Zientek MA, Goosen TC, Tseng E, Lin J, Bauman JN, Walker GS, Kang P, Jiang Y, Freiwald S, Neul D and Smith BJ. *In Vitro* Kinetic Characterization of Axitinib Metabolism. *Drug Metab Dispos.*

Supplemental Methods:

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

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

volume of 200 µL. The reactions were terminated after 60 minutes by the addition of 0.5 mL CH_3CN. Samples were immediately capped, vortexed, and centrifuged at 1900 x *g* (3600 rpm using a Beckman-Coulter GH3.8A rotor) for 25 minutes at 4°C. The supernatant was transferred into a new tube and dried under a stream of nitrogen. Extracts were reconstituted with 100-800 µL of 60:40 or 70:30 aqueous (0.1% formic acid in water):organic (0.1% formic acid in acetonitrile) mobile phase for radiodetection and LC-MS/MS analysis.

Metabolite profiling utilizing [^{14}C]axitinib was performed using HPLC coupled in-line with radiochemical detection (ARC/β-RAM) and MS detection with electrospray (ESI) source in positive mode. The instrumental components are specified as follows: HPLC Pumps/Autosampler Agilent 1100 (Agilent Technologies, Wilmington, DE); Column COSMOSIL, 5PYE (150 x 4.6 mm, Waters, Milford, MA); Guard Cartridge C18 (4.0 x 3.0 mm, Phenomenex, Torrance, CA); Quadrupole Ion Trap MS LCQ-Deca XP (ThermoFinnigan, San Jose, CA); ARC StopFlow System Model C (AIM Research Company, Newark, DE); RadioDetector (500 µL Cell) Model 3 β-RAM (IN/US Systems, Tampa, FL); Scintillation Cocktail StopFlow AD (AIM Research Company, Newark, DE). The mobile phase consisted of 20 mM ammonium acetate in water adjusted to pH 4.0 with acetic acid (Solvent A) and CH_3CN (Solvent B). The flow rate was maintained at 1.0 mL/min. The drug and metabolites were eluted using a linear gradient in mobile phase composition summarized as follows: 0% B for 10 min; linear gradient to 50% B from 10 to 60 min; linear gradient to 0% B from 60 to 61 min; 0% B from 61 to 70 min. The HPLC effluent was split so that 20% of the flow was introduced into the mass spectrometer via the ESI source while 80% was diverted to the β-RAM detector. The analog outputs from the ARC/β-RAM and MS detectors were recorded in real time by the ARC data system version 2.4 (AIM Research Company, Newark, DE). The major operating parameters for the ion-trap ESIMS method are shown below: ionization mode positive; spray voltage 5.0 kV; capillary voltage 5.0 V; tube lens offset 55 V; capillary temperature 200°C sheath gas flow rate 80 (arbitrary); auxiliary gas flow rate 20 (arbitrary). LC-MS spectra were acquired over a mass range of 200-

1400 m/z for all samples. Mass spectra were processed using either Xcalibur version 1.4 (ThermoFinnigan, San Jose, CA) or the ARC data system. Radiochromatographic data was processed using the ARC data system only. Metabolite screening was performed to manually search for molecular ions representing possible metabolites.

Oxidative biotransformation LC/MS metabolite profiling.

Metabolite profiling was performed using HPLC (Agilent 1100, Agilent Technologies, Wilmington, DE) coupled in-line with MS detection (LCQ-Deca XP, ThermoFinnigan, San Jose, CA) with ESI source in positive ion mode with reverse phase chromatography (two different HPLC columns were used: COSMOSIL 5PYE(100 mm x 2 mm, Phenomenex, Torrance, CA) for human liver microsome study, and Phenomenex C4 (150 mm x 2 mm, Phenomenex, Torrance, CA) for recombinant CYP study. For positive-mode MS detection, the mobile phase consisted of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in CH3CN (Solvent B). Parent drug and its metabolites were eluted at flow rate of 0.2 mL/min using a linear gradient in mobile phase composition as follows: 0% B for 5 min; linear gradient to 30% B from 5 to 20 min; held at 50% B from 25 to 28 min; linear gradient to 90% B from 28 to 40 min; held at 90% B from 40 to 43 min; linear gradient to 0% B from 43 to 45 min; held at 0% B from 45 to 50 min. After passing through the DAD detector, the HPLC effluent was introduced into the mass spectrometer. The analog outputs from the DAD and MS detectors were recorded in real time by the data system provided with the mass spectrometer (Xcalibur version 1.4, ThermoFinnigan, San Jose, CA). The MS detection showed a delayed (0.2 - 1.3 minutes) response, compared to the DAD detector as a result of plumbing. The major operating parameters for the ion-trap ESI-MS methods are shown below: ionization mode positive; spray voltage 4.5 kV; capillary voltage 2.6 V; tube lens offset 20 V; capillary temperature 270°C sheath gas flow rate 90 (arbitrary); auxiliary gas flow rate 30 (arbitrary).

The Xcalibur software was used to control both HPLC and MS systems to acquire and process all spectral data. LC-MS spectra were acquired over a mass range of 150-1500 m/z. Data dependent scanning was used to trigger MS2 and MS3 analysis of molecular ions and product ions over a threshold intensity of 1.0 x 106 and 1.0 x 105 counts, respectively. Only the most intense ions from MS and MS2 scans were selected for MS2 and MS3 scans, respectively. The settings used were: isolation width 2.50; normalized collision energy 40.0 eV; activation Q 0.25; activation time 30.00 ms; exclusion mass width to 0.5 Da. Searching of LC-MSn data for molecular ions representing possible metabolites was performed manually. In cases where the signal strength was of sufficient intensity to trigger MSn scanning, the subsequent MS2 and MS3 spectra were examined to further confirm the identity and structure of possible metabolites.

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

Analyte	Q1 m/z	Q3 m/z
Axitinib	387	356
Axitinib sulfoxide	403	372
Axitinib glucuronide	563	387
Buspirone	386	122

Supplemental Table 2: Intersystem extrapolation factors (ISEFs) predetermined

	CYP1A2	*CYP2C9*	*CYP2C19*	*CYP2D6*	*CYP3A4*	*CYP3A4*	*CYP2C8*	CYP2B6
Vendor:	Panvera	Panvera	Panvera	Panvera	Panvera	Gentest	Gentest	Panvera
Lot No.:	30248A	29897A	29896A	21248	29736A	59	8	19191
	Phenacetin	Diclofenac	S-Mephenytoin	Bufuralol	Testosterone	Midazolam	Amodiaquine	Bupropion
rCYPs:								
K_m	36.0	3.90	32.0	2.30	40.0	3.10	0.730	67.0
V_{max} (pmol/min/pmol)	33.0	26.0	9.70	110.0	140.0	21.58	3700	760.0
V_{max}/K_m (µL/min/pmol rCYP)	0.93	6.60	0.30	48	3.4	6.96	5100	11.0
HL-MIX-102 (350 pmol P450/mg):								
K_m (µM)	20	2.9	53	6.5	88	2.32	1.3	95
V_{max} (nmol/min/mg)	0.78	1.3	0.05	0.12	7.6	0.5	1.8	0.45
V_{max} (pmol/min/mg HLM protein)	780	1300	55	120	7600	498.9	1800	450
V_{max} (pmol/min/pmol)	15	17.0	3.90	29.0	56.0	7.81	73.0	41.0
V_{max}/K_m (µL/min/pmol CYP)	0.740	5.90	0.070	4.5	0.630	1.79	55.0	0.430
CYP abundance (pmol CYP/mg HLM protein)	52	73	14	4.20	137	137	24	11
V_{max} ISEF (unitless)	0.45	0.66	0.40	0.27	0.41	0.19	0.02	0.05
CL_{int} ISEF (unitless)	0.79	0.90	0.24	0.09	0.18	0.26	0.01	0.04

Supplemental Table 3: Pilot experiment to examine the formation of M7 and other glucuronide conjugates of [^{14}C]axitinib (100 µM) by human liver microsomes and rUGTs in the presence of UDPGA

Enzyme	Axitinib	Glu1	Glu2 (M7)	Glu3
HLM	87.5	1.8	0.8	2.4
UGT1A1	90.7	ND	1.2	0.1
UGT1A4	82.9	3.3	0.2	5.4

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

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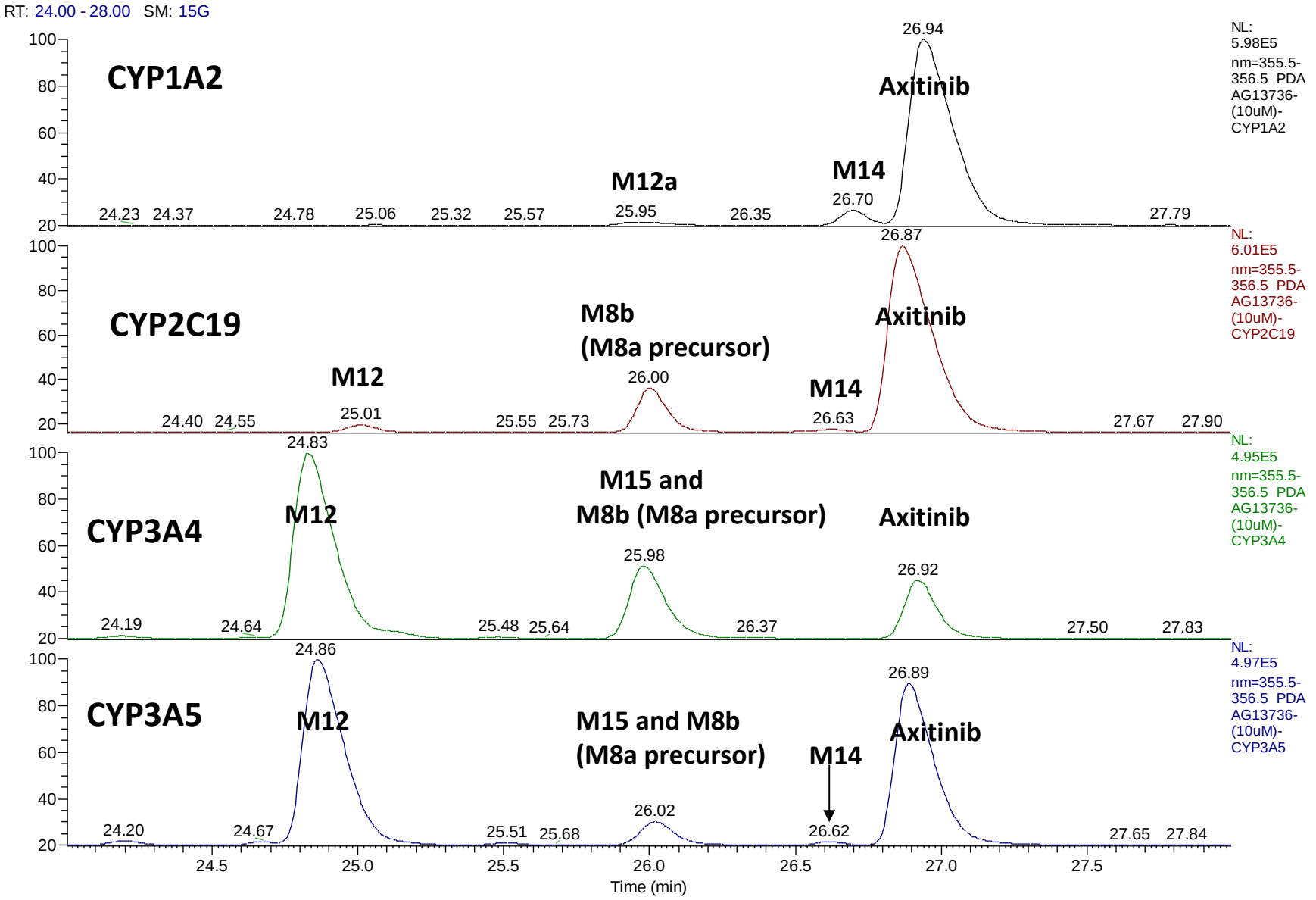
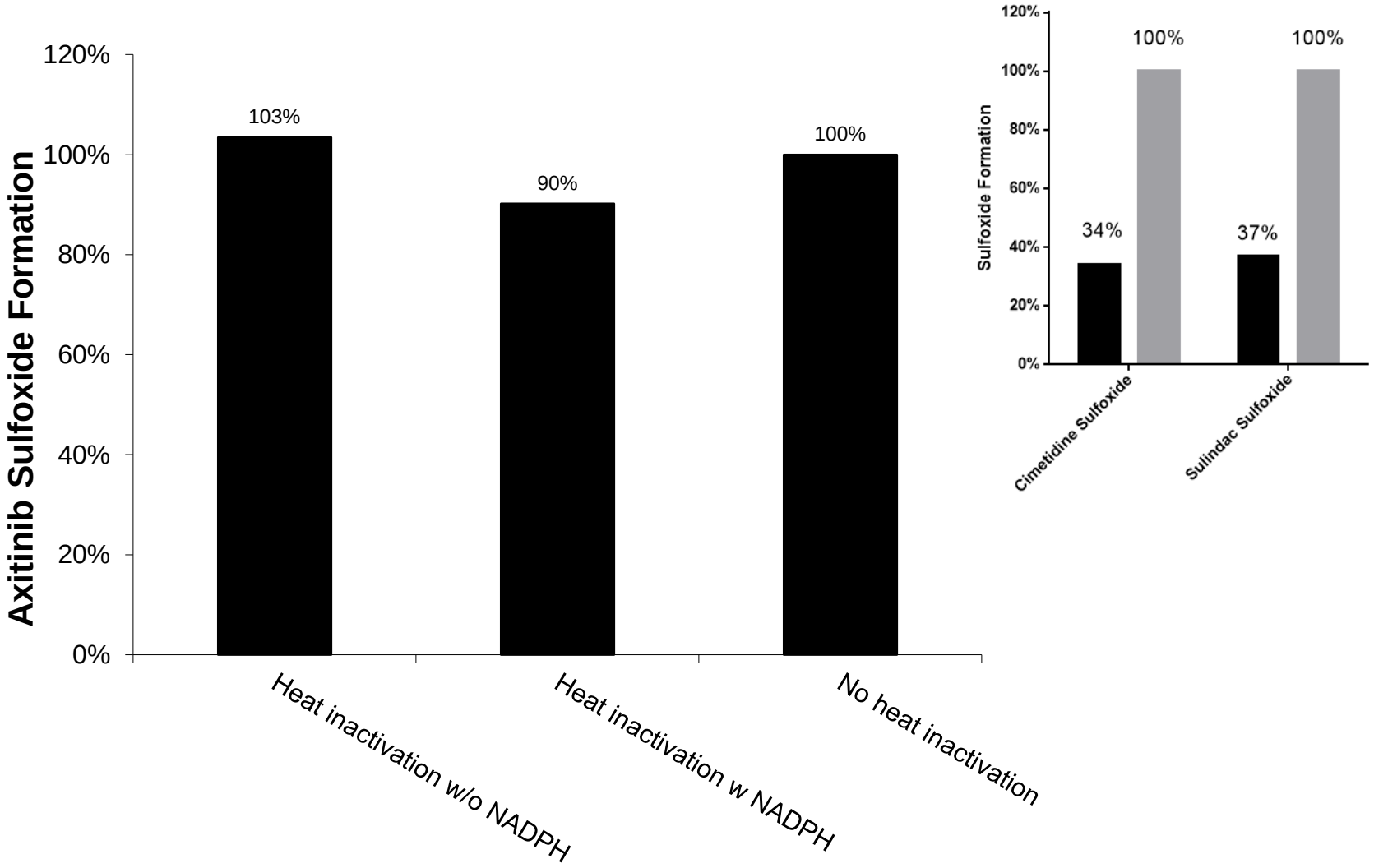


Supplemental Figure 1: Metabolite profiles of axitinib in recombinant CYP3A4, CYP3A5, CYP1A2, and CYP2C19 (UV Chromatograms at 356 nm)



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

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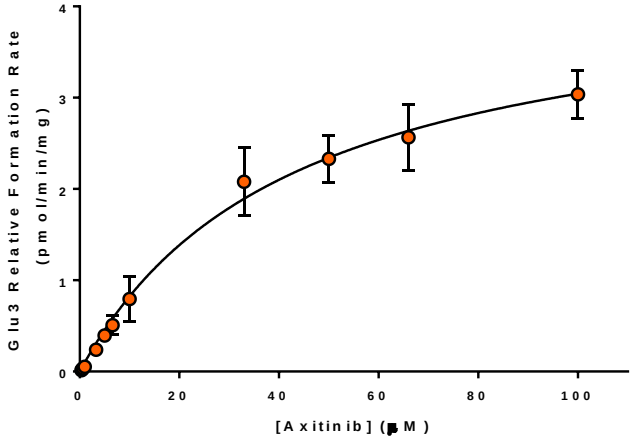

Supplemental Figure 3: Enzyme kinetics of axitinib glucuronidation (Glu3) by pooled HLMs in Tris-HCl buffer (pH = 7.4) displaying typical Michaelis-Menten kinetics.