Cynomolgus Monkey as a Surrogate for Human Aldehyde Oxidase Metabolism of the EGFR Inhibitor BIBX1382

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ABSTRACT

BIBX1382 was an epidermal growth factor receptor inhibitor under clinical investigation for treatment of cancer. This candidate possessed an attractive preclinical absorption, distribution, metabolism, and excretion 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 with the reported human pharmacokinetics of BIBX1382. Estimated hepatic clearance of BIBX1382 in rhesus (42 ml/min per kg) and cynomolgus monkey (43 ml/min per kg) liver cytosol was comparable to human (≥93% of liver blood flow). Metabolite identification after 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. After intravenous and oral administration of BIBX1382 to cynomolgus monkeys, high plasma clearance (118 ml/min per kg) and low oral exposure (Cmax = 12.7 nM and 6% oral bioavailability) was observed, with the exposure of M1 exceeding BIBX1382 after oral dosing. This pharmacokinetic profile compared favorably with the human clinical data of BIBX1382 (plasma clearance 25–55 ml/min per 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 [N8-(3-chloro-4-fluorophenyl)-N2-(1-methylpiperidin-4-yl)pyrimido[5,4-day]pyrimidine-2,8-diamine] (Fig. 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 was previously described and was reported to be relatively stable in vitro in human liver microsomes, 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 profile, the clearance of BIBX1382 from plasma after intravenous infusion dosing to humans was 25–55 ml/min per kg, a rate in excess of liver blood flow (20.7 ml/min per kg). In addition, after 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. (2002) provided evidence that BIBX1382 was metabolized by hepatic aldehyde oxidase (AO), although supporting data were not included. In a recent report, our laboratory demonstrated that the predominant metabolite after 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-day]pyrimidin-2(4aH)-one), with the position of oxidation occurring on the pyrimido-pyrimidine core (Fig. 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 coincubated (Hutzler et al., 2012), an in vitro phenotyping methodology also reported by Strelevitz et al. (2012).

Aldehyde oxidase is a molybdenum cofactor-containing drug-metabolizing enzyme that is active as a homodimer 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 absorption, distribution, metabolism, and excretion screening paradigms, where metabolic stability of new chemical entities are evaluated using liver microsomal fractions, do not capture the contribution of AO, because 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, 1987; Garattini et al., 2008). A recent review by Garattini and Terao (2012) suggested that rhesus monkey may be an appropriate surrogate for human AO activity. This

ABBREVIATIONS: AO, aldehyde oxidase; Clint, intrinsic clearance; EGFR, epidermal growth factor receptor; LC-HRMS, liquid chromatography-high-resolution mass spectrometry; MeCN, acetonitrile; LC-MS/MS, liquid chromatography-tandem mass spectrometry; WME, William’s Medium E; XO, xanthine oxidase.
The metabolism of BIBX1382 was investigated in Wistar-Han rat, beagle dog, cynomolgus monkey, rhesus monkey, human, and canine liver samples. The in vitro metabolism of BIBX1382 was studied using liver 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 hour. In selected incubations, the AO inhibitor raloxifene (50–100 μM) or xanthine oxidase (XO) inhibitor allopurinol (50 μM) was coincubated 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 acetonitrile (MeCN), followed by mixing and centrifugation at 3000g for 10 minutes at 4°C. The supernatant was 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/McCN (0.1% formic acid)] and centrifuged again at 13,000g for 10 minutes before bioanalysis by liquid chromatography–high-resolution mass spectrometry (LC-HRMS).

Hepatocytes. The in vitro metabolism of BIBX1382 was evaluated after incubation of 1 and 10 μM in cryopreserved human and cynomolgus monkey hepatocytes. Cryopreserved hepatocytes were stored in liquid nitrogen until use. Immediately before incubation, sufficient aliquots of hepatocytes were thawed rapidly (–2 minutes) in a shaking water bath at 37°C. The contents of each vial were diluted 1/50 in prewarmed (37°C) cryopreserved hepatocytes recovery medium (human) or cell maintenance medium (CM3000, cynomolgus monkey) and gently mixed before centrifugation at 100g for 5 minutes at room temperature. After centrifugation, the supernatant was discarded and the hepatocyte pellet was resuspended 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 × 10⁶ cells/ml and prewarmed at 37°C for 15 minutes. Stock solutions of BIBX1382 (10% MeCN in H₂O) were also diluted into prewarmed 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 × 10⁴ cells/ml). After a 60-minute incubation in borosilicate glass tubes in a water bath (37°C), reactions were terminated by addition of two volumes of cold McCN, and the resulting mixture underwent centrifugation at 3000g for 10 minutes 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/McCN (0.1% formic acid)] and centrifuged again at 13,000g for 10 minutes before bioanalysis by LC-HRMS.

Hepatic Cytosol and S9 Fractions. The in vitro metabolism of BIBX1382 was studied 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 hour. In selected incubations, the AO inhibitor raloxifene (50–100 μM) or xanthine oxidase (XO) inhibitor allopurinol (50 μM) was coincubated to identify the enzyme responsible for generation of the oxidative metabolite BIBU1476 in cynomolgus monkey (previously confirmed in human).

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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 minutes 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 3000g for 10 minutes at 4°C to precipitate proteins, and the supernatant was transferred to clean 96-well plates for bioanalysis by 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) 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 minutes. 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 an aliquot of 0.5 ml of blood and centrifuging at 3000g for 5 minutes at 4°C. From each blood and plasma incubation sample, 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 3000g for 10 minutes at 4°C to precipitate proteins, and the supernatant was transferred to clean 96-well plates for bioanalysis by 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% polyethylene glycol-400 in sterile water (1–3 minutes), and the orbital dose (via nasogastricavage) 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 predose at 5 and 30 minutes and 1, 2, 4, 6, 8, 12, and 24 hours postdose from the femoral vein (rotating). Approximately 1.0 ml of blood was collected at each time point into tubes containing K2-EDTA, mixed thoroughly, and chilled on ice. Plasma was obtained by centrifugation at 3000g for 10 minutes at 4°C. In addition, urine samples were collected at 0- to 4-, 4- to 8-, and 8- to 24-hour intervals postdose, and volumes were 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. 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 after 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-Ultraviolet-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 interface in positive ion mode and coupled with a Waters Acuity UPLC system (Waters, Milford, MA). Separation of analytes was performed using a Waters Acuity BEH high-pressure C18 1.7-μm (2.1 × 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 minute, then ramped linearly to 5:95 A:B and held for 1.3 minutes, followed by returning to initial conditions (3 minutes total run time). The multiple reaction monitoring transitions for each analyte from in vitro assays were as follows: BIBX1382 (m/z 380.0 > 98.0), BIBU1476 (m/z 406.0 > 99.0), chloroquine (m/z 320.0 > 142.0), eucatropine (m/z 292.1 > 109.1), and tolbutamide (m/z 271.3 > 91.1). The standard curves for each analyte were prepared in appropriate matrices from the various in vitro experiments with a concentration range of 0.0005–2.0 μM. The limit of quantitation 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 (after protein precipitation using 85/15 MeCN/water with 0.1% acetic acid) were measured using an AB Sciex (Applied Biosystems) 4000 QTRAP triple quadrupole mass spectrometer. The mass spectrometer was equipped with an atmospheric-pressure chemical ionization 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 × 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 minute. The gradient was then ramped to 100% B over the next 1.3 minutes and held for 0.7 minute, after which it was ramped back to 100% A over 0.2 minute and held for the next 0.8 minute for a total run time of 3 minutes. Mass spectral analysis was performed using multiple reaction monitoring, 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 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 were analyzed using Analyst 1.5.2 software.

**In Vitro Metabolite Profiling.** Metabolite profiling of in vitro samples after 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). The Orbitrap was equipped with an electro-spray ionization 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 set at 70 U, aux gas at 15 U, and sweep gas flow at 5 U. Analytes were loaded (20 μl injection) onto a SUPELCO Discovery C18 (5 μm, 2.1 × 150 mm; Supelco) 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 minutes, and then ramped linearly from 5% B to 48% B over 10 minutes and then to 95% B over the next minute and held for 2 minutes. The gradient was then returned to initial conditions, and column was allowed to re-equilibrate for 3 minutes (total run time 22 minutes). The high-pressure liquid chromatography eluent was introduced first into a photodiode array detector set at 244 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 Higher Energy Collisional Dissociation and Collision Induced Dissociation, respectively. Resolution was set at 30,000 for Fourier Transform MS and 15,000 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 (CI_{int}) was estimated from liver cytosol using substrate depletion methodologies and eq. 1:

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

A scaling factor of 81 mg cytosolic protein per gram of human liver (Cubit 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 Kishnara et al. (2001). An additional scaling factor of 25.7 g of liver per kg body weight (human) and 30 g of liver per kg body weight (monkey) was used for intrinsic clearance estimation (Davies and Morris, 1993). Hepatic clearance (Cl_{int}) was predicted using Cl_{int} values and the well-stirred model, uncorrected for fraction unbound in plasma, according to eq. 2:
Fig. 2. (A) In vitro metabolic stability of BIBX1382 after incubation (1 μM) in 0.5 mg/ml liver cytosol from multiple species and (B) formation of BIBU1476 after 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.

\[ Cl_h = \frac{Q_h \times Cl_{int}}{Q_h + (Cl_{int})} \]

where \( Q_h \) is hepatic blood flow (20.7 ml/min per kg for human and 45 ml/min per kg for monkey) (Davies and Morris, 1993), and \( Cl_{int} \) is the 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 Fig. 2A, no depletion of BIBX1382 was observed when incubated with rat and dog liver cytosol, whereas rapid depletion (half-life ≤ 7 minutes) 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 (Fig. 2B). In vitro substrate depletion half-life data were 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 (Fig. 3).

#### BIBX1382

The protonated molecular ion [M+H]+ for BIBX1382 was observed at \( m/z \) 388.14 with a retention time of 12.8 minutes. 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), \( m/z \) 361.0861 (calculated \( m/z \) 361.0869), and \( m/z \) 291.0549 (calculated \( m/z \) 291.0556), which all corresponded to ions after fragmentation of the methyl-piperidine moiety (Fig. 3A).

#### BIBU1476 (M1)

For the predominant oxidative metabolite of BIBX1382, which had a retention time of 10.9 minutes 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), \( m/z \) 361.0693 (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 (Fig. 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 minutes and produced a protonated molecular ion [M+H]+ at \( m/z \) 420.14 (e.g., +32 Da from BIBX1382). Among the daughter ions observed after 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 (Fig. 3C).

### In Vitro Biotransformation of BIBX1382

#### Cynomolgus Monkey Liver Cytosol

The liquid chromatography–mass spectrometry extracted ion chromatogram, demonstrating the formation of BIBU1476 (M1) after incubation of BIBX1382 (10 μM) with cynomolgus monkey liver cytosol, is shown in Fig. 4. Although 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 coincubating BIBX1382 with the selective AO inhibitor raloxifene at 50 \( \mu \)M, a roughly 66% decrease in peak area of BIBU1476 was observed. Meanwhile, after coincubation with the xanthine oxidase (XO) inhibitor allopurinol (50 \( \mu \)M), negligible inhibition of BIBU1476 metabolite formation was observed (Fig. 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. After incubation of BIBX1382 at both 1 and 10 μM for 60 minutes, 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 laboratory (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 (Fig. 5). The LC-MS/MS fragmentation of M2 is shown in Fig. 3C.

**Cynomolgus Monkey S9 Fraction.** In effort to characterize the origin (e.g., molybdenum hydroxylase versus cytochrome P450) of the secondary oxidation to produce M2, in vitro studies were conducted...
using S9 fraction from cynomolgus monkey liver, with and without supplementation of NADPH. After incubation of BIBX1382 (10 μM) in cynomolgus monkey liver S9 fraction (5 mg/ml) for 60 minutes, 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 (Fig. 6). It was apparent that formation of M2 was not dependent on the presence of NADPH, because the liquid chromatography-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 coincubated with raloxifene, there was a 41% reduction in M1, with essentially complete inhibition of M2 formation.

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

Fig. 5. Representative UV chromatogram demonstrating metabolite profile after 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.
(Supplemental Fig. 1). In addition, when allopurinol was coincubated, 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 versus time profile of BIBX1382 and the metabolite BIBU1476 after intravenous bolus (1 mg/kg) and oral (2 mg/kg) administration of BIBX1382 to male cynomolgus monkeys is shown in Fig. 7, A and B, respectively. Total plasma clearance of BIBX1382 (118 ml/min per kg) was higher than hepatic blood flow (Table 2), which suggests either an extrahepatic 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 2 hours (data not shown). In addition, it was found that the blood-to-plasma ratio of BIBX1382 in cynomolgus monkey blood was 2.1, whereas 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 per kg). When measuring the amount of parent BIBX1382 excreted in urine over a 24-hour period after intravenous administration, only 0.2% of the dose was recovered. Exposure after 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 (Fig. 8).
Although activity (measured by BIBU1476 formation) was relatively low in the extrahepatic human tissues evaluated, activity in cynomolgus monkey lung S9 fraction was substantially high (Fig. 8). When coincubating with the AO inhibitor raloxifene, a significant decrease in the formation of BIBU1476 was observed, whereas allopurinol had a minimal effect (Supplemental Fig. 2).

**Discussion**

Aldehyde oxidase 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, because of 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, because 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 link for this metabolic pathway, considering the sequence homology to human AOX1 (Hoshino et al., 2007), as well as the 99% homology to the rhesus monkey enzyme (Dittrich et al., 2002), consistent with the suggestions of Garattini and Terao (2012). However, within drug discovery and development, cynomolgus monkey is a more commonly used 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 humans.

The expression of AO in cynomolgus monkey (Macaca fascicularis) liver was previously established (Sugihara et al., 2000). In this report, cynomolgus monkey liver AO demonstrated high oxidase activity toward several known substrates, including benzaldehyde, phthalazine, and N\(^2\)-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; cyto, ACO73553.1; rhesus, ATF136988.1). 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 (Fig. 2). Furthermore, through use of specific inhibitors of molybdenum hydroxylases, our data show that this metabolic pathway is primarily catalyzed by AO in cynomolgus monkey (Fig. 4). To evaluate the in vitro-to-in vivo link for this metabolic pathway, a pharmacokinetic study was also conducted in cynomolgus monkeys.
After noncompartamental 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 (Blood to plasma ratio ~ 2.1) (Table 2). In addition, after oral administration, bioavailability was low and comparable to the pharmacokinetic data reported from human studies (Table 2), likely because of high first-pass metabolism by aldehyde oxidase. Corroborating this conclusion are data showing that the primary metabolite BIBU1476 was circulating in plasma at levels 160% of parent BIBX1382 (Fig. 7 and Table 2).

Similar to our findings with BIBX1382, the AO-mediated metabolism of SGX523, zaleplon, and RS-8359 has been reported to be comparable between cynomolgus monkey and human (Kawashima et al., 1999; Itoh et al., 2006; Diamond et al., 2010). 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 after dosing to cynomolgus monkeys. In another reported example, the metabolism and pharmacokinetics of SB-277011 were 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, although 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 predominately 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 preclinical 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; Nishimura and Naïto, 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. Although low but measurable activity was observed in human kidney, intestine, and lung S9 fractions, relatively high activity was observed in monkey lung S9 fraction (Fig. 8). The role of AO in cynomolgus monkey lung S9 was confirmed by use of raloxifene as an inhibitor of AO (Supplemental Fig. 2). Although 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 extrahepatic clearance observed in cynomolgus monkey. More studies would need to be conducted to confirm the relevance of this in vivo 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 (2013) when investigating zoniporide metabolism by AO across multiple species. 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 appear 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 representative of the human metabolism, both from a pharmacokinetic as well as a 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 with rhesus monkey for human AO from a practical standpoint. Rhesus monkey would require more compound for dosing (the 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, and Frederick.
Conducted experiments: Yang, Asher, and Frederick.
Performed data analysis: Hutzler, Yang, Asher, Wong, Frederick, and Gilpin.
Wrote or contributed to the writing of the manuscript: Hutzler, Cerny, Yang, Wong, Frederick, and Gilpin.

References

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolic Pathway</th>
<th>Metabolite</th>
<th>Matrix</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zaleplon</td>
<td>Oxidation of pyrazolo-pyrimidine</td>
<td>5-Oxo zaleplon</td>
<td>Liver S9 fraction</td>
<td>Kawashima et al., 1999</td>
</tr>
<tr>
<td>SB-277011</td>
<td>Oxidation of quinoline</td>
<td>M11</td>
<td>Liver S9 fraction, plasma, urine</td>
<td>Diamond et al., 2010</td>
</tr>
<tr>
<td>RS-8359</td>
<td>Oxidation of pyrimidine</td>
<td>Vanillic acid</td>
<td>Liver cytosol, hepatocytes</td>
<td>Sahi et al., 2008</td>
</tr>
<tr>
<td>Zebularine</td>
<td>Oxidation of pyrimidine</td>
<td>Liver S9 fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>Liver cytosol, hepatocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGX523</td>
<td>Oxidation of quinoline</td>
<td>M11</td>
<td>Liver cytosol, hepatocytes</td>
<td>Diamond et al., 2010</td>
</tr>
<tr>
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<td>M11</td>
<td>Liver S9 fraction, plasma, urine</td>
<td>Diamond et al., 2010</td>
</tr>
<tr>
<td>Zoniporide</td>
<td>Oxidation of quinoline</td>
<td>2-Oxo zoniporide</td>
<td>Liver S9 fraction</td>
<td>Dalvie et al., 2013</td>
</tr>
</tbody>
</table>


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Drug Metabolism and Disposition

Cynomolgus Monkey as a Surrogate for Human Aldehyde Oxidase Metabolism of the EGFR Inhibitor BIBX1382

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Supplemental Figure 1.

Inhibition of M1 (BIBU1476) and M2 formation in cynomolgus monkey liver S9 fraction (minus NADPH) by the AO inhibitor raloxifene and XO inhibitor allopurinol. Substantial inhibition of M1 and M2 by raloxifene but not allopurinol suggests that AO is the predominant enzyme involved in formation of M1 and M2 in cynomolgus monkey.
Supplemental Figure 2.

10μM BIBX1382 Incubated in Cyno Lung S9 (Formation of BIBU1476)

Inhibition of BIBU1476 (M1) formation in cynomolgus monkey lung S9 fraction (minus NADPH) by raloxifene following incubation of BIBX1382, implicating AO as the active enzyme.