The Pharmacokinetics, Metabolism, and Clearance Mechanisms of Abrocitinib, a Selective Janus Kinase Inhibitor, in Humans

Jonathan N. Bauman, Angela C. Doran, Amanda King-Ahmad, Raman Sharma, Gregory S. Walker, Jian Lin, Tsung H. Lin, Jean-Baptiste Telliez, Sakambari Tripathy, Theunis C. Goosen, Christopher Banfield, Bimal K. Malhotra, and Martin E. Dowty

Drug Metabolism and Disposition

DMD-AR-2022-000829

Supplemental Methods

Cytokine-induced STAT Phosphorylation Assays. A total of 14 cytokines was used to induce signal transducer and activator of transcription (STAT) phosphorylation in human whole blood, human keratinocytes, or human acute monocytic leukemia (THP-1) cells. The method of cytokine-induced STAT phosphorylation in human whole blood was previously described in detail by Dowty et al (2019). Human primary keratinocytes were used in the IL-4, IL-12, and IL-22-induced STAT phosphorylation assays. Human primary keratinocytes were cultured in DermaLife medium with supplement kit (Lifeline Cell Technology) to expand cell populations. Cell passages 2 to 5 were used in this study. Keratinocytes were suspended in warm DermaLife medium and aliquoted in 96-well, deep-well, V-bottom plates. Cells were treated with compounds (0.0003 to 20 μM) for 60 minutes and followed by stimulation with IL-4 (2 ng/mL) or IL-13 (20 ng/mL) for 15 minutes. For the IL-22 assay, keratinocytes were seeded in 24-well plates and cultured overnight. Cells were switched to DermaLife medium, treated with compounds (0.0003 to 20 μM) for 60 minutes, and then stimulated with IL-22 for 30 minutes. Cells were detached by the treatment of 0.25% trypsin/EDTA. Cytokine stimulated cells were fixed by 2% paraformaldehyde and permeabilized by 90% methanol. Fixed and permeabilized cells were stained with AlexaFluor647 labeled anti-pSTAT6 antibody for IL-4- and IL-13-treated cells, and AlexaFluor647 labeled anti-pSTAT3 antibody for IL-22-treated cells. THP-1 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 50 mM 2-mercaptoethanol, 50 U/mL penicillin, 50 mg/mL streptomycin, and 2 mM L-glutamine. THP-1 cells were treated with interferon (IFN)γ (20 ng/mL) for 18 hours. IFNγ-primed THP-1 cells were resuspended in fresh RPMI 1640 medium, treated with compounds (0.0003-20 μM) for 60 minutes, followed by stimulation with IL-31(1 mg/mL) for additional 10 minutes. Cells were

then fixed by 2% paraformaldehyde and permeabilized by 90% methanol. Fixed and permeabilized cells were stained with AlexaFluor647 labeled anti-pSTAT3 antibody. Fluorescence was analyzed with a LSRFortessa equipped with a plate-based autosampler. Data was analyzed using FACSDiva version 8.0.

Table S1. LC-MS/MS Conditions for Metabolite Profiling and ^{14}C-Abrocitinib Quantitation

Parameter	**Description**			
Mass spectrometer	Q Exactive™ Hybrid Quadrupole-Orbitrap (Thermo Fisher Scientific, Waltham, MA)			
UHPLC	Acquity UHPLC system (Waters, Milford, MA)			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	Waters Acquity UHPLC-HSS C18 2.1x100 1.8 μm, (Waters Corporation) maintained at 40°C.			
Mobile phase	A: 2 mM ammonium acetate:acetonitrile:methanol (95:2.5:2.5 % v/v)			
Mobile phase	B: 2 mM ammonium acetate:acetonitrile:methanol (10:45:45 % v/v)			
Gradient	Time (min)	Flow Rate (μL/min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.0	400	95	5
	1.25	400	95	5
	5.0	400	80	20
	7.5	400	80	20
	8.75	400	65	35
	12.5	400	55	45
	13.75	400	0	100
	15.65	400	0	100
	16.0	400	95	5
	28.50	400	95	5
Software	Xcalibur v2.2 sp1.48 (Thermo Fisher Scientific)			

Table S2. LC-MS/MS Conditions for Isolation of Metabolites M6 and M7 - Initial Assessment

Parameter	**Description**			
Mass spectrometer	LTQ Orbitrap XL™ Hybrid Ion Trap (Thermo Fisher Scientific)			
HPLC	Surveyor HPLC System (Thermo Fisher Scientific)			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	Luna C18 150 × 4.6 mm column (Agilent, Santa Clara, CA)			
Mobile phase	C 10 mM ammonium acetate			
Mobile phase	D methanol:acetonitrile (50:50 % v/v)			
Gradient	Time (min)	Flow Rate (μL/min)	Mobile Phase C (%)	Mobile Phase D (%)
	0.0	500	95	5
	5.0	500	95	5
	20	500	5	95
	25	500	5	95
	26	500	95	5
	30	500	95	5
Software	Xcalibur v2.2 sp1.48			

Table S3. LC-MS/MS Conditions for Isolation of Metabolites M6 and M7 - Initial Fractionation

Parameter	**Description**			
Mass spectrometer	LTQ Orbitrap XL™ Hybrid Ion Trap Mass Spectrometer			
HPLC	Surveyor HPLC System			
Fraction Collector	Gilson FC 203B			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	YMC Ph-Pack 250 × 4.6 mm column (YMC America, Devens, MA)			
Mobile phase	A 0.1% formic acid in water			
Mobile phase	D methanol:acetonitrile (50:50 % v/v)			
Gradient	Time (min)	Flow Rate (μL/min)	Mobile Phase A (%)	Mobile Phase D (%)
	0.0	1000	95	5
	5	1000	95	5
	45	1000	60	40
	50	1000	5	95
	54	1000	5	95
	55	1000	95	5
	60	1000	95	5
Software	Xcalibur v2.2 sp1.48			

Table S4. LC-MS/MS Conditions for Isolation of Metabolites M6 and M7 - Analysis of Fractions

Parameter	**Description**			
Mass spectrometer	LTQ Orbitrap XL™ Hybrid Ion Trap (Thermo Fisher Scientific)			
HPLC	Surveyor HPLC System (Thermo Fisher Scientific)			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	Zorbax SB-Phenyl 150 × 4.6 mm column (Agilent)			
Mobile phase	A 0.1% formic acid in water			
Mobile phase	D methanol:acetonitrile (50:50 % v/v)			
Gradient	Time (min)	Flow Rate (μL/min)	Mobile Phase A (%)	Mobile Phase D (%)
	0.0	500	95	5
	1	500	95	5
	12	500	5	95
	15	500	5	95
	16	500	95	5
	20	500	95	5
Software	Xcalibur v2.2 sp1.48			

Table S5. LC-MS/MS Conditions for Isolation of Metabolites M6 and M7 – Final Fractionation

Parameter	**Description**			
Mass spectrometer	LTQ Orbitrap XL™ Hybrid Ion Trap Mass Spectrometer			
HPLC	Surveyor HPLC System			
Fraction Collector	Gilson FC 203B			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	Zorbax SB-Phenyl 150 × 4.6 mm column (Agilent)			
Mobile phase	C 10 mM ammonium acetate			
Mobile phase	D methanol:acetonitrile (50:50 % v/v)			
Gradient	Time (min)	Flow Rate (μL/min)	Mobile Phase C (%)	Mobile Phase D (%)
	0.0	1000	98	2
	5	1000	98	2
	45	1000	80	20
	50	1000	5	95
	54	1000	5	95
	55	1000	98	2
	60	1000	98	2
Software	Xcalibur v2.2 sp1.48			

Table S6. LC-MS/MS Conditions for Abrocitinib in Plasma

Parameter	**Description**			
Mass spectrometer	SCIEX Triple Quad™ API-5500 (SCIEX, Framingham, MA)			
HPLC	Acquity UHPLC system			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	Aquasil C18 2.1 × 50 mm, 3 μm (Thermo Fisher Scientific)			
Mobile phase	A 0.1% formic acid and 10 mM ammonium acetate in acetonitrile:water (10:90 % v/v)			
Mobile phase	B 0.1% formic acid and 10 mM ammonium acetate in acetonitrile:water (90:10 % v/v)			
Gradient	Time (min)	Flow Rate (μL/min)	Mobile Phase A (%)	Mobile Phase D (%)
	0.0	1000	85	15
	0.99	1000	85	15
	1.0	1000	0	100
	2.0	1000	0	100
	2.01	1000	85	15
	3.0	1000	85	15
Software	Analyst TM version 1.4.2 (SCIEX)			
	Watson LIMS 7.2.0.02 (Thermo Fisher Scientific)			

MRM transitions

Analyte	Q1	Q3
Abrocitinib (PF-04965842)	324.4	134.2
d2-Abrocitinib (PF-06651703	327.2	178.2

Table S7. LC-MS/MS Conditions for In Vitro Hepatocyte Fractional Metabolism

Parameter	**Description**			
Mass spectrometer	SCIEX TripleTOF® 6600 quadrupole time-of-flight (QTOF) mass analyzer (SCIEX)			
HPLC	Agilent 1290 HPLC System (Agilent)			
Fraction Collector	PAS HTS-xt fraction collector (Leap Technologies, Morrisville, NC)			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	Acquity UHPLC-HSS C18 (2.1 × 100 mm, 1.7 μm) maintained at 40°C			
Mobile phase A	A: 2 mM ammonium acetate:acetonitrile:methanol (95:2.5:2.5 % v/v)			
Mobile phase B	B: 2 mM ammonium acetate:acetonitrile:methanol (10:45:45 % v/v)			
Gradient	Time (min)	Flow Rate (μL/min)	Mobile Phase A (%)	Mobile Phase D (%)
	0.0	400	95	5
	1.25	500	95	5
	5.0	400	80	20
	7.5	500	80	20
	8.75	400	65	35
	12.5	400	55	45
	13.75	400	0	100
	15.65	400	0	100
	16.0	400	95	5
	28.0	400	95	5

Parameter	Description
Software	Analyst TF 1.7.1 (SCIEX)

Table S8. LC-MS/MS conditions for in vitro CYP450 assignment (Enzyme kinetics and chemical inhibition)

Parameter	**Description**			
Mass spectrometer	SCIEX Triple Quad™ API-5500			
HPLC pump	Shimadzu LC-30AD (Shimadzu, Columbia, MD)			
Autosampler	Leap CTC HTS PAL (Leap Technologies)			
Detection mode	Electrospray ionization (ESI), Positive Mode			
Column	ACQUITY UHPLC BEH C18 column, 130Å, 1.7 µm, 2.1 mm × 50 mm (Waters)			
Injection volume	3 µL			
Mobile phase	A: 0.1% formic acid in water			
Mobile phase	B: 0.1% formic acid in acetonitrile			
Flow rate (mL/min)	0.5			
Gradient	Time (min)	Flow Rate (µL/min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.01	400	95	5
	0.16	500	95	5
	0.63	400	80	20
	0.94	500	80	20
	1.09	400	65	35
	1.56	400	55	45
	1.72	400	0	100
	3	400	0	100

Parameter	Description			
	3.01	400	95	5
	4	400	95	5
MRM transitions	Analyte	Q1	Q3	Polarity
	Abrocitinib (PF-04965842)	324.2	149.1	Positive
	M1 (PF-06471658)	340.1	149.1	Positive
	M2/M3 (PF-07055087/PF-07055090)	340.1	149.1	Positive
	M4 (PF-07058474)	340.1	217.1	Positive
	Diclofenac (IS)	296.0	215.0	Positive
	372-1	372.2	328.1	Positive
	358-1	358.3	340.0	Positive
	356-1	356.3	181.0	Positive
	M7 (PF-067377821)	354.3	149.0	Positive
Analyte concentration range	1.0-2000 nM			
Software	Analyst 1.6			

Q1, first quadrupole; Q3, third quadrupole.

Table S9. LC-MS/MS conditions for abrocitinib in plasma protein binding and blood-to-plasma ratio studies

Mass Spectrometer and Source Type	AB Sciex API 4000 Triple Quadrupole Electrospray		
HPLC pumps	Agilent 1290		
Autosampler	CTC HTS PAL		
Injection volume	25 µL		
Loading column	Halo Fused-core 2.7µ C18 100Å (5 x 4.6 mm ID)		
Loading solvent	90% Water, 10% Methanol (containing 0.1% Trifluoroacetic Acid)		
Mobile phase A	5 mM Ammonium Acetate (containing 0.075% Formic Acid)		
Mobile phase B	Acetonitrile		
Flowrate (mL/min)	1.0		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	90	10
	0.40	90	10
	1.20	10	90
	2.20	10	90
	2.25	90	10
	2.80	90	10
Column	Kinetex XB 2.6µ C18 100Å (50 x 3 mm ID)		
Detection mode	Positive ion MRM		
Data collection software/version	Analyst 1.6.2		
Data analysis software/version	Analyst 1.6.2		
MRM transitions	Compound	Q1	Q3
	Abrocitinib	324.0	149.1
	Chloroquine (PF-00345351)	320.1	247.0
	Internal standard (Tolbutamide)	271.2	172.0

HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring.

Table S10. LC-MS/MS conditions for M1 in plasma protein binding and blood-to-plasma ratio studies

Mass Spectrometer and Source Type	AB Sciex API 4000 Triple Quadrupole Electrospray		
HPLC pumps	Shimadzu LC-20AB Binary Pumps		
Autosampler	CTC HTS PAL		
Injection volume	50 µL		
Loading column	Halo Fused-core 2.7µ C18 100Å (5 x 4.6 mm ID)		
Loading solvent	90% Water, 10% Methanol (containing 0.1% Trifluoroacetic Acid)		
Mobile phase A	5 mM Ammonium Acetate (containing 0.075% Formic Acid)		
Mobile phase B	Acetonitrile		
Flowrate (mL/min)	1.0		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	90	10
	0.30	90	10
	1.00	10	90
	2.00	10	90
	2.05	90	10
	3.00	90	10
Column	Kinetex XB 2.6µ C18 100Å (50 x 3 mm ID)		
Detection mode	Positive ion MRM		
Data collection software/version	Analyst 1.6.2		
Data analysis software/version	Analyst 1.6.2		
MRM transitions	Compound	Q1	Q3
	M1	340.2	149.1
	Chloroquine (PF-00345351)	320.2	247.0
	Internal standard (Tolbutamide)	271.2	172.0

HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring.

Table S11. LC-MS/MS conditions for M2 in plasma protein binding and blood-to-plasma ratio studies

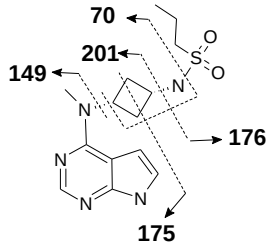
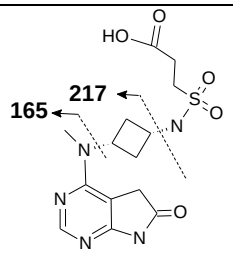
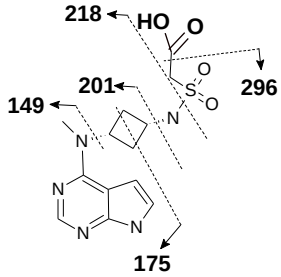
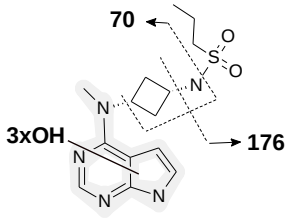
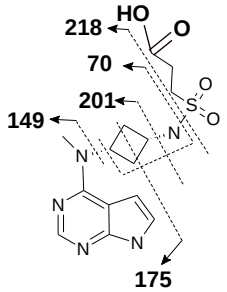
Mass Spectrometer and Source Type	AB Sciex API 4000 Triple Quadrupole Electrospray		
HPLC pumps	Shimadzu LC-20AB Binary Pumps		
Autosampler	CTC HTS PAL		
Injection volume	50 µL		
Loading column	Halo Fused-core 2.7µ C18 100Å (5 x 4.6 mm ID)		
Loading solvent	90% Water, 10% Methanol (containing 0.1% Trifluoroacetic Acid)		
Mobile phase A	5 mM Ammonium Acetate (containing 0.075% Formic Acid)		
Mobile phase B	Acetonitrile		
Flowrate (mL/min)	1.0		
Gradient	Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
	0.00	90	10
	0.30	90	10
	1.00	10	90
	2.00	10	90
	2.05	90	10
	3.00	90	10
Column	Kinetex XB 2.6µ C18 100Å (50 x 3 mm ID)		
Detection mode	Positive ion MRM		
Data collection software/version	Analyst 1.6.2		
Data analysis software/version	Analyst 1.6.2		
MRM transitions	Compound	Q1	Q3
	M2	340.1	149.1
	Chloroquine (PF-00345351)	320.2	247.0
	Internal standard (Tolbutamide)	271.2	172.0

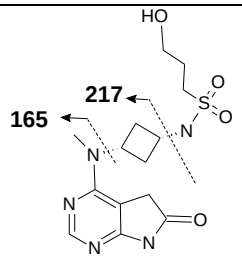
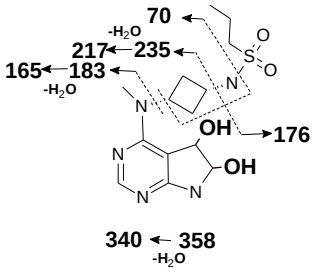
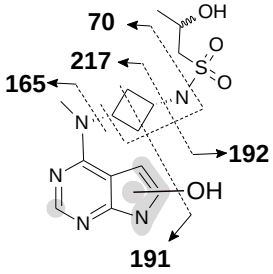
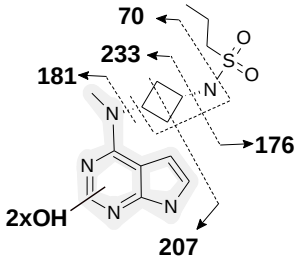
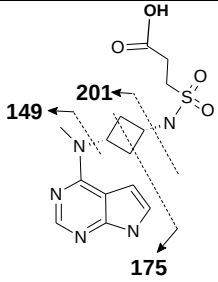
HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring.

Table S12. Incidence of treatment-emergent mild adverse events

Number of Subjects with AE by MedRA Preferred Term	**Period A (N=6)**	**Period B (N=5)**
Dizziness	3	2
Headache	1	0
Nausea	2	2
Dyspepsia	1	0
Oral paresthesia	1	0

Table S13. Diagnostic MS product ions used for abrocitinib and proposed metabolite structures

ID	Structure and Product Ions	Ion m/z	Diagnostic Fragment Ions
Abrocitinib		324.1490	201, 176, 175, 149, 70
M6		370.1179	217, 165
340-5		340.1074	296, 218, 201, 175, 149
372-1		372.1334	176, 70
M7		354.1230	218, 201, 175, 149, 70

M8		356.1387	217, 165
358-1		358.1543	235, 217, 183, 176, 165, 70
356-1a		356.1386	217, 192, 191, 165
356-2		356.1384	233, 207, 181, 176, 70
M1		340.1438	201, 175, 149

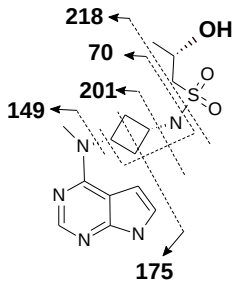
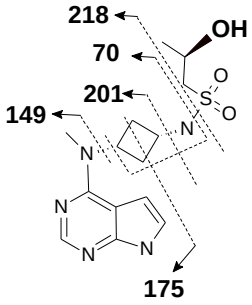
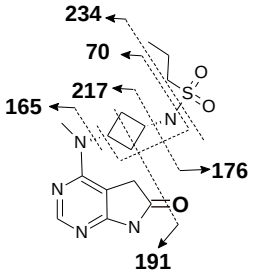
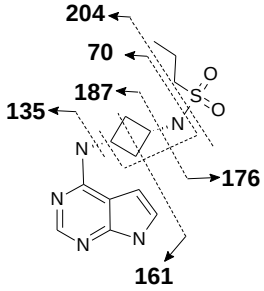
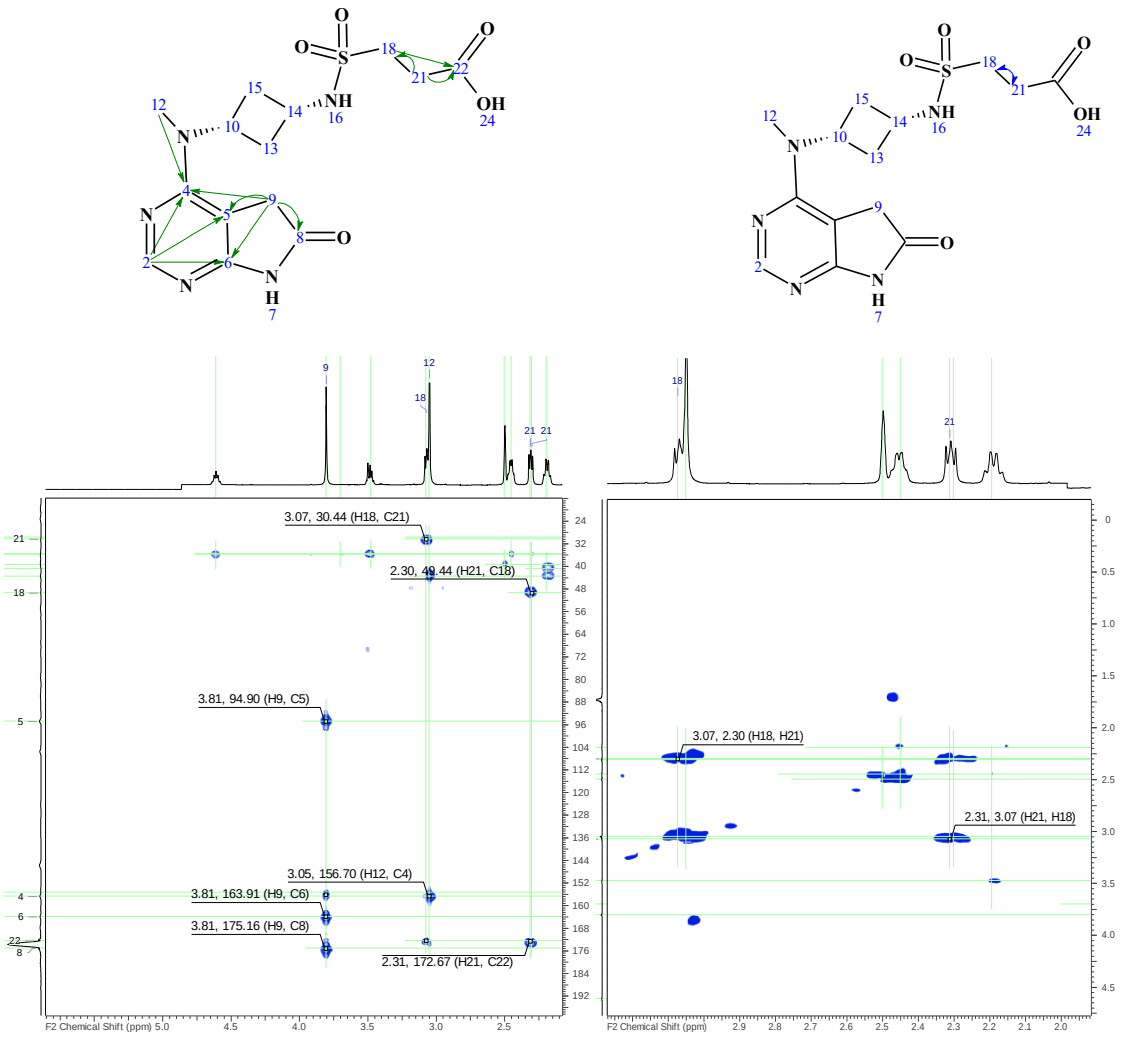
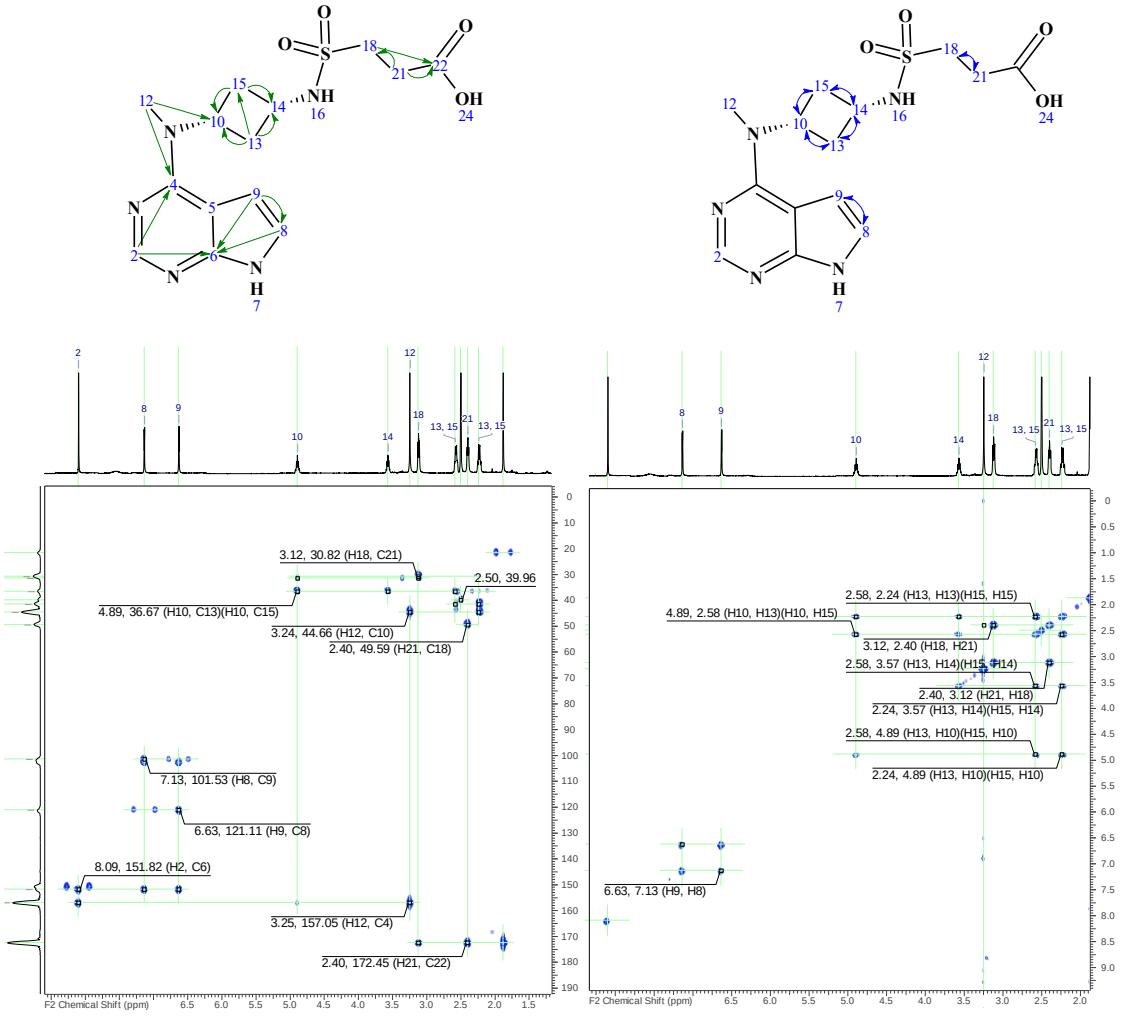
M2		340.1438	218, 201, 175, 149, 70
M3		340.1438	218, 201, 175, 149, 70
M4		340.1438	234, 217, 191, 176, 165, 70
M5		310.1332	204, 187, 176, 161, 135, 70

Figure S1. ^{1}H-^{13}C HMBC spectrum (left) and ^{1}H-^{1}H COSY spectrum (right) of M6 (PF-07095462, 370, m/z 370) metabolite isolated from pooled human urine



COSY, homonuclear correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy.

Figure S2. ^{1}H-^{13}C HMBC spectrum (left) and ^{1}H-^{1}H COSY spectrum (right) of M7 (PF-06737821, 354-1, m/z 354) metabolite isolated from pooled human urine



COSY, homonuclear correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy.

Figure S3. Radiochromatogram following 30-minute incubation of 1 µM ^{14}C-abroctinib (PF-04965842) in human hepatocytes (0.75 million cells/mL)

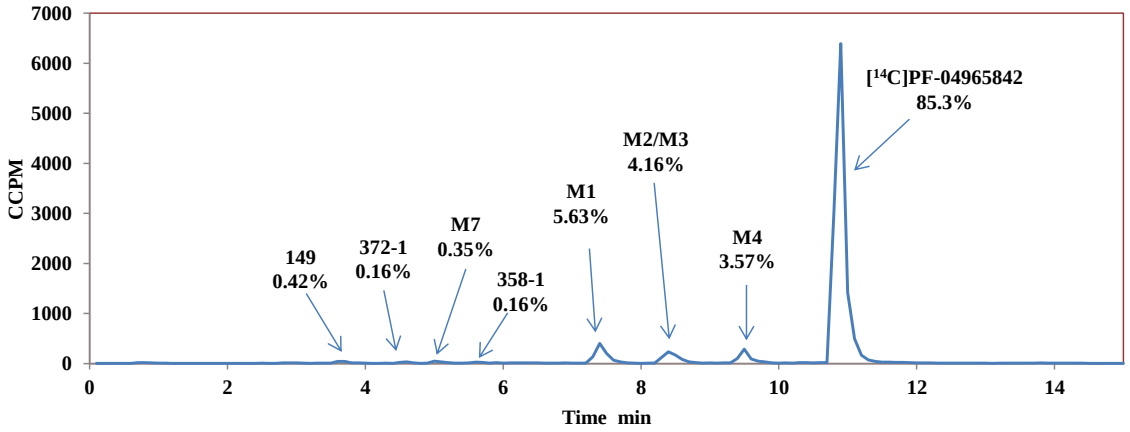
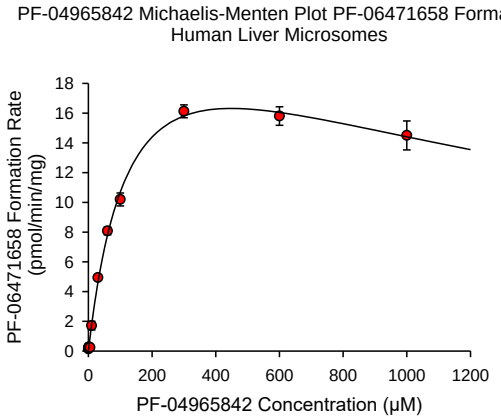
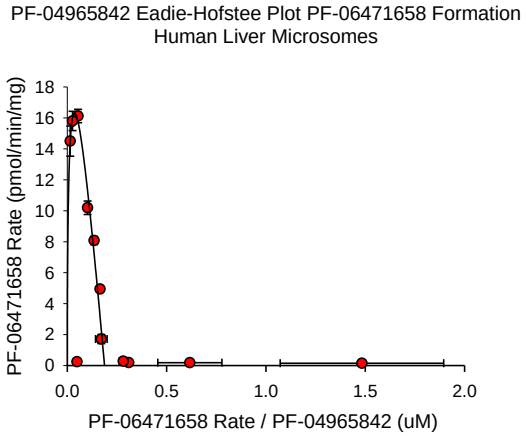


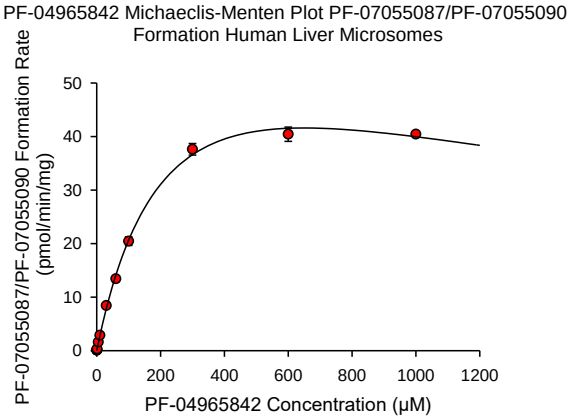
Figure S4. Enzyme kinetic plots for primary metabolites M1 (A), M2/M3 (B), M4 (C), and 149 (D) and secondary metabolites M7 (E), 358-1 (F), and 372-1 (G) from abrocitinib metabolism

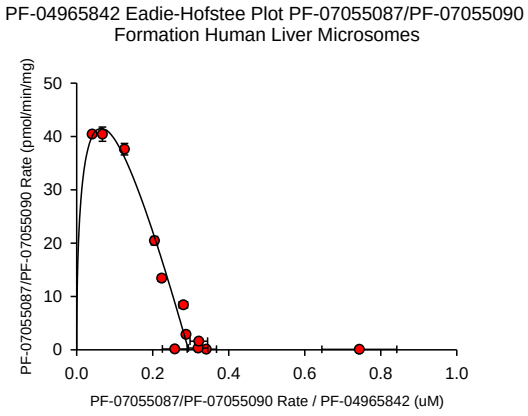
A. Enzyme kinetics of primary metabolite M1 (PF-06471658) formation following incubation of abrocitinib (PF-04965842) with human liver microsomes



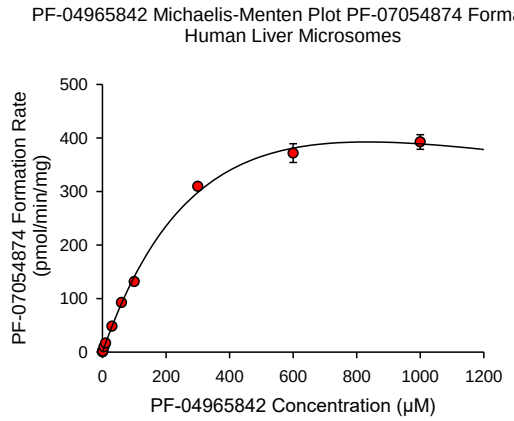


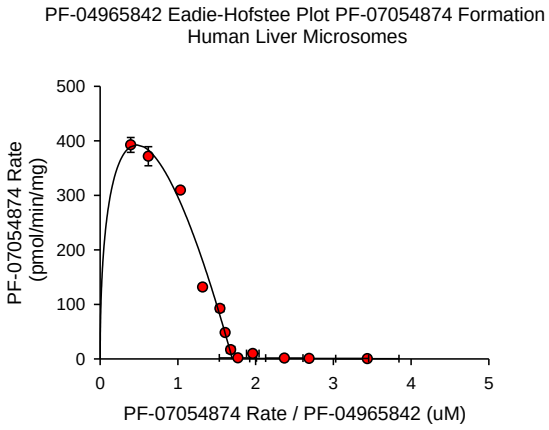
B. Enzyme kinetics of primary metabolite M2/M3 (PF-07055087/PF-07055090) formation following incubation of abrocitinib (PF-04965842) with human liver microsomes



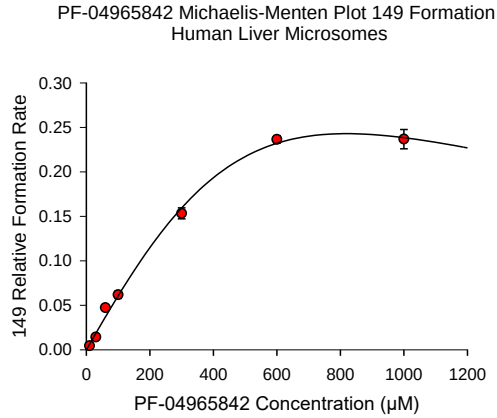


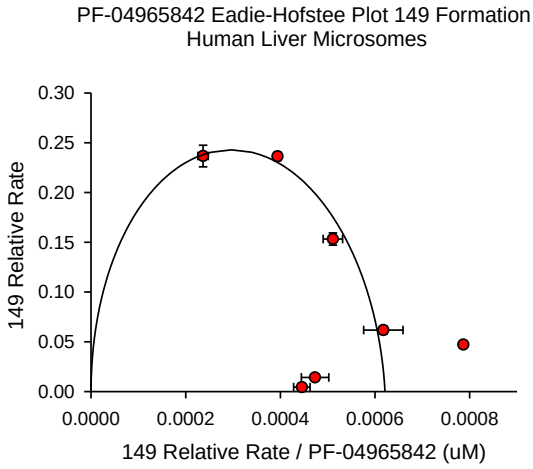
C. Enzyme kinetics of primary metabolite M4 (PF-07054874) formation following incubation of PF-04965842 with human liver microsomes



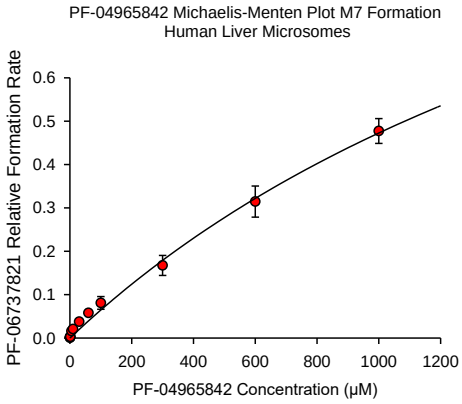


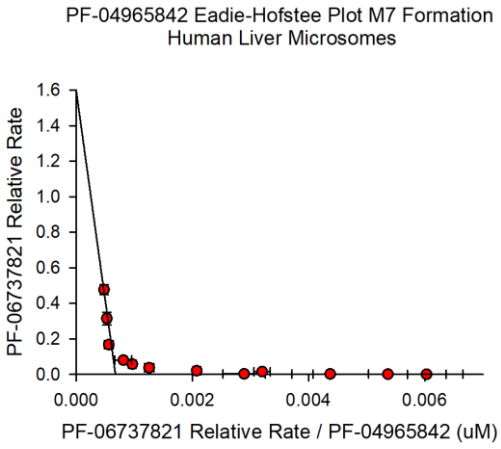
D. Enzyme kinetics of primary metabolite m/z 149 formation following incubation of abrocitinib (PF-04965842) with human liver microsomes



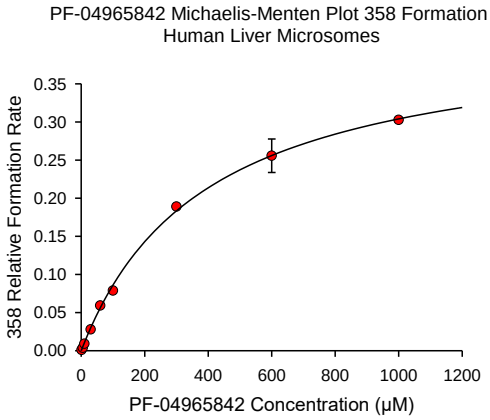


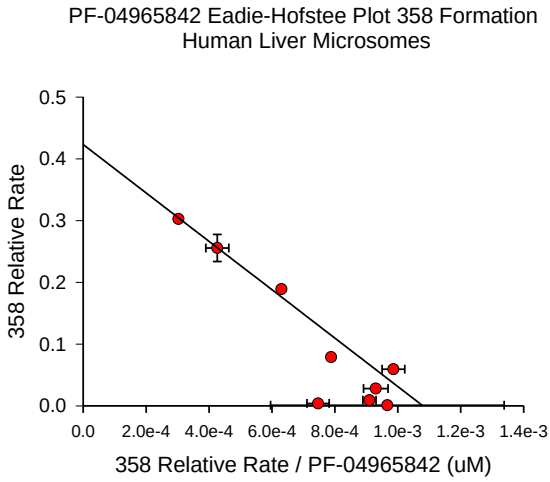
E. Enzyme kinetics of secondary metabolite M7 (PF-06737821) formation following incubation of abrocitinib (PF-04965842) with human liver microsomes





F. Enzyme kinetics of secondary metabolite 358-1 formation following incubation of abrocitinib (PF-04965842) with human liver microsomes





G. Enzyme kinetics of secondary metabolite 372-1 formation following incubation of abrocitinib (PF-04965842) with human liver microsomes