ABSTRACT

N-Methyl-2-[3-((E)-2-pyridin-2-yl-vinyl)-1H-indazol-6-ylsulfanyl]-benzamide (axitinib) is an oral inhibitor of vascular endothelial growth factor receptors 1–3, which is approved for the treatment of advanced renal cell cancer. Human [14C]-labeled clinical studies indicate aitxinib’s primary route of clearance is metabolism. The aims of the in vitro experiments presented herein were to identify and characterize the enzymes involved in axitinib metabolic clearance. In vitro biotransformation studies of axitinib identified a number of metabolites including an axitinib sulfoxide, several less abundant oxidative metabolites, and glucuronide conjugates. The most abundant NADPH- and UDPGA-dependent metabolites, axitinib sulfoxide (M12) and axitinib N-glucuronide (M7) were selected for phenotyping and kinetic study. Phenotyping experiments with human liver microsomes (HLMs) using chemical inhibitors and recombinant human cytochrome P450s demonstrated axitinib was predominately metabolized by CYP3A4/5, with minor contributions from CYP2C19 and CYP1A2. The apparent substrate concentration at half-maximal velocity (Km) and Vmax values for the formation of axitinib sulfoxide by CYP3A4 or CYP3A5 were 4.0 or 1.9 μM and 9.6 or 1.4 pmol min⁻¹ pmol⁻¹, respectively. Using a CYP3A4-specific inhibitor (Cyp3cide) in liver microsomes expressing CYP3A5, 66% of the axitinib intrinsic clearance was attributable to CYP3A4 and 15% to CYP3A5. Axitinib N-glucuronidation was primarily catalyzed by UDP-glucuronosyltransferase (UGT) UGT1A1, which was verified by chemical inhibitors and UGT1A1 null expressers, with lesser contributions from UGTs 1A3, 1A9, and 1A4. The Km and Vmax values describing the formation of the N-glucuronide in HLM or rUGT1A1 were 2.7 μM or 0.75 μM and 8.9 or 8.3 pmol min⁻¹ mg⁻¹, respectively. In summary, CYP3A4 is the major enzyme involved in axitinib clearance with lesser contributions from CYP3A5, CYP2C19, CYP1A2, and UGT1A1.

In Vitro Kinetic Characterization of Axitinib Metabolism

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Introduction

N-Methyl-2-[3-((E)-2-pyridin-2-yl-vinyl)-1H-indazol-6-ylsulfanyl]-benzamide (axitinib) (International Union of Pure and Applied Chemistry) is a small molecule inhibitor of signaling through vascular endothelial growth factor receptors 1, 2, and 3. Axitinib inhibits the kinase activity of vascular endothelial growth factor receptors by binding to the ATP binding site of the tyrosine kinase domain (McTigue et al., 2012). Inhibiting the vascular endothelial growth factor pathway either through the receptor/ligand interaction or the kinase activity is an effective approach to anticancer therapy via preventing tumor angiogenesis. Angiogenesis is necessary for the growth and metastasis of all solid tumors, in that newly formed vessels provide nutrients for growing tumors and serve as an escape route for metastatic cells (Folkman, 1995; Folkman and D’Amore, 1996; Bergers and Benjamin, 2003). The approval of antiangiogenic agents such as bevacizumab [colorectal cancer, nonsmall cell lung cancer, glioblastoma, cervical cancer, ovarian cancer, and renal cell carcinoma (RCC)], sorafenib (RCC and hepatocellular carcinoma), and sunitinib (RCC and gastrointestinal stromal tumor) confirms the hypothesis that disrupting the vascular endothelial growth factor signaling pathway has utility in the treatment of a variety of human malignancies. Axitinib (Inlyta) is approved for use in the United States and other countries throughout the world for the second line treatment of patients with advanced renal cell cancer [Pfizer Laboratories, 2012, prescribing information for Inlyta (axitinib) tablets for oral administration; http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/202324lbl.pdf].

The pharmacokinetics and absorption, distribution, metabolism, and excretion (ADME) properties of axitinib were characterized as part of the drug development program. Axitinib is well absorbed after oral administration of a 5-mg dose in humans with an absolute bioavailability of 58%. The T1/2 value is reached by 2.5–4.1 hours and the elimination half-life is reached by 2.5–6.1 hours [Pfizer Laboratories, 2012, prescribing information for Inlyta (axitinib) tablets for oral administration; http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/202324lbl.pdf]. The mass balance following an oral dose of 5 mg [14C]axitinib was evaluated in healthy human subjects (Smith et al., 2014). Axitinib appeared to be extensively metabolized and eliminated in most subjects by 48 hours. Metabolism appeared to be the primary route of axitinib clearance since only 12% of unchanged drug was detected in feces and no parent drug was observed in the urine. Key elimination pathways based on primary metabolites detected in excreta were sulfoxide (M12), N-glucuronide (M7), hydroxymethyl (M8a precursor), oxidation products (M12a/M14), and sulfoxide/N-glucuronide (M9). Two metabolites, which were
pharmacologically inactive, were observed in human plasma, axitinib N-glucuronide (M7) and axitinib sulfoxide (M12), accounting for 50.4% and 16.2% of circulating radioactivity, respectively. Clinical drug interaction studies with axitinib as an object or victim drug have been conducted in combination with ketoconazole and rifampin as strong inhibitors and inducers, respectively, of CYP3A and the respective changes in axitinib plasma exposure suggested a predominant role for CYP3A in the clearance of axitinib since ketoconazole increased the area under the plasma versus time curve (AUC) ratio relative to control by 2.1-fold and rifampin pretreatment decreased this AUC ratio to 0.21 (Pithavala et al., 2010, 2012). The axitinib plasma exposure in cancer patients is variable (e.g., interindividual variability expressed as %CV is on the order of ~80%), but has been effectively addressed in the clinic by use of a dose-tolerability titration schedule that essentially normalizes patient exposure over a dose range from 2 to 20 mg/day [Pfizer Laboratories, 2012, prescribing information for Inlyta (axitinib) tablets for oral administration] (Chen et al., 2013; Rini et al., 2013). Therefore, better understanding of the factors that contribute to axitinib exposure variability could impact therapy through identification of factors that could be used to predict dose prior to initiation of drug treatment. The objective of the in vitro studies was to thoroughly investigate the metabolic pathways responsible for the metabolism of axitinib using human reagents and to characterize the individual enzymes contributing to its metabolism. This included state of the art methods to delineate contributions of CYP3A4 and CYP3A5 using microsomes from genotyped donors and selective inhibitors, UDP-glucuronosyltransferase (UGT) phenotyping, and enzyme kinetic analyses (Tseng et al., 2014). This detailed characterization of the enzymology of axitinib biotransformation contributes to the integrated understanding of the clinical implications of axitinib metabolism.

Materials and Methods

Materials

The characterization of axitinib, [14C]axitinib, axitinib N-glucuronide (M7), axitinib sulfoxide (M12), and benzylninvinol synthesis or biosynthesis (M7) is described below and has been described previously (Smith et al., 2014). [14C] axitinib GMP material was prepared at Amersham (Buckinghamshire, United Kingdom) for Pfizer, La Jolla, CA. Acetonitrile (CH3CN) was obtained from Mallinckrot Baker (Phillipsburg, NJ). (E)-3-[4-(25,35,45,SR)-5-[1-(3-chloro-2,6-difluorobenzoxy)oximinomethyl]-3,4-dihydroxytetrahydrofurufuran-2-yl]oxyoxy-phenyl)-2-methyl-N3a,4SR,6S,7aR]-4,6,7-trihydroxytetrahydrobenzol[1,3,5]dioxol-5-yl] acrylamide, a Pfizer analytical internal standard, was acquired from Pfizer’s internal compound library (Lee et al., 2012). Alachemthixan, UDP glucurononic acid (UDPGA), Triaza hydrochloride, Tetramethylsilane, MgCl2, NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, sulindac sulfide, cimetidine, buspirone, diclofenac, furafylline, sulphanapholoze, quinidine, azatavanur, erlotinib, hecogenin, digoxin, ketoconazole, CYP3cide (a specific small molecule mechanism-based inhibitor of CYP3A4), 1 M potassium phosphate dibasic solution, 1 M potassium phosphate monobasic solution, and dimethylsulfoxide (DMSO) were obtained from Sigma Aldrich (St. Louis, MO). Montelukast was purchased from Cayman Chemical (Ann Arbor, MI).

Pooled human liver microsomes (HLMs) prepared from 50 mixed-gender donors [21.7 mg of microsomal protein/ml or 0.350 nmol of total cytochrome P450 (P450)/mg of microsomal protein] were obtained from BD Gentest (Woburn, MA). Rat cryopreserved hepatocytes were purchased from Celsis, Inc. (Baltimore, MD). All deuterated solvents were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). All other materials were commercially available and of highest analytical grade.

Oxidative Biotransformation of Axitinib Using HLMs.
Pilot single incubation mixtures consisted of HLM (2 mg/ml) and axitinib (10 μM) in 100 mM potassium phosphate (KH2PO4), pH 7.4. The reaction was initiated by addition of NADPH (final 1 mM) with preincubation (3 minutes) or without preincubation. The mixture (1 ml) was then incubated at 37°C for 1 hour to drive the reaction to completion, regardless of enzyme degradation.

To evaluate the role of flavin-containing monoxygenase (FMO) in the formation of axitin sulfoxide in HLMs, a FMO stabilization/destabilization protocol was employed. The incubation mixtures consisted of HLM (2 mg/ml) in 100 mM potassium phosphate (pH 7.4), preincubated with or without NADPH (1 mM) at 50°C for 90 seconds, and the reaction was initiated by addition of axitinib and incubated for 1 hour at 37°C to produce sufficient quantifiable amounts of the metabolites formed. The reactions were terminated by the addition of CH3CN (5 ml) followed by vortexing for 1 minute and then centrifuging (1900 g). The supernatants were transferred into conical glass tubes for evaporation to dryness under N2 at 30 °C. The residues were reconstituted in 200 μl of 30:70 (v/v) CH3CN/water (0.1% formic acid), and aliquots (50 μl) were injected for high-performance liquid chromatography (HPLC)/mass spectrometry (MS) analysis. As positive controls, sulindac sulfide (10 μM) and cimetidine (1 mM) were incubated under the same conditions and analyzed by liquid chromatography (LC)/MS for the formation of the sulfoxide metabolite.

Metabolite profiling was performed using HPLC coupled in line with MS detection with the electrospray ionization source in the positive ion mode. For positive-mode MS detection, the mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in CH3CN (solvent B). Parent drug and its metabolites were eluted using a linear gradient in mobile phase composition. After passing through the diode array detector, the HPLC effluent was introduced into the mass spectrometer. The analog outputs from the diode array detector (Dionex, Sunnyvale, California) and MS detectors were recorded in real time by the data system provided with the mass spectrometer (Xcalibur version 1.4, ThermoFinnigan, San Jose, CA). The MS detection showed a delayed (0.2–1.3 minutes) response, compared with the diode array detector, as a result of plumbing. The major operating parameters for the ion-trap ESI-MS (electrospray ionization - Mass spectrometry) methods consisted of the following: spray voltage, 4.5 kV; capillary voltage, 2.6 V; tube lens offset, –20 V; capillary temperature, 270°C; sheath gas flow rate, 90 (arbitrary); and auxiliary gas flow rate, 30 (arbitrary). The Xcalibur software was used to control both HPLC and MS systems to acquire and process all spectral data. LC-MS spectra were acquired over a mass range of 150–1500 m/z. Data-dependent scanning was used to trigger MS2 and MS3 analysis of molecular ions and product ions over threshold intensities of 1.0 × 106 and 1.0 × 105 counts, respectively. Only the most intense ions from the MS and MS2 scans were selected for the MS2 and MS3 scans, respectively.

Oxidative Biotransformation of Axitinib with rCYPs.

A single incubation mixture of each rCYP (100 pmol/ml) included axitinib (10 μM) and 100 mM potassium phosphate (pH 7.4) in a final volume of 1 ml.
The reaction was initiated by addition of NADPH (final 1 mM) after a 3-minute preincubation and the reaction mixture (1 ml) was then incubated at 37°C for 1 hour to drive the reaction to completion, regardless of enzyme degradation. The reaction was terminated by the addition of CH3CN (5 ml), followed by vortexing for 1 minute before centrifugation (1900g). The supernatants were transferred into conical glass tubes for evaporation to dryness under N2 at 37°C. The residues were reconstituted in 200 μl of 30:70 (v/v) CH3CN:water (0.1% formic acid) and aliquots (50 μl) were injected for HPLC-MS analysis.

Glucuronidation of Axitinin with HLMs

Pilot in vitro experiments using human microsomes were conducted to investigate the formation of the human circulating metabolite axtinin-N-glucuronide (Glu2MT). These experiments also identified additional glucuronide (Glu) conjugates of axtinin termed Glu1 (minor) and in vitro glucuronide metabolite, Glu3 (major), an apparently major (Glu3) and minor (Glu1) glucuronide identified from in vitro incubations with [14C]axitinib. The incubation conditions and analytical methods for the metabolite profiling utilizing [14C]axitinib can be found in the Supplemental Material.

The definitive experiments conducted to characterize UGT enzyme kinetics are described subsequently. The linearity of product formation was evaluated using HLMs (0.1–3 mg/ml) for up to 45 minutes and was found to produce linear formation of product with protein (1 mg/ml) for up to 45 minutes. Thirteen axtinin concentrations (0.33–100 μM) were incubated with HLM (1 mg/ml) and fully activated with alamethicin (10 μg/ml) in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing magnesium chloride (5 mM). Final solvent (DMSO) concentrations were 1% (v/v) or less. Reactions were initiated with the addition of UDPGA (5 mM) in a final volume of 200 μl and incubated for 30 minutes before termination by transferring 50 μl of the incubation solution to a 96-well plate containing 200 μl CH3CN with dicyclofenac as the internal standard. Samples were mixed, centrifuged at 1900g [3600 rpm using a Beckman-Coulter (Carlsbad, California) GH3.8A rotor] for 10 minutes, and subjected to LC-MS/MS analysis to quantify the formation of axtinin N-glucuronide (M7) as described subsequently. Since authentic standards for Glu1 and Glu3 were not available, their formation was quantified relative to the axtinin N-glucuronide (M7) authentic standard. Definitive UGT1A1 reaction phenotyping experiments were performed similarly to the incubations described previously. Separate incubations were performed with pooled HLMs (N = 50) or genotyped HLMs pooled from UGT1A1*28 homozygous donors (N = 3) at a final protein concentration of 1 mg/ml with three axtinin concentrations (0.4, 2, and 10 μM), and were fully activated with alamethicin (10 μg/ml) in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing magnesium chloride (5 mM). Similarly, UGT isoform-selective chemical inhibitor experiments were performed by incubating axtinin (2 μM) at a concentration approximating the apparent substrate concentration at half-maximal velocity (Km), according to previous work, with pooled HLMs (1 mg/ml) in the presence and absence of atazanavir (1 and 10 μM), erlotinib (10 μM), and digoxin (10 and 60 μM). Inhibitor concentrations were selected based on unbound IC50 (IC50u) values for axtinin (UGT1A1 IC50u = 0.14 μM; UGT1A4 IC50u = 2.1 μM), erlotinib (UGT1A1 IC50u = 0.37 μM; UGT1A9 IC50u = 3.0 μM), and digoxin (UGT1A1 IC50u = 0.5–1 μM; UGT1A1 IC50 > 100 μM), and digoxin (UGT1A9 IC50u = 30 μM; UGT1A1 IC50 > 100 μM or ~20% inhibition at 60 μM) as characterized in our laboratories utilizing isoform-selective probe substrates (Walsky et al., 2012a). Reactions were initiated with UDPGA (5 mM) and at least three 40-μl aliquots were removed between 0 and 30 minutes by termination into a 96-well plate containing 200 μl CH3CN with dicyclofenac as the internal standard. Samples were mixed, centrifuged at 1900g (3600 rpm using a Beckman-Coulter GH3.8A rotor) for 10 minutes, and subjected to LC-MS/MS analysis to quantify the formation of axtinin N-glucuronide (M7) as described subsequently.

Biosynthesis and NMR Analysis or Quantification of Axitinin N-glucuronide (M7)

Axitinin N-glucuronide (M7) was obtained from biosynthesis and quantified for use as the analytical standard by quantitative NMR (Walker et al., 2011) with a Bruker Avance 600 MHz NMR Spectrometer controlled by TopSpin V3 with a TCI Cryo probe (1.7 or 5 mm) (Bruker BioSpin Corporation, Billerica, MA). A 20-ml reaction of Axitinin (20 μM) was incubated with cryopreserved isolated rat hepatocytes (750,000 cells/ml) in modified William’s E-media (pH 7.4). The incubation was conducted in a 250-ml Erlenmeyer flask in a shaking water bath maintained at 37°C for 4 hours. At the end of the incubation, 80 ml CH3CN was added; the mixture was transferred to two 50-ml polypropylene conical tubes and vigorously mixed on a vortex mixer. The tubes were spun in a Model CT422 centrifuge (Jouan, Saint-Herblain, France) at 1700g for 5 minutes and the supernatant was transferred to new 50-ml polypropylene conical tubes. The tubes were subjected to vacuum centrifugation for approximately 1 hour in an evaporator (Genevac, Valley Cottage, NY) to remove the CH3CN. To the remaining solution was added water, to a total volume of ~100 ml, and formic acid (1 ml). This mixture was subjected to centrifugation in a Beckmann (Carlsbad, California) centrifuge at 40,000g for 30 minutes to clarify the supernatant. The supernatant was transferred to a 100-ml graduated cylinder and directly applied onto a Varian (Santa Clara, California) Polaris C18 column (4.6 × 250 mm; 5 μm particle size) through a PU-980 HPLC pump (Jasco Analytical Instruments, Easton, MD) at a flow rate of 0.8 ml/min. Following application of the 100 ml, another ~10 ml of 0.1% formic acid was pumped onto the column to ensure that the HPLC lines were cleared of the supernatant.

The HPLC column was then transferred to a Thermo LTQ HPLC-UV-MS system containing a Surveyor Quaternary HPLC, Surveyor PDA Detector, and LTQ Mass Spectrometer (Thermo Fisher, Wilmington, DE) with the mass spectrometer operated in the positive ion mode. The effluent was split between the mass spectrometer and a FC-204 fraction collector (Gilson, Middleton, WI) at a ratio of approximately 1:15. The mobile phase consisted of 0.1% formic acid (mobile phase A) and CH3CN (mobile phase B) at a flow rate of 0.6 ml/min. The mobile phase composition commenced at 100% A/0% B and was held at that composition for 5 minutes, followed by a linear gradient to 50% A/50% B at 1 hour. The mobile phase composition was held at that composition for 5 minutes, and then re-equilibrated at the initial conditions for 14 minutes. The mobile phase program and data collection were started with an initial dummy injection of water from the autosampler. Fractions were collected every 20 seconds into a wide-welled polypropylene microliter plate. The fractions collected in the region of interest [i.e., where the mass spectrometer indicated the presence of m/z 563, the protonated molecular ion of the Glu metabolite (M7)] were injected (5 μl) onto a second Polaris C18 column using the same mobile phase gradient to test for purity. Those fractions containing the product of interest were combined into a 700-μl conical glass tube and the solvent was evaporated by vacuum centrifugation in the Genevac evaporator. Upon dryness these fractions were prepared for NMR analysis as described subsequently.

The isolated axtinin glucuronide (M7) was dissolved in 0.04 ml of DMSO-d6 (Cambridge Isotope Laboratories, Inc., Andover, MA) and placed in a 1.7-mm NMR tube under a dry argon atmosphere. The 1H and 13C spectra were referenced with residual DMSO-d6 (1H δ = 2.50 ppm relative to Tetramethylsilane, δ = 0.00 ppm, 13C δ = 39.50 ppm relative to Tetramethylsilane, δ = 0.00). The NMR spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer (Bruker BioSpin Corporation, Billerica, MA) controlled by TopSpin V3.1 and equipped with a 1.7-mm TCI Cryo probe. One-dimensional spectra were recorded using an approximate sweep width of 8400 Hz and a total recycle time of approximately 7 seconds. The resulting time-averaged free-induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance the signal to noise. The two-dimensional data were recorded using the standard pulse sequences provided by Bruker. At minimum, a 1K × 128 data matrix was acquired using a minimum of two scans and 16 dummy scans with a spectral width of 10,000 Hz in the F2 dimension. The two-dimensional data sets were zero-filled to at least a 1K data point. Postacquisition data processing was performed with TopSpin V3.1 and MestReNova V8.1 (Mestrelab Research, Escondido, California). The concentration of the isolated axtinin glucuronide (M7) was determined by quantitative NMR to be 5.2 ± 0.1 mM.

Reaction Phenotyping

P450 Phenotyping Studies with Chemical Inhibition. Chemical inhibition experiments were performed using pooled HLMs and chemical inhibitors of CYP 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4. All inhibitors were treated as reversible inhibitors and were demonstrated to completely inhibit each respective enzyme as follows: 30 μM furafline (1A2), 0.2 μM monteleukast (2C8), 5 μM sulphaphenazole (2C19), 5 μM benzylxirvin (2C19), 1 μM quinidine (2D6),...
and 1 µM ketonozazole (3A) (Newton et al., 1995; Zhang et al., 2007; Harper and Brassil, 2008; Emoto et al., 2010). Each phenotyping study was conducted in duplicate at an axitinib substrate concentration assumed to be at or below Km (1 µM final) in a final organic concentration of 0.01% DMSO and 0.6% CH3CN. Reaction mixtures (175 µl) containing the individual inhibitors axitinib (1 µM), HLM (0.833 mg/ml), and 100 mM phosphate buffer (pH 7.4) were preincubated for 5 minutes at 37°C in an incubator on the Beckman FX automation system. Reactions were initiated by the addition of 25 µl of freshly prepared NADPH regeneration system (the final NADPH regeneration system concentrations for HLM (0.833 mg/ml), and 100 mM phosphate buffer (pH 7.4) were preincubated for 5 minutes at 37°C in an incubator on the Beckman FX automation system. Reactions were initiated by the addition of 25 µl of freshly prepared NADPH regeneration system (the final NADPH regeneration system concentrations for NADP+, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase in KH2PO4 buffer (pH 7.4) were 1 mM, 5 mM, and 1 U/ml, respectively) or an equal volume of 100 mM KH2PO4 buffer (pH 7.4) for a negative control. At 0, 10, 20, and 45 minutes, 25-µl aliquots were removed and the reaction was quenched with 75 µl of cold methanol containing the internal standard buspirone (0.1 µM). Samples were centrifuged at 1900g (Beckman-Coulter GH3.8A rotor) for 15 minutes. Aliquots (50 µl) of supernatant were transferred to a clean, shallow 96-well plate and each aliquot was combined with 50 µl of Milli-Q water, mixed, and analyzed for axitinib substrate disappearance and axitinib sulfoxide appearance by LC-MS/MS.

Recombinant P450 Phenotyping

Similarly to the chemical inhibition phenotyping, P450 phenotyping experiments were performed in duplicate using rCYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A5, and HLM as a control and comparator reaction. Reaction mixtures (184 µl) containing the individual recombinant P450 or HLM, or axitinib, and 100 mM phosphate buffer (pH 7.4) were preincubated for 5 minutes at 37°C in an incubator on the Beckman Biomek FX automation system. Reactions were initiated by the addition of the NADPH regeneration system (16 µl) or an equal volume of buffer as a negative control. In the final reaction mixture, the addition of each rCYP resulted in a P450 content of 100 pmol/ml (20 pmol/reaction) or in the HLM reaction 0.8 mg/ml. Reaction concentrations were 1 mM for NADPH, 100 mM for phosphate buffer (pH 7.4), and 1 µM for axitinib. At 0.5, 10, 20, 30, and 45 minutes, the 25-µl aliquots were removed and the reaction was quenched with 75 µl of cold methanol containing 0.1 µM buspirone (internal standard). Samples were centrifuged at 1900g (Beckman-Coulter GH3.8A rotor) for 15 minutes. Aliquots (50 µl) of supernatant were transferred to a clean, shallow 96-well plate and each aliquot was combined with 50 µl of Milli-Q water, mixed, and analyzed for axitinib substrate disappearance and axitinib sulfoxide appearance by LC-MS/MS.

Delineation of the Fraction Metabolized via CYP3A4 versus CYP3A5 Using CYP3Cide

Axitinib (1.0 µM) was incubated, in triplicate, with pooled lots of HLM CYP3A5*1/*1 (from five donors, see the Materials section) and HLM CYP3A5*3/*3 (from six donors, see the Materials section) at a protein concentration of 1 mg/ml for measurement of intrinsic clearance (CLint) in the presence and absence of chemical inhibitors, essentially as previously described (Walsky et al., 2012b; Tseng et al., 2014). Incubations were carried out in triplicate in 100 mM KH2PO4 (pH 7.4) containing MgCl2 (3.3 mM), ketoconazole (2 µM), or CYP3Cide (1 µM) and NADPH (1.3 mM) in a total volume of 0.3 ml. Incubations were commenced with the addition of NADPH cofactor and carried out in a 96-well heating block at a temperature of 37°C. As a control, incubation absence of NADPH was included to express a baseline non-P450 metabolism in the presence of the HLM. At times ranging between 0 and 60 minutes, aliquots (0.03 ml) were removed and added to 120 µl of termination solution (100% CH3CN containing (E)-3-4-((2S,3S,4S,5R,5a)-1-(3-chloro-2,6-difluorobenzoyloxyimino)ethyl)-3,4-dihydroxytetrahydrofururan-2-yl)-3-hydroxy-phenyl)-2-methyl-N-(3aS,4R,5R,6S,7R,7aR)-4,6,7-trihydroxyhexahydrobenzo[d][1,3]dioxol-5-yl) acrylamide, was conducted in the same manner as reported in Lee et al. (2012).

Recombinant UGT (rUGT) Phenotyping and Enzyme Kinetics

Thirteen recombinantly expressed UGTs were evaluated for their ability to catalyze the formation of the circulating human N-glucuronide (M7) and the two in vitro axitinib glucuronides (Glul and Glux) identified following incubation with HLMs (Supplemental Table 1). Axitinib (100 µM) was incubated in triplicate with rUGTs (0.5 mg/ml) in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing magnesium chloride (5 mM). Final DMSO concentrations were 1% (v/v) or less. Reactions were initiated with the addition of UDPGA (5 mM) to a final volume of 200 µl and incubated for 30 minutes before termination by the addition of 0.4 ml CH3CN containing diclofenac as the internal standard. Samples were centrifuged, reconstituted in the mobile phase, and subjected to LC-MS/MS analysis to quantify the three glucuronides as described subsequently. Based on the results from the UGT reaction phenotyping (Fig. 4), the abundance of axitinib N-glucuronide (M7) justified further kinetic characterization with UGT 1A1, 1A3, and 1A9. Definitive enzyme kinetics were performed in triplicate following initial evaluation of the linearity of product (M7) formation with respect to protein (0.05–1 mg/ml) and time (10–45 minutes), which were found to be linear with protein (1 mg/ml) up to 45 minutes. Final incubations contained 13 axitinib concentrations (0.33–100 µM), which were incubated with rUGTs (0.5 mg/ml) in 100 mM Tris-HCl buffer (pH 7.4 at 37°C) containing magnesium chloride (5 mM). Final DMSO concentrations were 1% (v/v) or less. Reactions were initiated with the addition of UDPGA (5 mM) in a final volume of 200 µl and incubated for 30 minutes before termination by transferring 50 µl of the incubation solution to a 96-well plate containing 200 µl CH3CN with diclofenac as the internal standard. Samples were mixed, centrifuged at 1900g (3600 rpm using a Beckman-Coulter GH3.8A rotor) for 10 minutes, and subjected to LC-MS/MS analysis to quantify the formation of axitinib N-glucuronide (M7) as described subsequently.

Determination of Nonspecific Microsomal Binding

The free fraction of axitinib in human liver microsomal incubations (0.71 mg/ml) was determined by equilibrium dialysis essentially as previously described (Di et al., 2012). The resulting fmec was further applied and corrected across microsomal reaction concentrations by extrapolating the fraction unbound for these subsequent experiments using the equation derived by Austin et al. (2002). This method was applied due to the validity and applicability of the method Austin et al. (2002) verified and the sheer number of studies with differing levels of microsomal protein.

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Bioanalytical Methods for Reaction Phenotyping and Enzyme Kinetics

The system used for P450 reaction phenotyping and kinetic studies was comprised of an HTS PAL Autosampler (Leap Technologies, Carrboro, NC), a Synergy μPolar-RP 80A column (30 × 2.0 mm, Phenomenex, Torrance, CA), a 10ADvp series HPLC controller and pumps (Shimadzu Scientific Instruments, Columbia, MD), and an API 4000 triple quadrupole mass spectrometer (TurboIonSpray; Applied Biosystems/MDS Sciex, Foster City, CA). A binary gradient utilizing mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in CH3CN) eluted 15 μl (loop under-fill) injections. Samples were loaded onto the column with 1% B at 800 μl/min, held at 1% B until 0.3 minutes, ramped to 90% B at 1.50 minutes, held at 90% B until 1.70 minutes, and then ramped back to 1% B at 1.71 minutes and held for the remainder of the 2-minute method. Multiple reactions monitoring was used to quantify analytes. The m/z transitions for axitinib and its metabolites are listed in Supplemental Table 1. The peak area ratio of the analyte to the internal standard (buspirone) was determined for each injection and used to measure substrate depletion or product formation as appropriate. Sample concentrations of the metabolites were determined against standard curves prepared in the extract conditions matched to those in the experiment.

Sample analysis for glucuronide metabolism formation was carried out on an AB SCIEX 5500 Triple Quadrupole (Applied Biosystems) equipped with ACQUITY UPLC System (Waters Corporation, Milford, MA). Chromatographic separation was accomplished on an ACQUITY UPLC Column (C18, HSS T3, 1.8μm, 2.1 × 100 mm, Waters Corporation). The mobile phase consisted of two solvents, solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in CH3CN). The gradient started at 2% B (for 0.3 minutes), followed by a linear increase to 30% B in 8.7 minutes and then to 95% B in 0.2 minutes, kept at 95% B for 1.8 minutes, and then followed by a linear decrease to 2% B in 0.2 minutes. The total run time for each injection was 12 minutes. The flow rate was 0.4 ml/min (0-9.2 minutes) and 0.45 ml/min (9.2–12 minutes). The multiple reactions monitoring mode was employed for the quantification: m/z 563 → 387 for axitinib N-glucuronide and m/z 296 → 215 for diclofenac (internal standard). Under these conditions, Glu1, Glu2 (M7), Glu3, and the internal standard were separated at the retention times of 7.1, 7.2, 7.7, and 10 minutes, respectively.

Data Analysis

The half-life (t1/2) was calculated using the following equation:

\[ t_{1/2} = \frac{\ln 2}{k_{deg}} \]  
(1)

where \( k_{deg} \) = − (slope of ln of the average peak area ratio of the replicates versus time). For the rCYP phenotyping study, the in vitro CLint was calculated using the following equation:

\[ CL_{int}(\mu l/min/pmol) = k_{deg} \times \text{ml of incubation} / \text{pmol of rCYP} \times (1000 \mu l/ml) \]  
(2)

For scaling from a recombinant system to HLM CLint an intersystem extrapolation factor (ISEF) was applied in conjunction with the abundance of the P450 in the liver (eq. 3). The ISEF numbers can be found in Supplemental Table 2. It has been the experience of the authors that CLint ISEF values are preferred over \( V_{max} \) ISEF values when scaling CLint rates from rCYP enzymes to human liver microsomal data (not shown):

\[ CL_{int}(\mu l/min/mg) = \left[ CL_{int} \times P450 \text{ abundance per mg of HLM} / V_{max} \right] \times \text{ISEF[derived from CLint or } V_{max}/K_m] \]  
(3)

The % contribution of each rCYP to the overall metabolic CLint was calculated using the following equation:

\[ \% \text{ Contribution} = 100 \times CL_{int} \text{ of each contributing P450} / (\text{sum of the } CL_{int} \text{ of all of the contributing P450s}) \]  
(4)

Chemical Inhibition Phenotyping Assay

The human CLint was calculated using the following equation:

\[ CL_{int}(\text{ml/min/kg}) = \frac{\text{ln2}}{\text{microsomal } t_{1/2}} \times \frac{\text{ml incubation}}{\text{microsomes}} \times \frac{45 \text{ mg microsomes}}{\text{g of liver}} \times \frac{21 \text{ g of liver}}{\text{kg of body weight}} \]  
(5)

The % contribution of each P450 isozyme was calculated using the following equation:

\[ \% \text{ Contribution} = 100 \times \left( \frac{\text{[average of the control CLint]} - \text{CLint of the inhibited reaction}}{\text{[average of the control CLint]} - \text{CLint of the uninhibited reaction}} \right) \]  
(6)

The relative % contribution was calculated using the following equation:

\[ \text{Relative % contribution} = 100 \times \left( \% \text{ contribution of one P450} \times \text{summation of all the % contributions from the P450s tested} \right) \]  
(7)

Delineation of the Fraction Metabolized via CYP3A4 versus CYP3A5

The linear portion of the mean regression line (n = 3) was used to determine a single slope of each incubation condition (\( k_{deg} \)). The slope of the line was used to determine the t1/2 (minutes) at each incubation condition (eq. 1), which was then extrapolated to the CLint, similar to eq. 2, but replacing the concentration of rCYP with the mg of pooled human liver microsomal protein in the equation. The formation of axitinib sulfoxide was also measured in incubations performed with genotyped microsomes to further verify that the depletion rates were associated with the sulfoxide metabolite. It was found that approximately 20% of axitinib was consumed after 5 minutes of incubation, and thus violating the rules of first-order kinetics. However, since the earliest time point investigated was 5 minutes, and approximately 80% of the original axitinib substrate concentration was remaining, the amount of the sulfoxide (M12) metabolite formed at 5 minutes was used to determine the percent CYP3A5 contribution.

The contribution by CYP3A5 was calculated using the following equations (Tseng et al., 2014):

Calculated % CYP3A contribution = \( \frac{\text{CLint}_{NADPH} - \text{CLint}_{substrate}}{\text{CLint}_{NADPH}} \times 100\% \)  
(8)

Calculated % CYP3A4 contribution = \( \frac{\text{CLint}_{NADPH} - \text{CLint}_{CYP3A4}}{\text{CLint}_{NADPH}} \times 100\% \)  
(9)

Calculated % CYP3A5 contribution = %CYP3A - %CYP3A4  
(10)

Enzyme Kinetic Parameter Determination

Substrate concentration [S] and velocity (V) data were fitted to the appropriate enzyme kinetic model by nonlinear least-squares regression analysis (Sigmmaplot v13, Systat Software, Inc., Chicago, or GraphPad Prism version 5.0, GraphPad Software, Inc., San Diego, CA) to derive the apparent enzyme kinetic parameters \( V_{max} \) and \( K_m \) (substrate concentration at half-maximal velocity). The Michaelis-Menten model (eq. 11) and the substrate activation model (eq. 12), which incorporates the Hill coefficient (n), were evaluated for best-fit of the data:

\[ V = \frac{V_{max} \times S}{K_m + S} \]  
(11)

\[ V = \frac{V_{max} \times S^n}{(S_0^n + S^n)} \]  
(12)

where \( V_{max} \) is the maximal velocity; \( K_m \) is the substrate concentration at half-maximal velocity; and n is an exponent indicative of the degree of curve sigmoidicity (or Hill coefficient). The best fit was based on a number of criteria, including visual inspection of the data plots (Michaelis-Menten and
Eadie-Hofstee), distribution of the residuals, size of the sum of the squared residuals, and the S.E. of the estimates. The $CL_{int}$ value was calculated as the $V_{max}/Km$ value for the Michaelis-Menten kinetics, and where necessary for the sake of comparison, the unbound $CL_{int}$ ($CL_{int,u}$) was corrected for the fraction of unbound substrate in incubation ($f_{un}$) as $CL_{int,f_{un}}$.

For the calculation of unbound clearance based on rCYP scaling from a recombinant system to HLM clearance, an ISEF was applied in conjunction with the abundance of the P450 in the liver and the fraction unbound in microsomes ($f_{mic}^{(c)}$) (eq. 13). Equation 13 provides an advanced understanding of the kinetic parameters based on the contribution of individual enzymes, and is therefore considered a better estimate of individual CYP-mediated clearance when evaluated for agreement of $CL_{int}$ estimates obtained in HLM.

$$CL_{int,u}(\mu l/min/mg) = \left( \frac{V_{max}/K_m \times CYP \text{ abundance per mg of HLM}}{f_{mic}} \right)$$

(13)

Results

Metabolite Profiling of Axitinib in HLMs. When incubated with HLMs in vitro, axitinib showed a moderate in vitro metabolic turnover rate, which was sufficient to identify in vitro metabolites (Table 1). Axitinib was primarily metabolized through oxidative pathways. The metabolite profile of axitinib in HLMs is shown in Fig. 1. Axitinib was mainly metabolized in vitro to the sulfoxide (M12).

Oxidation of pyridine and methyl amide moieties of axitinib and further oxidation of M12 resulted in the formation of a couple minor metabolites [M8b (M8a glucuronide precursor) and M15]. The structures of the metabolites were identified based on MS data. Metabolite standards of M7, M12, M9, and M15 (Fig. 2), which were chemically synthesized for the [14C]axitinib human absorption, distribution, metabolism, and excretion study, were used to confirm the in vitro structures (Smith et al., 2014). These in vitro metabolites are in agreement with in vivo metabolites (e.g., M12, M8a, M12a/M14, and M15) observed in human excreta as determined from a human mass balance study following administration of [14C]axitinib (Smith et al., 2014).

Non-specific Binding of Axitinib in HLMs. At a concentration of 0.71 mg/ml in HLMs, the axitinib free fraction was measured to be 0.42.

P450 Phenotyping Studies of Axitinib. In the in vitro P450 phenotyping study, eight rCYP enzymes were used to identify the P450s involved in axitinib metabolism. CYP3A4 was primarily responsible for metabolism (~92%) of axitinib (Table 1), determined by using the $CL_{int}$ values estimated from the experimental half-life (substrate disappearance) from rCYP enzymes using $CL_{int}$ ISEF values (Supplemental Table 2). The CYP3A4 contribution was explored utilizing Panvera Corp. recombinant enzyme; therefore, the ISEFs appropriate for that enzyme were applied (Supplemental Table 2). The metabolism of axitinib, as determined by substrate disappearance, indicated contribution by the other rCYPs was negligible, except for CYP3A5, CYP1A2, and to a much lesser extent CYP2C19 and CYP2D6. The half-life and extrapolated $CL_{int,u}$ values adjusted using the $CL_{int}$ ISEF are summarized in Table 1. The predicted scaled $CL_{int,u}$ extrapolated from rCYP3A4 was approximately 2-3-fold greater than what was predicted from HLM stability within the assay (94 versus 291 μl/min·mg⁻¹). Due to the lack of known specific substrates for CYP3A5 to differentiate from CYP3A4 a putative ISEF value has not been clearly established for clearance scaling from recombinant CYP3A5. Therefore, it was not possible to scale up the contribution associated with CYP3A5 to axitinib metabolism in a meaningful way, and therefore additional mechanistic studies were performed. The uncorrected observed apparent $CL_{int}$ ($CL_{int,app}$) value resulting from the rCYP3A5 experiment equated to 0.335 μl/min/pmol of rCYP3A5, hence inferring a portion of axitinib metabolism is attributable to CYP3A5.

In the P450 phenotyping chemical inhibitor studies, six selective chemical inhibitors were used to identify the P450s responsible for in vitro axitinib metabolism in HLMs. These data indicate the CYP3A family is primarily responsible for metabolism of axitinib (~78%) with minor contributions from 2D6 (7.5%), C219 (6.4%), and C29 (5.4%). Based on chemical inhibition studies, the contributions from C28 (2.4%) and 1A2 (0%) toward the metabolism of axitinib were negligible. The $CL_{int,u}$ values are summarized in Table 1. There was no contribution to metabolism of axitinib from any of the other P450 isoforms tested in this assay. A summary of both phenotyping assays provides a reasonably consistent finding; the majority of axitinib clearance is associated with CYP3A4, with minor contributions from a few other P450s (CYP1A2 > C219 >> 2D6).

### TABLE 1

Definitive phenotyping and kinetic determination utilizing HLMs and recombinant P450

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chemical Inhibitor</th>
<th>CYP Phenotyping</th>
<th>Recombinant CYP Phenotyping</th>
<th>Kinetic Determination of Axitinib Sulfoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$CL_{int,app}$</td>
<td>$CL_{int,app}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td>Chemical Inhibitor</td>
<td>$\mu l/min\cdot mg^{-1}$</td>
<td>Relative Contribution</td>
<td>$\mu l/min\cdot mg^{-1}$</td>
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<tr>
<td>CYP1A2</td>
<td>30 μM Furafylline</td>
<td>108.1</td>
<td>0</td>
<td>24.4</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.2 μM Montelukast</td>
<td>101.8</td>
<td>2.60</td>
<td>N/A</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>5 μM Sulphaphenazole</td>
<td>98.4</td>
<td>5.30</td>
<td>N/A</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>5 μM Benzylviniranol</td>
<td>97.2</td>
<td>6.40</td>
<td>1.20</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>1 μM Quinidine</td>
<td>95.8</td>
<td>7.50</td>
<td>0.040</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>1 μM Ketaconazole</td>
<td>11.4</td>
<td>78.2</td>
<td>291</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>(F, 13)</td>
<td>104.6</td>
<td>N/A</td>
<td>94.5</td>
</tr>
<tr>
<td>HLM</td>
<td></td>
<td></td>
<td>N/A</td>
<td>104.6</td>
</tr>
</tbody>
</table>

N/A, not applicable; —, no ISEF available.

Kansas City, Missouri, 2014, CYP3A4 and CYP3A5.

The $V_{max}$ value for HLM is expressed as pmol/min·mg.
Oxidative Metabolites Formed by Recombinant Enzymes. The identification of P450 enzymes responsible for the generation of axitinib metabolites was conducted in vitro utilizing recombinant CYP3A4, CYP3A5, CYP1A2, and CYP2C19 based on the preliminary chemical inhibition reaction phenotyping results. Metabolite profiles of axitinib generated with recombinant CYP3A4, CYP3A5, CYP1A2, and CYP2C19 are shown in Supplemental Fig. 1. CYP3A4 and CYP3A5 showed a similar metabolite profile, where sulfoxide (M12) was the major metabolite and the di-oxygenated metabolite M15 and hydroxymethyl metabolite M8b were minor metabolites. M14 was not observed in CYP3A4 incubations. Incubation of axitinib with CYP2C19 produced mainly M8b and apparently lesser amounts of M12 and M14. CYP1A2 generated M12a and M14, and M12a appeared to be only formed by CYP1A2. A proposed metabolic scheme of the metabolites

Fig. 1. Metabolite profile of axitinib derived for HLM incubations in the presence of NADPH (UV chromatogram at 356 nm).

Fig. 2. Proposed in vitro metabolic biotransformation scheme for axitinib. Both Glu1 and Glu3 were not observed in vivo in the human [14C] absorption, distribution, metabolism, and excretion study; therefore, no further effort to elucidate the definitive structure of the glucuronide was undertaken.
produced by the P450s and specific P450s responsible for each metabolite is shown in Fig. 2.

The major in vitro oxidative metabolite of axitinib is the sulfoxide (M12) and was also identified as a significant circulating metabolite representing 16.2% of radioactivity in vivo in humans and a notable metabolite eliminated in urine (Chen et al., 2013). In general, sulfoxidation can be mediated by both P450s and FMOs. Therefore, the potential role of FMOs in the formation of axitinib sulfoxide in HLMs was evaluated. After heat inactivation of human liver FMOs, sulfoxidation of axitinib by HLMs remained unchanged, whereas the oxidation of two known FMO substrates, sulindac sulfoxide and cimetidine, decreased to 34% and 35% of those without FMO inactivation. The result suggested that FMO was not involved in the sulfoxidation of axitinib (Supplemental Fig. 2).

**Delineation of the Fraction Metabolized via CYP3A4 versus CYP3A5 Using CYP3cide.** Following a 1-mg/ml microsomal incubation using both a CYP3A5 *1/*1 and a *3/*3 genotyped pooled donor lots, tested at 1 μM of axitinib in the presence and absence of NADPH with the addition of ketoconazole or CYP3cide, the data in CYP3A5*1/*1 HLMs suggests that CYP3A4 and CYP3A5 contribute 66% and 15% to axitinib metabolism, respectively (Fig. 3A). As a comparator the pooled CYP3A5 *3/*3 microsomes showed the CYP3A4 contribution to metabolism to be 74% of the total oxidative CLint (Fig. 3B). The CLint_app,scaled (eq. 5) data used to calculate the CYP3A4 and CYP3A5 contributions is displayed in Table 2.

To ensure proper interpretation, the axitinib sulfoxide (M12) metabolite formation was measured in the presence and absence of CYP3cide and compared with ketoconazole at an early time point of 5 minutes to ensure minimal axitinib disappearance. The resulting contribution from each isozyme yielded a CYP3A5 contribution of 18% and a CYP3A4 contribution of 77% (Fig. 3C) to the formation of axitinib sulfoxide. The CYP3A5 *3/*3 pooled lot using the same method provided a very similar contribution using either ketoconazole or CYP3cide (93%) (Fig. 3D).

**Glucuronidation of Axitinib in HLMs and rUGTs.** In pilot experiments, incubation of [14C]axitinib in HLMs supplemented with UDPGA generated small quantities of three glucuronide metabolites in initial studies. These glucuronides were identified as Glu1, Glu2, and Glu3 (Supplemental Table 3). Glu2 was subsequently identified as axitinib N-glucuronide (M7), a major circulating metabolite in humans (Smith et al., 2014). Therefore, M7 was prepared biosynthetically as an authentic standard for further in vitro evaluation. In the pilot studies, UGT1A1 and UGT1A4 formed the three axitinib glucuronide conjugates (Supplemental Table 3), prompting additional investigation. Subsequent definitive reaction phenotyping studies with microsomes from cells expressing rUGTs revealed an involvement of UGT1A1, UGT1A3, UGT1A4, and UGT1A9 in the formation of M7 (Fig. 4). Formation of the other two in vitro glucuronides (Glu1 and Glu3) were mediated almost exclusively by UGT1A4 (Fig. 4). Since Glu1 and Glu3 were not identified as a metabolite present in human plasma, and Glu3 was apparently the most abundant glucuronide formed in in vitro incubations with [14C]axitinib (100 μM) and nonradioactive studies (Fig. 4), more detailed UGT enzyme kinetic studies were conducted to explain the apparent in vitro and in vivo differences as described subsequently. UGT1A4 was not included in these kinetic assessments due to two reasons: 1) the extremely low levels of M7 produced at the assumed Vmax concentration of axitinib; and 2) the limitations in axitinib solubility made it difficult to attain maximum velocity and estimate kinetic parameters for UGT1A4. A scheme of the glucuronide metabolites produced by the UGTs and the particular UGTs responsible for each metabolite is shown in Fig. 2.

Additional UGT1A1 phenotyping experiments with UGT1A1*28/*28 genotyped microsomes indicated an approximately 75% reduction in the CLint_app with UGT1A1*28 (0.72 μl·min⁻¹·mg⁻¹) compared with wild-type pooled HLMs (2.8 μl·min⁻¹·mg⁻¹) at an axitinib concentration of 2 μM (Fig. 5) (Fig. 6). The maximal activity following incubation with a high axitinib concentration (10 μM) was also significantly reduced from 9.55 to 3.34 pmol·min⁻¹·mg⁻¹ in wild-type
versus UGT1A1*28 genotyped HLMs, respectively. Axitinib glucuronidation at concentrations approximating $K_m$ (2 µM) were similarly reduced from 4.44 to 1.64 pmol·min$^{-1}$·mg$^{-1}$ in wild-type versus UGT1A1*28 genotyped HLMs, respectively. The potent UGT1A1 chemical inhibitor, atazanavir (Zhang et al., 2005), at 1 and 10 µM inhibited M7 formation by 61% and 80%, respectively, while the selective UGT1A4 inhibitor, hecogenin (Uchaipichat et al., 2006), at 10 µM did not inhibit M7 formation. Erlotinib, a potent UGT1A1 and weak UGT1A9 inhibitor (Lapham et al., 2012), at 3 and 10 µM inhibited M7 formation by 68% and 83%, respectively. In contrast, digoxin, a potent UGT1A9 and weak UGT1A1 inhibitor (Lapham et al., 2012), at 10 and 60 µM inhibited M7 formation by 11% and 40%, respectively, substantiating a lesser role for UGT1A9 in axitinib glucuronidation.

### P450 and UGT Kinetic Studies.

Since the biotransformation and human absorption, distribution, metabolism, and excretion studies of axitinib indicated that the sulfoxide (M12) and $N$-glucuronide (M7) metabolites of axitinib were the major circulating metabolites in plasma (Smith et al., 2014), additional enzyme kinetic studies were conducted in the microsomal and recombinant enzyme systems that formed these metabolites. Recombinant CYP3A4, 3A5, 2C19, HLMs, and rUGT1A1, 1A3, and 1A9 enzymes were used to estimate enzyme kinetic determinations of axitinib metabolism. The CYP3A4 results showed complete saturation kinetics via a direct plot of the sulfoxide metabolite formation (Fig. 5; Table 1). Therefore, all of the kinetic parameters could be estimated within the solubility limit of axitinib. The kinetic parameters for the sulfoxide suggest CYP3A4, 3A5, and 2C19 had very similar low $K_m$ values of 4.0, 1.9, and 5.9 µM, respectively. Recombinant CYP3A4 exhibited the highest maximum velocity at 9.6 pmol·min$^{-1}$·pmol$^{-1}$ of rCYP3A4, while CYP3A5 and 2C19 had $V_{max}$ values equal to 1.4 and 0.11 pmol·min$^{-1}$·pmol$^{-1}$, respectively. In HLMs, an apparent $K_m$ of 6.2 µM was observed, with a $V_{max}$ value of 1078 pmol/min/mg protein. Direct plots are shown in Fig. 7 and the kinetic parameter results are summarized in Table 1. The ISEF (0.26 for Gentest rCYP3A4 and HLM lot 102) for clearance, the fu,mic [0.87 at 0.08 mg/ml microsomal protein derived using the Austin et al. (2002) equation], and the CYP3A4 abundance for rCYP3A4 (137 pmol/mg) were applied to determine an CLint,u value of 97.4 ml·min$^{-1}$·Kg$^{-1}$.

The enzyme kinetics for formation of axitinib $N$-glucuronide (M7) in HLMs was best described by the Michaelis-Menten equation (Fig. 8; Table 3). Based on the apparent $K_m$ (2.7 µM), the substrate concentration data from 0.3 to 33 µM adequately described the in vitro formation of M7. In contrast, the formation of Glu3 indicated low affinity ($K_m$ ~42 µM) and the enzyme kinetic parameters did not reach full saturation over the substrate concentrations (0.3–100 µM) evaluated (Supplemental Fig. 3; Table 3). The CLint,app for the formation of axitinib $N$-glucuronide (M7) was approximately 33-fold higher than the formation of Glu3 (Table 3). Only trace amounts of

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**TABLE 2**

Intrinsic clearance values derived from axitinib substrate depletion, when determined with pooled CYP3A5 *1/*1 or CYP3A5 *3/*3 in the presence of NADPH only or NADPH with either ketoconazole or cyp3cide to delineate the contribution of CYP3A4 and CYP3A5 to axitinib metabolism

<table>
<thead>
<tr>
<th>Reaction Reagent Designation</th>
<th>CYP3A5 *1/*1</th>
<th>CYP3A5 *3/*3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NADPH</td>
<td>0.013</td>
<td>—</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.374</td>
<td>—</td>
</tr>
<tr>
<td>NADPH + Ketoconazole</td>
<td>0.071</td>
<td>81 CYP3A</td>
</tr>
<tr>
<td>NADPH + CYP3cide</td>
<td>0.126</td>
<td>66 CYP3A4</td>
</tr>
<tr>
<td>Calculated</td>
<td>0.025</td>
<td>74 CYP3A4</td>
</tr>
</tbody>
</table>

—, not applicable.

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![Fig. 4. Metabolite formation UDP-glucuronosyltransferase reaction phenotyping of 13 isoforms for (A) $N$-Glucuronide (M7), (B) Glu1, and (C) Glu3.](image-url)
In Vitro Metabolism of Axitinib

Wild-Type HLM

UGT1A1*28 HLM

Axitinib N-Glucuronide (M7) CL_{int}

\( (\mu\text{L/min/mg}) \)

Fig. 5. Axitinib N-glucuronide (M7) CL_{int} in pooled wild-type (N = 50) and UGT1A1*28 genotyped (N = 3) HLMs. Incubations were performed in triplicate without addition of bovine serum albumin as described in Materials and Methods.

Glu1 were formed and did not justify further characterization of
the enzyme kinetics. Based on the number of UGTs involved in
the formation of the human circulatory glucuronide (M7), the kinetic
parameters for the three UGTs primarily involved in its formation were
described (Fig. 8; Table 3). Based on the CL_{int,app}, UGT1A1 is the
major UGT contributing to M7 formation followed by lesser contribu-
tions from UGT1A9 and UGT1A3.

Discussion

Multiple characterization methods, as suggested in both the U.S.
Food and Drug Administration (2012) and European Medicines
Agency (2013) guidance documents for drug interactions, were used
to increase the accuracy of the assessments and provide confidence of
an appropriate evaluation of the enzymes responsible for axitinib
metabolism. Both the chemical inhibition studies and recombinant
P450 reaction phenotyping indicated that oxidative metabolism medi-
at by CYP3A is the predominant route of metabolism. Assignment of
P450 reaction phenotyping indicated that oxidative metabolism medi-
ates both the chemical inhibition studies and recombinant
P450 reaction phenotyping studies that the oxidative metabolism that produces the
axitinib sulfoxide is catalyzed mainly by CYP3A, with minor
contributions by CYP2C19 and CYP1A2.

The sulfoxide and N-glucuronide metabolites of axitinib, present in
human plasma, were further characterized in vitro to estimate the
enzyme kinetic parameters by monitoring the formation of each
metabolite. These kinetics were then associated with the contribu-
tion of each pathway, equating to the total clearance of axitinib. Initial
experiments using 100 μM [14C]axitinib or unlabeled axitinib indicated
the major in vitro glucuronide was Glu3 (Fig. 4; Supplemental Table 3),
as opposed to the circulating human glucuronide (M7). Detailed in vitro
experiments provided an explanation for the apparent in vitro/in vivo
disconnect. The detailed studies indicated the disconnect was attributed
to the [14C]axitinib metabolite identification, and early unlabeled
phenotyping studies had been conducted at a high and clinically
irrelevant concentration. The formation of M7 in HLMs displayed a
high-affinity (low K_{m}) process as opposed to the low-affinity formation
of Glu3, exhibiting a 33-fold lower CL_{int} than those derived from M7
formation. Since the peak axitinib exposure in humans is low (C_{max}
0.072 μM or 0.36 nM free) (Chen et al., 2013), these in vitro data
provide an explanation as to why at low exposures of axitinib, M7
would be the glucuronide with the highest formation rate and thus be
detected in human plasma and excreta. When comparing the axitinib
kinetic parameters using HLMs for both the sulfoxide and glucuroni-
dation and taking into account the recombinant enzymes, it was
apparent that the sulfoxide metabolite accounts for a major portion of
axitinib metabolism, and the glucuronidation pathway contributes only
a nominal portion toward the total metabolic clearance of axitinib
(Tables 1 and 3). However, due to the rather unusual case of the rUGT
kinetics indicating UGT1A1 as being the major contributor to the
N-glucuronidation of axitinib versus those commonly described in
literature (e.g., UGT1A4 or UGT2B10) (Kaivosari et al., 2011),
subsequent experiments were conducted. These subsequent experi-
ments utilizing genotyped UGT1A1*28 homozygous HLMs, and
parallel experiments using pooled liver microsomes combined with
UGT-specific chemical inhibitors, provided additional evidence that
UGT1A1 is the major contributor to the N-glucuronidation of axitinib
(M7) followed by lesser contributions from UGT1A9 and UGT1A3.
Additionally, the culmination of the in vitro data unequivocally
provided evidence that glucuronidation is a minor pathway to axitinib
metabolism.

With P450 confirmed as the major clearance pathway and sulfoxide
metabolite being the major metabolite, further analysis of the HLM
sulfoxide formation kinetics was undertaken. The HLM kinetics of
axitinib provided a CL_{int} of approximately 174 μL/min \( \cdot \) mg⁻¹
(Table 1). When the recombinant P450 kinetic data were corrected
for abundance using the ISEF, and the fraction unbound in microsomes,
one can compare the result against the clearance observed using HLMs.
When comparing the ISEF-derived recombinant kinetic data to the
HLM kinetics, the CYP3A4 contribution to the overall metabolism of
axitinib was estimated at approximately 50%, with less than 1%
associated with CYP2C19, therefore indicating the unassigned portion
of approximately 40% of the sulfoxide metabolism could be associated
with CYP3A5. Hence, further investigation of CYP3A5 contribution
was needed. When the original P450 phenotyping studies were
conducted in vitro tools were not available to quantify the CYP3A5

Fig. 6. Inhibition of axitinib N-glucuronide (M7) CL_{int} in pooled HLMs by the UGT
isoform-selective chemical inhibitors atazanavir (UGT1A1), erlotinib (UGT1A1),
hecogenin (UGT1A4), and digoxin (UGT1A9). Incubations were performed in
triplicate without addition of bovine serum albumin and inhibitor concentrations
were selected based on inhibition constants (IC_{50}) for UGTs as described in
Materials and Methods.
contribution. Therefore, genotyping of drug metabolizing enzymes during clinical studies in which axitinib was administered to healthy volunteers were conducted. Clinical results indicated no clear correlation of CYP3A5 polymorphisms with variability in axitinib pharmacokinetics (Brennan et al., 2012), indicating CYP3A5 may have a rather small clinical contribution to overall oral clearance. Recently, in vitro tools to confirm or refute the contribution of CYP3A5 have been made available via the use of CYP3cide, a specific CYP3A4 inhibitor (Walsky et al., 2012b). When axitinib was assessed using CYP3cide, in tandem with CYP3A5-genotyped donor pools of microsomes (CYP3A5 *1/*1 and *3/*3) and axitinib substrate loss was followed over time. It was shown that CYP3A5 contributed to approximately 15% of the overall in vitro oxidative metabolism. These CYP3cide substrate depletion studies were not conducted with a dilution step, which if diluted would minimize any time-dependent effects of CYP3cide on CYP3A5 activity. The absence of the dilution step method has been shown to be an effective method for delineating CYP3A5 contribution from CYP3A4 (Walsky et al., 2012b), especially when combined with quantifying the metabolite formation in the presence and absence of inhibitors at a time point where relatively little loss of the parent compound is observed. When comparing the data from the formation of the sulfoxide metabolite, a similar contribution of CYP3A5 (18%) was noted to that of the parent loss method. Thus, it was shown CYP3A4 contributed to the majority (~66%) of both parent loss and sulfoxide metabolite formation, which has also been supported by clinical observations on the relevance of CYP3A5 polymorphism in axitinib clearance (Brennan et al., 2012; Pithavala et al., 2015). Together these in vitro results suggest an insignificant clinical contribution (~30% contribution) of CYP3A5 to the in vivo metabolic clearance of axitinib (Tseng et al., 2014), which if conducted a priori

**Fig. 7.** Michaelis-Menten hyperbolic kinetic profile of axitinib sulfoxide formation activity derived from (A) pooled HLMs and recombinant (B) CYP3A4, (C) CYP3A5, and (D) CYP2C19. All data represented graphically were tested in triplicate and the S.D. is represented by the error bars.

| Analyte                  | Enzyme Condition | \( K_m \)  \\
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu M )</td>
<td>( \text{pmol min}^{-1} \text{mg}^{-1} )</td>
</tr>
<tr>
<td><em>N</em>-glucuronide (M7)</td>
<td>HLM</td>
<td>2.7 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>rUGT1A1</td>
<td>0.75 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>rUGT1A3</td>
<td>2.0 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>rUGT1A9</td>
<td>1.1 ± 0.06</td>
</tr>
<tr>
<td>Glu3</td>
<td>HLM</td>
<td>43 ± 65a</td>
</tr>
</tbody>
</table>

*The apparent \( K_m \) and \( V_{\text{max}} \) values are estimates since true saturation kinetics were not observed (the highest axitinib concentration in incubation was 100 \( \mu M \)).
would not justify clinical investigation. While the retrospective correlations with axitinib clinical genotyping data are interesting, perhaps the more important application will be the prospective prediction of pharmacokinetic variability due to CYP3A5 through a more rigorous quantitative understanding of the CYP3A5 contribution to the overall metabolic clearance for compounds that, like axitinib, are mixed CYP3A4/5 substrates.

The involvement of CYP3A4 and CYP3A5 in the metabolism of axitinib is supported by pharmacokinetic drug interactions studies. Following the administration of ketoconazole, a 2-fold increase in the AUC of axitinib 5 mg was observed (Pithavala et al., 2012). Furthermore, administration of rifampin decreased the AUC of axitinib 5 mg by 79% (Pithavala et al., 2010). Since ketoconazole is a strong inhibitor of CYP3A and rifampin is a strong inducer of both CYP3A and UGT1A1, the in vitro phenotyping, enzyme kinetics, and UGT1A1-genotyped HLM in vitro studies, accompanied by knowledge of the absolute bioavailability (58%) of axitinib, support the impact of CYP3A4 on the findings of clinical drug-drug interaction studies. Furthermore, a meta-analysis evaluated the correlation of genotypes of several drug metabolizing enzymes (CYP2C19, CYP3A4, CYP3A5, and UGT1A1) and transporters (ABCB1 and SLCO1B1) with pharmacokinetic variability using available data from healthy volunteer clinical studies (315 healthy human subjects) in which genotyping samples were collected. This analysis showed no statistically significant correlation with the genotype for any of the enzymes evaluated. These results are consistent with the in vitro findings that CYP2C19 and UGT1A1 play a minor role in the overall clearance from the body. While CYP3A enzymes, especially CYP3A4, are clearly important in the metabolism of axitinib, the lack of correlation of pharmacokinetic variability with specific genotypes of CYP3A is consistent with the lack of phenotype-genotype correlations reported for other CYP3A substrates such as diltiazem, midazolam, nifedipine, and oxycodone (Floyd et al., 2003; Kharasch et al., 2007; Tomalik-Scharte et al., 2008; Naito et al., 2011; Haas et al., 2013; Zheng et al., 2013), where the pharmacokinetic variability can be attributed to the inherent range of CYP3A4 expression in the population, regardless of polymorphism. Additionally, investigations of pharmacokinetic variability seen in the clinic for axitinib, in lieu of the putative in vitro data supporting the prominent CYP3A4 role in axitinib clearance, include CYP3A4*22 polymorphism assessment from the collected genotyping samples. These samples were used to correlate with the variability in axitinib plasma exposures in patients; however, the preliminary genotypic analysis indicates a lack of any correlation (Pithavala et al., 2015). Finally, one aspect that may have an effect on variability of axitinib in RCC patients is disease state. Axitinib has not been tested with latter lines of treatment in RCC; however, exposure variability of axitinib in patients is similar to what has been observed with other oral chemotherapeutic therapies (Hartvig et al., 1988; Regazzi et al., 1998; Wohl et al., 2002; Soepenberg et al., 2005; Preiss et al., 2006).

In conclusion, the in vitro metabolite identification reported herein provided specific routes of metabolism of axitinib and confirmed the major pathways responsible for axitinib metabolites observed in plasma and excreta. Utilizing multiple methods, the relative quantitative contribution of each pathway to axitinib metabolism could be assigned. The majority of the data from the three different experiments (chemical inhibition, rCYP phenotyping, and definitive kinetics) indicate CYP3A4 is responsible for more than 50% of the metabolism of axitinib and also provide evidence that CYP1A2, CYP2C19, CYP3A5, and UGT1A1 are minor contributors to the metabolism of axitinib. Despite this extensive enzymology study and clinical correlates, all factors contributing to axitinib exposure...
variability in patients have not been identified and remain the subject of ongoing investigations.

Acknowledgments

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Authorship Contributions


References


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Pilot experiments to investigate the glucuronidation of axitinib with human liver microsomes.

Preliminary experiments were conducted with radiolabeled axitinib to identify the potential for in vitro glucuronidation. Briefly, To examine the UGT isoforms involved in glucuronidation, axitinib was initially incubated with 12 UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) at 1.15 mg/mL protein with a range of activity (39-2300 pmol/mL). After initial screening, only UGT1A1 and UGT 1A4 protein concentrations were optimized for glucuronide turnover and the range of activity was 85-8000 pmol/sample. All UGT isoforms (supersomes) were quickly thawed and aliquoted into 39 x100 mm-polypropylene test tubes already containing alamethicin (100-200 μg/mg of microsomal protein) and potassium phosphate (100 mM, pH = 7.4). After gentle vortexing, the microsomal aliquots were kept on ice for 15 minutes to permeabilize the microsomal membrane and then magnesium chloride (1 mM) and [14C]AG-013736 (100 μM ) were introduced and the mixture was brought to 37°C over a 5-minute preincubation in a shaking water bath. The incubation reactions were initiated by the addition of UDPGA (5 mM) for a final volume of 200 L. The reactions were terminated after 60 minutes by the addition of 0.5 mL acetonitrile. Samples were immediately capped, vortexed, and centrifuged at 1900 x g (3600 rpm using a Beckman-Coulter GH3.8A rotor) for 25 minutes at 4°C. The supernatant of each sample was transferred into a new tube and dried under a stream of nitrogen. Extracts were reconstituted with appropriate 100-800 μL of 60:40 or 70:30 aqueous:organic mobile phase for radiodetection and LC-MS/MS analysis. For HLM evaluations, HLM (0.5 mg/mL) activated with alamethicin (100-200 μg/mg of microsomal protein) were incubated in potassium phosphate buffer (100 mM, pH = 7.4) containing saccharolactone (5 mM), magnesium chloride (1 mM), and [14C]axitinib (100 μM) in triplicate. The incubation reactions were initiated by the addition of UDPGA (5 mM) in a final
volume of 200 μL. The reactions were terminated after 60 minutes by the addition of 0.5 mL CH₃CN. Samples were immediately capped, vortexed, and centrifuged at 1900 x g (3600 rpm using a Beckman-Coulter GH3.8A rotor) for 25 minutes at 4°C. The supernatant was transferred into a new tube and dried under a stream of nitrogen. Extracts were reconstituted with 100-800 μL of 60:40 or 70:30 aqueous (0.1% formic acid in water):organic (0.1% formic acid in acetonitrile) mobile phase for radiodetection and LC-MS/MS analysis.

Metabolite profiling utilizing [¹⁴C]axitinib was performed using HPLC coupled in-line with radiochemical detection (ARC/β-RAM) and MS detection with electrospray (ESI) source in positive mode. The instrumental components are specified as follows: HPLC Pumps/Autosampler Agilent 1100 (Agilent Technologies, Wilmington, DE); Column COSMOSIL, 5PYE (150 x 4.6 mm, Waters, Milford, MA); Guard Cartridge C18 (4.0 x 3.0 mm, Phenomenex, Torrance, CA); Quadrupole Ion Trap MS LCQ-Deca XP (ThermoFinnigan, San Jose, CA); ARC StopFlow System Model C (AIM Research Company, Newark, DE); RadioDetector (500 μL Cell) Model 3 β-RAM (IN/US Systems, Tampa, FL); Scintillation Cocktail StopFlow AD (AIM Research Company, Newark, DE). The mobile phase consisted of 20 mM ammonium acetate in water adjusted to pH 4.0 with acetic acid (Solvent A) and CH₃CN (Solvent B). The flow rate was maintained at 1.0 mL/min. The drug and metabolites were eluted using a linear gradient in mobile phase composition summarized as follows: 0% B for 10 min; linear gradient to 50% B from 10 to 60 min; linear gradient to 0% B from 60 to 61 min; 0% B from 61 to 70 min. The HPLC effluent was split so that 20% of the flow was introduced into the mass spectrometer via the ESI source while 80% was diverted to the β-RAM detector. The analog outputs from the ARC/β-RAM and MS detectors were recorded in real time by the ARC data system version 2.4 (AIM Research Company, Newark, DE). The major operating parameters for the ion-trap ESIMS method are shown below: ionization mode positive; spray voltage 5.0 kV; capillary voltage 5.0 V; tube lens offset 55 V; capillary temperature 200°C sheath gas flow rate 80 (arbitrary); auxiliary gas flow rate 20 (arbitrary). LC-MS spectra were acquired over a mass range of 200-
1400 m/z for all samples. Mass spectra were processed using either Xcalibur version 1.4 (ThermoFinnigan, San Jose, CA) or the ARC data system. Radiochromatographic data was processed using the ARC data system only. Metabolite screening was performed to manually search for molecular ions representing possible metabolites.

**Oxidative biotransformation LC/MS metabolite profiling.**

Metabolite profiling was performed using HPLC (Agilent 1100, Agilent Technologies, Wilmington, DE) coupled in-line with MS detection (LCQ-Deca XP, ThermoFinnigan, San Jose, CA) with ESI source in positive ion mode with reverse phase chromatography (two different HPLC columns were used: COSMOSIL SPYE(100 mm x 2 mm, Phenomenex, Torrance, CA) for human liver microsome study, and Phenomenex C4 (150 mm x 2 mm, Phenomenex, Torrance, CA) for recombinant CYP study. For positive-mode MS detection, the mobile phase consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in CH3CN (Solvent B). Parent drug and its metabolites were eluted at flow rate of 0.2 mL/min using a linear gradient in mobile phase composition as follows: 0% B for 5 min; linear gradient to 30% B from 5 to 20 min; held at 50% B from 25 to 28 min; linear gradient to 90% B from 28 to 40 min; held at 90% B from 40 to 43 min; linear gradient to 0% B from 43 to 45 min; held at 0% B from 45 to 50 min. After passing through the DAD detector, the HPLC effluent was introduced into the mass spectrometer. The analog outputs from the DAD and MS detectors were recorded in real time by the data system provided with the mass spectrometer (Xcalibur version 1.4, ThermoFinnigan, San Jose, CA). The MS detection showed a delayed (0.2 - 1.3 minutes) response, compared to the DAD detector as a result of plumbing. The major operating parameters for the ion-trap ESI-MS methods are shown below: ionization mode positive; spray voltage 4.5 kV; capillary voltage 2.6 V; tube lens offset 20 V; capillary temperature 270°C sheath gas flow rate 90 (arbitrary); auxiliary gas flow rate 30 (arbitrary).
The Xcalibur software was used to control both HPLC and MS systems to acquire and process all spectral data. LC-MS spectra were acquired over a mass range of 150-1500 m/z. Data dependent scanning was used to trigger MS2 and MS3 analysis of molecular ions and product ions over a threshold intensity of 1.0 x 10^6 and 1.0 x 10^5 counts, respectively. Only the most intense ions from MS and MS2 scans were selected for MS2 and MS3 scans, respectively. The settings used were: isolation width 2.50; normalized collision energy 40.0 eV; activation Q 0.25; activation time 30.00 ms; exclusion mass width to 0.5 Da. Searching of LC-MSn data for molecular ions representing possible metabolites was performed manually. In cases where the signal strength was of sufficient intensity to trigger MSn scanning, the subsequent MS2 and MS3 spectra were examined to further confirm the identity and structure of possible metabolites.

Supplemental Table 1: m/z ratios for axitinib and the metabolites

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Q1 m/z</th>
<th>Q3 m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axitinib</td>
<td>387</td>
<td>356</td>
</tr>
<tr>
<td>Axitinib sulfoxide</td>
<td>403</td>
<td>372</td>
</tr>
<tr>
<td>Axitinib glucuronide</td>
<td>563</td>
<td>387</td>
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<tr>
<td>Buspirone</td>
<td>386</td>
<td>122</td>
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### Supplemental Table 2: Intersystem extrapolation factors (ISEFs) predetermined

<table>
<thead>
<tr>
<th>rCYPs:</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
<th>CYP3A4</th>
<th>CYP2C8</th>
<th>CYP2B6</th>
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</thead>
<tbody>
<tr>
<td>Lot No.:</td>
<td>Panvera 30248A</td>
<td>Panvera 29897A</td>
<td>Panvera 29896A</td>
<td>Panvera 21248</td>
<td>Panvera 29736A</td>
<td>Gentest 59</td>
<td>Gentest 8</td>
<td>Panvera 19191</td>
</tr>
<tr>
<td><strong>K_m (pmol/min/pmol)</strong></td>
<td>36.0</td>
<td>3.90</td>
<td>32.0</td>
<td>2.30</td>
<td>40.0</td>
<td>3.10</td>
<td>0.730</td>
<td>67.0</td>
</tr>
<tr>
<td><strong>V_max (pmol/min/pmol)</strong></td>
<td>33.0</td>
<td>26.0</td>
<td>9.70</td>
<td>110.0</td>
<td>140.0</td>
<td>21.58</td>
<td>3700</td>
<td>760.0</td>
</tr>
<tr>
<td><strong>V_max/K_m (µL/min/pmol rCYP)</strong></td>
<td>0.93</td>
<td>6.60</td>
<td>0.30</td>
<td>48</td>
<td>3.4</td>
<td>6.96</td>
<td>5100</td>
<td>11.0</td>
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<table>
<thead>
<tr>
<th>HL-MIX-102</th>
<th>(350 pmol P450/mg):</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K_m (µM)</strong></td>
<td>20</td>
</tr>
<tr>
<td><strong>V_max (nmol/min/mg)</strong></td>
<td>0.78</td>
</tr>
<tr>
<td><strong>V_max (pmol/min/mg HLM protein)</strong></td>
<td>780</td>
</tr>
<tr>
<td><strong>V_max/K_m (pmol/min/pmol)</strong></td>
<td>15</td>
</tr>
<tr>
<td><strong>V_max/K_m (µL/min/pmol CYP)</strong></td>
<td>0.740</td>
</tr>
<tr>
<td><strong>CYP abundance (pmol CYP/mg HLM protein)</strong></td>
<td>52</td>
</tr>
</tbody>
</table>

| **V_max, ISEF (unitless)** | 0.45 | 0.66 | 0.40 | 0.27 | 0.41 | 0.19 | 0.02 | 0.05 |
| **CL_int, ISEF (unitless)** | 0.79 | 0.90 | 0.24 | 0.09 | 0.18 | 0.26 | 0.01 | 0.04 |
Supplemental Table 3: Pilot experiment to examine the formation of M7 and other glucuronide conjugates of \[^{14}C\]axitinib (100 \(\mu\)M) by human liver microsomes and rUGTs in the presence of UDPGA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Axitinib</th>
<th>Glu1</th>
<th>Glu2 (M7)</th>
<th>Glu3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>87.5</td>
<td>1.8</td>
<td>0.8</td>
<td>2.4</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>90.7</td>
<td>ND</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>82.9</td>
<td>3.3</td>
<td>0.2</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Note: values are % dose, average of 4 to 6 experiments. ND = not detected

Supplemental Figure 1: Metabolite profiles of axitinib in recombinant CYP3A4, CYP3A5, CYP1A2, and CYP2C19 (UV Chromatograms at 356 nm)

**Supplemental Figure 2:** Main figure: Effect of heat inactivation of human liver FMO on sulfoxidation of axitinib. Inset depicts effect of heat inactivation of human liver FMO on the positive controls: cimetidine and sulindac to cimetidine sulfoxide and sulindac sulfoxide, respectively. Black bars of the inset represent heat inactivation without NADPH and gray bars represent the FMO formation of the sulfoxide metabolite when heated in the presence of NADPH to stabilize the FMO enzyme.

**Supplemental Figure 3:** Enzyme kinetics of axitinib glucuronidation (Glu3) by pooled HLMs in Tris-HCl buffer (pH = 7.4) displaying typical Michaelis-Menten kinetics.