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

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ABSTRACT

Compound 1 [(E)-4-fluoro-N-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)-1-((1S,4S)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzo[d][1,2,3]imidazol-2(3H)-ylidene)benzamide], 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 i.v. pharmacokinetics studies in mice showed that compound 1 had high clearance (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 as candidate to advance into efficacy studies in mice and showed decreased CL corresponding to roughly 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)-1-((1S,4S)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzo[d][1,2,3]imidazol-2(3H)-ylidene)benzamide] had high clearance (CL) and a short half-life. Oral pharmacokinetics studies in mice showed that compound 1 had high clearance (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 as candidate to advance into efficacy studies in mice and showed decreased CL corresponding to roughly investigate the processes, including the contribution of other metabolic pathways and the possibility of saturation kinetics.

Abbreviations: ALK, anaplastic lymphoma kinase; AUC, area under the curve; AUC₀–inf, area under the plasma concentration-time curve extrapolated to infinity; CL, clearance; CL₀–inf, intrinsic clearance; compound 1, (E)-4-fluoro-N-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)-1-((1S,4S)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzo[d][1,2,3]imidazol-2(3H)-ylidene)benzamide; compound 2, (E)-3,5-difluoro-N-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)-1-((1S,4S)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzo[d][1,2,3]imidazol-2(3H)-ylidene)benzamide; compound 3, (E)-3-chloro-5-fluoro-N-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)-1-((1S,4S)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzo[d][1,2,3]imidazol-2(3H)-ylidene)benzamide; compound 4, (E)-N-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)-1-((1S,4S)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzo[d][1,2,3]imidazol-2(3H)-ylidene)-3-(trifluoromethyl)benzamide; DFP, diisopropyl fluorophosphate; F, bioavailability; fub, fraction unbound in blood; fup, fraction unbound in plasma; IVIVE, in vitro–in vivo extrapolation; LC–MS/MS, liquid chromatography–tandem mass spectrometry; M1, (1S,4S)-4-(2-amino-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)methyl)-1H-benzo[d][1,2,3]imidazol-1-yl)-N-isopropylcyclohexanecarboxamide; DFP, diisopropyl fluorophosphate; F, bioavailability; fub, fraction unbound in blood; fup, fraction unbound in plasma; IVIVE, in vitro–in vivo extrapolation; LC–MS/MS, liquid chromatography–tandem mass spectrometry; M1, (1S,4S)-4-(2-amino-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)methyl)-1H-benzo[d][1,2,3]imidazol-1-yl)-N-isopropylcyclohexanecarboxamide; MRT, mean residence time; P450, cytochrome P450; PK, pharmacokinetics; QC, quality control; t₁/₂, half-life; VSS, volume at steady state.
imidazol-2(3H)-ylidene]benzamide], is a member of a new class of potent and selective ALK 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 (IC_{50} = 1 nM) and ALK expressing Karpas-299 cells (4 nM) (Lewis et al., 2012). Rat in vivo studies showed acceptable pharmacokinetics (PK) with a clearance (CL) of 0.8 l/h per kg, a half-life (t_{1/2}) of 5.6 hours, and mean residence time (MRT) of 8 hours (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. Enzyme-mediated hydrolytic metabolism of compound 1 was examined to determine whether it would be a liability in the further development of this molecule for potential use in humans.

Although not as common as cytochrome P450 (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, thiesters, 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 preceded. For example, considerable species differences in extent of hydrolysis were observed for lidocaine (Lewis et al., 1997), pranlukast, (Luan et al., 1997), piroxicam (Hobbs and Twomey, 1998), 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 (R)-N-(3-(6-(4-(1,4-dimethyl-3-oxopiperazin-2-yl)phenylamino)-4-methyl-5-oxo-4,5-dihydropyrazin-2-yl)-2-methylphenyl)-4,5,6,7-tetrahydrobenzo[h]thiophene-2-carboxamide (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 vitro assays that may contain the hydrolytic enzymes, and instability in bioanalytical matrices such as blood or plasma. Instability 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 PK before advancement of the compound to the clinic. For example, although they performed kinetic studies to determine V\text{max} and K\text{m} through formation of the hydrolysis metabolite in multiple species, Liu et al. (2011) used the substrate depletion method at a single arbitrary concentration to arrive at the predicted CL. They reported substantial uncertainty in the in vitro–in vivo extrapolation (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.

In a discovery setting, extrahepatic metabolism, such as hydrolysis in plasma, is occasionally proposed as a possible explanation for poor predictivity of IVIVE based on data obtained from liver microsomes or hepatocytes. In no knowledge, there are no reports in the literature that deal with an IVIVE method for hydrolysis that take into account plasma hydrolysis, saturation kinetics, and contribution of NADPH-dependent metabolism. In this article we report our experiences with the hydrolysis of compound 1, 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 humans (n = 50) were purchased from BD Biosciences (San Jose, CA). Plasma from male Sprague-Dawley rats, CD-1 mice, beagle dogs, and humans was purchased from Bioreclamation, Inc. (Westbury, CT). All fresh plasma and blood were 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 hours. For initial stability screening in mouse plasma, test compounds were incubated at a concentration of 0.83 μM. For determination of metabolism kinetics in plasma, plasma samples were incubated at a compound concentration range of 0.0023–5 μM. At various time points, samples were collected from the incubations into acetone containing internal standard (tolbutamide), centrifuged at 3500g for 15 min, and analyzed by liquid chromatography-tandem mass spectrometry (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-min 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 following relationship shown in eq. 1 (Obach and Reed-Hagen, 2002):

\[
CL_{int, obs} = \frac{0.693}{t_{1/2}} \times \frac{1}{M}
\]  

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 Microsomes.** 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 3500g for 15 min, and analyzed by LC–MS/MS. Subsequent metabolism kinetics (V\text{max} and K\text{m}) for compound 1, compound 2 (E)-(3,5-difluoro-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, compound 3 (E)-3-chloro-5-fluoro-N-(6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)methyl)-1-(1s,4s)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzod[3H,4H]imidazol-2(3H)-ylidene]benzamide, and compound 4 (E)-N-(6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)methyl)-1-(1s,4s)-4-(isopropylcarbamoyl)cyclohexyl)-1H-benzox[d]imidazol-2(3H)-ylidene]-3-(trifluoromethyl)benzamide) in mouse liver microsomes were determined as above in the presence and absence of NADPH at a compound concentration range of 0.0023–5 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, 0.5, or 2 μM test compound were dialyzed against 500 μl of phosphate-buffered saline for 7 hours at 37°C in an incubator maintained at 5% CO₂. Preliminary experiments
with selected compounds indicated that a 7-hour incubation time was sufficient to achieve equilibrium. Since some test compounds were rapidly hydrolyzed in plasma, plasma was preincubated 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 that DFP did an adequate job of preventing hydrolysis. Aliquots of the postdialysis buffer were transferred in 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), and 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 postdialysis 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 microsome solution in phosphate buffer.

**Blood to Plasma Ratio.** The blood to plasma ratio 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 hour, 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 preincubation for 20 min with 100 μM 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. The blood to plasma ratio 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 (n = 60) were housed in a humidity- and temperature-controlled environment subject to a 12/12-hour 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 hours postdose. After a 5-day acclimation period, the mice were administered a single dose of test material by bolus i.v. injection through the lateral tail vein (2 mg/kg) or by oral gavage (20, 50, 100 mg/kg). Mice were euthanized by CO2 at 150 min. Blood to Plasma Ratio.

**Analysis of Pharmacokinetic Samples.** The LC–MS/MS system consisted of a Acquity UltraHPLC Performance Liquid Chromatography system (Waters, Milford, MA) and an API4000 mass spectrometer (Applied Biosystems, Foster City, CA) equipped with either an atmospheric pressure chemical ionization source or electrospray ionization source. For each analyte, the mass ionization source or electrospray ionization source. For each analyte, the mass of 1,578.4 → m/z 435.3; compound 2, m/z 596.4 → m/z 453.3; compound 3, m/z 612.3 → m/z 469.2; and 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 10,000 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 UltraHPLC Performance Liquid Chromatography Phenyl 1.7 μm Bridged Ethane-silicon Hybrid column (2.1 x 50 mm Waters) with 0.5 μM Poly-Ethylene-Ether-Ketone 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, and then maintained at 95% for an additional 0.5 min; flow rate was 0.6 ml/min.

**Noncompartmental PK Analysis.** The noncompartmental PK parameters were calculated using SMDA Watson software (version 7.0.0.01; Thermo Fisher Scientific). The area under the plasma concentration-time curve from time 0 to the last time point (t) with a quantifiable concentration (CA) was calculated using the linear trapezoidal rule. The area under the plasma concentration-time curve was extrapolated to infinite time (AUC0–inf) by the following equation: AUC0–inf = AUC0–t + C/t/k, where k is the elimination rate constant determined by linear regression of the last 2–4 quantifiable data points in the log plasma concentration-time curve. Plasma clearance (CL) was calculated using the following equation: MRT = V CL. Bioavailability (F) was calculated using the following equation: F = (AUCinf/C0) × (Dose/C0). Where AC and AUCinf, are area under the curve (AUC) estimates after the i.v. and oral administration, respectively, and Dose is the administered dose for i.v. 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 high-performance liquid chromatography pumps and a DGU-14A degasser (Shimadzu, Columbia, MD), a High-Throughput- Screening Prep-And-Load autoinjector (Leap Technologies, Carboro, 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; and 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 Poly-Ethylene-Ether-Ketone guard filter. Analytes were separated using a gradient solvent system consisting of two 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 0 to 95% over 1 min; the flow rate was 0.4 ml/min.

**Metabolite Identification.** The LC–MS/MS system consisted of two Shimadzu LC-20AD high-performance liquid chromatography pumps, a DGU-14A degasser, and an autosampler (Shimadzu, Columbia, MD), and a Therm LTQ-Orbitrap (ThermoElectro 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 Vmax and Km for the hydrolysis of test compounds in plasma or liver microsomes by assuming that Michaelis-Menten kinetics was sufficient to describe the metabolic processes. Kinetic constants for the hydrolysis pathways in plasma or liver microsomes in the absence of NAPDH were determined using the relationship shown in eq. 2:

\[
\text{CL}_{\text{int,hydrolysis}} = \frac{\text{V}_{\text{max,hydrolysis}}}{K_{\text{m,hydrolysis}}} + C
\]

where CL_{int,hydrolysis} is the observed CL_{int} due to hydrolysis in plasma or in liver microsomes in the absence of NAPDH, and C is the concentration of test compound in the incubation.

CL_{int,observed} is the observed in incubations with mouse liver microsomes in the presence of NAPDH (CL_{int,NAPDH}) reflects the contribution of hydrolysis together with NAPDH-dependent processes. Therefore, the apparent kinetic constants for NAPDH-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 eq. 3 to estimate the apparent \( V_{\text{max}} \) and \( K_m \) for NADPH-dependent metabolism in the presence of NADPH, as shown in the following relationship:

\[
\text{CL}_{\text{int,NADPH}} = \text{CL}_{\text{int,hydrolysis}} + \frac{V_{\text{max,NADPH}}}{K_m,\text{NADPH} + C}
\]  

(3)

where \( \text{CL}_{\text{int,hydrolysis}} \) is also concentration dependent and determined by fitting eq. 2 to results from incubations in the absence of NADPH. Equations 2 and 3 were fit by nonlinear regression using GraphPad Prism software (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 shown in eq. 4:

\[
v = \frac{V_{\text{max}} \times C}{K_m + C}
\]  

(4)

**Prediction of CL from In Vitro Data.** CL caused by metabolism in the plasma and liver was predicted separately using the corresponding apparent kinetic constants. Maximum \( \text{CL}_{\text{int}} \) (\( \text{CL}_{\text{int,max}} \)) for hydrolysis in plasma, hydrolysis in liver, or NADPH-dependent metabolism in liver was calculated using eq. 5:

\[
\text{CL}_{\text{int,max}} = \frac{V_{\text{max}}}{K_m}
\]  

(5)

CL caused by hydrolysis in plasma (\( \text{CL}_{\text{plasma}} \)) was considered independent of blood flow and was predicted by the relationship shown in eq. 6:

\[
\text{CL}_{\text{plasma}} = \text{CL}_{\text{int,max}} \times SF
\]  

(6)

where SF is a scaling factor with a value of 48.8 ml plasma per kg body weight and was predicted 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.

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. QC samples processed under similar conditions (see Materials and Methods) were within 20% of nominal concentrations across the standard curve (relative standard deviations of 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 i.v. 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 hour and CL of 5.5 l/h-kg.

**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. Fig. 1 shows the chromatogram of mouse plasma incubated with 5 \( \mu \)M 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 M1 [(1S,4S)-4-(2-amino-6-(4-(2-hydroxypropan-2-yl)piperidin-1-yl)methyl)-1H-benzo[d]imidazol-1-yl)-N-isopropylcyclohexanecarboxamide] with \( \text{MH}^+ \) ion at \( m/z \) 456.3324 and the same chromatographic retention time as the chemically synthesized authentic standard. The product ion of the \( \text{MH}^+ \) ion of compound 1 shows fragments of mass 435.2179 and 268.0879 together with the fluorobenzacyl cleavage fragments of the \( \text{MH}^+ \) ion at 313.2025 and 146.0711 (Fig. 2). Furthermore, the product ion spectra of M1 and the authentic standard were identical.

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![Fig. 1. Compound 1 in mouse plasma after 0 min incubation (A), compound 1 in mouse plasma after 20 min incubation (B), and authentic standard of hydrolysis product, M1, in phosphate buffer solution (C).](image-url)
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 CLint 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. Fig. 3 shows the plasma levels of compound 1 and its hydrolysis product after oral 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 (Fig. 4). Bulky electron withdrawing groups appear to decrease the rate, whereas no substitution or substitution with electron donating groups appears to increase the rate. Fig. 5 shows the PK profiles of three selected compounds (compounds 2, 3, and 4) with half-lives of 4–8 hours. Compound 1 was included for comparison. Table 1 shows the noncompartmental PK parameters of the four compounds. A decrease in CL associated with increased plasma stability was observed.

### Kinetics of Hydrolysis in Mouse Plasma

Fig. 6 shows the kinetics of metabolism of selected ALK inhibitors versus concentration in mouse plasma. The Vmax and Km values for the hydrolysis of these compounds in mouse plasma are shown in Table 4. The Vmax for compound 1 hydrolysis in plasma was significantly higher than

![Product ion mass spectrum of compound 1 (A), hydrolysis product from plasma (B), and authentic hydrolysis product (C).](image)

**Fig. 2.** Product ion mass spectrum of compound 1 (A), hydrolysis product from plasma (B), and authentic hydrolysis product (C).

### Table 1

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<th>Compound</th>
<th>AUC0-&gt;t_C</th>
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<td>l/h kg</td>
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</table>

A total of 27 animals (3 per time point) were used to generate the data for each compound.

### Table 2

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

CLint in rat, dog, and human −NADPH was <5 μl/min·mg.

### Table 3

Intrinsic liver microsomal clearance (CLint, μl/min·mg) of compound 1 in liver microsomes.
other compounds, whereas the \( K_m \) values for all were comparable and lower than 1 \( \mu M \).

**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 (Fig. 7). The \( V_{max} \) 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 \( V_{max} \) for the hydrolysis of compound 1 is significantly higher than the other compounds, whereas the \( K_m \) is comparable. As seen from Fig. 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 compared with the contribution of the NADPH-dependent pathways. The \( K_m \) values for NADPH-dependent metabolism are larger than those for the hydrolysis pathway (approximately 6–30× 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 \( V_{max} \) 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 (\( \geq 90\% \)) 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

**Fig. 3.** Plasma concentrations of compound 1 (A) and metabolite M1 (B) after oral administration of compound 1 to mice.

**TABLE 3**

Mean noncompartmental pharmacokinetics parameters for compound 1 and M1 after oral administration of compound 1 to mice

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>( C_{max} ) (( \mu M ))</th>
<th>( T_{max} ) (h)</th>
<th>( t_{1/2} ) (h)</th>
<th>AUC\text{0-inf} (( \mu M\text{h} ))</th>
<th>CL\text{/F} (l/h • kg)</th>
<th>F%</th>
</tr>
</thead>
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<tr>
<td>Compound 1</td>
<td>20</td>
<td>0.782</td>
<td>0.25</td>
<td>1.23</td>
<td>1.31</td>
<td>26.2</td>
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<tr>
<td></td>
<td>50</td>
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<td>3.65</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15.2</td>
<td>1.0</td>
<td>2.17</td>
<td>37.4</td>
<td>4.63</td>
</tr>
<tr>
<td>M1</td>
<td>20</td>
<td>0.881</td>
<td>ND</td>
<td>1.0</td>
<td>2.99</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.75</td>
<td>ND</td>
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<td>10.9</td>
<td>ND</td>
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<td>3.27</td>
<td>ND</td>
<td>4.0</td>
<td>23.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined; \( T_{max} \) is defined as the time at which the maximum concentration of drug in plasma \( (C_{max}) \) is observed; \( V_{ss} \), volume at steady state.

* Relative to 2 mg/kg i.v. dose.

**Fig. 4.** Structure activity relationship of hydrolysis for selected ALK inhibitors.

**Fig. 5.** Plasma concentrations of ALK inhibitor analogs after 2 mg/kg i.v. administration to mice.
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 \( \text{CL}_{\text{int, max}} \) values (Table 5). 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 BP 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. The blood to plasma concentration ratio for each compound was determined to be close to 1 (0.90–1.02 with S.D. of 0.02–0.19); therefore, \( f_{\text{up}} \) was taken to be equivalent to \( f_{\text{up}} \). Table 5 shows the results of the predictions from in vitro data and the comparison with observed in vivo CL. Maximal compound 1 \( \text{CL}_{\text{int}} \) values in plasma and microsomes via hydrolysis were 71.4 \( \mu \text{l/min}\times\text{ml} \) and 1876 \( \mu \text{l/min}\times\text{mg} \), respectively. The maximal NADPH-dependent \( \text{CL}_{\text{int}} \) for compound 1 was estimated to be 173 \( \mu \text{l/min}\times\text{mg} \). 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

![Fig. 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 V_{max} and K_{m} values.](image-url)
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 after an i.v. 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 $\mu$/min-mg, respectively. The maximal CL$_{int}$ in plasma was 10.7 $\mu$/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/h/kg, suggesting that 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) was within approximately 2-fold of the predicted CL. CL for these analogs appeared to be primarily driven by NADPH-dependent metabolic pathways.

**Discussion**

**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 design 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 after 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 samples. 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 (Hobbs and Twomey, 1981; Potts et al., 1989; Luan et al., 1997; Martin et al., 1997; Eng et al., 2010; Liu et al., 2011). High CL due to hydrolysis in mouse was problematic because of the desire to use readily available immunocompromised animals for the evaluation of in vivo efficacy in xenograft models of disease. Examining the oral 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. Exposure levels achieved in 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 was described by Douch and Ghagahan (1977) in the metabolism of niclosamide. 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 likely due to the distortion of the coplanar conformation that 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 a clear structure activity relationship. Disubstitutions 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 hours, 2.9 hours, 8.2 hours, and 8.5 hours, respectively. The i.v. 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 whether 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 $\mu$/M was significantly higher than that at 1 $\mu$/M (898 $\mu$/min-mg versus 110 $\mu$/min-mg). This suggested that the hydrolytic pathway possessed a very low $K_m$ and therefore experiments at 1 $\mu$/M could dramatically underpredict the in vivo CL observed after a low i.v. dose. Indeed, more detailed experiments revealed that the $K_m$ values 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hydrolysis</th>
<th>NADPH-Dependent Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Liver Microsomes</td>
</tr>
<tr>
<td>V$_{max}$</td>
<td>$K_m$</td>
<td>V$_{max}$</td>
</tr>
<tr>
<td>1</td>
<td>19.7 ± 5.6</td>
<td>0.276 ± 0.088</td>
</tr>
<tr>
<td>2</td>
<td>5.81 ± 0.85</td>
<td>0.357 ± 0.088</td>
</tr>
<tr>
<td>3</td>
<td>0.305 ± 0.133</td>
<td>0.146 ± 0.072</td>
</tr>
<tr>
<td>4</td>
<td>1.14 ± 0.16</td>
<td>0.680 ± 0.103</td>
</tr>
</tbody>
</table>
a single substrate concentration might not always produce CL\text{int} values that can be used for reasonable in vivo prediction. Further investigation of saturation kinetics is recommended.

Second, since these ALK inhibitors were also hydrolyzed in mouse plasma, in vitro plasma CL\text{int} values were also experimentally obtained. The 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 Riegelman (1968) have 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

![Intrinsic clearance (CL\text{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\text{int} or v in the presence of NADPH (hydrolysis + NADPH-dependent metabolism). Squares are CL\text{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 V\text{max} and K\text{m} values for hydrolysis and NADPH-dependent metabolism.](image-url)
hydrolysis in plasma to CL relative to the contribution of elimination or metabolism by other organs.

Third, for microsomal CL_{mic} experiments in mouse, both hydrolysis and NADPH-dependent metabolic pathways were considered. Metabolite identification clearly showed that the only metabolite formed in the absence of NADPH was the hydrolysis product. Obach and Reed-Hagen (2002) showed 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 (approximately 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 with 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 underprediction, 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, aside hydrolysis, was saturation limited, and that plasma levels of free drug exceeding in vitro IC_{50} 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. Finally, it can be concluded from the prediction results 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

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

Impact of Hydrolysis on the PK of ALK Inhibitors

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<tr>
<th>Compound</th>
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<th>f_{u,mic}</th>
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<tr>
<td></td>
<td>µl/min ml</td>
<td>µl/min mg</td>
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<tr>
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</table>

<table>
<thead>
<tr>
<th>µl/min mg</th>
<th>l/h kg</th>
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<tbody>
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<td>1.66</td>
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<td>1.4</td>
<td>2.2</td>
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Authorship Contributions

Participated in research design: Tuffera, Brake, Lewis, Safran, Zhao.

Conducted experiments: Berry, Moore, Liu.

Performed data analysis: Tuffera, Berry, Moore, Liu.

Wrote or contributed to the writing of the manuscript: Tuffera, Berry, Lewis, Zhao.

References


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