Cynomolgus Monkey as a Surrogate for Human Aldehyde Oxidase

Metabolism of the EGFR Inhibitor BIBX1382

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Non-Standard Abbreviations:

EGFR, epidermal growth factor receptor; AO, aldehyde oxidase; ADME, absorption, distribution, metabolism, and excretion; MeCN, acetonitrile; P450, cytochrome P450; Cl_{int}, intrinsic clearance; Cl_{h}, hepatic clearance; HLM, human liver microsomes; Q_{h}, liver blood flow
Abstract

BIBX1382 was an epidermal growth factor receptor (EGFR) inhibitor under clinical investigation for treatment of cancer. This candidate possessed an attractive pre-clinical ADME profile, yet failed in clinical studies due in part to poor oral exposure, resulting from extensive metabolism by aldehyde oxidase (AO). In vitro metabolism studies were performed in liver cytosol and cryopreserved hepatocytes from multiple species. In addition, a pharmacokinetic study was performed in cynomolgus monkey for comparison to the reported human pharmacokinetics of BIBX1382. Estimated hepatic clearance of BIBX1382 in rhesus (42 ml/min/kg) and cynomolgus monkey (43 ml/min/kg) liver cytosol was comparable to human (≥93% of liver blood flow). Metabolite identification following incubation of BIBX1382 in liver cytosol fortified with the AO inhibitor raloxifene confirmed that AO is involved in the formation of the predominant metabolite (BIBU1476, M1) in cynomolgus monkey. Following intravenous and oral administration of BIBX1382 to cynomolgus monkeys, high plasma clearance (118 ml/min/kg), and low oral exposure (Cmax=12.7 nM and 6% oral bioavailability) was observed, with the exposure of M1 exceeding BIBX1382 following oral dosing. This pharmacokinetic profile compared favorably with the human clinical data of BIBX1382 (plasma clearance 25-55 ml/min/kg and 5% oral bioavailability). Thus, it appears that cynomolgus monkey represents a suitable surrogate for the observed human AO metabolism of BIBX1382. To circumvent clinical failures due to uncharacterized metabolism by AO, in vitro studies in the appropriate subcellular fraction, followed by pharmacokinetic and toxicokinetic studies in the appropriately characterized surrogate species should be conducted for substrates of AO.
Introduction

BIBX1382 (N\textsuperscript{8}-(3-chloro-4-fluorophenyl)-N\textsuperscript{2}-(1-methylpiperidin-4-yl)pyrimido[5,4-d]pyrimidine-2,8-diamine) (Figure 1) was a clinical drug candidate under investigation as an inhibitor of the tyrosine kinase epidermal growth factor receptor (EGFR) for the treatment of cancer (Solca et al., 2004). The disposition of BIBX1382 has been previously described, and was reported to be relatively stable in vitro in human liver microsomes (HLMs), although metabolized to some degree by cytochrome P450 2D6 (Dittrich et al., 2002). In addition, preclinical pharmacokinetic studies in rats and mice revealed absolute oral bioavailability to be high, ranging from 50 to 100%. Despite this attractive preclinical drug metabolism-pharmacokinetic (DMPK) profile, the clearance of BIBX1382 from plasma following intravenous (i.v.) infusion dosing to humans was 25-55 ml/min/kg, a rate in excess of liver blood flow (20.7 ml/min/kg). In addition, following oral administration, plasma levels of BIBX1382 were well below target concentrations expected for efficacy (5% mean absolute oral bioavailability), resulting in the rapid attrition of this drug candidate (Dittrich et al., 2002).

Metabolite scouting investigations uncovered an oxidative metabolite circulating in human plasma at concentrations exceeding that of BIBX1382 (Dittrich et al., 2002). Retrospective experiments by Dittrich et al. provided evidence that BIBX1382 was metabolized by hepatic aldehyde oxidase (AO), although supporting data were not included. In a recent report, our lab demonstrated that the predominant metabolite following incubation of BIBX1382 in cryopreserved human hepatocytes had a retention time and fragmentation pattern matching that of the authentic standard metabolite, BIBU1476 (4-((3-chloro-4-fluorophenyl)amino)-6-((1-methylpiperidin-4-yl)amino)pyrimido[5,4-d]pyrimidin-2(4aH)-one), with the position of oxidation occurring on the pyrimido-pyrimidine core (Figure 1) (Hutzler et al., 2012). The role
of aldehyde oxidase in the production of BIBU1476 was confirmed by a decrease in the observed in vitro clearance in cryopreserved human hepatocytes when the AO-selective inhibitor hydralazine was co-incubated (Hutzler et al., 2012), an in vitro phenotyping methodology also reported by Strelevitz et al. (Strelevitz et al., 2012).

Aldehyde oxidase is a molybdenum cofactor-containing drug-metabolizing enzyme that is active as a homo-dimer and is composed of two identical ~150 kDa subunits. AO is responsible for metabolizing a variety of aldehydes and heterocyclic-containing drug molecules (Beedham, 1987; Kitamura et al., 2006; Garattini et al., 2008). Current in vitro ADME screening paradigms, where metabolic stability of new chemical entities are evaluated using liver microsomal fractions, do not capture the contribution of AO, since this enzyme is present in the cytosolic fraction. An additional complication with the early identification of AO as a relevant human metabolic pathway is profound species differences in activity, where rats in general possess low activity (depending on strain), and dogs are completely devoid of activity (Beedham et al., 1987; Garattini et al., 2008). A recent review by Garattini and Terao suggested that rhesus monkey may be an appropriate surrogate for human AO activity (Garattini and Terao, 2012). This proposal is supported by findings with BIBX1382, where the AO metabolic mechanism was confirmed in rhesus monkey (Dittrich et al., 2002). Other reports have demonstrated that the activity of AO in cynomolgus monkey may also be representative of the human situation (Diamond et al., 2010; Morrison et al., 2012). Thus, the objective of these studies was to evaluate the cynomolgus monkey as a surrogate species for the AO-mediated metabolism of BIBX1382 observed in humans.
Materials and Methods

Reagents. BIBX1382, BIBU1476 and BIBU1361 (N-(3-chloro-4-fluorophenyl)-6-(4-((diethylamino)methyl)piperidin-1-yl)pyrimido[5,4-d]pyrimidin-4-amine) were acquired from the internal compound library at Boehringer-Ingelheim Pharmaceuticals (Ridgefield, CT). Potassium phosphate buffer, raloxifene, allopurinol, chloroquine, and eucatropine were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human (10 donor pool, mixed gender), wistar-han rat (15 donor pool, male), beagle dog (3 donor pool, male), and cynomolgus monkey (3 donor pool, male) liver cytosol were purchased from BioreclamationIVT (Baltimore, MD). Rhesus monkey liver cytosol (3 donor pool, male), and S9 fractions from human kidney (4 donor pool, mixed gender), lung (4 donor pool, mixed gender) and intestine (18 donor pool, mixed gender), and cynomolgus monkey kidney (5 donor pool, male), lung (5 donor pool, male), and intestine (9 donor pool, male), were purchased from Xenotech LLC (Lenexa, KS). Cryopreserved human (19 donor pool, mixed gender), and cynomolgus monkey (3 donor pool, male) hepatocytes were purchased from BioreclamationIVT (Baltimore, MD). Hepatocyte thawing and incubation media and reagents were purchased from BioreclamationIVT (Baltimore, MD) and Invitrogen (Carlsbad, CA). All other reagents and chemicals were of the highest purity available.

Metabolism of BIBX1382 in Liver Cytosol and Extrahepatic S9 Fraction. The metabolism of BIBX1382 was investigated in wistar-han rat, beagle dog, cynomolgus monkey, rhesus monkey, and human liver cytosol using a 96-well assay plate design. Incubations were conducted in 100 mM potassium phosphate pH 7.4 buffer and 0.5 mg/ml liver cytosol in a 37°C Thermomixer® (Eppendorf, Hauppauge, NY) with a final incubation volume of 0.2 ml. Reactions were initiated by addition of BIBX1382 (1 µM, final organic <0.1%), and subsequently quenched at 0, 2, 5, 10,
15, 20 and 30 min (in triplicate) by addition of 600 μL of ice cold MeCN containing 0.25 μM tolbutamide as the analytical internal standard. Quenched samples were then centrifuged at 3000 x g for 10 min at 4°C to precipitate proteins, and the supernatant was transferred to a clean 96-well plate for analysis by tandem LC-MS/MS. Standard curves were prepared in potassium phosphate pH 7.4 buffer at concentrations ranging from 0 to 2 μM for quantitation of both BIBX1382 and BIBU1476, and were treated the same as incubation samples. The metabolism of BIBX1382 was also evaluated in cynomolgus monkey and human liver S9 fractions from kidney, lung, and intestine using a 96-well assay plate design. Incubations were conducted in 100 mM potassium phosphate pH 7.4 buffer and 1.0 mg/ml S9 fractions in a 37°C Thermomixer® (Eppendorf, Hauppauge, NY) with a final incubation volume of 0.2 ml. Reactions were initiated by addition of BIBX1382 (1 or 10 µM, final organic <0.1%), and subsequently 25 µl was removed and quenched at 0, 5, 15, 30 and 60 min (in triplicate) by addition of 150 μL of ice cold MeCN containing 0.25 μM tolbutamide as the analytical internal standard. Quenched samples were then centrifuged at 3000 x g (4°C) for 10 min to precipitate proteins, and the supernatant was transferred to a clean 96-well plate for analysis by tandem LC-MS/MS. Standard curves were prepared in potassium phosphate pH 7.4 buffer at concentrations ranging from 0 to 2 μM for quantitation of BIBU1476, and were treated the same as incubation samples.

**In Vitro Biotransformation of BIBX1382. Hepatic Cytosol and S9 Fractions.** The in vitro metabolism of BIBX1382 was investigated using hepatic cytosol and S9 fraction from cynomolgus monkey. BIBX1382 (10 μM) was incubated in cynomolgus monkey liver cytosol (5 mg/ml) or S9 fraction (5 mg/ml) diluted in 100 mM potassium phosphate pH 7.4 buffer at 37°C in borosilicate glass tubes. Reactions were initiated by addition of BIBX1382, and allowed to proceed for 1 hr. In select incubations, the AO inhibitor raloxifene (50-100 μM) or xanthine
oxidase (XO) inhibitor allopurinol (50 µM) were co-incubated to identify the enzyme responsible for generation of the oxidative metabolite BIBU1476 in cynomolgus monkey (previously confirmed in human). Incubations using S9 fraction from cynomolgus monkey were conducted both with and without addition of NADPH. Cytosol and S9 fraction protein was precipitated by addition of 2 volumes of cold MeCN, followed by mixing and centrifugation at 3000 x g for 10 min at 4°C. The supernatant was subsequently transferred to a clean glass borosilicate tube and dried under a gentle stream of nitrogen (N₂) gas. Dried samples were then reconstituted in 200 µl of mobile phase (85:15 (v/v) water/MeCN (0.1% formic acid)) and centrifuged again at 13,000 x g for 10 min prior to bioanalysis by LC-high resolution mass spectrometry (HRMS).

**Hepatocytes.** The in vitro metabolism of BIBX1382 was evaluated following incubation at 1 and 10 µM in cryopreserved human and cynomolgus monkey hepatocytes. Cryopreserved hepatocytes were stored in liquid nitrogen until use. Immediately prior to incubation, sufficient aliquots of hepatocytes were thawed rapidly (~2 min) in a shaking water bath at 37°C. The contents of each vial were diluted 1/50 in pre-warmed (37°C) cryopreserved hepatocytes recovery medium (CHRM, human) or Cell Maintenance medium (CM3000, cynomolgus monkey) and gently mixed prior to centrifugation at 100 x g for 5 min at room temperature. Following centrifugation, the supernatant was discarded and the hepatocyte pellet was re-suspended in William’s Medium E (WME) by repeated gentle inversion in a capped tube, and the cell number and viability were determined using a hemocytometer after staining with trypan blue (viabilities ≥80%). The cell suspension was then diluted into WME to provide 5 x 10⁶ cells/ml and pre-warmed at 37°C for 15 min. Stock solutions of BIBX1382 (10% MeCN in H₂O) were also diluted into pre-warmed WME (final organic solvent ≤0.01%). Incubations were
initiated by addition of BIBX1382 solution (500 µl) to the hepatocyte suspension (500 µl, final cell density 2.5 x 10^6 cells/ml). Following 60 min incubation in borosilicate glass tubes in a water bath (37°C), reactions were terminated by addition of two volumes of cold MeCN, and the resulting mixture underwent centrifugation at 3000 x g for 10 min at 4°C. The supernatants were then transferred to clean glass test tubes, and subsequently dried under a gentle stream of nitrogen (N₂) gas. Dried samples were then reconstituted in 200 µl of mobile phase (85:15 (v/v) water/MeCN (0.1% formic acid)) and centrifuged again at 13,000 x g for 10 min prior to bioanalysis by LC-high resolution mass spectrometry (HRMS).

**Plasma Stability and Red Blood Cell Partitioning.**

**Plasma Stability.** Incubations containing BIBX1382 or the metabolite BIBU1476 were conducted in previously frozen human and cynomolgus monkey plasma (Bioreclamation, LLC, Westbury, NY) in a 37°C Thermomixer® (Eppendorf, Hauppauge, NY). Reactions were initiated by addition of BIBX1382, BIBU1476, or eucatropine as positive control (1 µM, final organic <0.1%) with a final incubation volume of 1 ml. Reactions were terminated at 0, 5, 15, 30, 60 and 120 min by taking a 50 µL aliquot of incubate and placing into 150 µl of cold MeCN containing internal standard (0.25 µM tolbutamide). Quenched samples were then centrifuged at 3000 x g for 10 min at 4°C to precipitate proteins, and the supernatant was transferred to clean 96-well plates for bioanalysis by tandem LC-MS/MS.

**Red Blood Cell Partitioning.** BIBX1382 or chloroquine (1 µM, final DMSO <0.1%) was added to 2 ml aliquots of cynomolgus monkey (Bioreclamation, LLC, Westbury, NY) and human (donor program, Boehringer-Ingelheim Pharmaceuticals Inc) blood in a 20 ml glass vial. Capped vials were then mixed manually and placed in 37°C water bath and incubated for 2 hours, with manual mixing every 30 min. At the end of the incubation, the blood sample was collected by
taking a 150 μl aliquot of blood, and diluting into an equal volume of water. The plasma sample was prepared by taking aliquot of 0.5 ml of blood and centrifuging at 3000 x g for 5 minutes at 4°C. From each blood and plasma incubation samples, a 50 μl aliquot was placed into 150 μl of cold MeCN containing internal standard (0.25 μM tolbutamide). Quenched samples were then centrifuged at 3000 x g for 10 min at 4°C to precipitate proteins, and the supernatant was transferred to clean 96-well plates for bioanalysis by tandem LC-MS/MS.

Pharmacokinetic Studies. Male cynomolgus monkeys (2–5 years of age and weighing between 2.9 and 4.2 kg at study initiation) were obtained and dosed externally at Charles River Laboratories (Reno, NV). Monkeys (n=3) were dosed intravenously using a butterfly tubing set and syringe into the cephalic vein. The intravenous bolus dose was administered at 1 mg/kg (1 mg/ml) in 70% PEG-400 in sterile water (1-3 minutes), and the oral dose (via nasogastric gavage) was administered at 2 mg/kg (1 mg/ml) in 50 mM citric acid at pH 3.0 (adjusted with 1M NaOH), followed by a 5 ml tap water flush. Dose formulations were prepared the same day as administered and the volume of each dose delivered was based on the individual animal body weight. Monkeys had access to food, and water was available ad libitum. Blood was collected pre-dose, and at 5 and 30 min and 1, 2, 4, 6, 8, 12, and 24 hours post-dose from the femoral vein (rotating). Approximately 1.0 ml of blood was collected at each time point into tubes containing K$_2$-EDTA, mixed thoroughly, and chilled on ice. Plasma was obtained by centrifugation at 3000 x g for 10 min at 4°C. In addition, urine samples were collected at 0 to 4, 4 to 8, and 8 to 24 hour intervals post-dose and volumes recorded to evaluate extent of direct urinary excretion. Plasma and urine samples were stored at -70°C until shipped on dry ice to Boehringer-Ingelheim Pharmaceuticals Inc. (Ridgefield, CT) for bioanalysis by tandem LC-MS/MS. All animal studies were approved by the Charles River Institutional Animal Care and Use Committee. The animal
care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

**Pharmacokinetic analysis.** Pharmacokinetic parameters following intravenous and oral administration to male cynomolgus monkeys were obtained by noncompartmental analysis of individual plasma concentration-time profiles using Phoenix™ WinNonlin® Version 6.1 (Pharsight, Cary, NC).

**Liquid Chromatography-UV-Mass Spectrometry Bioanalysis.** *In Vitro Studies.* Quantitation of all analytes was performed using an AB Sciex (Applied Biosystems, Foster City, CA) API-5000 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) interface in positive ion mode and coupled with a Waters Acquity UPLC® system (Waters, Milford, MA). Separation of analytes was performed using a Waters Acquity BEH high-pressure C18 1.7µm (2.1 x 50 mm) column held at 50°C. Mobile phase was flowed at 0.5 ml/min with a rapid gradient starting at 95% A (0.1% formic acid in water) and 5% B (0.1% formic acid in MeCN), held for 0.5 min, then ramped linearly to 5:95 A:B, and held for 1.3 min, followed by returning to initial conditions (3 minutes total run time). The multiple reaction monitoring (MRM) transitions for each analyte from in vitro assays were as follows: BIBX1382 ($m/z$ 388.0 > 98.0), BIBU1476 ($m/z$ 404.0 > 98.0), chloroquine ($m/z$ 320.0 > 142.0), eucatropine ($m/z$ 292.1 > 109.1) and tolbutamide ($m/z$ 271.3 > 91.1). Standard curves for each analyte were prepared in appropriate matrices from the various in vitro experiments with a concentration range of 0.0025-2.0 μM. The limit of quantitation (LOQ) for both BIBX1382 and BIBU1476 was 0.0025 μM. All data were analyzed using AB Sciex Analyst 1.4.2 software.

*Cynomolgus Monkey Pharmacokinetic Studies.* BIBX1382 and BIBU1476 concentrations in plasma and urine (following protein precipitation using 85/15 MeCN/water with 0.1% acetic...
acid) were measured using an AB Sciex (Applied Biosystems, Foster City, CA) 4000 Q-trap triple quadrupole mass spectrometer. The mass spectrometer was equipped with an atomospheric-pressure chemical ionization (APCI) probe (source temp 450°C) in positive ion mode, and was connected with an Agilent 1200 liquid chromatography system and a CTC PAL autosampler (LEAP Technologies, Carrboro, NC). Analytes were chromatographically separated using a Phenomenex 4 µm Synergi Polar-RP 50 x 2 mm column using a gradient elution profile. The flow rate was set at 600 µl/min and the gradient was initiated and held at 100% A (A, 95% 10 mM ammonium acetate/5% MeCN/0.1% formic acid; B 95% MeCN/5% 10 mM ammonium acetate/0.1% formic acid) for 0.05 min. The gradient was then ramped to 100% B over the next 1.3 min and held for 0.7 min, after which it was ramped back to 100% A over 0.2 min and held for the next 0.8 min for a total run time of 3 min. Mass spectral analysis was performed using multiple reaction monitoring (MRM), with transitions as follows: BIBX1382 (m/z 388.2 > 98.2), BIBU1476 (m/z 404.2 > 98.2) and internal standard BIBU1361 (m/z 444.2 > 371.2). Standard curves for both analytes were prepared in blank plasma or urine at concentrations between 1 and 5000 ng/mL, and the limit of quantitation (LOQ) for both BIBX1382 and BIBU1476 was 1 ng/mL. Low, medium and high quality control samples were included in plasma bioanalysis and all were within 25% of nominal concentrations. All data was analyzed using Analyst 1.5.2 software.

In Vitro Metabolite Profiling. Metabolite profiling of in vitro samples following incubation with liver cytosol, S9 fractions, or cryopreserved hepatocytes was performed using a high resolution LTQ-Orbitrap XL (Thermo Scientific, Waltham, MA) mass spectrometer connected to a Thermo Accela UPLC pump and CTC PAL auto-sampler (Leap Technologies, Carrboro, NC). The Orbitrap was equipped with an electro-spray ionization (ESI) source in positive polarity, and was
calibrated using ProteoMass LTQ/FT-Hybrid Calibration Mix (Supelco, Bellefonte, PA) for mass accuracies < 5 ppm in external calibration mode. The source voltage was set at 5 kV, tube lens voltage at 90 V, and capillary voltage at 5 V, with the capillary temperature at 325°C. Sheath gas flow was at 70 U, aux gas at 15 U, and sweep gas flow at 5. Analytes were loaded (20 µL injection) onto a SUPELCO Discovery C_{18} (5 µm, 2.1 x 150 mm; Supelco, Bellefonte, PA) column and separated using a gradient elution profile consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in MeCN). The gradient was initiated at an 95%A:5%B ratio at a flow rate of 0.4 mL/min, held for 5 min, then ramped linearly from 5% B to 48% B over 10 min, then to 95%B over the next minute, and held for 2 min. The gradient was then returned to initial conditions, and column was allowed to re-equilibrate for 3 min (total run time 22 min). The HPLC eluent was introduced first into a photodiode array detector (PDA) set at 254 nm, and subsequently via electrospray ionization directly into the Orbitrap. Mass spectrometry analyses were carried out utilizing full-scan MS with a mass range of 99-1000 Da and MS/MS spectra were collected in a data-dependent fashion with relative collision energies of 50 and 35% for HCD and CID, respectively. Resolution was set at 30000 for FT MS, and 15000 for FT MS/MS. All mass spectral data were analyzed using Xcalibur version 2.1 software, and chemical structures and calculated exact mass estimates for fragments were generated using ChemDraw Ultra Version 12 software (Cambridge, MA).

**Intrinsic Clearance Estimates.** In vitro intrinsic clearance (Cl_{in}) was estimated from liver cytosol using substrate depletion methodologies and Equation 1:

\[
Cl_{in} = 0.693 \times \frac{1}{t_{1/2}} \times \frac{ml \; incubation}{mg \; cytosol} \times \frac{gm \; liver \; wt}{kg \; body \; wt} \times \frac{mg \; cytosolic \; protein}{gm \; liver \; wt}
\]

A scaling factor of 81 mg cytosolic protein per gram of human liver (Cubitt et al., 2011) was used, while a scaling factor of 92 mg cytosolic protein per gram of monkey liver was estimated.
based on a report by (Kitamura et al., 2001). An additional scaling factor of 25.7 gram of liver per kg body weight (human) and 30 gram of liver per kg body weight (monkey) was used for intrinsic clearance estimation (Davies and Morris, 1993). Hepatic clearance ($C_l_h$) was predicted using $Cl_{int}$ values and the well-stirred model, uncorrected for fraction unbound in plasma, according to Equation 2:

$$C_l_h = \frac{Q_h \times Cl_{int}}{Q_h + (Cl_{int})}$$

where $Q_h$ is hepatic blood flow (20.7 mL/min/kg for human and 45 mL/min/kg for monkey) (Davies and Morris, 1993), and $Cl_{int}$ is in vitro intrinsic clearance derived from in vitro experiments in liver cytosol.
Results

Metabolic Stability in Liver Cytosol. The metabolic stability of BIBX1382 was investigated in wistar-han rat, beagle dog, rhesus monkey, cynomolgus monkey and human liver cytosol using a substrate depletion methodology. As demonstrated in Figure 2A, no depletion of BIBX1382 was observed when incubated with rat and dog liver cytosol, while rapid depletion (half-life ≤ 7 min) was observed in rhesus monkey, cynomolgus monkey, and human liver cytosol (Table 1). Subsequent to BIBX1382 substrate depletion studies, BIBU1476 formation was also confirmed and found to be comparable in rhesus monkey, cynomolgus monkey, and human liver cytosol (Figure 2B). In vitro substrate depletion half-life data was scaled to estimate intrinsic and hepatic clearance in rhesus monkey, cynomolgus monkey, and human (Table 1), where for each species, high clearance relative to liver blood flow was observed (≥93% of liver blood flow).

Characterization of BIBX1382 and In Vitro Metabolites by LC-MS/MS. The predominant oxidative metabolite of BIBX1382 generated by aldehyde oxidase (BIBU1476) has been previously characterized in human hepatocytes (Hutzler et al., 2012). In the current studies, LC-MS/MS methods were used to characterize BIBX1382 and in vitro metabolites from liver cytosol, S9 fraction, and hepatocytes (Figure 3).

BIBX1382. The protonated molecular ion [M+H]⁺ for BIBX1382 was observed at m/z 388.14 with a retention time of 12.8 min. Fragmentation of BIBX1382 produced four predominant HRMS fragment ions at m/z 357.1017 (calculated m/z 357.1025), m/z 345.1018 (calculated m/z 345.1025), m/z 331.0861 (calculated m/z 331.0869), and m/z 291.0549 (calculated m/z 291.0556), which all corresponded to ions following fragmentation of the methyl-piperidine moiety (Figure 3A).
**BIBU1476 (M1).** For the predominant oxidative metabolite of BIBX1382, which had a retention time of 10.9 min and produced a protonated molecular ion [M+H]^+ at m/z 404.14 (BIBU1476), each of the aforementioned fragment ions for BIBX1382 shifted 16 Da to m/z 373.0966 (calculated m/z 373.0974), m/z 361.0963 (calculated m/z 361.0974), m/z 307.0496 (calculated m/z 307.0505), and m/z 292.0396 (calculated m/z 292.0396), respectively (Figure 3B). The fragmentation pattern of metabolically generated M1 of BIBX1382 was consistent with the synthetically prepared standard BIBU1476 (data not shown).

**Metabolite M2.** A secondary oxidation resulted in formation of M2 which eluted at 10.7 min and produced a protonated molecular ion [M+H]^+ at m/z 420.14 (e.g. +32 Da from BIBX1382). Among the daughter ions observed following fragmentation, m/z 308.0335 (calculated m/z 308.0345) and m/z 323.0443 (calculated m/z 323.0454) were useful in determining that the secondary oxidation likely occurred on either the pyrimido-pyrimidine core or the 3-chloro-4-fluorophenyl moiety (Figure 3C).

**In Vitro Biotransformation of BIBX1382.** *Cynomolgus Monkey Liver Cytosol.* The LC-mass spectrometry extracted ion chromatogram (XIC) demonstrating the formation of BIBU1476 (M1) following incubation of BIBX1382 (10 µM) with cynomolgus monkey liver cytosol is shown in Figure 4. While formation of BIBU1476 has been previously confirmed to be a result of oxidation by aldehyde oxidase in humans, this has yet to be demonstrated in cynomolgus monkey. When co-incubating BIBX1382 with the selective AO inhibitor raloxifene at 50 µM, a roughly 66% decrease in peak area of BIBU1476 was observed. Meanwhile, following co-incubation with the xanthine oxidase (XO) inhibitor allopurinol (50 µM), negligible inhibition of BIBU1476 metabolite formation was observed (Figure 4), which suggests that AO is the principle enzyme involved in formation of BIBU1476 in cynomolgus monkey.
Human and Cynomolgus Monkey Hepatocytes. To investigate the metabolic pathways of BIBX1382 more thoroughly in human and cynomolgus monkey, incubations were conducted using cryopreserved hepatocytes. Following incubation of BIBX1382 at both 1 and 10 µM for 60 min, the principal metabolite in human was BIBU1476 (M1), matching the retention time and mass spectral fragmentation of the authentic standard, consistent with previous studies in our lab (Hutzler et al., 2012). When BIBX1382 was incubated with cryopreserved hepatocytes from cynomolgus monkey, BIBU1476 (M1) was also the principal metabolite, but a secondary metabolite (M2) was also observed, which had a mass consistent with a dual oxidation (+32 Da) mechanism (Figure 5). The LC-MS/MS fragmentation of M2 is shown in Figure 3C.

Cynomolgus Monkey S9 Fraction. In effort to characterize the origin (e.g. molybdenum hydroxylase vs. cytochrome P450) of the secondary oxidation to produce M2, in vitro studies were conducted utilizing S9 fraction from cynomolgus monkey liver, with and without supplementation of NADPH. Following incubation of BIBX1382 (10 µM) in cynomolgus monkey liver S9 fraction (5 mg/mL) for 60 min, a metabolite profile similar to that observed in hepatocytes was observed, where BIBU1476 (M1) was the predominant metabolite, with the secondary oxidative metabolite M2 also formed (Figure 6). It was apparent that formation of M2 was not dependent on the presence of NADPH, as the LC-UV metabolite profile was essentially identical with or without NADPH present. Thus, the generation of M2 is likely not cytochrome P450-mediated. In a subsequent study, it was found that when BIBX1382 was co-incubated with raloxifene, there was a 41% reduction in M1, with essentially complete inhibition of M2 formation (Supplemental Figure 1). In addition, when allopurinol was co-incubated, M1 was unaffected, and M2 was only inhibited moderately, which suggests a potential small role for
xanthine oxidase (XO) in the formation of M2 from M1. More definitive studies need to be conducted to characterize this secondary pathway, and will be the focus of future investigations.

**Pharmacokinetic Studies.** The mean plasma concentration vs. time profile of BIBX1832 and the metabolite BIBU1476 following i.v. bolus (1 mg/kg) and oral (2 mg/kg) administration of BIBX1382 to male cynomolgus monkeys are shown in Figure 7A and 7B, respectively. Total plasma clearance of BIBX1382 (118 ml/min/kg) was higher than hepatic blood flow (Table 2), which suggests either an extra-hepatic contribution to total clearance, a partitioning into blood cells, or instability in plasma. Subsequent in vitro studies demonstrated that BIBX1382 and BIBU1476 are stable when incubated in human and cynomolgus monkey plasma for two hours (data not shown). In addition, it was found that the blood-to-plasma ratio of BIBX1382 in cynomolgus monkey blood was 2.1, while the blood-to-plasma ratio was 1.4 in human. When accounting for the observed partitioning into blood cells, blood clearance in monkey still remained higher than liver blood flow (56 ml/min/kg). When measuring the amount of parent BIBX1382 excreted in urine over 24 hour period following i.v. administration, only 0.2% of the dose was recovered. Exposure following oral administration to cynomolgus monkeys was low, with absolute oral bioavailability ranging from 3 to 10% (average 6%) (Table 2). Implicating AO-mediated metabolism as the likely contributor to the observed high clearance and low oral bioavailability, the total exposure of the metabolite BIBU1476 was 160% that of parent BIBX1382 (Table 2).

**Extrahepatic Biotransformation of BIBX1382.** To investigate the possible contribution of AO-mediated metabolism in extrahepatic tissues, BIBX1382 was incubated with S9 fractions from human and cynomolgus monkey kidney, lung, and intestine (Figure 8). While activity (measured by BIBU1476 formation) was relatively low in the extrahepatic human tissues
evaluated, activity in cynomolgus monkey lung S9 fraction was substantially high (Figure 8).
When co-incubating with the AO inhibitor raloxifene, a significant decrease in the formation of
BIBU1476 was observed, whereas allopurinol had a minimal effect (Supplemental Figure 2).
Discussion

Aldehyde oxidase (AO) is a cytosolic drug-metabolizing enzyme that has been highlighted recently as playing a prominent role in the metabolism of heterocyclic-containing drug molecules (Pryde et al., 2010; Garattini and Terao, 2011, 2012; Hutzler et al., 2013). Numerous clinical programs have been impacted by rapid metabolism, and thus, poor pharmacokinetics, due to aldehyde oxidase (Kaye et al., 1984; Dittrich et al., 2002; Akabane et al., 2011). One cause for the failure to predict the human pharmacokinetic properties of these AO substrates is the profound species differences noted for this enzyme. In particular, routine pharmacokinetic studies rat and dog in early drug discovery will likely not adequately capture AO-mediated contributions to metabolic clearance, as rats generally have low AO activity, and dogs are devoid of activity (Beedham, 1987; Garattini et al., 2008). In a recently published review on aldehyde oxidase, it was reported that rhesus monkey and guinea pig may represent the best experimental in vivo models for studies with AO substrates (Garattini and Terao, 2012). BIBX1382 was a clinical candidate being evaluated as an inhibitor of epidermal growth factor receptor (EGFR) in the treatment of cancer. Following the observation of extremely low oral exposure in humans (Table 2), it was discovered that rapid metabolism by AO was the root cause, and it was further noted that the AO pathway and pharmacokinetic properties of BIBX1382 were effectively reproduced retrospectively in rhesus monkey (Dittrich et al., 2002), consistent with the suggestions of Garattini and Terao. However, within drug discovery and development, cynomolgus monkey is a more commonly utilized primate for pharmacokinetic/toxicokinetic assessment. Thus, we chose to investigate if cynomolgus monkey would have been an equally suitable surrogate for the AO-mediated metabolism and pharmacokinetics of BIBX1382 observed in human.
The expression of AO in cynomolgus monkey (Macaca fascicularis) liver has been previously established (Sugihara et al., 2000). In this report, cynomolgus monkey liver AO demonstrated high oxidase activity towards several known substrates, including benzaldehyde, phthalazine, and N^1-methylnicotinamide (Sugihara et al., 2000). It was first proposed in this report that monkey may be a model of human for drugs metabolized by AO. In addition to the high oxidase activity, high reductase activity by AO for drugs such as zonisamide, sulindac, and imipramine N-oxidase has been reported in cynomolgus monkey liver (Kitamura et al., 2001). In later studies, the full male cynomolgus monkey AO cDNA was cloned and sequenced and found to have 96% amino acid identity with the human enzyme (Hoshino et al., 2007). Our findings with BIBX1382 are thus not entirely surprising when considering the sequence homology to human AOX1 (Hoshino et al., 2007), as well as the 99% homology to the rhesus monkey enzyme (accession numbers for the AO genes (human, NP_001150; cyno, ACQ73553.1; rhesus, AFI36988.1). BIBX1382 metabolism to the oxidative product BIBU1476 was demonstrated in our in vitro studies to be comparable in human, rhesus monkey, and cynomolgus monkey liver cytosol (Figure 2). Further, through use of specific inhibitors of molybdenum hydroxylases, our data shows that this metabolic pathway is primarily catalyzed by AO in cynomolgus monkey (Figure 4). To evaluate the in vitro-to-in vivo link for this metabolic pathway, a pharmacokinetic study was also conducted in cynomolgus monkeys. Following non-compartmental pharmacokinetic analysis, it was demonstrated that total clearance was high (greater than liver blood flow), even after accounting for observed distribution into red blood cells (B/P~2.1) (Table 2). In addition, following oral administration, bioavailability was low, and comparable to the pharmacokinetic data reported from human studies (Table 2), likely due to high first-pass metabolism by aldehyde oxidase. Corroborating this conclusion is data showing that the primary
metabolite BIBU1476 was circulating in plasma at levels 160% of parent BIBX1382 (Figure 7 and Table 2).

Similar to our findings with BIBX1382, the AO-mediated metabolism of SGX523, zaleplon, and RS-8359 have been reported to be comparable between cynomolgus monkey and human (Diamond et al., 2010; Kawashima et al., 1999; Itoh et al., 2006). Of particular relevance to metabolites and drug safety is the example of SGX523, where the AO-mediated metabolic pathway reportedly led to the generation of a highly insoluble 2-quinolinone metabolite that precipitated in the kidney and caused acute renal toxicity in humans (Diamond et al., 2010). Interestingly, this metabolic pathway and the observed renal findings were reproduced following dosing to cynomolgus monkeys. In another reported example, the metabolism and pharmacokinetics of SB-277011 was profoundly different across rat, dog, and monkey (Austin et al., 2001). Namely, in vitro metabolism data in microsomes suggested that SB-277011 was relatively stable in rat, dog, cynomolgus monkey, and human. However, in vivo, this compound displayed low clearance in rat, moderate clearance in dog, and high clearance in cynomolgus monkey (Austin et al., 2001). In addition, while oral bioavailability was acceptable in rat and dog (~40%), it was extremely low in cynomolgus monkey (~2%). It was uncovered that the metabolism in cynomolgus monkey was catalyzed predominantly by AO, and thus it was predicted that the oral bioavailability of SB-277011 in human would likely also be unacceptable (Austin et al., 2001). This is consistent with the reported species differences for AO activity in standard pre-clinical species.

Regarding extrahepatic expression of aldehyde oxidase, it is known that AOX1 is expressed in tissues such as adrenal gland, kidney, pancreas, respiratory system and intestine (Moriwaki et al., 2001, and Nishimura et al., 2006). To investigate the possibility of extrahepatic
tissue contributing to metabolism, and perhaps total clearance in vivo, BIBX1382 was incubated in S9 fractions from various tissues from human and cynomolgus monkey. While low but measurable activity was observed in human kidney, intestine, and lung S9 fractions, relatively high activity was observed in monkey lung S9 fraction (Figure 8). The role of AO in cynomolgus monkey lung S9 was confirmed by use of raloxifene as an inhibitor of AO (Supplemental Figure 2). While no scaling to estimate the contribution to total systemic clearance was attempted, it is plausible that in a highly perfused tissue such as lung, metabolism may contribute to the high extra-hepatic clearance observed in cynomolgus monkey. More studies would need to be conducted to confirm the relevance of this in vitro data.

Despite our findings for the AO substrate BIBX1382, caution should be taken in that cynomolgus monkey may not always be a suitable species for the human situation for all substrates of AO, as was reported with the p38 kinase inhibitor RO1 (Zhang et al., 2011). The relative activities of AO across species more likely depend on the substrate being investigated. This point was recently highlighted by Dalvie and colleagues when investigating zoniporide metabolism by AO across multiple species (Dalvie et al., 2013). Thus, appropriate in vitro studies need to be conducted to first identify which species may form the relevant human AO-derived metabolite(s), which may then inform as to the most appropriate species in which to conduct in vivo studies.

In summary, based on in vitro studies in liver fractions as well as the pharmacokinetic properties of BIBX1382 in cynomolgus monkey, metabolism and clearance appears to be mediated primarily by AO, which is analogous to the reported human situation. This example underscores the importance of characterizing the metabolism of drug candidates in the proper subcellular fractions, to identify the predominant metabolic pathways for human, and to then
subsequently consider conduct of pharmacokinetic studies in a species that is known to be representational of the human metabolism, both from a pharmacokinetic, as well as toxicokinetic (e.g. metabolites in safety testing, MIST) standpoint. Also, with our observations, and the additional literature precedence (summarized in Table 3), it seems that cynomolgus monkey may be more ideal as a possible surrogate species compared to rhesus monkey for human AO from a practical standpoint. Rhesus monkey would require more compound for dosing (rhesus monkey typically weighs 6 to 10 kg, whereas the cynomolgus monkey weighs 3 to 5 kg), is more expensive, and is a less often used species for conducting routine pharmacokinetic and toxicokinetic studies.
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Authorship Contributions

Participated in research design: Hutzler, Cerny, Yang, Frederick

Conducted experiments: Yang, Asher, Frederick

Contributed new reagent or analytic tools: n/a

Performed data analysis: Hutzler, Yang, Asher, Wong, Frederick, Gilpin

Wrote or contributed to the writing of the manuscript: Hutzler, Cerny, Yang, Wong, Frederick, Gilpin
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Footnotes

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Legends for Figures

Figure 1. BIBX1382 metabolism by aldehyde oxidase (AO) to the oxidative metabolite BIBU1476.

Figure 2. (A) In vitro metabolic stability of BIBX1382 following incubation (1 µM) in 0.5 mg/ml liver cytosol from multiple species and (B) formation of BIBU1476 following incubation in liver cytosol from human, rhesus monkey, and cynomolgus monkey. No measurable formation of BIBU1476 was observed in wistar-han rat and beagle dog liver cytosol.

Figure 3. LC-MS/MS spectra of product ions obtained by mass fragmentation of BIBX1382 at m/z 388 (A), the oxidative metabolite M1 (BIBU1476) at m/z 404 (B), and M2 at m/z 420 (C). RT, retention time; NL, normalized level; ESI, electrospray ionization.

Figure 4. Extracted ion chromatograms (XIC) demonstrating formation of BIBU1476 (M1) in cynomolgus monkey liver cytosol following incubation of BIBX1382, and inhibition of metabolism by co-incubation with 50 µM raloxifene (selective inhibitor of AO). Co-incubation with allopurinol (selective inhibitor of xanthine oxidase, XO) did not result in appreciable inhibition of BIBU1476 formation.

Figure 5. Representative UV chromatogram demonstrating metabolite profile following incubation of BIBX1382 (10 µM) in human and cynomolgus monkey hepatocytes for 60 minutes. M1 represents the authentic standard oxidative metabolite BIBU1476, and M2 is a metabolite consistent with addition of 32 Da.

Figure 6. Representative UV chromatogram demonstrating metabolite profile following incubation of BIBX1382 (10 µM) in cynomolgus monkey S9 fraction (5 mg/mL) for 60 minutes. Formation of M1 and M2 in S9 fraction without supplementation of NADPH suggests that formation of M2 is also mediated by a molybdenum hydroxylase.
Figure 7. Plasma pharmacokinetic profile of BIBX1382 and metabolite BIBU1476 (M1) in cynomolgus monkey following intravenous (1 mg/kg) and oral (2 mg/kg) administration. Pharmacokinetic parameters are reported in Table 1.

Figure 8. Formation of BIBU1476 (M1) following incubation of BIBX1382 in various extra-hepatic tissues from human (A) and cynomolgus monkey (B). Incubations were conducted in S9 fractions from kidney, lung and intestine.
Table 1. In vitro metabolic clearance of BIBX1382 in human, cynomolgus monkey, and rhesus monkey liver cytosol.

In vitro intrinsic and hepatic clearance was calculated as described in *Materials and Methods*.

| Parameter                        | Human | Cynomolgus Monkey | Rhesus Monkey |
|----------------------------------|-------|-------------------|---------------|
| In vitro half-life (min)         | 6.0   | 4.3               | 6.7           |
| mg cytosol/gram liver            | 81    | 92                | 92            |
| gram liver/kg body weight        | 25.7  | 30                | 30            |
| $Cl_{int}$ (ml/min/kg)           | 484   | 882               | 573           |
| $Cl_{hepatic}$ (ml/min/kg)       | 20    | 43                | 42            |
| Liver blood flow (ml/min/kg)     | 21    | 45                | 45            |
| Percent (%) of liver blood flow  | 95    | 95                | 93            |

$Cl_{int}$, intrinsic clearance; $Cl_{hepatic}$, predicted hepatic clearance
Table 2. Pharmacokinetic properties of BIBX1382 in human and male cynomolgus monkey. Data are reported as a range (human) or as mean (standard deviation, SD) from n=3 monkeys.

| Species          | Subjects     | Route       | Dose         | Plasma Clearance (SD) | Volume of Distribution (SD) | C<sub>max</sub> (SD) | AUC (SD) | Absolute Bioavailability (range) |
|------------------|--------------|-------------|--------------|------------------------|-----------------------------|----------------------|----------|---------------------------------|
| Human<sup>a</sup> | 11           | i.v. infusion | 25-200 mg    | 25-55                  | 10-20                       | -                    | 8.5-34.5 | -                               |
| (4M, 7F)         | p.o.         | 25-200 mg   | -            | -                      | -                           | 0.03-0.41             | 0.1-2.5  | 5 (2-12)                        |
| Cynomolgus       | 3 (M)        | i.v. bolus  | 1 mg/kg      | 118 (28)               | 39 (6)                      | -                    | 350 (80) | -                               |
| Monkey<sup>b</sup> | 3 (M)       | p.o.        | 2 mg/kg      | -                      | -                           | 12.7 (3.8)            | 43 (25)  | 6 (3-10)                        |

C<sub>max</sub>, maximal plasma concentration; AUC, area under the concentration-time curve; i.v., intravenous; M, male; F, female

<sup>a</sup>Data reported in Dittrich et al., 2002 (C<sub>max</sub> and AUC<sub>(0-24)</sub> values are dose normalized)

<sup>b</sup>AUC reported as AUC<sub>last</sub>
Table 3. Literature reports of aldehyde oxidase mediated metabolism in cynomolgus monkey.

| Substrate | Metabolic Pathway | Metabolite          | Matrix                     | Reference                  |
|-----------|-------------------|---------------------|----------------------------|---------------------------|
| Zaleplon  | Oxidation of pyrazolo-pyrimidine | 5-Oxo Zaleplon | Liver S9 Fraction          | Kawashima et al., 1999   |
| SB-277011 | Oxidation of quinoline | SB-369912          | Liver Homogenate           | Austin et al., 2001       |
| RS-8359   | Oxidation of pyrimidine | 2-Keto RS-8359     | Liver Cytosol              | Itoh et al., 2006         |
| Zebularine| Oxidation of pyrimidine | Uridine            | Liver Cytosol              | Klecker et al., 2006      |
| Vanillin  | Vanillic Acid formation | Vanillic Acid      | Liver Cytosol, Hepatocytes | Sahi et al., 2008         |
| SGX523    | Oxidation of quinoline | M11                | Liver S9 fraction, Plasma, Urine | Diamond et al., 2010   |
| VU0409106 | Oxidation of pyrimidine | M1                 | Hepatocytes                | Morrison et al., 2012     |
| Zoniporide| Oxidation of quinoline | 2-Oxo Zoniporide   | Liver S9 Fraction          | Dalvie et al., 2013       |
Figure 1.
Figure 2
Figure 5
Figure 7

Graph A:
- IV, 1mg/kg BIBX1382
- PO, 2mg/kg BIBX1382

Graph B:
- IV, 1mg/kg BIBX1382
- PO, 2mg/kg BIBX1382

Concentration (nM) vs Time (hr)
Figure 8

A

- Kidney S9 Fraction
- Lung S9 Fraction
- Intestinal S9 Fraction

B

- Kidney S9 Fraction
- Lung S9 Fraction
- Intestinal S9 Fraction

BIBU476 Formation (µM)

Time (min)