**In Vitro Kinetic Characterization of Axitinib Metabolism**

Michael A. Zientek, Theunis C. Goosen, Elaine Tseng, Jian Lin, Jonathan N. Bauman, Gregory S. Walker, Ping Kang, Ying Jiang, Sascha Freiwald, David Neul, and Bill J. Smith


Received May 26, 2015; accepted October 27, 2015

**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 axitinib’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 \( [K_m] \) and \( V_{max} \) values for the formation of axitinib sulfoxide by CYP3A4 or CYP3A5 were 4.0 or 1.9 \( \mu \)M and 9.6 or 1.4 pmol min\(^{-1}\) \( \mu \)mol\(^{-1}\), 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 \( K_m \) and \( V_{max} \) values describing the formation of the N-glucuronide in HLM or rUGT1A1 were 2.7 \( \mu \)M and 0.75 \( \mu \)M and 8.9 or 8.3 pmol min\(^{-1}\) \( \mu \)mol\(^{-1}\), respectively. In summary, CYP3A4 is the major enzyme involved in axitinib clearance with lesser contributions from CYP3A5, CYP2C19, CYP1A2, and UGT1A1.

**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 \( T_{1/2} \) 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/glucuronide (M12/M14). Two metabolites, which were

dx.doi.org/10.1124/dmd.115.065615.

This article has supplemental material available at dmd.aspetjournals.org.

**ABBREVIATIONS:** AUC, area under the plasma concentration versus time curve; axitinib, N-Methyl-2-{[3-([E]-2-pyridin-2-yl-vinyl)-1H-indazol-6-ylsulfanyl]-benzamide; CH3CN, acetonitrile; CL\(_{\text{in}}\), intrinsic clearance; CL\(_{\text{unbound}}\), unbound intrinsic clearance; DMSO, dimethylsulfoxide; FMO, flavin-containing monooxygenase; Glu, glucuronide; HLM, human liver microsome; HPLC, high-performance liquid chromatography; ISEF, intersystem extrapolation factor; KH\(_2\)PO\(_4\), potassium phosphate; K\(_{\text{app}}\), apparent substrate concentration at half-maximal velocity; LC, liquid chromatography; MS, mass spectrometry; P450, cytochrome P450; RCC, renal cell carcinoma; rCYP, recombinant P450; rUGT, recombinant UDP-glucuronosyltransferase; \( t_{1/2} \), half-life; UDPGA, UDP glucuronic acid; UGT, UDP-glucuronosyltransferase.
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 benzynirvinol 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 Mallinckrodt Baker (Phillipsburg, NJ). (E)-3-(4-(25,35,45,5)-m-1-(3-chloro-2,6-difluorobenzoyl oxoiminomethyl)-3,4-dihydroxytetrahydrofuran-2-yloxy)-3 hydroxyphenyl)-2-methyl-([3a,4,8,6,7,5R)-N(3-chloro-2,6 difluorobenzyloxyimino)ethyl)-3,4-dihydroxytetrahydrofuran-2-yloxy)-3 hydroxy-1,3-dioxol-5-yl) acrylamide, a Pfizer analytical internal standard, was acquired from BD Gentest. Inc Lots BD HH47, HH86, HH107, BD HH785, and BD HH867 (designated as *1/*1) were further genotyped for *6 and *7 alleles and were verified as not possessing those alleles except for lot HH86, in which the microsomes did not yield an adequate DNA sample. Pooled lots of HLM CYP3A5 *1/*1 (from five donors) and HLM CYP3A5 *3/*3 (from six donors) were prepared by pooling equal amounts of microsomal protein of each of the aforementioned lots, such that the resulting enzymatic activity (measured by 6-b-OH testosterone formation) was similar in the two pooled lots. Recombinant heterologously expressed CYP3A4 and CYP3A5 were prepared under contract by Panvera Corp. (Madison, WI).

Recombinant P450s (rCYPs) were obtained from Panvera Corp. and BD Gentest. cDNA-expressed UGts were procured from BD Gentest. The following cDNA-expressed UGT enzymes were used in the activity screening studies: UGT1A1, UGT1A3, UGT1A4, UGT1A16, UGT1A7, UGT1A8, UGT1A91, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B15, and UGT2B17, and also a nonactive UGT control protein was used to provide a uniform protein concentration among UGT incubation reactions (from BD Gentest). Pooled UGT1A1*28 genotyped HLM prepared from three donors—HH9 (Caucasian male), HH81 (Caucasian male), and HH82 (Caucasian female)—were obtained from BD Gentest. Rat cryopreserved isolated 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.

Biotransformation

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 axitinib 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 (19000 g). 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 Axitinib with HLMs**

Pilot in vitro experiments using human microsomes were conducted to investigate the formation of the human circulating metabolite axitinib-N-glucuronide (Glu2M7). These experiments also identified additional glucuronide (Glu) conjugates of axitinib termed Glu1 (minor) and in vitro glucuronide metabolite, Glu3 (major), an apparently major (Glu3) and minor (Glu1) glucuronide identified from in vitro incubations with [1C]axitinib. The incubation conditions and analytical methods for the metabolite profiling utilizing [1C]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 axitinib 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 dicyclofem 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 axitinib-N-glucuronide (M7) as described subsequently. Since authentic standards for Glu1 and Glu3 were not available, their formation was quantified relative to the axitinib-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 axitinib 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 axitinib (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 (3 and 10 μM), hecogenin (10 μM), or digoxin (10 and 60 μM).

Inhibitor concentrations were selected based on unbound IC50 (IC50u) values for axitinib (UGT1A1 IC50u = 0.14 μM; UGT1A4 IC50u = 2.1 μM), erlotinib (UGT1A1 IC50u = 0.37 μM; UGT1A4 IC50u = 3.0 μM), hecogenin (UGT1A4 IC50u = 5.0–1 μM; UGT1A1 IC50u > 100 μM), and digoxin (UGT1A9 IC50u = 30 μM; UGT1A1 IC50u > 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 dicyclofem 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.

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

Axitinib-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 Axitinib (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 mM). 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 x 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 (Gibson, Middletown, 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 25 minutes and held at this 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-walled polypropylene microtiter 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 axitinib 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 to residual DMSO-d6 (1H δ = 2.5 ppm, relative to Tetramethylsilane, δ = 0.0 ppm) and 11B δ = 0 ppm. 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 x 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 axitinib glucuronide (M7) was determined by quantitative NMR to be 5.2 ± 0.1 mM.

**Reaction Phenytoping**

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 furafylline (1A2), 0.2 μM monteleukast (2C8), 5 μM sulphaphenazole (2C9), 5 μM benzylnirval (2C19), 1 μM quinidine (2D6),
and 1 μM ketonazole (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 NADP+, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase in regeneration system (the final NADPH regeneration system concentrations for 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 sulf oxide 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 3A4 as a control and comparator reaction. Reaction mixtures (184 μl) containing the individual recombinant P450 or HLM, 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 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 axitinib. Incubations were carried out in triplicate in 100 mM KH2PO4 (pH 7.4) containing MgCl2 (3.3 mM), ketoconazole (2 mM), or CYP3cide (1 mM) containing magnesium chloride (5 mM). Final DMSO concentrations were 1% (v/v) or less. Reactions were initiated with the addition of NADPH (1 mM final), incubated with shaking at 900 rpm for 20 minutes at 37°C for HLM, rCYP3A4, and rCYP3A5 and for 45 minutes at 37°C for CYP 2C19. Reactions were terminated using 500 μl ice cold (4°C) CH3CN containing 0.01 μM buspirone final (internal standard). After mixing, the plates were centrifuged at 1900g (Beckman-Coulter GH3.8A rotor) for 15 minutes and 200-μl aliquots/well were transferred to a clean 96-well polystyrene plate for LC-MS/MS analysis.

**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), ketonazole (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-((2S,3S,4S,5R)5-1-(3-chloro-2,6-difluorobenzoxylimino)ethyl)-3,4-dihydroxytetrahydrofuran-2-yl)oxy)-3 hydroxy-phenyl)-2-methyl-N(3aS,4R,5S,7R,7aR)-4,6,7-trihydroxyhexahydrobenzo[\[1,3\]dioxol-5-y]) acrylamide, was conducted in the same manner as reported in Lee et al. (2012).

**P450 Enzyme Kinetic Studies**

Enzyme kinetic studies were performed in triplicate using pooled HLMs and rCYPs (3A4, 3A5, and 2C19). Preliminary linearity of product formation with protein and time (e.g., substrate depletion time-course studies) were conducted to assure that assumptions for Michaelis-Menten first-order rate kinetics were met. Reaction mixtures (500 μl) contained axitinib with final concentrations of axitinib ranging from 0 to 50 μM. Reaction mixtures (470 μl) had a final protein concentration of 0.08 mg/ml HLM or rCYP3A4 (10 pmol/ml), 0.095 mg/ml rCYP3A5 (10 pmol/ml), or 0.94 mg/ml CYP2C19 (100 pmol/ml). The mixtures were preincubated for 5 minutes at 37°C and mixed using an Eppendorf microplate mixer (Hamburg, Germany) at 900 rpm throughout the incubation period. The reactions were initiated with 25 μl of 20 mM NADPH (1 μM final), incubated with shaking at 900 rpm for 20 minutes at 37°C for HLM, rCYP3A4, and rCYP3A5 and for 45 minutes at 37°C for CYP 2C19. Reactions were terminated using 500 μl ice cold (4°C) CH3CN containing 0.01 μM buspirone final (internal standard). After mixing, the plates were centrifuged at 1900g (Beckman-Coulter GH3.8A rotor) for 15 minutes and 200-μl aliquots/well were transferred to a clean 96-well polystyrene plate for LC-MS/MS analysis.

**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 Glu3) 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 dicyclofenac 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 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 axitinib N-glucuronide (M7) as described subsequently.

**Determination of Non specificity 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 fm,unc was further applied and corrected across microsomal reaction concentrations by extrapolating the fraction unbound for 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.
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/e transitions for axitinib and its metabolites are listed in Supplemental Table 1. The peak area ratio of the analyze 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 metabolite formation was carried out on an AB SCIEX 5500 Triple Quadrupole (Applied Biosystems) equipped with ACQUITY UPLC Column (C18, 90 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), 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/e 563 →387 for axitinib N-glucuronide and m/e 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:

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

where \( k_{deg} = - (\text{slope of } \ln(\text{of the average peak area ratio of the replicates versus time}) \).

For the rCYP phenotyping study, the in vitro CLint was calculated using the following equation:

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

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

\[ \text{% Contribution} = 100 \times \text{CL}_{\text{int}} \times \text{of each contributing P450} \times (\text{sum of the CL}_{\text{int}} of all of the contributing P450s}) \] (3)

Chemical Inhibition Phenotyping Assay

The human CLint was calculated using the following equation:

\[ \text{CL}_{\text{int}}(\text{ml/min/kg}) = \frac{\ln(2) \times \text{ml incubation}}{\text{microsomal } t_{1/2} \times \text{microsomes}} \times \frac{45 \text{ mg microsomes}}{\text{g of liver}} \times \frac{21 \text{ g of liver}}{\text{kg of body weight}} \] (4)

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

\[ \text{Relative } \% \text{ contribution} = 100 \times \frac{\text{sum of all the } \% \text{ contributions from the P450s tested}} {\text{CL}_{\text{int}} \text{ of the inhibited reaction}} \] (5)

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 (\( k_{\text{app}} \)). The slope of the line was used to determine the \( t_{1/2} \) (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):

\[ \text{Calculated } \% \text{ CYP3A contribution} = \frac{\text{CL}_{\text{int}} \times \text{NADPH} - \text{CL}_{\text{int}} \times \text{NADPH}} {\text{CL}_{\text{int}} \times \text{NADPH}} \times 100\% \] (6)

\[ \text{Calculated } \% \text{ CYP3A4 contribution} = \frac{\text{CL}_{\text{int}} \times \text{NADPH} - \text{CL}_{\text{int}} \times \text{CYP3A4}} {\text{CL}_{\text{int}} \times \text{NADPH}} \times 100\% \] (7)

\[ \text{Calculated } \% \text{ CYP3A5 contribution} = \% \text{ CYP3A} - \% \text{ CYP3A4} \] (8)

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 (Sigmaplot v13, Systat Software, Inc., Chicago, or GraphPad Prism version 5.0, GraphPad Software, Inc., San Diego, CA) to derive the apparent enzyme kinetic parameters \( V_{\text{max}} \) and \( K_{S} \) (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:

\[ V = \frac{V_{\text{max}} \times S}{K_{S} + S} \] (9)

\[ V = \frac{V_{\text{max}} \times S^{n}}{K_{S}^{n} + S^{n}} \] (10)

where \( V_{\text{max}} \) is the maximal velocity; \( K_{S} \) 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 \( V_{max}/K_m \) value was calculated as the \( V_{max}/\text{U} \times \text{CYP abundance per mg of HLM} \)
\[
\times \text{ISEF}(\text{CL}_{int})
\]
(13).

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 (\( f_{u,mic} \)) (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 \( \text{CL}_{int} \) estimates obtained in HLM.

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

### 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 (M12). Oxidation of pyridine and methyl amide moieties of axitinib hence inferring a portion of axitinib metabolism is attributable to CYP3A5. 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 [\(^{13}\)C]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 [\(^{13}\)C]axitinib (Smith et al., 2014).

#### Nonspecific 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 \( \text{CL}_{int} \) values estimated from the experimental half-life (substrate disappearance) from rCYP enzymes using \( \text{CL}_{eq} \) 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 \( \text{CL}_{int,u} \) values adjusted using the \( \text{CL}_{eq} \) ISEF are summarized in Table 1. The predicted scaled \( \text{CL}_{int,u} \) extrapolated from rCYP3A4 was approximately 2- to 3-fold greater than what was predicted from HLM stability within the assay (94 versus 291 \( \mu\text{mol/min}/\text{mg} \)). 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 \( \text{CL}_{int} \) (\( \text{CL}_{int,app} \)) value resulting from the rCYP3A5 experiment equated to 0.335 \( \mu\text{mol/min}/\text{mg} \) 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%), 2C19 (6.4%), and 2C9 (5.4%). Based on chemical inhibition studies, the contributions from 2C8 (2.4%) and 1A2 (0%) toward the metabolism of axitinib were negligible. The \( \text{CL}_{int,u} \) 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 CYP3A, with minor contributions from a few other P450s (CYP1A2 > 2C19 >> 2D6).

#### TABLE 1

Definitive phenotyping and kinetic determination utilizing HLMs and recombinant P450

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Chemical Inhibitor</th>
<th>Chemical Inhibition CYP Phenotyping</th>
<th>Recombinant CYP Phenotyping</th>
<th>Kinetic Determination of Axitinib Sulfoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu\text{mol/min/mg} )</td>
<td>Relative Contribution(^a)</td>
<td>( \mu\text{mol/min/mg} )</td>
<td>Relative Contribution</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>30 ( \mu\text{M} )</td>
<td>108.1</td>
<td>24.4</td>
<td>No</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>5 ( \mu\text{M} )</td>
<td>98.4</td>
<td>12.0</td>
<td>Yes</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>5 ( \mu\text{M} )</td>
<td>97.2</td>
<td>12.0</td>
<td>Yes</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>1 ( \mu\text{M} )</td>
<td>95.8</td>
<td>0.040</td>
<td>No</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>1 ( \mu\text{M} )</td>
<td>2.4</td>
<td>18.4</td>
<td>Yes</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>1 ( \mu\text{M} )</td>
<td>11.4</td>
<td>2.1</td>
<td>Yes</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>(N.A.)</td>
<td>94.5</td>
<td>6.2</td>
<td>13.9</td>
</tr>
</tbody>
</table>

\(\text{N.A.} \), not applicable; \(\sim\), no ISEF available.

\(^a\)Percent contribution for each isozyme was converted to relative % contribution due to the small overlap of some inhibitors on multiple isozymes. In cases where total % contribution is >100%, there is a requirement to normalize the data to 100% while still retaining the same proportional contribution by each CYP.

\(^b\)CYP clearances require scaling from \( \mu\text{mol/min/mg} \) to \( \mu\text{mol/min/mg} \) microsomal protein, using the most appropriate CYP ISEFs.

\(\text{CL}_{int,u}(\mu\text{l/min/mg}) \) extrapolated from rCYP3A4 was approximately 2- to 3-fold greater than what was predicted from HLM stability within the assay (94 versus 291 \( \mu\text{mol/min/mg} \)).

\(\text{CL}_{int,u}(\mu\text{l/min/mg}) \) extrapolated from rCYP3A5 experiment equated to 0.335 \( \mu\text{mol/min/mg} \) of rCYP3A5, hence inferring a portion of axitinib metabolism is attributable to CYP3A5.

\(\text{CL}_{int,u}(\mu\text{l/min/mg}) \) extrapolated from rCYP3A5 experiment equated to 0.335 \( \mu\text{mol/min/mg} \) of rCYP3A5, hence inferring a portion of axitinib metabolism is attributable to CYP3A5.
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...
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 sulfide 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 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 sulfide 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).

**In Vitro Metabolism of Axitinib.** 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 Glu1 was apparently the most abundant glucuronide formed 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.

**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 Glu1 was apparently the most abundant glucuronide formed 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 $\mu$M) 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 $\mu$M inhibited M7 formation by 61% and 80%, respectively, while the selective UGT1A4 inhibitor, hecogenin (Uchaipichat et al., 2006), at 10 $\mu$M did not inhibit M7 formation (Fig. 6). Erlotinib, a potent UGT1A1 and weak UGT1A9 inhibitor (Lapham et al., 2012), at 3 and 10 $\mu$M 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 $\mu$M 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 $\mu$M, respectively. Recombinant CYP3A4 exhibited the highest maximum velocity at $9.6 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of rCYP3A4, while CYP3A5 and 2C19 had $V_{\max}$ values equal to 1.4 and 0.11 pmol·min$^{-1}$·mg$^{-1}$, respectively. In HLMs, an apparent $K_m$ of 6.2 $\mu$M 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 $f_{\text{u,mic}}$ [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 $CL_{\text{int,u}}$ value of 97.4 $\text{ml} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

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 $\mu$M), the substrate concentration data from 0.3 to 33 $\mu$M adequately described the in vitro formation of M7. In contrast, the formation of Glu3 indicated low affinity ($K_m \sim 42 \mu$M) and the enzyme kinetic parameters did not reach full saturation over the substrate concentrations (0.3–100 $\mu$M) evaluated (Supplemental Fig. 3; Table 3). The $CL_{\text{int,app}}$ for the formation of axitinib N-glucuronide (M7) was approximately 33-fold higher than the formation of Glu3 (Table 3). Only trace amounts of

---

**Table 2**

<table>
<thead>
<tr>
<th>Reaction Reagent Designation</th>
<th>CYP3A5 *1/*1</th>
<th>Contribution</th>
<th>CYP3A5 *3/*3</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NADPH</td>
<td>0.013 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>—%</td>
<td>0.025 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>—%</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.374 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>—%</td>
<td>All P450 0.374 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>—%</td>
</tr>
<tr>
<td>NADPH + Ketoconazole</td>
<td>0.071 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>81% CYP3A</td>
<td>0.079 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>79% CYP3A</td>
</tr>
<tr>
<td>NADPH + CYP3cide</td>
<td>0.126 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>66% CYP3A4</td>
<td>0.098 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>74% CYP3A4</td>
</tr>
<tr>
<td>Calculated</td>
<td>0.25 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>15% CYP3A5</td>
<td>0.025 $\text{ml} \cdot \text{min}^{-1} \cdot \text{Kg}^{-1}$</td>
<td>5% CYP3A5</td>
</tr>
</tbody>
</table>

—, not applicable.

Fig. 4. Metabolite formation UDP-glucuronosyltransferase reaction phenotyping of 13 isoforms for (A) N-Glucuronide (M7), (B) Glu1, and (C) Glu3.
Glu1 were formed and did not justifiably 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 CLint,app, UGT1A1 is the major UGT contributing to M7 formation followed by lesser contributions 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 mediated by CYP3A is the predominant route of metabolism. Assignment of P450s that have a minor contribution to axitinib metabolism required some judgment due to disparate results when complementary approaches were applied. Chemical inhibition studies suggested minor roles for CYP2C19 and CYP2D6; however, when using recombinant enzymes only CYP2C19 was shown to form metabolites at a CLint contribution greater than 0.3%. Conversely, a role for CYP1A2 could not be shown through inhibition by furafylline in HLMs, but studies using recombinant enzymes have demonstrated that CYP1A2 was the only enzyme that formed M12a, an in vivo metabolite of axitinib in humans (Smith et al., 2014). Axitinib metabolism in HLMs was inhibited by <10%, each by montelukast and sulfaphenazole, selective inhibitors for CYP2C8 and CYP2C9, respectively. Follow-up studies of this result using recombinant enzymes could not verify a significant contribution from CYP2C8 or CYP2C9 to axitinib metabolism. No contribution of FMO to the formation of axitinib sulfoxide could be demonstrated. Therefore, it was concluded from the P450 and FMO 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 contribution 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. 3; 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 Km) process as opposed to the low-affinity formation of Glu3, exhibiting a 33-fold lower CLint than those derived from M7 formation. Since the peak axitinib exposure in humans is low (Cmax 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 glucuronidation 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 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 experiments 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 CLint of approximately 174 µL·min⁻¹·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. 5. Axitinib N-glucuronide (M7) CLint 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.

Fig. 6. Inhibition of axitinib N-glucuronide (M7) CLint 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 (IC50) 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.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Enzyme Condition</th>
<th>$K_m$ μM</th>
<th>$V_{max}$ pmol/min/mg</th>
<th>Clint $μM$·min$^{-1}$·mg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-glucuronide (M7)</td>
<td>HLM</td>
<td>2.7 ± 0.24</td>
<td>8.9 ± 0.25</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>rUGT1A1</td>
<td>0.75 ± 0.08</td>
<td>8.3 ± 0.19</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>rUGT1A3</td>
<td>20 ± 3.2</td>
<td>1.9 ± 0.10</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>rUGT1A9</td>
<td>1.1 ± 0.06</td>
<td>1.6 ± 0.02</td>
<td>1.5</td>
</tr>
<tr>
<td>Glu3</td>
<td>HLM</td>
<td>43 ± 6.5a</td>
<td>4.3 ± 0.29b</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*The apparent $K_m$ and $V_{max}$ values are estimates since true saturation kinetics were not observed (the highest axitinib concentration in incubation was 100 μM).
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, nefedipine, 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

The authors thank Dr. Deepak Dalvie, Dr. James Ferrero, Dr. Caroline Lee, Dr. Yazidi Pithaval, and Dr. Ellen Wu for useful contributions, conversations, and review of this work.

Authorship Contributions


Wrote or contributed to the writing of the manuscript: Zientek, Goosen, Tseng, Bauman, Walker, Kang, Freiwald, Smith.

References


Uchaipichat V, Mackenzie PJ, Elliot DJ, and Miners JO (2006) Selectivity of substrate (tri- fluoromethoxyamide) and inhibitor (amitriptyline, androsterone, canrenoic acid, hecogenin, phenylbuta- zone, quinidine, quinine, and sulfipyrazone) “probes” for human UDP-glucuronosyltransferases.

Drug Metabolism Dispos 34:449–456.


Address correspondence to: Michael A. Zientek, PharmD, Pharmacokinetics, Dynamics and Metabolism—New Biological Entities, Pfizer Worldwide Research and Development, Pfizer, Inc., 10646 Science Center Drive, La Jolla, CA 92121, E-mail: michael.zientek@pfizer.com


Atypical Phenytoin Cross-Resistance in Patients with Genetic Polymorphisms of CYP3A4 and CYP3A5, pharmacogenetics, metabolism, and excretion of [3H]Cytosine, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in humans. Drug Metab Dispos 23:154–158.
