
<td>0.680 ± 0.103</td>
<td>5.38 ± 0.32</td>
</tr>
</tbody>
</table>

$^a$ Model fit parameters for NADPH-dependent metabolism of compound 1 yielded >100% error. Please see text for more information.
Table 5. Prediction of *in vivo* CL in mouse from *in vitro* data for 4 ALK inhibitor analogs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\text{CL}_{\text{int, max}}$</th>
<th>$f_{\text{uninc}}$</th>
<th>$f_{\text{lab}}$</th>
<th>Predicted CL</th>
<th>In vivo CL</th>
<th>Ob/Pr*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plasma</td>
<td>liver microsomes</td>
<td>plasma</td>
<td>hepatic</td>
<td>plasma</td>
<td>hepatic</td>
</tr>
<tr>
<td></td>
<td>µL/(min*mg)</td>
<td>µL/(min*mg)</td>
<td>µL/(min*mg)</td>
<td>L/(h*kg)</td>
<td>L/(h*kg)</td>
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<tr>
<td>2541103</td>
<td>71.4</td>
<td>1876</td>
<td>173</td>
<td>0.76</td>
<td>0.020</td>
<td>0.21</td>
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<tr>
<td>2559588</td>
<td>10.7</td>
<td>175</td>
<td>153</td>
<td>0.53</td>
<td>0.046</td>
<td>0.031</td>
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<tr>
<td>2560139</td>
<td>2.09</td>
<td>47.0</td>
<td>147</td>
<td>0.22</td>
<td>0.011</td>
<td>0.0061</td>
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<tr>
<td>2560142</td>
<td>1.68</td>
<td>20.6</td>
<td>99.5</td>
<td>0.22</td>
<td>0.012</td>
<td>0.0049</td>
</tr>
</tbody>
</table>

*Ob/Pr-Observed/Predicted*
Figure 3
Figure 5

Plasma Concentration (μM) vs. Time (h)

- Compound 1
- Compound 2
- Compound 3
- Compound 4
Figure 6