Pharmacokinetics, metabolism and excretion of [14C]axitinib, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in humans.

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Running Title: Axitinib Human Metabolism

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Number of Pages: 37 (text)
Number of Tables: 4
Number of Figures: 8
Number of References: 33
Number of words in Abstract: 250
Number of words in Introduction: 452
Number of words in Discussion: 1539

Abbreviations: ADME (absorption, distribution, metabolism and excretion), EDTA (ethylenediaminetetraacetic acid), VEGFR (vascular endothelial growth factor receptor), \(T_{\text{max}}\) (time to maximal plasma concentration), \(T_{1/2}\) (elimination half life), \(C_{\text{max}}\) (maximal plasma concentration), AUC (area under the plasma concentration-time curve), \(\text{AUC}^\prime\) (area under the plasma concentration-time curve in the presence of an interacting drug), dpm (disintegrations per minute), \(\text{AUC}_{\text{inf}}\) (area under the plasma concentration-time curve from time zero to infinity), LC/MS (liquid chromatography mass spectrometry), NMR (nuclear magnetic resonance), nOe (nuclear Overhauser effect), MCPBA (meta-chloroperbenzoic acid), \([\text{M+H}^+]\) (protonated molecular ion), \(\text{MS}^n\) (multistage mass spectrometry product ion spectra to the \(n^{\text{th}}\)-generation product ions).
Abstract

The disposition of a single oral dose of 5 mg (100 μCi) of [14C]axitinib was investigated in fasted healthy human subjects (N=8). Axitinib was rapidly absorbed with a median plasma T\(_{\text{max}}\) of 2.2 h, and a geometric mean C\(_{\text{max}}\) and half-life of 29.2 ng/mL and 10.6 h, respectively. The plasma total radioactivity-time profile was similar to axitinib but the AUC was greater, suggesting the presence of metabolites. The major metabolites in human plasma (0-12 h) identified as axitinib N-glucuronide (M7) and axitinib sulfoxide (M12), were pharmacologically inactive, and with axitinib, comprised 50.4%, 16.2% and 22.5% of the radioactivity, respectively. In excreta, the majority of radioactivity was recovered in most subjects by 48 h post dose. The median radioactivity excreted in urine, feces and total recovery was 22.7%, 37.0%, and 59.7%, respectively. The recovery from feces was variable across subjects (range 2.5-60.2%). The metabolites identified in urine were M5 (carboxylic acid), M12 (sulfoxide), M7 (N-glucuronide), M9 (sulfoxide/N-oxide) and M8a (methylhydroxy glucuronide), accounting for 5.7%, 3.5%, 2.6%, 1.7% and 1.3% of the dose, respectively. The drug-related products identified in feces were unchanged axitinib, M14/15 (mono-oxidation/sulfone), M12a (epoxide) and an unidentified metabolite, comprising 12%, 5.7%, 5.1% and 5.0% of the dose, respectively. The proposed mechanism to form M5 involved a carbon-carbon bond cleavage via M12a, followed by rearrangement to a ketone intermediate and subsequent Baeyer-Villiger rearrangement, possibly through a peroxide intermediate. In summary, the study characterized axitinib metabolites in circulation and primary elimination pathways of the drug, which were mainly oxidative in nature.
Introduction

Axitinib (AG-013736), N-methyl-2-[[3-[1E]-2-(pyridin-2-yl)ethenyl]-1H-indazol-6-yl]sulfanyl]benzamide (Figure 1), is an oral medication that has been approved in the US and multiple countries as a treatment for renal cancer. In the US axitinib is indicated for the treatment of advanced renal cell carcinoma in patients who have failed one prior systemic therapy (Inlyta®, 2012). Axitinib is an inhibitor of ATP binding to the kinase domain of vascular endothelial growth factor receptors (VEGFR)-1, 2 and 3 with subnanomolar affinity (Bender et al., 2004; Hu-Lowe et al., 2008; McTigue et al., 2012). The VEGFR signaling network is a regulator of angiogenesis, which is necessary to supply tumors with oxygen and nutrients during phases of rapid growth (Folkman et al., 1971). Solid tumors in many organ systems have high expression of VEGFR compared to normal tissues (Hanahan and Folkman, 1996).

During phase I clinical trials the pharmacokinetics of axitinib were evaluated over a dose range of 2-20 mg BID in cancer patients (Rugo et al., 2005). Time to maximal plasma concentration ($T_{max}$) and half-life ($T_{1/2}$) ranged from 1.7-6.0 hr and 1.7-4.8 hr, respectively. Exposure increased linearly as assessed by maximal plasma concentration ($C_{max}$) and area under the plasma concentration-time curve (AUC). In vitro studies suggested CYP3A was responsible for substrate consumption of axitinib in human liver microsomes (Zientek et al., 2010). The role of CYP3A in the metabolism of axitinib was supported by results from clinical drug interaction studies where ketoconazole increased the axitinib plasma AUC ratio relative to a control arm by 2-fold, and rifampin pretreatment decreased this ratio to 0.2 (Pithavala et al., 2010; Pithavala et al., 2012b).

Human radiolabeled mass balance studies are usually performed during drug development to identify and quantify the exposure to metabolites circulating in plasma, elucidate metabolite structures, determine the primary clearance mechanisms of the drug and understand the rate and route of excretion of parent drug and their metabolites, and to validate the species used in safety studies. These
studies generate important knowledge regarding the disposition of the drug in humans. Furthermore, the studies are typically conducted during drug development as part of drug registration packages and are seldom repeated due to the cost, complexity and challenges of performing human studies with radioactivity. During the conduct of human ADME studies challenges may arise that could compromise the ability to achieve the study objectives defined above. The human ADME study of axitinib, conducted during early clinical development, is an example of a study that advanced our knowledge of the disposition of the drug, but had the limitation of low and variable recovery of radioactivity. Herein we describe the knowledge gained during the conduct of the radiolabel mass balance and metabolite structure elucidation of a single oral 5 mg dose of [14C]axitinib to healthy volunteers.

Materials and Methods

Chemicals. Axitinib was synthesized by Pfizer Global Research and Development, La Jolla Laboratories (San Diego, CA). [14C]Axitinib was prepared by Pfizer Global Research and Development Radiosynthesis Group (Groton, CT) using the same synthetic route as used for unlabeled axitinib (Borchardt et al., 2006). This route yielded 125 mg of [14C]axitinib with a specific activity of 58.3 mCi/mmol and the position of the incorporation of 14C is shown in Figure 1. The resulting [14C]axitinib was mixed with 811 mg of non-radiolabeled axitinib and subjected to polymorph control (crystal form IV) (Campeta et al., 2010) yielding 800 mg of [14C]axitinib drug substance mixture with a specific activity of 7.1 mCi/mmol. The purity of [14C]axitinib drug substance was 99% by HPLC. Reference standards for M9 (axitinib sulfoxide/N-oxide), M12 (axitinib sulfoxide), M15 (axitinib sulfone) were prepared biosynthetically using microorganisms and isolated by HPLC and the structures confirmed by LC/MS and NMR (Supplemental data). M7 (axitinib N-glucuronide) was isolated from urine from this study and characterized by NMR.

Study Design, Dosing and Sample Collection. This was a single dose, open label study conducted at a single center in eight healthy male nonsmoker subjects (6 Caucasian, 2 African American) with a median
age of 34 years (range 25-46 years) and median weight of 83 Kg (range 79-96 Kg) who met inclusion/exclusion criteria. The study was conducted in accordance with the Declaration of Helsinki, approved by an institutional review board, and all subjects provided voluntary informed consent. All subjects received a single 5-mg oral dose of axitinib containing 100 μCi of $[^{14}\text{C}]$axitinib supplied as a powder for reconstitution. On the day of dosing, 20 mL of cranberry juice was added to each plastic container containing $[^{14}\text{C}]$axitinib powder. The container was capped tightly and then shaken vigorously by hand 10 to 12 times until the powder was suspended in the cranberry juice. Subjects drank the resulting suspension directly from the plastic container. The container was then rinsed 4 times using 20 mL of additional cranberry juice each time. At each rinse, the container was shaken vigorously. Subjects immediately drank the rinsed solutions directly from the container. After drinking the final rinse solution, the subjects swallowed at least 200 mL of tepid water. The oral cavity of each subject was examined after dosing to ensure that all study medication was swallowed. After each drug administration, the empty dosing container was capped and retained for assay of residual radioactivity and, if necessary, for residual drug. Radioactivity determinations of the dose solution and residual remaining in the container confirmed that all subjects received >98% of the expected radioactive dose. Recovery calculations were based on the actual amount of radioactivity administered. Blood samples (7 mL) were collected in K$_3$-EDTA containing glass Vacutainer tubes prior to dosing and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 36, 48, 72, 96, 120, 144, and 168 h after dosing. Additional blood volume (20 mL) was collected at 1, 4, 8 and 12 h after dosing for metabolite profiling and identification. Following collection of blood samples, plasma was prepared by centrifugation for drug and metabolite analyses and stored frozen. Urine was collected over 4 h intervals through 12 h, 12 h intervals through 48 h and every 24 h thereafter. Feces was collected over 24 h intervals. Per protocol, excreta samples were collected and tested daily until less than 1% of the administered dose was recovered, at which point the subject could be discharged from the study. Samples were stored frozen prior to analysis. Precautions
were taken during sample collection, processing and analysis to protect all samples from exposure to visible light.

**Measurement of Radioactivity.** Plasma (0.25 mL) and urine (1 mL) aliquots were added to Ultima Gold scintillation fluid to a final volume of 20 mL for radioactivity determination by liquid scintillation counting, in triplicate and duplicate, respectively. The concentration of the radioactivity in the whole blood and red blood cell samples containing potassium EDTA was determined by oxidizing triplicate 0.25 mL aliquots of well mixed whole blood as described below for fecal samples. To each fecal collection was added an equal weight of water followed by homogenization. Three aliquots of each fecal homogenate (0.25-0.47 g) were burned in a Packard 307A oxidizer, which captured the evolved CO\textsubscript{2} from each aliquot into Carbosorb (10 mL). Permafluor (10 mL) was added by the oxidizer into each 20 mL scintillation vial. Whole blood (0.25 mL) was processed similarly using the sample oxidizer. All radioactivity measurements were determined using a Tricarb 2100 counter for 20 min or a period of time that yielded a counting precision of 95%. Only values greater than two times the background were reported. The disintegrations per min (dpm) were converted to ng-equivalents/mL or g for each matrix using the known specific activity. For urine and feces, the data were also converted to the percentage of dose for each time interval.

**Determination of Axitinib in Plasma and Urine.** Axitinib was determined in human plasma and urine by LC-MS/MS with plasma methods as described previously (Rugo et al., 2005; Pithavala et al., 2012b). The analysis of axitinib in urine was similar to that described for plasma with the exception of glass tubes instead of the use of a 96-well plate for extraction. These methods employed sample extraction by an ethyl acetate/hexanes liquid/liquid extraction followed by evaporation, reconstitution and final extract analyses by LC-MS/MS. The methods were validated for the analysis of axitinib over a concentration range of 0.100 to 25.0 ng/mL using a 200 μL extraction volume for plasma samples and 1.00 to 500
ng/mL using a 100 μL extraction volume for urine samples. Accuracy, evaluated by % bias of QC samples, ranged between -2.0% to 4.0% for plasma and -0.5% to 1.3% for urine across concentrations and analytical runs. Precision, evaluated by %CV of QC samples, ranged from 3.8% to 7.9% for plasma and 1.9% to 3.5% for urine across concentrations and analytical runs.

**Calculation of Pharmacokinetic Parameters.** Plasma pharmacokinetic parameters (AUC, C, T, T) were estimated by using WinNonLin software, Version 3.2, and summarized with descriptive statistics as appropriate using the Statistical Analysis Systems software, Version 8.2. All other calculations, including the estimation of urinary, fecal, and total recovery, were also performed using SAS. As outlined in the study protocol, no formal statistical analyses were performed for this human mass balance study conducted in 8 healthy volunteers.

**Sample Preparation for Plasma, Urine and Fecal Extracts for Metabolite Profiling.** Plasma samples collected at 0, 1, 4, 8, and 12 h post-dosing from each subject were pooled based on the algorithm described by Hamilton and coworkers (Hamilton et al., 1981) to generate a plasma pool (7.2 mL). Plasma proteins were precipitated by the addition of 4 volumes of acetonitrile:methanol (1:1 v/v), vortexed, and centrifuged. The supernatant was removed and retained. The pellet was reextracted as above and the two supernatants were combined and transferred into glass tubes for evaporation to dryness under N₂ at 40°C. The residues were reconstituted in 1 mL of 30:70 (v/v) methanol:20 mM ammonium acetate (pH 4) and the solutions were transferred into autosampler vials for analysis. The injection volume was 900 μL.

Urine samples collected over pre-specified time intervals post dosing from each subject were pooled on a percent weight basis to generate a urine pool (2 mL). After centrifugation, each pooled urine sample was transferred into autosampler vials for analysis. The injection volume was 900 μL.
The fecal homogenates selected for metabolite profiling varied by subject to cover time intervals where the majority of the radioactivity was excreted. The selected fecal homogenates from each subject were pooled on a percent weight basis to generate a homogenate pool (2 mL). Each pooled sample was precipitated by the addition of 4 volumes of acetonitrile, vortexed, and centrifuged. The supernatant was removed and retained. The pellet was reextracted as above and the supernatants were combined and transferred into glass tubes for evaporation to dryness under \( \text{N}_2 \) at 40°C. The residues were reconstituted in 1 mL of 30:70 (v/v) methanol:20 mM ammonium acetate (pH 4) and the solutions were transferred into autosampler vials for analysis. The injection volume was 800 \( \mu \text{L} \).

To investigate causes of observed variable fecal recovery of radioactivity in this study, the fecal homogenization procedure was also put to additional scrutiny. In an effort to ascertain if an alternate homogenization procedure might produce better recovery of radioactivity from the fecal samples, a series of experiments were undertaken. Briefly, the following alternate procedures were evaluated using blank fecal samples as well as actual fecal samples from this study. 1) Manual vigorous shaking of the fecal homogenate slurry for 1 to 2 min before sampling for radioactivity counting; 2) sonication of the fecal homogenate slurry after manual vigorous shaking and before sampling for radioactivity counting; 3) liquid extraction of the axitinib–related material from the fecal homogenate slurry (using an acetonitrile-acidified water system that is routinely used for solubilizing axitinib), involving 3 successive extractions of the fecal homogenate, followed by the pooled supernatant and extraction pellet being counted for radioactivity; and 4) evaluation of any radioactivity adsorbed to the polyethylene bag used for the homogenization by oxidation of 2 cut sections of the polyethylene bag that held the fecal homogenate.

**Metabolic Profiling and Structure Elucidation.** All metabolite profiling and structure elucidation were performed using HPLC coupled in-line with radiochemical detection (ARC/\( \beta \)-RAM) and MS detection with
electrospray (ESI) source in positive mode. The instrumental components were as follows: Agilent 1100 HPLC Pumps/Autosampler (Agilent Technologies, Wilmington, DE), 4.0 x 3.0 mm guard column C18 (Phenomenex, Torrance, CA), Cosmosil SPYE column 150 x 4.6 mm (Waters, Milford, MA), LCQ-Deca XP Ion Trap MS (ThermoFinnigan, San Jose, CA), Model C ARC StopFlow System (AIM Research Company, Newark, DE), Model 3 β-RAM Radiodetector (500 µL Cell) (IN/US Systems, Tampa, FL), StopFlow AD Scintillation Cocktail (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 acetonitrile (Solvent B). The flow rate was maintained at 1.0 mL/min. Axitinib and metabolites were eluted using a linear gradient in mobile phase composition summarized as follows: 100% Solvent A for 10 min, a linear gradient to 50% Solvent A/50% Solvent B at 60 min, a linear gradient to 100% Solvent A at 61 min and held at 100% Solvent A 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 ESI/MS method were as follows: positive ionization mode, 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) and negative ionization mode, spray voltage (5.0 kV), capillary voltage (-47.0 V), tube lens offset (-60 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. Ion-trap LC-MS² (n = 2-4) experiments were performed to generate multi-stage mass spectra for the selected molecular ions representing possible metabolites of
axitinib. Helium at a constant pressure of 40 psi was used as the damping and collision gas for all MS$^n$ experiments. The mobile phase, gradient, guard cartridge, column, and source conditions used were identical to the ion-trap LC-ESI/MS method. Precursor isolation window, activation amplitude, activation Q, and activation time were set at 1.5 amu, 30%-50%, 0.25 ms and 30 ms, respectively. The chemical structures of the axitinib metabolites were proposed based on metabolite prediction and mass spectral interpretation. Definitive structures were assigned for isolated metabolites using nuclear magnetic resonance (NMR) spectroscopy or by comparison to authentic synthetic standards.

**Metabolite Biosynthesis and Isolation and NMR Spectral Characterization.**

**Isolation of carboxylic acid metabolite (M5) and N-glucuronide of Axitinib (M7):**

Metabolites M5 and M7 were isolated from human urine. Briefly, pooled urine samples from each subject were combined to generate a mixed urine pool and treated with acetonitrile. Following vortexing and centrifugation of the mixture, the supernatant was separated, concentrated and further lyophilized. The residue was reconstituted in 1 mL of mobile phase and injected onto a 900 µL sample loop and separated by HPLC using the same analytical column and the gradient system used for profiling the metabolites. The fractions containing the glucuronide conjugate were collected (detection of the metabolite was done by UV signal) and evaporated under a steady stream of N$_2$. The structures of M5 and M7 were characterized by $^1$H NMR. Metabolite M7 was also investigated using 1D nuclear Overhauser effect (nOe) spectroscopy. Approximately 3 µg of the isolated M7 was dissolved into 120 µL of dimethyl sulfoxide-d6 (DMSO-d6, Cambridge Isotope Laboratories, Inc.) and transferred to a 3 mm NMR tube (Wilmad 335-PP). The sample of axitinib was prepared by dissolving 51 mg into 0.5 mL of DMSO-d6 and transferring the material to a 5 mm NMR tube (Wilmad 535-PP). NMR measurements were made on a Bruker Avance DRX spectrometer operating at 500.13 MHz $^1$H frequency at 30°C (rt 0.01) using a 5 mm TXI Zgradient CryoProbe™. The M7 $^1$H-NMR spectrum was acquired with 4000 scans
and 32768 complex data points using a spectral width of 10,000 Hz and an acquisition time of 1.64 seconds. An additional relaxation delay of 1.0 second was added between pulses to allow for T1 relaxation. NMR data for axitinib was acquired under the same conditions, with the exception that only 16 scans were needed. For the M7 nOe data, 20,000 scans were acquired using a 1D nOe experiment and selectively inverting the glucuronic acid anomic proton resonance. Spectra were acquired and processed using Bruker’s XWTNNMR software, version 3.5-patch level 6. Chemical shifts were referenced to an internal standard of tetramethylsilane.

Preparation of metabolites M9, M12 and M15:

Authentic metabolite standards of M9, M12 and M15 were prepared either using microbes or chemically using metaperchlorobenzoic acid (MCPBA) in support of definitive structure determination. For microbial synthesis, 40 fungi and 40 bacteria, the majority obtained from American Type Culture Collection (ATCC) and a few from in house sources (Pfizer Inc., Groton, CT) were screened for M12 formation. The bacteria and fungi were grown in 20 g glucose, 5 g soy flour, 5 g yeast extract, 5 g K2HPO4, 5 g NaCl, and 1 g MgSO4.7H2O, adjusted to a final volume of 1 L with water, adjusted to pH 7.2, and sterilized. All strains were grown at 28°C and 250 RPM on a rotary shaker. After 2 and 3 days, 0.2 mg of axitinib from a 10% ethanol solution was added. After 3 and 6 days, the reactions were analyzed by LC-MS using a COSMOSIL, 5PYE column (150 x 4.6 mm, Waters, Milford, MA) with a mobile phase of acetonitrile:50 mM ammonium acetate (pH 4.5), at a flow rate of 1.0 mL/min and UV detection at 254 nm. For positive-mode MS detection, the mobile phase consisted of 20 mM ammonium acetate in water adjusted to pH 4.0 with acetic acid and acetonitrile. Among all strains screened, Cunninghamella echinulata and Streptomyces griseus showed the highest oxygenation activity. M12 was produced in abundance of >80% and this was used to prepare a large batch of the metabolite.
For preparative transformation, *Cunninghamella echinulata* obtained from ATCC was grown from an agar plate into a 100 mL preculture using the screening medium and conditions. After 2 days, 10 mL of the preculture was inoculated into 1 L culture on a 4 L shake flask (x 5 L). The culture was grown for 24 h and then axitinib was added (0.2 g/L substrate for each 1 L culture). Oxidation was followed by reverse phase HPLC and the reaction was stopped after 8 days. The mycelium was removed from the culture by filtration and the oxidation products were extracted three times with 1 volume of chloroform used each time. After removal of CHCl₃ in vacuo, crude product (~500 mg) was obtained. The crude product was purified by silica gel flash chromatography, using CH₂Cl₂:acetone:MeOH (10:1:1) as eluent to afford 30 mg of the sulfoxide M12. For preparative transformation of axitinib to M12 by *Streptomyces griseus*, the microbe was grown from an agar plate into a 100 mL preculture using the screening medium and conditions. After 2 days, 10 mL of the preculture was inoculated into 1 L culture in a 4-L shake flask (x 10 L). The culture was grown for 24 h and axitinib was added (0.1 g/L substrate for each 1 L culture). Oxidation was followed by reverse phase HPLC and the reaction stopped after 8 days. The mycelium was removed from the culture by filtration and the oxidation products were extracted 3 times with 1 volume of chloroform each. After removal of CHCl₃ in vacuo, the crude product (1.5 g) was obtained. The crude product was purified by silica gel flash chromatography using CH₂Cl₂:acetone:MeOH (10:1:1) as eluent to afford 80 mg of sulfoxide and yielded 8-9 mg pure sulfoxide.

**Preparative transformation of axitinib to M15 by metaperchlorobenzoic acid (MCPBA):** MCPBA was added to 200 mg of axitinib suspended in 10 mL of methylene chloride at 0 °C. The reaction mixture was stirred at room temperature for 7 h. After removal of solvent, the crude products were purified by silica-gel chromatography using methylene chloride:acetone:methanol (10:1:1) as eluent to afford M15 (63 mg, 30%) and M9 (11 mg, 5%). The structures of M9, M12 and M15 were elucidated using ¹H- and ¹³C-NMR methods by dissolving the compounds into DMSO-d6. The chromatographic profiles and ¹H- and ¹³C-NMRs of M9, M12 and M15 are presented in Supplemental Data, Figures S1 to S7.
Evaluation of M7 and M12 for inhibition of kinases and hERG: Axitinib metabolites M7 (glucuronide) and M12 (sulfoxide) were evaluated in cellular assays for their ability to inhibit kinase activities according to the methods described in Hu-Lowe et al. (2008). M12 was tested for inhibition of VEGF-stimulated human vascular endothelial cell (HUVEC) survival, PDGFR-β (ELISA), bFGF-stimulated HUVEC survival, and KIT (ELISA) while M7 (glucuronide) was evaluated for inhibition of VEGFR2 (ELISA) in transfected porcine aorta endothelial (PAE) cells. M7 and M12 were also evaluated for the inhibition of human ether-a-go-go related gene (hERG) to assess their risk to prolong cardiac QTc interval. M7 was evaluated for inhibition of IKr using whole cell voltage clamp electrophysiology and M12 was evaluated for inhibition of ligand binding fluorescence polarization according to methods described by Deacon et al. (2007).

Results

Pharmacokinetics of Axitinib Radioactive Equivalents and Axitinib in Plasma. The mean plasma concentration time profile for total radioactivity and axitinib parent drug following the administration of 5 mg of $^{14}$C]axitinib to healthy human subjects is presented in Figure 2 and the derived pharmacokinetic parameters presented in Table 1. Total radioactivity and parent axitinib reached maximal concentration in plasma at approximately 2-3 h post dose. The mean axitinib plasma $C_{\text{max}}$ and AUC was about 32% and 15% of total radioactivity, suggesting that total circulating axitinib metabolites exceeded the parent drug. The terminal elimination half-life after oral dosing was similar for axitinib and total radioactivity suggesting similar parent and total metabolite elimination rates from the body. The total $^{14}$C]axitinib-derived radioactivity whole blood exposure, assessed by $C_{\text{max}}$ and AUC, was about half of the plasma values, indicating that the combination of parent and metabolite was preferentially distributed in plasma (data not shown).
**Excretion of Axitinib Radioactive Equivalents in Urine and Feces.** The urine and feces were collected for 144 h and up to 288 h post dose from eight healthy male subjects following an oral 5 mg dose of radiolabeled axitinib. The excretion of total radioactivity in urine and feces in each subject is shown in Figure 2. The recovery of total radioactivity was lower than expected especially in subjects 1001 and 1003, and variable between subjects. The total recovery was 37.9% and 16.0% in subject 1001 and 1003, respectively. In the remaining 6 subjects, the overall recovery of radioactivity ranged from 51.3% to 77.9% of the administered dose. The median recovery (all subjects) was 59.7%. In all but one subject (1001) with low recovery, the excretion of radioactivity in feces was greater than urine. The median recovery across all subjects in feces and urine was 37.0% and 22.7 %, respectively. Urinary recovery was consistent across the 8 subjects however fecal recovery was quite variable (2.5-60%). An investigation into potential causes of the poor recovery was conducted. This included confirmation of the administered radioactivity in the dose and additional homogenization and sampling of fecal homogenates. In subjects 1002 and 1003 a change in bowel habits was noted with no fecal sample obtained for several consecutive collection intervals (Figure 3). Fecal samples were homogenized and resampled for radioactivity determination in three randomly selected subjects however no difference was noted from the original counts (data not shown).

**Axitinib Plasma Metabolites.** Plasma samples collected at 1, 4, 8 and 12 h were combined according to the pooling method of Hamilton et al. (1981) to create a 0-12 h plasma pool for each subject. A representative radiochromatogram is presented in Figure 4. These samples were profiled by HPLC and the individual percentage of each axitinib derived component is presented in Table 2. The mean percentage of axitinib and related metabolites present in circulation was M7 (50.4%), M12 (16.2%) and axitinib (22.5%) with a coefficient of variation<27% for each component.
Axitinib Urinary and Fecal Metabolites. Also shown in Table 2 are the percentage of dose excreted in the urine and feces for each metabolite, and representative radiochromatograms are shown in Figure 4. The mean percent metabolite excreted in urine was largest for M5 (5.7%) followed by M12 (3.5%), M7 (2.6%), M9 (1.7%) and M8a (1.3%), while unchanged axitinib was not detectable. In addition, a more sensitive LC/MS assay was used to detect and quantify lower concentrations of unchanged axitinib in urine. In 5 out of 8 subjects there was no detectable axitinib (< 1 ng/mL) in any urine sample. In the remaining three subjects low concentrations of axitinib were noted (≤ 3.1 ng/mL) in urine samples collected from 0-12 h. This independently confirmed the results of radiochemical profiling that there was no appreciable renal clearance of unchanged axitinib. In fecal extracts the primary component was unchanged axitinib (12.0%) followed by roughly equal amounts of M14/15 (5.7%), M12a (5.1%) and an unidentified metabolite (5.0%).

Identification of Axitinib Metabolites. Axitinib gave a molecular ion at m/z 387 [M+H]+ in a positive ion mode. The mass spectrum of m/z 387 gave characteristic major fragment ions at m/z 356, 222 and 166 (Table 3). The fragment ion at m/z 356 was a result of the loss of the methylamine moiety from axitinib and subsequent formation of acylium ion whereas the fragment ions at m/z 222 and 166 were obtained from the cleavage of the sulfide linkage. The structures (confirmed or proposed) of metabolites of axitinib are shown in Figure 5, and Table 3 depicts the molecular and major fragment ions of all the detected metabolites.

Metabolite M5: Metabolite M5 showed a protonated molecular ion at m/z 342 in the full scan. The mass spectrum of m/z 342 gave major fragment ions at m/z 311, 296, 265 and 166 (Table 3). The presence of a radiolabel peak in the chromatogram suggested the metabolite was drug related. The molecular ion at m/z 342 suggested a loss of 45 amu from m/z 387 ([M+H]+ of axitinib). An even molecular ion of the metabolite suggested a loss of a nitrogen atom from the molecule. The fragment
ions at m/z 311 (Table 3) were consistent with a loss of a methylamine moiety from m/z 342, while the fragment ion at m/z 166 suggested a cleavage of the sulfide moiety. Both the loss of the methylamino group and the fragment ion of m/z 166 were observed in the mass spectral fragmentation of axitinib. Other fragment ions at m/z 296 and 265 showed a loss of 46 amu from m/z 342 and 311, respectively (Table 3). The loss of 46 amu, which is characteristic of the presence of a carboxylic acid group in the molecule (Ling et al., 2006; Dalvie et al., 2010), suggested that M5 was a carboxylic acid derivative (Table 3). The structure of the metabolite was further confirmed by 1H NMR after isolating the metabolite from human urine. The 1H NMR spectrum of M5 revealed a loss of the pyridine ring and the vinyl protons observed in axitinib (Table 4; the 1H NMR spectra of axitinib and M5 are shown in Supplemental Data, Figures S8 and S9 respectively). This was replaced by the singlet due to methylene protons at 3.8 ppm, sandwiched between the carboxy group and the indazole ring. The metabolite was therefore proposed as 2-(6-(2-(methylcarbamoyl)phenylthio)-1H-indazol-3-yl)acetic acid (Figure 5).

**Metabolite M7:** Metabolite M7 showed a protonated molecular ion at m/z 563 in the full scan and was consistent with addition of 176 amu to axitinib and suggested that M7 was a glucuronide conjugate of axitinib. The mass spectrum of m/z 563 gave fragment ions at m/z 387 (the molecular ion of axitinib) and 356 (Table 3). The ion at m/z 387 indicated a loss of 176 amu from m/z 563 and corresponded to the molecular ion of axitinib. The fragment ion at m/z 356 suggested a loss of the methylamine moiety from m/z 387 as observed in the mass spectrum of axitinib as described above. The exact site of glucuronidation could not be discerned from the mass spectral details. The site of glucuronidation was evaluated further by isolating the metabolite from human urine and subjecting it to NMR analysis. 1H NMR of M7 showed all the assigned resonances of axitinib in addition to the resonance signals from protons in the glucuronic acid moiety (Table 4; the 1H NMR is shown in Supplemental Data, Figure S10A and S10B). The key change in the 1H NMR spectrum of M7 was the shift of the resonance signal at 7.65 ppm for proton at the 7-position of the indazole ring to 7.95 ppm (Table
4) and suggested attachment of the glucuronic acid moiety to the indazole nitrogen. The point of attachment of glucuronic acid moiety to the indazole nitrogen was confirmed with additional 1D nuclear Overhauser effect (nOe) measurements. A selective nOe to the nearby proton at the 7-position was observed (Figure 6) upon selective inversion of resonance of the anomeric proton of glucuronic acid.

**Metabolite M8a**: Metabolite M8a gave a signal at m/z 579 suggesting an addition of 192 amu to the molecular ion of axitinib. A mass spectrum of M8a at m/z 579 showed major fragment ions at m/z 403, 385, 373 and 356 in the MS² spectrum (Table 3). The molecular ion at m/z 403 (loss of 176 amu) in the mass spectrum suggested that the metabolite was a glucuronide conjugate of an oxidative metabolite of axitinib. The modification was possibly on the N-methyl amide moiety since the mass spectrum also showed a fragment ion at m/z 356 which was consistent with the loss of methylamine group in axitinib. The fragment ion at m/z 385 and 373 suggested a loss of water and a hydroxymethyl moiety from m/z 403, respectively, further confirming that the methyl group on the methylamine moiety was the site of hydroxylation. Although the exact site of glucuronidation could not be discerned from the fragment ions the possible site of glucuronidation was either the nitrogen of the indazole moiety as observed in M7, or the hydroxyl group of the hydroxymethyl amide moiety.

**Metabolite M9**: Metabolite M9 showed a protonated molecular ion at m/z 419 in the full scan, which was 32 amu greater than axitinib. This indicated that M9 was a di-oxygenated axitinib metabolite. Its mass spectrum at m/z 419 gave major fragment ions at m/z 388, 324 and 296 (Table 3). The fragment ion at m/z 388 indicated a loss of 31 amu, which was consistent with the loss observed in the fragmentation of axitinib while the fragment ions at m/z 324 and 296 resulted from a loss of 64 amu from m/z 388 and a subsequent loss of 28 amu from m/z 324, similar to the losses observed in the spectrum of M15 (as described below). Although the loss of 64 amu generally indicates extrusion of sulfur dioxide and therefore the presence of a sulfone, the different retention times of M9 to M15 suggested that M9 was a different di-oxygenated metabolite of axitinib. The regiochemistry of
oxygenation could not be discerned from the mass spectral data. Comparison of retention time of M9 with N-oxide derivative of axitinib sulfoxide, prepared using MCPBA, suggested that the oxidation in M9 occurred on the sulfur as well as the pyridine nitrogen as shown in Figure 5.

**Metabolite M12:** Metabolite M12 showed a protonated molecular ion at m/z 403, suggesting an addition of 16 amu to 387 ([M+H]⁺ of axitinib). An addition of 16 amu indicated that M12 was a mono-oxygenated metabolite. The mass spectrum at m/z 403 gave fragment ions at m/z 372, 355, 344 and 327 (Table 3). The fragment ion at m/z 372 indicated a loss of 31 amu which was consistent with the loss observed in the fragmentation of axitinib. The fragment ion at m/z 355 resulted from a loss of 48 amu and was characteristic of a loss of a sulfoxide moiety from the molecule (Bu et al., 2007; Shimizu et al., 2009). The ions at m/z 344 and 327 indicated a loss of 28 amu from m/z 372 and 355, respectively, and suggested a loss of either a carbonyl group from the acylium intermediate that was formed from cleavage of the N-methylamide or loss of nitrogen from the indazole ring. The metabolite was confirmed by comparing it with the synthetic standard, which was prepared using microbial reactions.

**Metabolite M12a/M14:** Metabolites M12a and M14 gave a protonated molecular ion at m/z 403 in the full scan, which suggested that both the metabolites were formed via mono-oxygenation of axitinib. The mass spectrum of M12a gave major fragment ions at m/z 372, 354 and 344 (Table 3). The presence of the ion at m/z 372 indicated a loss of 31 amu from m/z 403, suggesting a loss of methylamine moiety and the ion at m/z 344 was 28 amu lower than m/z 372, corresponding to loss of N₂ from the indazole or a carbonyl moiety. These losses were similar to those observed in the fragmentation pattern of axitinib. The fragment ion at m/z 354 on the other hand indicated a loss of water molecule from m/z 372, which suggested that hydroxylation was at a site that was susceptible to dehydration and eliminated pyridine, or indazole or the phenyl group in the axitinib molecule as probable sites of oxygenation. It was therefore speculated that the site of oxidation was the alkene moiety in the molecule and the possibility of the metabolite being the epoxide of axitinib could not be
ruled out. The proposed mechanism leading to the loss of a water molecule from an epoxide is presented in Figure 7.

The mass spectrum of M14 at $m/z$ 403 gave major fragment ions at $m/z$ 372, 355 and 344 (Table 3). Like in the case of M12a, the fragment ions at $m/z$ 372 and 344 indicated that pyridine was the likely site of modification. The fragment ion at $m/z$ 355 indicated a loss of 17 amu from $m/z$ 372 which was characteristic of an N-oxide metabolite, especially the pyridine N-oxide. This suggested that M14 was most likely a pyridine N-oxide metabolite of axitinib. However, since neither M12a nor M14 were confirmed by NMR or their respective synthetic standards, these metabolites have been represented as Markush structures in Figure 5 and Table 3.

**Metabolite M15:** Metabolite M15 showed a protonated molecular ion at $m/z$ 419, suggesting an addition of 32 amu to 387 ([$M+H^+$] of axitinib). This indicated that M15 was a di-oxygenated metabolite. Its mass spectrum at $m/z$ 419 gave major fragment ions at $m/z$ 388, 324 and 327 (Table 3). The fragment ion at $m/z$ 388 indicated a loss of 31 amu, which was consistent with the loss observed in the fragmentation of axitinib. The fragment ion at $m/z$ 324 resulted from a loss of 64 amu from $m/z$ 388. Published reports indicate that compounds with a sulfone moiety such as the sulfonamide and/or sulfones show a characteristic loss of 64 amu due to extrusion of sulfur dioxide from the molecule (Wang et al., 2003; Bu et al., 2007). The fragment ion at $m/z$ 296 indicated a loss of 28 amu from $m/z$ 324 and suggested a loss of either a carbonyl group from the acylium intermediate that was formed from cleavage of the N-methylamine or loss of nitrogen from the indazole ring similar to that observed in the spectrum of the sulfoxide metabolite (M12). This suggested that M15 was a sulfone metabolite of axitinib. The modification of the sulfide to the sulfone was further confirmed by comparing the spectral properties and the retention time with the synthetic standard which was prepared using MCPBA.
Assessment of Kinase Activity of M7 and M12. Since M7 and M12 were the primary circulating metabolites in human plasma they were evaluated for biological activity in relevant assays. The activity of axitinib in the assays used to evaluate the metabolites has been reported previously (Hu-Lowe et al., 2008). M7 (axitinib N-glucuronide) was not suspected to be active since it lacks the indazole NH which forms a critical hydrogen bond in the ATP binding site of the VEGFR kinase domain (McTigue et al., 2012). The IC$_{50}$ for inhibition of VEGFR-2 autophosphorylation in KDR-PAE cells by M7 was 1990 nM (N=7) which was 8300-fold higher than axitinib. M12 (axitinib sulfoxide) was evaluated for inhibition of VEGF-stimulated HUVEC survival, and a few other related kinase activities. Unlike axitinib, M12 showed minimal activity against VEGF-stimulated HUVEC survival, PDGFR-β and KIT in cell assays with IC$_{50}$ values of >100, 810 and 310 nM which were greater than 400-, 470- and 290-fold higher than the IC$_{50}$ values for axitinib. The IC$_{50}$ for inhibition of bFGF-stimulated HUVEC survival by M12 was >1000 nM, an assay where axitinib also showed weak activity. These data indicated that M7 and M12 were far less potent against the target and some related kinases, and hence, were considered pharmacologically inactive metabolites. M7 and M12 had hERG inhibitory potencies of >30 µM and >79 µM in cellular voltage clamp and binding assays, respectively.

Discussion

The objectives of the study were to investigate the disposition of an oral 5 mg dose of [14C]axitinib in healthy human subjects, to identify and quantify the exposure to metabolites circulating in plasma, elucidate metabolite structures, determine the primary clearance mechanisms of axitinib, understand the rates and routes of excretion of axitinib and its metabolites from the body, and validate the species used in safety studies. The majority of these study objectives were met although not without some limitations.
Metabolism was the primary route of axitinib clearance since only 12% of the unchanged drug was observed in the feces and none was detected in the urine. Essentially, five primary metabolites detected in human excreta comprised the key pathways for axitinib clearance: sulfoxide (M12), N-glucuronide (M7), hydroxymethyl (M8a precursor), oxidation products (M12a/M14) and N-oxide (M9). In human plasma the only metabolites detected were axitinib sulfoxide and axitinib N-glucuronide, and together, they comprised about 66.6% of the drug-related plasma AUC. Many of the metabolites in excreta were not detected in plasma. This suggested that the urinary metabolites M5, M8a, and M9 were cleared by the kidney much more rapidly than their formation clearance, preventing accumulation to detectable concentrations in blood. Similarly, the fecal metabolites M12a, M14/15 and the unknown metabolite are likely rapidly transported into the bile and are not substrates for hepatic apical transporters that could facilitate their transfer to blood.

Assessment of circulating metabolites in humans contributes to an overall understanding of their potential impact on the safety and efficacy of a drug. From a safety point of view, gathering knowledge of the in vivo metabolic profile of a drug in humans has gained more importance since the publication of position papers on drug metabolites in safety testing as well regulatory guidance documents on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals (Davis-Bruno and Atrakchi, 2006; EMA, 2009; Nedderman et al., 2011). \(^{[14]}\)C\)axitinib disposition has been studied in the mouse and dog (manuscript in preparation), and the sulfoxide (M12), the metabolite of primary concern for such assessments, was detected in sufficient concentrations in the plasma of both species when compared to the results from the present study in humans. Further, neither M7 nor M12 were pharmacologically active for VEGFR, other kinases nor were they found to exert activity in hERG assays. Hence, both metabolites did not warrant further evaluation and quantification in nonclinical studies or clinical trials.
The enzymology of axitinib metabolism has been investigated in human liver microsomes and recombinant enzymes (Zientek et al., 2010). CYP3A4/5 were the major enzymes involved in the oxidative metabolism of axitinib. This was consistent with the results from several studies that investigated the clinical pharmacokinetics of axitinib. When ketoconazole 400 mg was given as an interacting drug with 5 mg axitinib as the object, a 2-fold increase in Cmax and AUC was observed (Pithavala et al., 2012b). Axitinib has a moderate intravenous clearance and an oral bioavailability of 58% (Inlyta®, 2012). Thus, a 2-fold interaction with ketoconazole is suggestive that CYP3A is the major clearance mechanism.

The formation of M7 was primarily catalyzed by UGT1A1 (Zientek et al., 2010). Given the polymorphic nature of UGT1A1, several genetic polymorphisms were investigated using a meta analysis of data from 11 axitinib healthy volunteer clinical studies in 315 subjects to determine if there was association of specific genotypes with the observed variability in axitinib pharmacokinetics (Brennan et al., 2012). However, none of the evaluated genotypes were found to significantly contribute to the observed between-subject variability in axitinib pharmacokinetics. This suggests that the conversion of axitinib to the N-glucuronide is a minor clearance mechanism even though it is a major metabolite in plasma. The factors that cause the apparent disconnect between metabolite abundance in plasma and clearance are known and have been well described by others (Lutz et al., 2010; Loi et al., 2013). Finally, taken together, these and other data suggest that liver is the primary organ involved in the clearance of axitinib from the body.

The formation of carboxylic acid metabolite (M5) from axitinib was unusual in that it involves loss of a pyridine ring via a cleavage of a carbon-carbon bond. While the mechanism for its formation is unknown, one possible pathway might involve oxidation of axitinib to an epoxide metabolite, which could undergo rearrangement to a putative ketone intermediate (Figure 8). Although not observed in
any matrices, this intermediate could undergo a carbon-carbon cleavage via a mechanism analogous to the Baeyer-Villiger rearrangement via a per oxy intermediate, which involves insertion of an oxygen atom into the carbon–carbon bond next to a carbonyl group (aldehyde or ketone) to form an ester. Formation of this intermediate could occur through a P-450-catalyzed -Fe(III)-OOH mediated attack on the carbonyl carbon of the putative ketone metabolite to form an enzyme-bound peracid intermediate (Ortiz de Montellano and DeVoss, 2005). Some evidence suggests that mammalian flavin mono-oxygenases may also catalyze these rearrangements in the metabolism of xenobiotics (Chen et al., 1995; Lai et al., 2011). Hydrolysis of the corresponding ester would result in the carboxylic acid metabolite M5. Radical decomposition of the peroxyhemiacetal intermediate can also lead to M5. A similar Baeyer-Villiger mechanism has been proposed previously by Mayol and co-workers in the formation of the triazolodione metabolite of nefazadone and Dalvie and co-workers in the formation of a cleaved product of trovafloxacin (Mayol et al., 1994; Dalvie et al., 1996).

Another important objective of this study was to determine the rates and routes of excretion of $^{14}$C-axitinib in humans. In most subjects the majority of the radioactive dose was recovered in the first 48 h. The median overall recovery in all subjects was 59.7% (16.0% to 77.9%) with 22.7% (13.5%-28.2%) in urine and 37.0% (2.5%-60.2%) in feces. When assessing the rates and routes of excretion, the excretion of $^{14}$C-axitinib-equivalents in the feces was the major route in three subjects, whereas in other subjects fecal excretion was lower and/or the excretion pattern was unusual. The overall recovery of $^{14}$C-axitinib was low across subjects and this result did not meet expectations, initiating an investigation as to the potential causes. We confirmed that all subjects received the entire dose by counting the radioactivity before and after administration. Further, the plasma axitinib Cmax and AUC presented in Table 1 was similar to what has been observed in other studies involving healthy human subjects receiving the same 5 mg single oral dose of the drug (Pithavala et al., 2010; Pithavala et al., 2012a; Pithavala et al., 2012b). The recovery of radiolabeled axitinib-equivalents in urine was consistent
between subjects and the urinary excretion rate was similar and aligned with the plasma pharmacokinetics of total radioactivity and parent drug. These results indicated that the recovery of radioactivity from feces was variable and warranted further investigation. Several different procedures were evaluated to determine if homogenization of the fecal samples might have been incomplete and thus contributed to the recovery variability. Fecal samples from three subjects were rehomogenized and sampled in triplicate; however, the results did not differ from the original sampling (data not shown). We did note that the fecal output was low in some subjects. In particular, one subject with the lowest fecal excretion of radioactivity only had reported fecal collections on 3 out of 10 days during the study (Figure 3). Another two subjects also had an unusual profile of generated fecal samples. Total fecal output collected during the first 5 days of the study was lower in the subjects with the lowest recovery (data not shown). Finally, the radiolabel recovery in mouse and dog mass balance studies with $[^{14}\text{C}]$axitinib was $>90\%$, which suggested that the radiolabel was likely incorporated in a metabolically stable position in the molecule (manuscript in preparation), and thus radiolabel instability was an unlikely contributor to recovery variability. The exact reason(s) for incomplete total recovery is unknown, but it was determined that it was not due to dosing errors or suboptimal fecal homogenization techniques, and may have to do with irregular and incomplete/sparse bowel movements. Beumer et al. (2006) conducted an extensive review of human mass balance studies for anticancer drugs and found a range of recovery for anticancer antibiotics (28-70%), topoisomerase inhibitors (49-102%) and tubulin inhibitors (27-85%). Roffey et al. (2007) reviewed human mass balance studies from 27 internal (recovery range 61-105%) and 171 literature compounds (recovery range 43%-105%) from a broad range of therapeutic indications. The recovery of axitinib in this study, while not optimal, was within the range of that reported for these anticancer and other therapeutic drug classes.

In summary, the disposition of 5 mg $[^{14}\text{C}]$axitinib in humans was investigated. While the overall recovery of radioactivity was low, the majority of the study objectives were met. The plasma
concentration time profile of total radioactivity was similar to parent drug. Only two metabolites were present in circulation, axitinib N-glucuronide (M7) and axitinib sulfoxide (M12), both pharmacologically inactive. The axitinib human ADME results were consistent with hepatic metabolism being the main clearance mechanism with excretion of parent drug and metabolites in the feces as the main route of excretion and excretion of axitinib metabolites in the urine as the secondary route. The metabolite structures for the majority of the metabolites were elucidated with a proposed mechanism for the formation of an unusual carboxylic acid metabolite.
Acknowledgements

The authors thank Dr. David Hoelscher at PPD Development in Austin, TX for the conduct of the clinical mass-balance study, Andy Marquardt from PPD Development for radioactivity counting, Adrienne Manning from Charles River Laboratories (Worcester, MA) for the bioanalysis of axitinib in plasma and urine. Dr. Shanghui Hu from the Pfizer Pharmaceutical Sciences group contributed to the structure elucidation of axitinib N-glucuronide. Dr. Dana Hu-Lowe assessed pharmacologic activity of axitinib metabolites. Maxwell Parker and Dr. Jill Steidl-Nichols evaluated M7 and M12 for hERG interaction. Finally, Dr. Dennis Smith is acknowledged for helpful discussions on the recovery of the radiolabel.
Authorship Contributions.

Participated in research design: Bu, Klamerus, Pithavala, Pool, Wu

Conducted experiments: Bu, Deese, Kang

Contributed new reagents or analytic tools: Bu, Deese

Performed data analysis: Bu, Dalvie, Deese, Hee, Kang, Klamerus, Pithavala, Pool

Wrote or contributed to the writing of the manuscript: Bu, Dalvie, Deese, Kang, Pithavala, Pool, Smith, Wu
References


potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. Clin Cancer Res 14:7272-7283.

Inlyta® (2012) Prescribing Information: Inlyta® (axitinib) tablets for oral administration, Pfizer Labs, Division of Pfizer Inc., NY, NY, 10017.


Footnotes

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This study was sponsored by Pfizer Inc.
Legends for Figures

Figure 1. Structure of axitinib. Asterisk denotes position of [14C] incorporation.

Figure 2. Plasma concentration of [14C]axitinib total radioactivity and axitinib with time following a 5 mg oral dose to healthy subjects. Each point represents the mean and standard deviation (N=8). The inset graph shows the same results from 0-12 h.

Figure 3. Cumulative urinary, fecal excretion and total recovery of [14C]axitinib associated radioactivity in individual healthy subjects following a 5 mg oral dose. The asterisk represent collection intervals where no fecal sample was obtained.

Figure 4. A typical reverse phase HPLC radiochromatogram of plasma (0-12 h), urine (0-36 h) and fecal extracts (varied time interval) following a 5 mg oral dose of [14C]axitinib (100 µCi).

Figure 5. Proposed biotransformation scheme of [14C]axitinib in humans.

Figure 6. Comparison plot of 1D nOe difference spectrum and normal 1D 1H NMR spectrum of M7.

Figure 7. Proposed mechanism for the formation of fragment ion m/z 354 from an epoxide metabolite.

Figure 8. Proposed mechanism for formation of the carboxylic acid metabolite (M5).
### Tables

Table 1. Plasma pharmacokinetic parameters of axitinib and total radioactivity after a 5 mg oral dose of $^{14}$C]axitinib to healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>Axitinib</th>
<th>Total Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{\text{max}}$</td>
<td>$C_{\text{max}}$</td>
</tr>
<tr>
<td>Geo Mean</td>
<td>2.22</td>
<td>29.2</td>
</tr>
<tr>
<td>Upper 90% CI</td>
<td>2.72</td>
<td>43.4</td>
</tr>
<tr>
<td>Lower 90% CI</td>
<td>1.81</td>
<td>19.7</td>
</tr>
</tbody>
</table>

Geomean, geometric mean; $C_{\text{max}}$, maximum plasma concentration; $T_{\text{max}}$, time of maximum plasma concentration; AUC$_{\text{inf}}$, area under the plasma concentration versus time profile from time zero to infinity; $T_{\frac{1}{2}}$, plasma terminal elimination half life.
Table 2. Individual and mean percentages of radioactivity present in plasma, urine and feces in healthy male subjects following oral administration of 5 mg [14C]axitinib (100 µCi).

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Percentage in Radiochromatogram or Dose</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolite</td>
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<td></td>
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</tr>
<tr>
<td><strong>Plasma (0-12h)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>59.2 50.3 40.5 45.9 42.2 50.7 50.9 63.5 50.4 7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M12</td>
<td>9.3 15.9 20.5 20.2 15.9 17.7 21.5 8.7 16.2 4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Axitinib</td>
<td>18.9 22.0 30.4 23.7 32.4 18.9 17.2 16.8 22.5 6.0</td>
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<td></td>
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<tr>
<td><strong>Urine (0-36h)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>4.1 5.5 3.3 7.6 5.1 7.3 6.6 6.2 5.7 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>5.5 2.0 1.1 2.1 1.5 3.4 2.7 2.2 2.6 1.4</td>
<td></td>
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</tr>
<tr>
<td>M8a</td>
<td>2.3 1.3 0.6 1.4 0.5 1.0 1.7 1.8 1.3 0.6</td>
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</tr>
<tr>
<td>M9</td>
<td>2.1 1.8 1.1 1.8 1.4 1.7 1.9 1.8 1.7 0.3</td>
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<td></td>
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<tr>
<td>M12</td>
<td>6.3 3.8 0.9 1.9 3.2 6.6 3.3 2.2 3.5 2.0</td>
<td></td>
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<tr>
<td><strong>Feces (varied time interval)</strong></td>
<td></td>
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<tr>
<td>UNK*</td>
<td>0.7 6.2 0.2 8.6 6.0 6.8 7.8 3.3 5.0 3.2</td>
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<td>M12a</td>
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<td>M14/15</td>
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<tr>
<td>Axitinib</td>
<td>6.2 10.0 1.5 5.7 36.5 10.5 12.2 13.8 12.0 10.6</td>
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</tbody>
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* Unidentified metabolite.
Table 3. Mass spectral fragmentation and structures of axitinib and proposed metabolites.

<table>
<thead>
<tr>
<th>Structure</th>
<th>[M+H]^+</th>
<th>Fragment Ions</th>
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<tr>
<td>Axitinib</td>
<td>387</td>
<td>356 222 166</td>
</tr>
<tr>
<td>M5</td>
<td>342</td>
<td>311 296 265 166</td>
</tr>
<tr>
<td>M7</td>
<td>563</td>
<td>387 356</td>
</tr>
<tr>
<td>M8a</td>
<td>579</td>
<td>403 385 373 356</td>
</tr>
<tr>
<td>M9</td>
<td>419</td>
<td>388 324 296</td>
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<tr>
<td>M12</td>
<td>403</td>
<td>372 355 344 327</td>
</tr>
<tr>
<td>M12a</td>
<td>403</td>
<td>372 354 344</td>
</tr>
</tbody>
</table>
M14

M15

DMD Fast Forward. Published on March 7, 2014 as DOI: 10.1124/dmd.113.056531
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DMD #56531
Table 4. $^1$H NMR chemical shift (ppm) assignments for axitinib, M5 and M7 after an NMR analysis following isolation from human urine.

<table>
<thead>
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<th>Protons</th>
<th>Axitinib</th>
<th>M5</th>
<th>M7</th>
</tr>
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<tbody>
<tr>
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<td>2.8 ($d$)</td>
<td>2.8 ($d$)</td>
<td>2.8 ($d$)</td>
</tr>
<tr>
<td>2</td>
<td>8.4 ($q$)</td>
<td>8.4 ($q$)</td>
<td>8.4 ($q$)</td>
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<td>3</td>
<td>7.51 ($d$)</td>
<td>7.4 ($d$)</td>
<td>7.51 ($d$)</td>
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<tr>
<td>4</td>
<td>7.28 ($m$)</td>
<td>7.25 ($d$)</td>
<td>7.28 ($m$)</td>
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<td>7.6 ($s$)</td>
<td><strong>7.95</strong> ($s$)</td>
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<td>7.00 ($d$)</td>
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<td>nd</td>
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<td>nd</td>
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<td>7.27 ($m$)</td>
<td>nd</td>
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<tr>
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<td>nd</td>
<td>8.21 ($m$)</td>
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<td>Glucuronic Acid</td>
<td>COOH</td>
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s (Deacon et al.), d (doublet), q (quartet), m (multiplet), nd (not detected)

All NMR spectra are presented in Supplemental Data, Figures S8, S9 and S10A,B for axitinib, M5 and M7, respectively. The values in bold indicate changes in the NMR spectra.
Figure 1

Axitinib
Figure 4

Plasma

Retention Time (min)

Urine

Retention Time (min)

Feces

Retention Time (min)
Figure 6

1D nOe

Aromatic Region of
$^1$H NMR of M7
Figure 8

Axitinib → M12a → 

O
H
<table>
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\( \text{[O]} \) 

Baeyer-Villiger Rearrangement

Hydrolysis

M5

\(+\)

\( \text{H}_2\text{O} \)