Supporting Information

Design and Synthesis of Novel Deuterated Ligands Functionally Selective for the γ-Aminobutyric Acid Type A Receptor ($GABA_AR$) α6 Subtype with Improved Metabolic Stability and Enhanced Bioavailability

Daniel E. Knutson[a], Revathi Kodali[a], Branka Divović[b], Marco Treven[e], Michael R. Stephen[a], Nicolas M. Zahn[a], Vladimir Dobričić[c], Alec T. Huber[a], Matheus A. Meirelles[a], Ranjit S. Verma[a], Laurin Wimmer[d], Christopher Witzigmann[a], Leggy A. Arnold[a], Lih-Chu Chiou[f,g,h], Margot Ernst[e], Marko D. Mihovilovic[d], Miroslav M. Savić[b], Werner Sieghart[e], and James M. Cook[a]*

[a]Department of Chemistry and Biochemistry, Milwaukee Institute for Drug Discovery, University of Wisconsin-Milwaukee, 3210 N. Cramer St., Milwaukee, Wisconsin 53211, USA.
[b]Department of Pharmacology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia.
[c]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia.
[d]TU Wien—Institute of Applied Synthetic Chemistry, Getreidemarkt 9/163, A-1060 Vienna, Austria.
[e]Department of Molecular Neurosciences, Center for Brain Research, Medical University of Vienna, Spitalgasse 4, A-1090 Vienna, Austria.
[f]Department of Pharmacology, [g]Graduate Institute of Brain and Mind Sciences, College of Medicine, National Taiwan University, Taipei 10051, Taiwan.
[h]Graduate Institute of Acupuncture Science, China Medical University, Taichung 40402, Taiwan

Table of Contents

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Serotonin 5-HT$_{1A}$	Serotonin 5-HT$_{1B}$	Serotonin 5-HT$_{1D}$	Serotonin 5-HT$_{1E}$	Serotonin 5-HT$_{2A}$	Serotonin 5-HT$_{2B}$
Radioligand	[^{3}H]8-OH-DPAT	[^{3}H]GR127543	[^{3}H]GR127543	[^{3}H]5-HT	[^{3}H]Ketanserin	[^{3}H]LSD
8a[p]	9.7	12	27.5	1.3	12.8	28.6
8b[b,p]	-5.4	21.6	11.3	0	-2.8	46.3
8c[b,p]	11.1	22.8	28	-16.5	0.4	37.9
8d[b,p]	0.7	25.5	23.5	-18.9	1.2	47
8e[b,m]	-13.7	1.8	2.4	-4.2	-5.2	22.6
8f[b,o]	-6	-4.2	-10.2	-7.8	-13.7	21
8g[b,m]	-2.1	-8.2	-8.4	7.2	2.7	15.9
8h[b,o]	0.3	-8.6	-9.2	-0.8	-11.8	20.1
13a[b,e]	-2.6	-5	11.2	-1.5	3.5	25.6
13b[b,e]	-5.7	4.1	-1.4	1.8	-6.8	8
13c[b,e]	-0.7	-4.1	0.3	8.6	0.2	48.7
13i[b,e]	1.5	-4	-3.6	-5.1	5.9	27.3
8i[m]	-1.3	-14.8	5.6	1	12.2	8.3
8j[c,m]	-15.8	-8.5	-6.5	-5.4	-2.5	37.7
8l[c,p]	7.4	8	13.7	-11.3	-1.4	49.9
8m[c,o]	-8.2	-12.8	-10.2	9.1	-1.5	9.9
13e[c,e]	8.9	2.4	4.9	-1.3	9.4	37.1
13f[c,e]	-1.3	2.6	-8.3	-24.2	-11.1	3.9
8n[p]	-16.1	1.4	4.5	-1	5.7	15.3
8o[d,p]	1.8	9.7	6.5	-9.7	-2.6	**72.2**
8p[d,m]	-6.3	-9.5	-9.1	-3.5	-8.8	23.6
8q[d,o]	32.6	6.9	-11.7	-9.9	-15.5	45.5
13g[d,e]	2.7	-5.3	0.9	-25.2	-5.5	13.4
13h[d,e]	-10.7	-2.8	-5.9	-29.3	-6.4	23
8r[p]	33.8	13.1	5.9	1.4	-1.9	28.8
8s[m]	6.6	-0.1	3.1	-3.3	1.8	21
8t[o]	3.2	-8.6	-5.1	5.6	0.8	9

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Serotonin 5-HT$_{2C}$	Serotonin 5-HT$_3$	Serotonin 5-HT$_{5a}$	Serotonin 5-HT$_6$	Serotonin 5-HT$_7$	Adrenergic α_{1A}
Radioligand	[^{3}H]Mesulergine	[^{3}H]LY278584	[^{3}H]LSD	[^{3}H]LSD	[^{3}H]LSD	[^{3}H]Prazosin
8a[p]	36.2	33.4	5.8	24.5	**78.6**	-14.2
8b[b,p]	7.8	14.6	3.9	2.4	**60.2**	-3.6
8c[b,p]	37.5	35.4	31.2	23.3	**69**	-1.9
8d[b,p]	7.1	21.3	20.2	1.4	**74.4**	-4.4
8e[b,m]	-3.6	14.4	-14.9	1.5	34.7	-20.1
8f[b,o]	-3.3	1.9	-4.1	-13.3	20.8	19.7
8g[b,m]	-14.6	8.1	-11.6	-9.3	-4.8	-17.9
8h[b,o]	-5.8	-8.4	-19.5	0.6	13.2	0
13a[b,e]	5.2	1.8	6.2	11.5	22.4	-18.9
13b[b,e]	-1.4	-6.3	-16.5	-3.8	15.6	-13.6
13c[b,e]	5	-7.4	-12.1	13	26.5	-7.9
13i[b,e]	5.6	10.4	-10.5	9.6	32.7	-9.2
8i[m]	10.3	24.2	-2.8	13.5	41.7	-9.6
8j[c,m]	-3.7	3.1	-9.1	1.9	20.7	-16.8
8l[c,p]	10.1	22.5	21.1	1.1	12.8	-6.8
8m[c,o]	-1.9	-6.4	-9.7	-7.1	0.4	-6.9
13e[c,e]	5.8	6.2	8.7	15.2	6.5	-6.9
13f[c,e]	-7.5	15.9	-7	-4.7	6.3	5.7
8n[p]	4.9	20.9	-0.4	10.4	**61.5**	-9.7
8o[d,p]	13.9	9.7	13.5	0.2	**54.6**	-6.9
8p[d,m]	-0.8	1.8	-10.2	-8.7	12.6	5.7
8q[d,o]	**73.8**	43.8	3	-16.4	15.9	2.5
13g[d,e]	**67.9**	-14.9	-14	-6.9	-13.7	-12.1
13h[d,e]	7.9	18.9	-1.5	-5.3	30.6	2.7
8r[p]	**76.4**	45.8	18.1	13.4	20.1	-20.1
8s[m]	0	-4.8	11.6	3.9	-4.3	-14.2
8t[o]	-0.3	5.7	11.2	6.9	1.1	-25.1

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Adrenergic α_{2B}	Adrenergic α_{1D}	Adrenergic α_{2A}	Adrenergic α_{2B}	Adrenergic α_{2C}	Adrenergic β_1
Radioligand	**[^{3}H]Prazosin**	**[^{3}H]Clonidine**	**[^{3}H]Clonidine**	**[^{3}H]Clonidine**	**[^{3}H]Clonidine**	**[^{125}I]Iodopindolol**
8a[p]	11.9	-3.2	-8.7	6.4	2.6	-10.4
8b[b,p]	30.9	8.8	9.6	6.4	19.8	-7.8
8c[b,p]	20.8	16	18.7	18.9	26.4	-2.7
8d[b,p]	24.2	14.3	31.1	9.2	26.7	21.5
8e[b,m]	17.8	0.2	-4.8	6.8	0.5	-9.1
8f[b,o]	36.9	-3.3	16.3	3.3	17.5	-16.9
8g[b,m]	21.2	-1.6	-13.4	-30.3	-7.1	-15.8
8h[b,o]	31.6	-4.6	11	17.3	14.9	-4.7
13a[b,e]	37.8	3	-3.4	23.4	-7.5	2
13b[b,e]	14.7	-17	-102.5	-21.5	-48.2	-12.1
13c[b,e]	13.3	5.6	10.4	20	17	-16.2
13i[b,e]	8	9.2	14.6	33	10	-13.6
8i[m]	24.6	-3.9	-0.7	-5.7	5.5	7.7
8j[c,m]	23.6	-18.1	-7.9	-1.1	1.7	11.4
8l[c,p]	25	2.8	23.6	14.2	18.6	-3.4
8m[c,o]	26.5	-2.8	18.1	-2.3	20	1.6
13e[c,e]	22.7	11.7	6.4	37.1	13	44.1
13f[c,e]	39.4	-13.2	-6.3	19.5	-7.2	-13.6
8n[p]	25.1	-7.8	10.7	9.9	-7.1	-7.3
8o[d,p]	21.7	15.1	26.6	4.8	22.8	-6.6
8p[d,m]	39.4	-13.2	-6.3	19.5	-7.2	-13.6
8q[d,o]	25.9	1.8	18.9	11.7	17.5	-4.4
13g[d,e]	14.6	-14.1	-123.2	-37.9	14.3	7.3
13h[d,e]	12.6	9.6	-129.3	7.2	3.9	-16.7
8r[p]	5.4	0.4	-19.1	-1.4	-1.2	1
8s[m]	1	12.9	-17.6	-6.8	4.4	-10.2
8t[o]	11.4	4.9	-6.5	8.5	-0.1	1.7

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Adrenergic β_2	Adrenergic β_3	BZP[f] Rat Brain Site	Dopamine D_1	Dopamine D_2	Dopamine D_3
Radioligand	**[^{125}I]Iodopindolol**	**[^{125}I]Iodopindolol**	**[^{3}H]Flunitrazepam**	**[^{3}H]-SCH233930**	**[^{3}H]-N-methyl-spiperone**	**[^{3}H]-N-methyl-spiperone**
8a[p]	6.9	4	**92.4**	-0.1	-20.8	3.2
8b[b,p]	-19.7	-5.7	**96.2**	-17.8	-16.5	-7.4
8c[b,p]	-9.5	-19	**96.7**	13.5	-5	-5.8
8d[b,p]	8.6	-9.3	**94.5**	5.7	-18.3	-7.7
8e[b,m]	3.9	-11.6	**95**	10.1	-4.9	-1.3
8f[b,o]	-3.3	-7.3	**80.5**	-6.2	-3.9	13.8
8g[b,m]	7.4	-18.7	**99.3**	17.4	11.3	-10
8h[b,o]	1.4	-17.5	41.3	-2.4	-16	-5
13a[b,e]	-4.2	-6.3	**91.3**	6.1	-9	7.9
13b[b,e]	-6.3	-13.6	**93.7**	-15.2	9.5	5.5
13c[b,e]	3.4	-5.7	**98.2**	-3.9	-14.9	-3.6
13i[b,e]	7	-7.1	**96.9**	12.7	-3.1	0.6
8i[m]	12.6	-9.3	**94.1**	-12.1	-15.1	-5.1
8j[c,m]	-4.1	-3.6	**96.3**	4.2	-18.1	3.1
8l[c,p]	-8.7	5.2	**95**	-5.1	-11.2	-11.2
8m[c,o]	-8	-15.2	**88.7**	-16.2	-11.2	-3.3
13e[c,e]	-3.9	-2.7	**100.6**	-0.4	-2.6	7.2
13f[c,e]	5.3	-2.7	**95.6**	-1.8	15.1	16.6
8n[p]	1.7	-9.6	**93.2**	-1.8	-12.4	-0.8
8o[d,p]	-10.3	-19.7	**97.4**	-0.7	-14.4	-6.6
8p[d,m]	5.3	-2.7	**95.6**	-0.1	-13.8	6.3
8q[d,o]	-9.1	-23.3	42.2	11.9	-24.2	11.9
13g[d,e]	-17.8	9.8	**101.4**	2.2	19.1	-5.6
13h[d,e]	-5.1	-1.1	**93.3**	10.5	3.9	10.8
8r[p]	11.7	0.7	**91.5**	0	-19	8.6
8s[m]	4.5	1.9	**96.7**	-1.4	-2.9	-2.8
8t[o]	-9.9	5.9	**78**	-2.5	-11	7.6

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Dopamine D_4	Dopamine D_5	Transporter Dopamine (DAT)	DOR[f]	$GABA_A$	Histamine H_1
Radioligand	[^{3}H]-N-methyl-spiperone	[^{3}H]-SCH233930	[^{3}H]WIN35428	[^{3}H]DADLE	[^{3}H]Muscimol	[^{3}H]Pyrilamine
8a[p]	-0.4	**57.9**	-7.8	10.8	13.1	52.4
8b[b,p]	-0.5	14.7	-6.7	6.3	6.8	48.7
8c[b,p]	5.4	8.5	-14	23.2	8.5	25.5
8d[b,p]	1.4	12.3	-7.7	5.9	2.6	28.9
8e[b,m]	-3	30.9	4.4	3.6	7.6	64.3
8f[b,o]	2.2	14.3	-5.2	28.6	1	14.8
8g[b,m]	-15	12.2	-32.4	9.5	-1.6	41.7
8h[b,o]	2	1.7	-1	-12.3	-5.2	49.8
13a[b,e]	-7.9	4	10.3	26	14.5	57.6
13b[b,e]	-9.5	7.5	12.4	-2.3	-9.2	48.7
13c[b,e]	-7.3	9	-10.4	-8.2	17.3	62.1
13i[b,e]	1.2	9.2	-9.5	-5.7	9.8	74.7
8i[m]	-6.7	44.9	5.3	-0.1	-0.5	64.7
8j[c,m]	-0.5	23.2	0.9	14.3	-2.9	56.2
8l[c,p]	-6.7	9	2	**50.8**	7.5	38.9
8m[c,o]	-4.1	-1.9	-7.1	0.2	-3.2	27.9
13e[c,e]	5.9	4.9	-1	**72.4**	18.5	51.9
13f[c,e]	5.4	-3.9	5.2	**58.6**	24.5	-12
8n[p]	3.7	40.8	2.8	-1	-9.9	71.8
8o[d,p]	1	17	-3.5	14.1	9.2	55.9
8p[d,m]	1.4	21.8	2.5	0.6	-4.8	31.4
8q[d,o]	-6.9	10	-9.2	-16.9	-3.1	37.8
13g[d,e]	2	0.1	-10.9	47.7	45.5	44.4
13h[d,e]	17.9	1.8	-12	20.1	**66.8**	-15
8r[p]	-0.3	**59.1**	5.2	31.4	-15.7	45.1
8s[m]	14.7	4.3	6.7	7.1	2.7	40.5
8t[o]	-0.8	5.7	15.4	-10	2.6	54.1

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Histamine H_2	Histamine H_3	Histamine H_4	hERG binding	KOR[f]	Muscarinic M_1
Radioligand	[^{3}H]Tiotidine	[^{3}H]alpha-methylhistamine	[^{3}H]Histamine	N/A	[^{3}H]U69593	[^{3}H]QNB
8a[p]	-1.4	-1.5	-12.2	7.4	7.1	20.6
8b[b,p]	20.8	-1.3	-5.9	-0.1	11.9	8.5
8c[b,p]	-0.7	-3	0.6	6.9	22.8	16.9
8d[b,p]	-2.6	-6.3	13.2	8.7	10.1	17.6
8e[b,m]	23	-2	0.2	5.2	1.8	0
8f[b,o]	-5	2.4	1.3	-29.3	26.3	19.1
8g[b,m]	-1.5	-2.9	-9.7	-5.4	20.5	-7
8h[b,o]	-6.5	2.9	-9.9	-1	23.5	5.2
13a[b,e]	-1.8	10.9	-1.1	5.1	8.5	-19.5
13b[b,e]	1.5	11.7	-8.4	6.4	-4.7	-8
13c[b,e]	23.5	12	1.1	8.6	8.6	-0.8
13i[b,e]	24.1	32	-0.5	9.1	20.3	-1.2
8i[m]	7.5	3.9	-4.6	0.1	10.2	4.5
8j[c,m]	-0.5	2	0	-16.1	-3.8	-8.1
8l[c,p]	2.4	-9	-8.9	-2.5	15.9	-0.1
8m[c,o]	-12.1	-1.9	-7.8	-1.9	21.4	8
13e[c,e]	10.2	6.3	3.8	-1.2	21.2	9.3
13f[c,e]	7.7	-11	7.9	8.1	-2.1	-5.6
8n[p]	8.5	-4.6	-3.1	-2.7	14.5	3.9
8o[d,p]	13.8	2.6	13.8	8.8	26.5	5.6
8p[d,m]	-4.1	0.5	1.5	-15.1	1.2	18.2
8q[d,o]	-0.4	-4.7	-11.1	-4.1	30.9	-5.8
13g[d,e]	-7.4	-1.7	2.3	0.3	-3.8	-4.1
13h[d,e]	31.4	-10	-0.1	-10.3	-8.1	-9.3
8r[p]	-3.3	26.5	18.7	17.8	8.9	-4.5
8s[m]	-3.3	12.8	-5.3	4.6	4.7	-4.8
8t[o]	0.2	16.7	26.8	31.3	9.1	-1.7

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Muscarinic M_2	Muscarinic M_3	Muscarinic M_4	Muscarinic M_5	MOR[f]
Radioligand	**[^{3}H]QNB**	**[^{3}H]QNB**	**[^{3}H]QNB**	**[^{3}H]QNB**	**[^{3}H]DAMGO**
8a[p]	-8.4	-4.5	10.9	-5.4	38.3
8b[b,p]	6	27	19.2	-4.8	**53.1**
8c[b,p]	6.9	13.6	-14	2.1	**50.8**
8d[b,p]	8.4	18.1	9.2	3.9	48.9
8e[b,m]	-2.6	5.2	-0.7	-6.6	3.2
8f[b,o]	15.4	7.3	-7.5	14.8	-11
8g[b,m]	-8.1	-15	-10	4.4	18.5
8h[b,o]	14.9	15.2	-37	-6	18.8
13a[b,e]	-16.6	-20	**53.2**	31.8	5.5
13b[b,e]	-24.1	-20	-12	-29.1	-4.5
13c[b,e]	10	-7.5	7	-13.6	4
13i[b,e]	17.8	-4	7.3	2.9	-1.6
8i[m]	-2.7	-0.3	-1.7	0.1	-0.6
8j[c,m]	2.6	15.2	-3.5	-5.2	-0.2
8l[c,p]	16.2	16.2	21.6	3.5	46.5
8m[c,o]	5.3	21.6	-26	9.4	-6
13e[c,e]	8.8	-16	15.6	48.7	21.4
13f[c,e]	-19.4	13.1	-2	4.3	10.8
8n[p]	-0.2	2.9	1.9	2.3	9.7
8o[d,p]	8	-3.2	31.5	0.1	**57.2**
8p[d,m]	4	6.9	-1.6	25.8	-1.7
8q[d,o]	14	-1.5	-22	-4.8	11.3
13g[d,e]	-17.8	-15	-8.1	-27.1	10.5
13h[d,e]	13.3	-1.1	-4.6	-2.4	24.3
8r[p]	-4.5	-21.3	-11	2.6	1.7
8s[m]	-4.8	-15.1	-13	23.9	5.8
8t[o]	-1.7	-29.7	-19	42.8	17.2

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S1: Primary radioligand binding assays. Compound-induced radioligand displacement assays for 46 receptors, transporters and channels conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Transporter, Norepinephrine (NET)	Peripheral Benzodiazepine Receptor (PBR)	Transporter, Serotonin (SERT)	Sigma σ_1	Sigma σ_2
Radioligand	**[^{3}H]Nisoxetine**	**[^{3}H]PK11195**	**[^{3}H]Citalopram**	**[^{3}H]Pentazocine**	**[^{3}H]DTG**
8a[p]	18.1	8.1	1.3	-14.2	15.7
8b[b,p]	9.6	8	7.8	20	21.2
8c[b,p]	21.9	7.7	11.4	-5.3	20.7
8d[b,p]	18.8	9.2	16.4	-4.3	22.4
8e[b,m]	6.1	8.2	3.3	-5.1	1
8f[b,o]	25.2	-6.3	13.9	31.4	38.3
8g[b,m]	15	4.8	-12.7	10.6	6
8h[b,o]	39.9	8	27.7	17.5	29.3
13a[b,e]	2.1	-0.4	-6.1	-13.3	12.2
13b[b,e]	1.7	8.7	-20.7	-11.5	3.7
13c[b,e]	5.3	-7.1	6.3	15.6	16.5
13i[b,e]	5.6	9.9	-1.6	39	16.5
8i[m]	27.7	7.3	1.4	-2.7	10.8
8j[c,m]	0.5	12	-1.1	-3.4	-5.7
8l[c,p]	20.5	23	5.8	-5.4	8.8
8m[c,o]	36.6	-1.3	33.7	-7.4	42.2
13e[c,e]	8.6	10.7	-1.9	-1.5	15.1
13f[c,e]	-3.2	16.5	-7.5	-16.2	28.5
8n[p]	21.2	32.8	7.1	-5.3	6.8
8o[d,p]	3.7	42.5	11.2	-0.6	4.9
8p[d,m]	15.9	14	15.2	20	-5.5
8q[d,o]	29.7	8.7	18.6	-10.5	26.7
13g[d,e]	-8.3	2.4	-10	-13.5	15.1
13h[d,e]	-2.8	17.8	-18.5	-7.8	8.8
8r[p]	11.3	20	-6.6	-17.8	11.8
8s[m]	8.5	29.6	-10.9	-14.1	8.3
8t[o]	4.1	5	-6.1	-23.5	15.9

[a]Data are the percent inhibition induced by 10 μM of each respective compound on the specific binding at the screened target. The higher the number the more the radioligand was displaced. The number higher than 50% inhibition is considered meaningful and is shown in **bold** and highlighted. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S2: Secondary radioligand binding assays. Binding affinity (Ki, nM) values obtained from non-linear regression of radioligand competition binding isotherms. Testing conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	Serotonin 5-HT$_{2B}$	Serotonin 5-HT$_{2C}$	Serotonin 5-HT$_{7}$	BZP[f] Rat Brain Site	Dopamine D$_5$
8a[p]	NA	NA	**2599.0**	**2.7**	**10,000**
8b[b,p]	NA	NA	**2039.0**	**2.1**	NA
8c[b,p]	NA	NA	**2616.3**	**2.0**	NA
8d[b,p]	NA	NA	**2371.3**	**2.3**	NA
8e[b,m]	NA	NA	NA	**11.3**	NA
8f[b,o]	NA	NA	NA	**343.3**	NA
8g[b,m]	NA	NA	NA	**32.3**	NA
8h[b,o]	NA	NA	NA	NA	NA
13a[b,e]	NA	NA	NA	**28.3**	NA
13b[b,e]	NA	NA	NA	**21.7**	NA
13c[b,e]	NA	NA	NA	**8.16**	NA
13i[b,e]	NA	NA	NA	**2.6**	NA
8i[m]	NA	NA	NA	**1.4**	NA
8j[c,m]	NA	NA	NA	**1.2**	NA
8l[c,p]	NA	NA	NA	**0.4**	NA
8m[c,o]	NA	NA	NA	**98.0**	NA
13e[c,e]	NA	NA	NA	**2.0**	NA
13f[c,e]	NA	NA	NA	**2.2**	NA
8n[p]	NA	NA	**7603.7**	**12.2**	NA
8o[d,p]	**5193.0**	NA	**5836.3**	**5.5**	NA
8p[d,m]	NA	NA	NA	**50.4**	NA
8q[d,o]	NA	**10,000**	NA	NA	NA
13g[d,e]	NA	**10,000**	NA	**1.8**	NA
13h[d,e]	NA	NA	NA	**6.2**	NA
8r[p]	NA	**10,000**	NA	**0.4**	**10,000**
8s[m]	NA	NA	NA	**1.6**	NA
8t[o]	NA	NA	NA	**205.0**	NA

[a]Ki values are calculated from best fit IC_{50} values using the Cheng-Prusoff equation. Values are an average of n = 3. NA are for compounds/receptors not testing for Primary Screening results of < 50% inhibition (**Table S1**). [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S2: Secondary radioligand binding assays. Binding affinity (Ki, nM) values obtained from non-linear regression of radioligand competition binding isotherms. Testing conducted by the National Institute of Medical Health Psychoactive Drugs Screening Program (B. Roth et al., UNC, available at http://pdsp.med.unc.edu).[a]

Compound	DOR[f]	$GABA_A$	Muscarinic M_4	MOR[f]
8a[p]	NA	NA	NA	NA
8b[b,p]	NA	NA	NA	**3524.3**
8c[b,p]	NA	NA	NA	**4431.0**
8d[b,p]	NA	NA	NA	NA
8e[b,m]	NA	NA	NA	NA
8f[b,o]	NA	NA	NA	NA
8g[b,m]	NA	NA	NA	NA
8h[b,o]	NA	NA	NA	NA
13a[b,e]	NA	NA	**7852.0**	NA
13b[b,e]	NA	NA	NA	NA
13c[b,e]	NA	NA	NA	NA
13i[b,e]	NA	NA	NA	NA
8i[m]	NA	NA	NA	NA
8j[c,m]	NA	NA	NA	NA
8l[c,p]	**3640.7**	NA	NA	NA
8m[c,o]	NA	NA	NA	NA
13e[c,e]	**3820.7**	NA	NA	NA
13f[c,e]	**8499.3**	NA	NA	NA
8n[p]	NA	NA	NA	NA
8o[d,p]	NA	NA	NA	**6663.5**
8p[d,m]	NA	NA	NA	NA
8q[d,o]	NA	NA	NA	NA
13g[d,e]	NA	NA	NA	NA
13h[d,e]	NA	**1458.0**	NA	NA
8r[p]	NA	NA	NA	NA
8s[m]	NA	NA	NA	NA
8t[o]	NA	NA	NA	NA

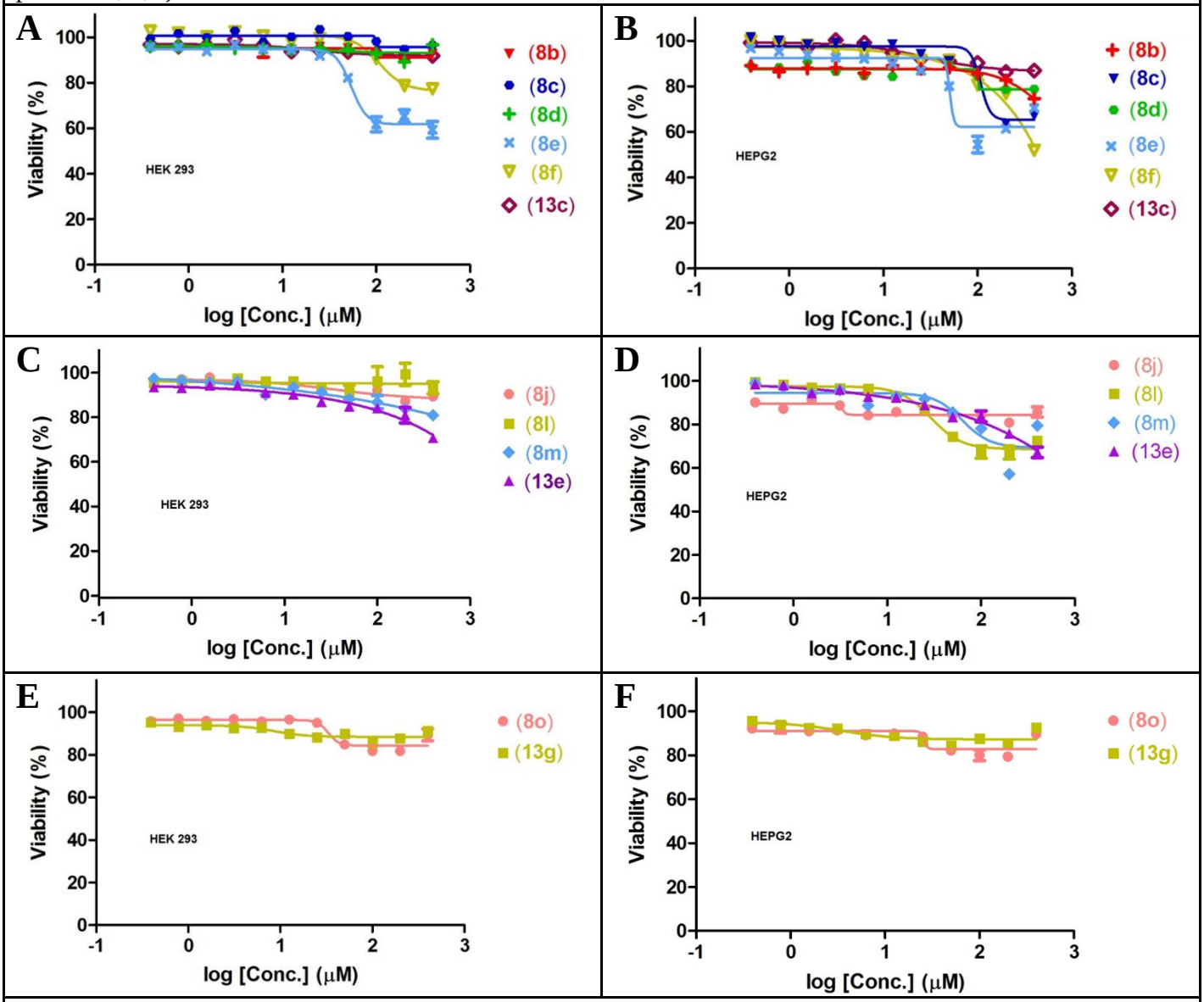
[a]Ki values are calculated from best fit IC_{50} values using the Cheng-Prusoff equation. Values are an average of n = 3. NA are for compounds/receptors not testing for Primary Screening results of < 50% inhibition (**Table S1**). [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]"A-ring" or "D-ring" N-hetero analogs. [f]BZP = benzodiazepine, DOR = δ-opioid receptor, KOR = κ-opioid receptor, MOR = μ-opioid receptor. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

Table S3: Results of Cytotoxicity Studies on HEPG2 and HEK293 Cell Lines[a]

Compound	Toxicity in HEK293 (Kidney) LD_{50} (µM)	Toxicity in HEPG2 (Liver) LD_{50} (µM)
8b[b,p]	>400	>400
8c[b,p]	>400	>200
8d[b,p]	>400	>400
8e[b,m]	>200	>200
8f[b,o]	>400	>200
13c[b,e]	>400	>400
8j[c,m]	>400	>400
8l[c,p]	>400	>200
8m[c,o]	>400	>400
13e[c,e]	>400	>400
8o[d,p]	>400	>400
13g[d,e]	>400	>400

[a]Cellular viability studies confirmed that the ligands were not cytotoxic to HEPG2 or HEK293 Cell Lines. The compounds were incubated at different concentrations with specified cells for 48 h, followed by detection of viability using Cell-Titer Glo (Promega). The results were normalized using DMSO (negative) and 3-dibutylamino-1-(4-hexyl-phenyl)-propan-1-one (150 mM in DMSO final concentration, positive). Data were acquired by three independent experiments carried out in quadruplet. [b]**8a** related analogs in **red**. [c]**8i** related analogs in **blue**. [d]**8n** related analogs in **green**. [e]Select "D-ring" N-hetero analogs. [o]D-ring "*ortho*". [m]D-ring "*meta*". [p]D-ring "*para*".

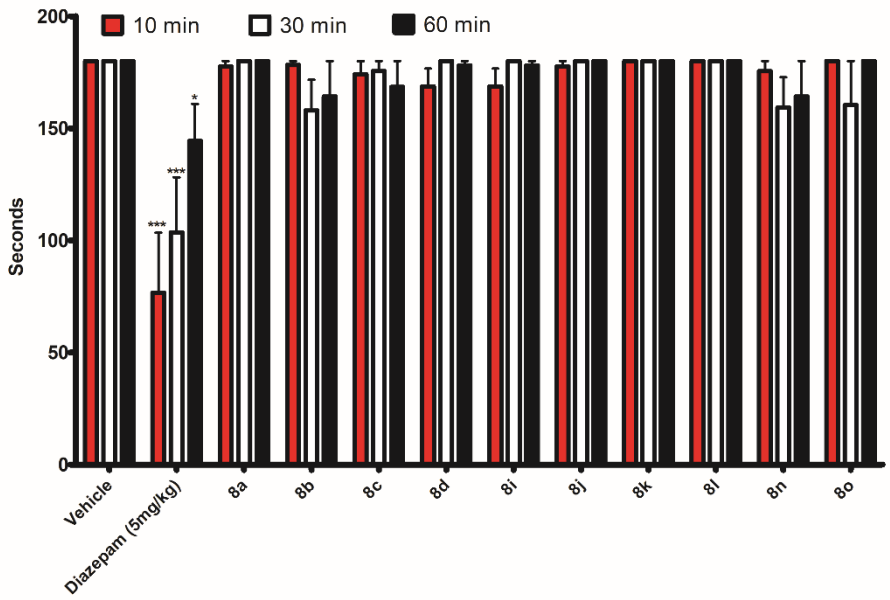
Figure S1: Cytotoxicity on HEK293 (Kidney) Cells (left panels: A,C,E) and HEPG2 (Liver) Cells (right panels: B,D,F)[a]



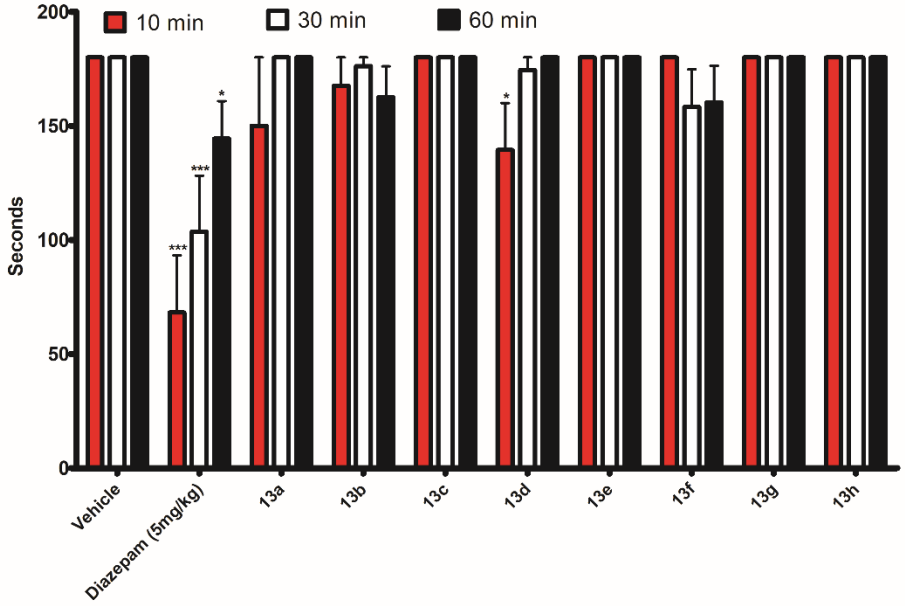
[a]**8a** related analogs (**A**, **B**). **8i** related analogs (**C**, **D**). **8n** related analogs (**E, F**). HEK 293 = kidney cells. (**A**, **C**, **E**). HEPG2 = liver cells (**B**, **D**, **F**). Cellular viability studies confirmed that the ligands were not cytotoxic to HEPG2 or HEK293 Cell Lines. The compounds were incubated at different concentrations with specified cells for 48 h, followed by detection of viability using Cell-Titer Glo (Promega). The results were normalized using DMSO (negative) and 3-dibutylamino-1-(4-hexyl-phenyl)-propan-1-one (150 mM in DMSO final concentration, positive). Data were acquired by three independent experiments carried out in quadruplet.

Figure S2: Effect of Selected Ligands on Motor Coordination[a]

A

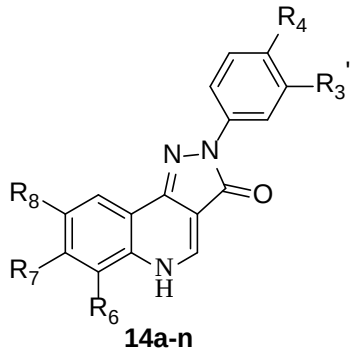


B



[a]Rotorod studies confirmed that deuteration of the methoxy groups (**A**) and N-hetero substitution and deuteration of the methoxy groups (**B)** of the parent ligands had no effect on motor coordination in contrast to the effects of diazepam at 5mg/kg. Female Swiss Webster mice were tested on a rotarod at 15 rpm for 3 min at 10, 30, and 60 min following compound exposure. Mice (N = 10) received a single injection via oral gavage (PO) of the test compounds (40 mg/kg), diazepam (5 mg/kg), or vehicle (2% polyethylene glycol, 2.5% hydroxypropylmethyl cellulose solution). The time of fall was recorded if it occurred prior to 3 min. Data are expressed as mean ± SEM (N = 10). ∗ ($p < 0.05$), ∗∗ ($p < 0.01$) or ∗∗∗ ($p < 0.001$) significance compared to vehicle-treated mice. All compounds dissolved in the oral vehicle.

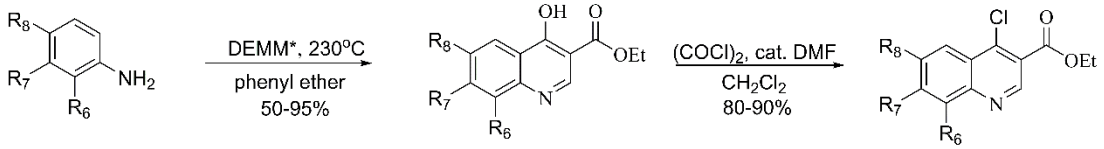
Table S4: Additional Ligands Synthesized via the Chemistry in **Scheme S1** for Future SAR Studies[a]



14a-n

Compound	R_8	R_7	R_6	R_4'	R_3'
14a	H	OCD_3	H	H	H
14b	H	OCH_3	CH_3	OCH_3	H
14c	H	OCH_3	CH_3	H	H
14d	H	OCH_3	CH_3	OCF_3	H
14e	H	OCH_3	H	OCF_3	H
14f	Br	H	F	OCH_3	H
14g	Br	H	F	Cl	H
14h	Br	H	F	F	H
14i	H	CF_3	H	OCH_3	H
14j	H	CF_3	H	Cl	H
14k	H	CF_3	H	NO_2	H
14l	H	CF_3	H	OCF_3	H
14m	H	CF_3	H	F	H
14n	H	CF_3	H	H	OCH_3

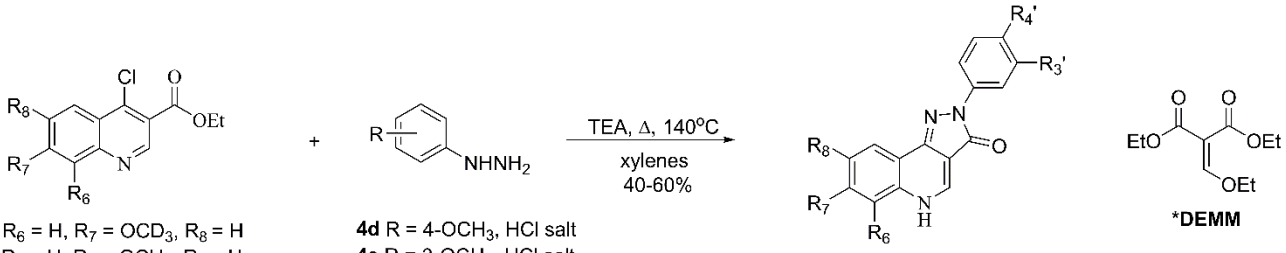
Scheme S1: Synthesis of Additional Analogs



5g $R_6 = CH_3$, $R_7 = OCH_3$, $R_8 = H$
5h $R_6 = F$, $R_7 = H$, $R_8 = Br$
5i $R_6 = H$, $R_7 = CF_3$, $R_8 = H$

6g $R_6 = CH_3$, $R_7 = OCH_3$, $R_8 = H$
6h $R_6 = F$, $R_7 = H$, $R_8 = Br$
6i $R_6 = H$, $R_7 = CF_3$, $R_8 = H$

7g $R_6 = CH_3$, $R_7 = OCH_3$, $R_8 = H$
7h $R_6 = F$, $R_7 = H$, $R_8 = Br$
7i $R_6 = H$, $R_7 = CF_3$, $R_8 = H$



7b $R_6 = H$, $R_7 = OCD_3$, $R_8 = H$
7e $R_6 = H$, $R_7 = OCH_3$, $R_8 = H$
7g $R_6 = CH_3$, $R_7 = OCH_3$, $R_8 = H$
7h $R_6 = F$, $R_7 = H$, $R_8 = Br$
7i $R_6 = H$, $R_7 = CF_3$, $R_8 = H$

4d R = 4-OCH_3, HCl salt
4e R = 3-OCH_3, HCl salt
4f R = 4-OCF_3, HCl salt
4h R = 4-Cl, HCl salt
4i R = 4-F, HCl salt
4j R = 4-NO_2, HCl salt
4k R = H, HCl salt

14a-n

Supporting Experimental

Chemistry. All reactions were performed in oven-dried round-bottom flasks with magnetic stir bars or overhead mechanical stirrers under an argon atmosphere unless the reaction conditions were supposed to contain water. Organic solvents were purified when necessary by standard methods[36] or purchased from Sigma-Aldrich.™ Chemicals were purchased from either Sigma Aldrich™, Oakwood Chemical, Alfa Aesar, Matrix Scientific, or Acros Organic. The progress of the reactions was monitored by TLC on a silica gel plate (25% EtOAc in hexanes or 10% MeOH in DCM). The ^{1}H and ^{13}C NMR data were obtained on Bruker Spectrospin 300 MHz and GE 500 MHz instruments with the chemical shifts in δ (ppm) reported relative to TMS. The HRMS spectral data was obtained on a LCMS-IT-TOF by Shimadzu Scientific. Purity of all final compounds was 98% or higher and was determined by HPLC on a LC-MS with Shimadzu LCMS 2020, (Shimadzu Scientific Instruments, Columbia, MD) using a PDA detector at 254 nm. The column was a Shimadzu C18 3 µm 50 x 4.6mm reversed phase LC column. LC mobile phase: 90% acetonitrile (w. 0.1% TFA) and 10% H_2O (w/ 0.1% TFA) with a flow rate of 1 mL min^{-1}, column temperature: 25 ^{o}C, injection size: 1.0µL.

Ethyl 4-hydroxy-7-methoxy-8-methyl quinoline-3-carboxylate (6g). A mixture of 3-methoxy-2-methylaniline **5g** (25 g, 182.2 mmol), diethyl ethoxymethylenemalonate (39.4 g, 182.2 mmol) and diphenyl ether (100 mL) was slowly heated to 230 ^{o}C. The EtOH, which evolved, was collected in a Dean-Stark trap. Once the EtOH formation had ceased, the reaction mixture was heated for an additional 30 min at 230 ^{o}C. The reaction mixture was then cooled to 80 ^{o}C and diluted with ethanol (100 mL). Upon cooling to 20-25 ^{o}C the solid, which formed,

was collected by filtration and washed with ethanol (50 mL x 2) and then hexanes (50 mL x 2). The solid was dried under vacuum at 40 °C to afford **6g** as a light brown solid (41.9 g, 88%); ^{1}H NMR (300 MHz, TFA) δ 11.66 (s, 1H), 9.13 (s, 1H), 8.53 (d, *J* = 9.3 Hz, 1H), 7.60 (d, *J* = 9.4 Hz, 1H), 4.60 (q, *J* = 7.1 Hz, 2H), 4.11 (s, 3H), 2.52 (s, 3H), 1.46 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, TFA) δ 172.57, 167.57, 164.66, 144.78, 139.23, 124.90, 114.60, 114.12, 113.61, 102.73, 64.14, 55.62, 11.94, 6.58; HRMS (ESI) m/z calculated for $C_{14}H_{16}NO_4$ $(M+H)^+$ 262.1074; found 262.1097.

Ethyl 6-bromo-8-fluoro-4-hydroxyquinoline-3-carboxylate (6h). A mixture of 4-bromo-2-fluoroaniline **5h** (10 g, 52.6 mmol), diethyl ethoxymethylenemalonate (11.4 g, 52.6 mmol) and diphenyl ether (40 mL) was slowly heated to 230 °C. The EtOH, which evolved, was collected in a Dean-Stark trap. Once the EtOH formation had ceased, the reaction mixture was heated for an additional 30 min at 230 °C. The reaction mixture was then cooled to 80 °C and diluted with ethanol (40 mL). Upon cooling to 20-25 °C the solid, which formed, was collected by filtration and washed with ethanol (10 mL x 2) and then hexanes (10 mL x 2). The solid was dried under vacuum at 40 °C to afford **6h** as a light brown solid (41.9 g, 88%); ^{1}H NMR (300 MHz, TFA) δ 11.65 (s, 1H), 9.37 (s, 1H), 8.61 (s, 1H), 8.07 (d, *J* = 9.3 Hz, 1H), 4.68 (q, *J* = 7.0 Hz, 2H), 1.52 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, TFA) δ 172.48 (s), 166.96 (s), 151.48 (d, $^{1}J_{CF}$ = 261.7 Hz), 145.48 (s), 128.08 (d, $^{2}J_{CF}$= 14.9 Hz), 125.44 (d, $^{2}J_{CF}$ = 19.3 Hz), 124.22 (d, $^{3}J_{CF}$ = 8.1 Hz), 123.00 (d, $^{3}J_{CF}$ = 4.7 Hz), 121.87 (s), 106.18 (s), 65.01 (s), 11.92 (s); HRMS (ESI) m/z calculated for $C_{12}H_{10}BrFNO_3$ $(M+H)^+$ 313.9823; found 313.9822.

Ethyl 4-hydroxy-7-(trifluoromethyl)quinoline-3-carboxylate (6i). A mixture of 3-(trifluoromethyl)aniline **5i** (25 g, 155.1 mmol), diethyl ethoxymethylenemalonate (33.6 g, 155.1

mmol) and diphenyl ether (100 mL) was slowly heated to 230 ºC. The EtOH, which evolved, was collected in a Dean-Stark trap. Once the EtOH formation had ceased, the reaction mixture was heated for an additional 30 min at 230 ºC. The reaction mixture was then cooled to 80 ºC and diluted with ethanol (100 mL). Upon cooling to 20-25 ºC the solid, which formed, was collected by filtration and washed with ethanol (25 mL x 2) and then hexanes (25 mL x 2). The solid was dried under vacuum at 40 ºC to afford **6i** as an off-white solid (35.6 g, 80%); ^{1}H NMR (300 MHz, TFA) δ 11.65 (s, 1H), 9.44 (s, 1H), 8.81 (d, *J* = 8.7 Hz, 1H), 8.50 (s, 1H), 8.16 (d, *J* = 8.7 Hz, 1H), 4.68 (q, *J* = 7.1 Hz, 2H), 1.52 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, TFA) δ 173.51 (s), 167.08 (s), 146.62 (s), 139.51 (q, $^{2}J_{CF}$ = 35.7 Hz), 138.80 (s), 126.21 (s), 125.92 (q, $^{3}J_{CF}$ = 2.9 Hz), 121.94 (q, $^{1}J_{CF}$ = 263.7 Hz), 121.61 (s), 117.70 (q, $^{3}J_{CF}$ = 4.0 Hz), 105.92 (s), 64.87 (s), 11.91 (s); HRMS (ESI) m/z calculated for $C_{13}H_{11}F_3NO_3$ $(M+H)^+$ 286.0686; found 286.0688.

Ethyl 4-chloro-7-methoxy-8-methyl quinoline-3-carboxylate (7g). A mixture of ethyl-4-hydroxy-7-methoxy-8-methyl quinoline-3-carboxylate **6g** (41.0 g, 156.9 mmol), N,N-dimethylformamide (0.5 mL, 6.5 mmol), and DCM (500 mL) was heated to 35-40ºC. Oxalyl chloride (21.9 g, 172.6 mmol) was added dropwise to the reaction mixture over 30 min. The reaction mixture was heated for 6 h at reflux (38-40 ºC). The pale yellow solution, which resulted, was then allowed to cool to 20-25 ºC. The reaction mixture was brought to pH = 10 (pH paper) by slowly adding a 25% solution of aq potassium carbonate (62.5 g) in H_2O (250 mL). The layers were then separated and the aq layer was extracted with DCM (250 mL). The combined organic layers were then washed with a 25% solution of aq potassium carbonate (62.5 g) in H_2O (250 mL). The combined organic layers were dried ($MgSO_4$). The solvents were then removed under reduced pressure and the residue was slurried with cold hexanes (200 mL). The

solid, which formed, was filtered and washed with cold hexanes (50 mL x 2). The solid was dried under vacuum at 40 ^{o}C to afford **7g** as an off-white solid (42.9 g, 97%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.21 (s, 1H), 8.27 (d, *J* = 9.4 Hz, 1H), 7.41 (d, *J* = 9.4 Hz, 1H), 4.49 (q, *J* = 7.1 Hz, 2H), 4.02 (s, 3H), 2.66 (s, 3H), 1.46 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.78, 159.33, 149.82, 149.23, 143.84, 124.25, 122.51, 120.88, 119.92, 114.35, 61.74, 56.20, 14.25, 10.00; HRMS (ESI) m/z calculated for $C_{14}H_{15}ClNO_3$ $(M+H)^+$ 280.0735; found 280.0746.

Ethyl 6-bromo-4-chloro-8-fluoroquinoline-3-carboxylate (7h). A mixture of ethyl-6-bromo-4-hydroxy-8-fluoro quinoline-3-carboxylate **6h** (11.0 g, 35.0 mmol), N,N-dimethylformamide (0.1 mL, 1.3 mmol), and DCM (200 mL) was heated to 35-40^{o}C. Oxalyl chloride (4.9 g, 38.5 mmol) was added dropwise to the reaction mixture over 30 min. The reaction mixture was heated for 6 h at reflux (38-40 ^{o}C). The pale yellow solution, which resulted, was then allowed to cool to 20-25 ^{o}C. The reaction mixture was brought to pH = 10 (pH paper) by slowly adding a 25% solution of aq potassium carbonate (25 g) in H_2O (100 mL). The layers were then separated and the aq layer was extracted with DCM (100 mL). The combined organic layers were then washed with a 25% solution of aq potassium carbonate (25 g) in H_2O (100 mL). The combined organic layers were dried ($MgSO_4$). The solvents were then removed under reduced pressure and the residue was slurried with cold hexanes (50 mL). The solid, which formed, was filtered and washed with cold hexanes (10 mL x 2). The solid was dried under vacuum at 40 ^{o}C to afford **7h** as a pale yellow solid (9.7 g, 83%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.21 (s, 1H), 8.36 (d, *J* = 1.3 Hz, 1H), 7.68 (dd, *J* = 9.1, 1.6 Hz, 1H), 4.52 (q, *J* = 7.1 Hz, 2H), 1.48 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.79 (s), 157.55 (d, $^{1}J_{CF}$ = 263.7 Hz), 150.38 (s), 142.17 (s), 138.63 (d, $^{2}J_{CF}$ = 12.4 Hz), 128.50 (s), 124.90 (s), 123.64 (d, $^{3}J_{CF}$ = 5.0 Hz), 121.59 (d, $^{3}J_{CF}$ = 9.4 Hz),

120.15 (d, $^{2}J_{CF}$ = 22.0 Hz), 62.54 (s), 14.21 (s); HRMS (ESI) m/z calculated for $C_{12}H_9BrClFNO_2$ $(M+H)^+$ 331.9484; found 331.9485.

Ethyl 4-chloro-7-(trifluoromethyl)quinoline-3-carboxylate (7i). A mixture of ethyl-4-hydroxy-7-(trifluoromethyl) quinoline-3-carboxylate **6i** (35.0 g, 122.7 mmol), N,N-dimethylformamide (0.5 mL, 6.5 mmol), and DCM (500 mL) was heated to 35-40ºC. Oxalyl chloride (17.1 g, 135.0 mmol) was added dropwise to the reaction mixture over 30 min. The reaction mixture was heated for 6 h at reflux (38-40 ºC). The pale yellow solution, which resulted, was then allowed to cool to 20-25 ºC. The reaction mixture was brought to pH = 10 (pH paper) by slowly adding a 25% solution of aq potassium carbonate (62.5 g) in H_2O (250 mL). The layers were then separated and the aq layer was extracted with DCM (250 mL). The combined organic layers were then washed with a 25% solution of aq potassium carbonate (62.5 g) in H_2O (250 mL). The combined organic layers were dried ($MgSO_4$). The solvents were then removed under reduced pressure and the residue was slurried with cold hexanes (50 mL). The solid, which formed, was filtered and washed with cold hexanes (10 mL x 2). The solid was dried under vacuum at 40 ºC to afford **7i** as an off-white solid (26.1 g, 70%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.28 (s, 1H), 8.54 (d, *J* = 8.8 Hz, 1H), 8.45 (s, 1H), 7.87 (dd, *J* = 8.8, 1.6 Hz, 1H), 4.53 (q, *J* = 7.1 Hz, 2H), 1.49 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.98 (s), 151.40 (s), 148.60 (s), 143.38 (s), 133.46 (q, $^{2}J_{CF}$ = 33.1 Hz), 127.80 (s), 127.64 (q, $^{3}J_{CF}$ = 4.4 Hz), 126.90 (s), 124.65 (s), 123.97 (q, $^{3}J_{CF}$ = 3.0 Hz), 123.41 (q, $^{1}J_{CF}$ = 272.9 Hz), 62.44 (s), 14.20 (s); HRMS (ESI) m/z calculated for $C_{13}H_{10}ClF_3NO_2$ $(M+H)^+$ 304.0347; found 304.0344.

7-Methoxy-*d*$_3$-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14a). A mixture of ethyl-4-chloro-7-methoxy-*d*$_3$-quinoline-3-carboxylate **7b** (2.0 g, 7.4 mmol), phenylhydrazine hydrochloride **4k** (1.29g, 89.3 mmol), and triethylamine (1.8 g, 17.8 mmol) in xylenes (16mL) was heated to reflux (138^{o}C) for 2 hr. The yellow-orange slurry, which resulted, was cooled to 100 ^{o}C and diluted with EtOH (16 mL). The reaction mixture was heated at reflux at 80^{o}C for 30 min and then allowed to cool to 20-25^{o}C. The solids, which remained, were collected by filtration and washed with a 1:1 mixture of EtOH (2.5 mL x 2) and hexanes (2.5 mL x 2) and then washed with hexanes (5 mL x 2). The solid was dried under vacuum at 40 ^{o}C to afford **14a** as a yellow powder (1.7g, 78%): ^{1}H NMR (300 MHz, DMSO) δ 12.63 (s, 1H), 8.68 (s, 1H), 8.21 (d, *J* = 8.0 Hz, 2H), 8.12 (d, *J* = 9.1 Hz, 1H), 7.43 (t, *J* = 7.6 Hz, 2H), 7.16 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (75 MHz, DMSO) δ 162.05, 160.97, 143.55, 140.62, 139.61, 137.56, 129.11, 124.24, 124.12, 118.98, 115.82, 112.62, 106.79, 102.31; HRMS (ESI) m/z calculated for $C_{17}H_{11}D_3N_3O_2$ $(M+H)^+$ 295.1274; found 295.1272; HPLC purity, 99.9%.

7-Methoxy-2-(4-methoxyphenyl)-6-methyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14b). A mixture of ethyl 4-chloro-7-methoxy-8-methylquinoline-3-carboxylate **7g** (2 g, 7.1 mmol), 4-methoxyphenylhydrazine hydrochloride **4d** (1.5 g, 8.6 mmol), triethylamine (1.7 g, 17.2 mmol) and xylenes (16 mL) was heated to reflux (138 ^{o}C) and held at reflux for 2 h. The yellow-orange slurry, which resulted, was cooled to 100 ^{o}C and diluted with EtOH (16 mL). The reaction mixture was heated at reflux at 80^{o}C for 30 min and then allowed to cool to 20-25 ^{o}C. The solids, which remained, were collected by filtration and washed with a 1:1 mixture of EtOH (2.5 mL x 2) and hexanes (2.5 mL x 2) and then washed with hexanes (5 mL x 2). The solid was dried under vacuum at 40 ^{o}C to afford **14b** as a yellow powder (1.7 g, 70%): ^{1}H NMR (300 MHz, DMSO) δ 11.80 (s, 1H), 8.37 (s, 1H), 8.08 (dd, *J* = 8.9, 5.1 Hz, 3H), 7.29 (d, *J* = 9.0 Hz, 1H),

7.01 (d, *J* = 9.1 Hz, 2H), 3.92 (s, 3H), 3.78 (s, 3H), 2.36 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 161.39, 158.24, 156.22, 143.58, 139.11, 135.37, 134.09, 121.18, 120.66, 114.33, 114.25, 112.80, 111.11, 106.53, 56.65, 55.68, 10.03; HRMS (ESI) m/z calculated for $C_{19}H_{18}N_3O_3$ $(M+H)^+$ 336.1343; found 336.1342; HPLC purity, 97.8%.

7-Methoxy-6-methyl-2-phenyl-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14c). A mixture of ethyl 4-chloro-7-methoxy-8-methylquinoline-3-carboxylate **7g** (2 g, 7.1 mmol), phenylhydrazine hydrochloride **4k** (1.2 g, 8.6 mmol), triethylamine (1.7 g, 17.2 mmol) and xylenes (16 mL) was heated to reflux (138 ºC) and held at reflux for 2 h. The yellow-orange slurry, which resulted, was cooled to 100 ºC and diluted with EtOH (16 mL). The reaction mixture was heated at reflux at 80ºC for 30 min and then allowed to cool to 20-25 ºC. The solids, which remained, were collected by filtration and washed with a 1:1 mixture of EtOH (2.5 mL x 2) and hexanes (2.5 mL x 2) and then washed with hexanes (5 mL x 2). The solid was dried under vacuum at 40 ºC to afford **14c** as a yellow powder (2.0 g, 92%): ^{1}H NMR (500 MHz, DMSO) δ 11.86 (s, 1H), 8.42 (s, 1H), 8.22 (d, *J* = 8.2 Hz, 2H), 8.11 (d, *J* = 8.8 Hz, 1H), 7.45 (t, *J* = 7.9 Hz, 2H), 7.33 (d, *J* = 8.9 Hz, 1H), 7.17 (t, *J* = 7.3 Hz, 1H), 3.95 (s, 3H), 2.39 (s, 3H); ^{13}C NMR (126 MHz, DMSO) δ 162.00, 158.41, 144.04, 140.60, 139.43, 135.49, 129.15, 124.29, 121.32, 118.98, 114.42, 112.77, 111.22, 106.48, 56.71, 10.08; HRMS (ESI) m/z calculated for $C_{18}H_{16}N_3O_2$ $(M+H)^+$ 306.1243; found 306.1237; HPLC purity, 99.8%.

7-Methoxy-6-methyl-2-(4-(trifluoromethoxy)phenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14d). A mixture of ethyl 4-chloro-7-methoxy-8-methylquinoline-3-

carboxylate **7g** (2 g, 7.1 mmol), 4-(trifluoromethoxy)phenylhydrazine hydrochloride **4f** (2.0 g, 8.6 mmol), triethylamine (1.7 g, 17.2 mmol) and xylenes (16 mL) was heated to reflux (138 ^{o}C) and held at reflux for 2 h. The yellow-orange slurry, which resulted, was cooled to 100 ^{o}C and diluted with EtOH (16 mL). The reaction mixture was heated at reflux at 80^{o}C for 30 min and then allowed to cool to 20-25 ^{o}C. The solids, which remained, were collected by filtration and washed with a 1:1 mixture of EtOH (2.5 mL x 2) and hexanes (2.5 mL x 2) and then washed with hexanes (5 mL x 2). The solid was dried under vacuum at 40 ^{o}C to afford **14d** as a yellow powder (1.9 g, 97%): ^{1}H NMR (300 MHz, DMSO) δ 11.87 (s, 1H), 8.34 (dd, *J* = 16.0, 11.7 Hz, 3H), 8.07 (d, *J* = 8.7 Hz, 1H), 7.44 (d, *J* = 8.0 Hz, 2H), 7.29 (d, *J* = 8.8 Hz, 1H), 3.92 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 162.09 (s), 158.47 (s), 144.48 (s), 144.41 (s), 139.63 (s), 139.57 (s), 135.45 (s), 121.97 (s), 121.29 (s), 120.66 (q, $^{1}J_{CF}$ = 256.6 Hz), 120.09 (s), 114.44 (s), 112.60 (s), 111.22 (s), 106.09 (s), 56.64 (s), 10.01 (s); HRMS (ESI) m/z calculated for $C_{19}H_{15}F_3N_3O_3$ $(M+H)^+$ 390.1066; found 390.1068; HPLC purity, 99.9%.

7-Methoxy-2-(4-(trifluoromethoxy)phenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one 14e). A mixture of ethyl-4-chloro-7-methoxyquinoline-3-carboxylate **7e** (2 g, 7.5 mmol), 4-(trifluoromethoxy)phenylhydrazine hydrochloride **4f** (2.1 g, 9.0 mmol), triethylamine (1.8, 18.1 mmol) and xylenes (16 mL) was heated to reflux (138 ^{o}C) and held at reflux for 2 h. The yellow-orange slurry, which resulted, was cooled to 100 ^{o}C and diluted with EtOH (16 mL). The reaction mixture was heated at reflux at 80^{o}C for 30 min and then allowed to cool to 20-25 ^{o}C. The solids, which remained, were collected by filtration and washed with a 1:1 mixture of EtOH (2.5 mL x 2) and hexanes (2.5 mL x 2) and then washed with hexanes (5 mL x 2). The solid was dried under vacuum at 40 ^{o}C to afford **14e** as a yellow powder (1.9 g, 67%)**:** ^{1}H NMR (300

MHz, DMSO) δ 12.71 (s, 1H), 8.71 (s, 1H), 8.33 (s, 2H), 8.12 (s, 1H), 7.45 (s, 2H), 7.18 (s, 2H), 3.88 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 162.18, 161.10, 144.50, 144.48, 143.97, 139.93, 139.60, 137.61, 124.15, 121.98, 120.12, 115.95, 112.52, 106.45, 102.37, 56.00; HRMS (ESI) m/z calculated for $C_{18}H_{13}F_3N_3O_3$ $(M+H)^+$ 376.0909; found 376.0914; HPLC purity, 99.9%.

8-Bromo-6-fluoro-2-(4-methoxyphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14f). A mixture of ethyl 6-bromo-4-chloro-8-fluoroquinoline-3-carboxylate **7h** (0.5 g, 1.5 mmol), (4-methoxyphenyl)hydrazine hydrochloride **4d** (0.31 g,1.8 mmol)**,** and triethylamine (2 mL) in xylenes (8 mL) was heated to reflux (138ºC) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was washed several times with hexane and water. Then, the solid was dissolved in a basic solution of 3 N NaOH and stirred for 15 min. The basic solution was brought to pH = 7 (pH paper) with 3 N HCl and filtered. The solid was recrystallized using hot ethanol and dried in vacuo to afford a yellow solid **14f**, (0.28 g, 48%)**:** mp 333-334°C; ^{1}H NMR (300 MHz, DMSO) δ 13.05 (s, 1H), 8.51 (s, 1H), 8.09 (s, 1H), 8.03 (d, *J* = 8.9 Hz, 2H), 7.89 (d, *J* = 10.5 Hz, 1H), 7.01 (d, *J* = 9.0 Hz, 2H), 3.78 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 161.11 (s), 156.65 (s), 152.72 (d, $^1J_{CF}$ = 254.3 Hz), 140.93 (s), 139.49 (s), 133.57 (s), 124.28 (d, $^2J_{CF}$ = 13.7 Hz), 122.02 (s), 121.00 (s), 120.62 (d, $^3J_{CF}$ = 2.4 Hz), 118.96 (d, $^2J_{CF}$ = 20.5 Hz), 118.28 (d, $^3J_{CF}$ = 9.0 Hz), 114.32 (s), 107.83 (s), 55.73 (s); HRMS (ESI) m/z calculated for $C_{17}H_{12}BrFN_3O_2$ $(M+H)^+$ 388.0097; found 388.0094; HPLC purity, 98.4%.

8-Bromo-2-(4-chlorophenyl)-6-fluoro-2,5-dihydro-3H-pyrazolo[4,3-*c*]quinolin-3-one (14g). A mixture of ethyl 6-bromo-4-chloro-8-fluoroquinoline-3-carboxylate **7h** (0.2 g, 0.64 mmol), (4-chlorophenyl)hydrazine hydrochloride **4g** (0.18 g, 1.2 mmol), and triethylamine (2 mL) in

xylenes (8 mL) was heated to reflux (138ºC) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was collected by filtration and washed with hexane and water. The solid was dissolved in DMSO (10mL). The solution was then poured into H_2O (30mL), and filtered in order to remove the triethylamine hydrochloride salt. After this, a recrystallization using EtOH (15mL) and H_2O (2mL) was employed to afford yellow crystals **14g**, (0.17 g, 70%)**:** ^{1}H NMR (300 MHz, DMSO) δ 12.96 (s, 1H), 8.49 (s, 1H), 8.18 (d, *J* = 8.4 Hz, 2H), 8.04 (s, 1H), 7.86 (d, *J* = 10.5 Hz, 1H), 7.46 (d, *J* = 8.5 Hz, 2H); ^{13}C NMR (75 MHz, DMSO) δ 161.66 (s), 152.70 (d, $^1J_{CF}$ = 254.4 Hz), 141.68 (s), 139.92 (s), 138.99 (s), 129.07 (s), 128.48 (s), 124.50 (d, $^2J_{CF}$ = 13.3 Hz), 121.86 (d, $^4J_{CF}$ = 3.3 Hz), 120.70 (d, $^3J_{CF}$ = 3.6 Hz), 120.35 (s), 119.19 (d, $^2J_{CF}$ = 20.3 Hz), 118.40 (d, $^3J_{CF}$ = 9.3 Hz), 107.55 (s); HRMS (ESI) m/z calculated for $C_{16}H_9BrClFN_3O$ $(M+H)^+$ 391.9596; found 391.9599; HPLC purity, 99.9%.

8-Bromo-6-fluoro-2-(4-fluorophenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14h). A mixture of ethyl 6-bromo-4-chloro-8-fluoroquinoline-3-carboxylate **7h** (0.2 g, 0.64 mmol), (4-fluorophenyl)hydrazine hydrochloride **4h** (0.22 g, 1.3 mmol) and triethylamine (2 mL) in xylenes (8mL) was heated to reflux (138ºC) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was collected by filtration and washed with hexane and water. After this, a recrystallization using EtOH (15mL) and H_2O (2mL) was employed to afford yellow crystals **14h**, (0.120 g, 50 %)**:** ^{1}H NMR (300 MHz, DMSO) δ 12.99 (s, 1H), 8.50 (s, 1H), 8.28 – 8.10 (m, 2H), 8.05 (s, 1H), 7.88 (d, *J* = 10.4 Hz, 1H), 7.27 (t, *J* = 8.7 Hz, 2H); ^{13}C NMR (75 MHz, DMSO) δ 161.45 (s), 159.29 (d, $^1J_{CF}$ = 241.3 Hz), 152.72 (d, $^1J_{CF}$ = 255.0 Hz), 141.38 (d, $^4J_{CF}$ = 2.5 Hz), 139.88 (d, $^4J_{CF}$ = 2.8 Hz), 136.64 (d, $^4J_{CF}$ = 2.3 Hz), 124.42 (d, $^2J_{CF}$ = 12.4 Hz), 121.95 (d, $^4J_{CF}$ = 3.0 Hz), 120.96 (d, $^3J_{CF}$ = 8.0 Hz), 120.69 (d, $^3J_{CF}$ = 3.1 Hz), 119.17 (d, $^2J_{CF}$ =

22.2 Hz), 118.40 (d, $^{3}J_{CF}$ = 8.9 Hz), 115.84 (d, $^{2}J_{CF}$ = 22.3 Hz), 107.65 (s); HRMS (ESI) m/z calculated for $C_{16}H_9BrF_2N_3O$ $(M+H)^+$ 375.9892; found 375.9891; HPLC purity, 99.9%.

2-(4-Methoxyphenyl)-7-(trifluoromethyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14i). A mixture of ethyl 4-chloro-7-(trifluoromethyl)quinoline-3-carboxylate **7i** (0.5 g, 1.5 mmol), (4-methoxyphenyl)hydrazine hydrochloride **4d** (0.57 g, 1.8 mmol), and triethylamine (2 mL) in xylenes (8mL) was heated to reflux (138ºC) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was collected by filtration and washed with hexane and water. After this, a recrystallization using EtOH (15mL) and H_2O (2mL) was employed to afford yellow crystals **14i**, (0.51 g, 82 %)**:** mp 315 – 316°C; ^{1}H NMR (300 MHz, DMSO) δ 12.93 (s, 1H), 8.84 (s, 1H), 8.40 (d, J = 8.4 Hz, 1H), 8.08 (d, J = 9.1 Hz, 2H), 8.03 (s, 1H), 7.83 (d, J = 8.1 Hz, 1H), 7.03 (d, J = 9.1 Hz, 2H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 161.38 (s), 156.60 (s), 141.99 (s), 140.79 (s), 135.83 (s), 133.75 (s), 130.02 (q, $^{2}J_{CF}$ = 32.5 Hz), 124.15 (q, $^{1}J_{CF}$ = 272.7 Hz), 124.05 (s), 122.66 (q, $^{3}J_{CF}$ = 3.3 Hz), 122.12 (s), 121.00 (s), 117.21 (q, $^{3}J_{CF}$ = 4.2 Hz), 114.34 (s), 107.42 (s), 55.71 (s); HRMS (ESI) m/z calculated for $C_{18}H_{13}F_3N_3O_2$ $(M+H)^+$ 360.0954; found 360.0956; HPLC purity, 99.9%.

2-(4-Chlorophenyl)-7-(trifluoromethyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14j). A mixture of ethyl 4-chloro-7-(trifluoromethyl)quinoline-3-carboxylate **7i** (0.5 g, 1.6 mmol), (4-chlorophenyl)hydrazine hydrochloride **4g** (0.47 g, 3.2 mmol), and triethylamine (2 mL) in xylenes (8mL) was heated to reflux (138ºC) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was collected by filtration and washed with hexane and water. After this, a recrystallization using EtOH (15mL) and H_2O (2mL) was employed to afford yellow crystals **14j**, (0.44 g, 75%)**:** mp 346 – 347°C; ^{1}H NMR (300 MHz, DMSO) δ 12.98 (s, 1H), 8.84 (s, 1H), 8.34

(d, J = 8.3 Hz, 1H), 8.21 (d, J = 8.9 Hz, 2H), 7.98 (s, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.47 (d, J = 8.9 Hz, 2H); ^{13}C NMR (75 MHz, DMSO) δ 161.92 (s), 142.71 (s), 141.03 (s), 139.11 (s), 135.84 (s), 130.29 (q, $^{2}J_{CF}$ = 32.5 Hz), 129.08 (s), 128.45 (s), 124.11 (s), 124.08 (q, $^{1}J_{CF}$ = 272.8 Hz), 122.73 (q, $^{3}J_{CF}$ = 3.8 Hz), 121.92 (s), 120.40 (s), 117.15 (q, $^{3}J_{CF}$ = 4.2 Hz), 107.15 (s); HRMS (ESI) m/z calculated for $C_{17}H_{10}ClF_3N_3O$ $(M+H)^+$ 364.0459; found 364.0453; HPLC purity, 99.8%.

2-(4-Nitrophenyl)-7-(trifluoromethyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14k). A mixture of ethyl 4-chloro-7-(trifluoromethyl)quinoline-3-carboxylate **7i** (1.0 g, 3.3 mmol), (4-nitrophenyl)hydrazine hydrochloride **4i** (0.75 g, 3.9 mmol), and triethylamine (0.80g, 7.9 mmol) in xylenes (8 mL) was heated to reflux (138 ^{o}C) and held at reflux for 2 h. The yellow-orange slurry, which resulted, was cooled to 100 ^{o}C and diluted with EtOH (8 mL). The reaction mixture was heated at reflux at 80^{o}C for 30 min and then allowed to cool to 20-25 ^{o}C. The solids, which remained, were collected by filtration and washed with a 1:1 mixture of EtOH (2.5 mL x 2) and hexanes (2.5 mL x 2) and then washed with hexanes (5 mL x 2). The solid was dried under vacuum at 40 ^{o}C to afford **14k** as a yellow powder (0.7 g, 57%)**:** mp > 350 °C; ^{1}H NMR (300 MHz, DMSO) δ 13.73 (s, 1H), 8.74 (s, 1H), 8.35 (d, *J* = 9.0 Hz, 2H), 8.24 (dd, *J* = 17.0, 8.7 Hz, 3H), 8.11 (s, 1H), 7.75 (d, *J* = 8.3 Hz, 1H); ^{13}C NMR (75 MHz, DMSO) δ 162.70 (s), 145.21 (s), 144.09 (s), 142.95 (s), 141.13 (s), 136.15 (s), 130.50 (q, $^{2}J_{CF}$ = 33.2 Hz), 125.16 (s), 124.14 (s), 123.99 (q, $^{1}J_{CF}$ = 272.3 Hz), 122.78 (q, $^{3}J_{CF}$ = 3.7 Hz), 121.66 (s), 118.06 (s), 117.31 (q, $^{3}J_{CF}$ = 3.7 Hz), 106.46 (s); HRMS (ESI) m/z calculated for $C_{17}H_{10}F_3N_4O_3$ $(M+H)^+$ 375.0700; found 375.0706; HPLC purity, 99.9%.

2-(4-(Trifluoromethoxy)phenyl)-7-(trifluoromethyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one 14l). A mixture of ethyl 4-chloro-7-(trifluoromethyl)-quinoline-3-carboxylate

7i (0.3 g, 1 mmol), (4-(trifluoromethoxy)phenyl)hydrazine hydrochloride **4f** (0.48 g, 2 mmol), and triethylamine (2 mL) in xylenes (8 mL) of xylene was heated to reflux (138^{o}C) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was washed several times with hexane and water. Then, the solid was dissolved in a basic solution of 3 N NaOH and stirred for 15 min. The basic solution was brought to pH = 7 (pH paper) with 3 N aq HCl and filtered. The solid was recrystallized using hot ethanol and dried in vacuo to afford a yellow solid **14l**, (0.25 g, 60%)**:** mp 286 – 287°C; ^{1}H NMR (300 MHz, DMSO) δ 12.94 (s, 1H), 8.86 (s, 1H), 8.36 (d, *J* = 8.4 Hz, 1H), 8.29 (d, *J* = 9.0 Hz, 2H), 7.99 (s, 1H), 7.81 (d, *J* = 8.3 Hz, 1H), 7.43 (d, *J* = 8.8 Hz, 2H); ^{13}C NMR (75 MHz, DMSO) δ 161.98 (s), 144.85 (s), 142.85 (s), 141.22 (s), 139.27 (s), 135.96 (s), 130.33 (q, $^{2}J_{CF}$ = 33.0 Hz), 124.12 (s), 124.09 (q, $^{1}J_{CF}$ = 272.4 Hz), 122.78 (s), 122.29 (q, $^{3}J_{CF}$ = 3.2 Hz), 121.98 (s), 120.64 (q, $^{1}J_{CF}$ = 255.2 Hz), 120.37 (s), 117.26 (q, $^{3}J_{CF}$ = 5.3 Hz), 107.07 (s); HRMS (ESI) m/z calculated for $C_{18}H_{10}F_6N_3O_2$ $(M+H)^+$ 414.0672; found 414.0674; HPLC purity, 99.7%.

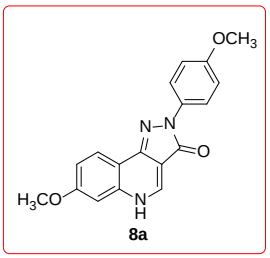
2-(4-Fluorophenyl)-7-(trifluoromethyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14m). A mixture of ethyl 4-chloro-7-(trifluoromethyl)quinoline-3-carboxylate **7i** (0.2 g, 0.66 mmol), (4-fluorophenyl)hydrazine hydrochloride **4h** (0.22 g, 1.3 mmol), and triethylamine (2 mL) in xylenes (8 mL) of xylene was heated to reflux (138^{o}C) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was collected by filtration and washed with hexane and water. After this, a recrystallization using EtOH (15mL) and H_2O (2mL) was employed to afford yellow crystals **14m**, (0.15 g, 65%) which were finally obtained by recrystallization from hot EtOH**:** mp 296 – 297 °C; ^{1}H NMR (300 MHz, DMSO) δ 12.98 (s, 1H), 8.84 (s, 1H), 8.36 (d, *J* = 8.3 Hz, 1H), 8.19 (dd, *J* = 9.0, 5.1 Hz, 2H), 8.00 (s, 1H), 7.81 (d, *J* = 8.4 Hz, 1H), 7.27 (t, *J* =

8.9 Hz, 2H); ^{13}C NMR (75 MHz, DMSO) δ 161.69 (s), 159.26 (d, $^{1}J_{CF}$ = 241.2 Hz), 142.41 (s), 140.94 (s), 136.76 (d, $^{3}J_{CF}$ = 2.5 Hz), 135.78 (s), 130.21 (q, $^{2}J_{CF}$ = 32.4 Hz), 124.09 (q, $^{1}J_{CF}$ = 272.3 Hz), 124.07 (s), 122.73 (q, $^{3}J_{CF}$ = 3.0 Hz), 121.98 (d, $^{4}J_{CF}$ = 0.6 Hz), 120.97 (d, $^{3}J_{CF}$ = 7.9 Hz), 117.14 (q, $^{3}J_{CF}$ = 3.9 Hz), 115.80 (d, $^{2}J_{CF}$ = 22.4 Hz), 107.21 (s); HRMS (ESI) m/z calculated for $C_{17}H_{10}F_4N_3O$ $(M+H)^+$ 348.0755; found 348.0766; HPLC purity, 99.8%.

2-(3-Methoxyphenyl)-7-(trifluoromethyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (14n). A mixture of ethyl 4-chloro-7-(trifluoromethyl)quinoline-3-carboxylate **7i** (0.5 g, 1.6 mmol), (3-methoxyphenyl)hydrazine hydrochloride **4e** (0.575 g, 4.1 mmol), and triethylamine (2 mL) in xylenes (15mL) was heated to reflux (138^{o}C) for 4 hr. The reaction was cooled to rt and filtered. The solid, which formed, was collected by filtration and washed with hexane and water. After this, a recrystallization using EtOH (15mL) and H_2O (2mL) was employed to afford yellow crystals **14n**, (0.762 g, 46 %)**:** mp > 350 °C; ^{1}H NMR (300 MHz, DMSO) δ 12.94 (s, 1H), 8.84 (s, 1H), 8.40 (d, *J* = 8.3 Hz, 1H), 8.02 (s, 1H), 7.89 – 7.71 (m, 3H), 7.35 (t, *J* = 8.2 Hz, 1H), 6.78 (dd, *J* = 8.2, 1.9 Hz, 1H), 3.81 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 161.97 (s), 159.98 (s), 142.41 (s), 141.40 (s), 140.97 (s), 135.93 (s), 130.23 (q, $^{2}J_{CF}$ = 31.5 Hz), 130.06 (s), 124.18 (s), 124.11 (q, $^{1}J_{CF}$ = 270.8 Hz), 122.75 (q, $^{3}J_{CF}$ = 3.8 Hz), 122.04 (s), 117.21 (q, $^{3}J_{CF}$ = 4.4 Hz), 111.50 (s), 110.14 (s), 107.40 (s), 105.01 (s), 55.58 (s); HRMS (ESI) m/z calculated for $C_{18}H_{13}F_3N_3O_2$ $(M+H)^+$ 360.0954; found 360.0958; HPLC purity, 99.6%.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8a**
Concentration: 10 µM
Date: 02-16-2015
Facility: Milwaukee Institute for Drug Discovery



8a

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in Acetonitrile.
   b. 0.5 µM Verapamil in Acetonitrile (ACN) as internal standard (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 48.75 µL of MAM into separate Eppendorf 1.5 mL vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes should be added to all time points except the zero time point.
6. For each time point add 1.25 µL microsomes (Final concentration of 0.5 mg/mL) and place in the incubator (37 °C) and record the time.
7. At the end of each time interval add 100 µL of ice cold Verapamil solution in ACN and sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 500 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	33951	601221	32355	541076	43992	555548	100.83	99.66	100.23
10	42583	640770	15626	226765	42530	564189	118.67	114.84	95.42
20	38151	561437	41959	594788	46868	582371	121.34	117.57	101.87
30	40481	622191	28975	614244	42095	608270	116.18	78.62	87.60
40	37748	595368	36594	602489	41311	584370	113.21	101.23	89.48
50	33524	640546	38790	658705	36637	554181	93.45	98.14	83.68
60	25781	629252	38607	576764	39742	602548	73.16	99.66	83.48

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	226304	837974	251250	676349	221151	719623	100.02	100.12	100.10
10	222165	845586	197063	750089	248601	823455	97.30	70.81	98.33
20	222847	820071	215134	732628	230949	796583	100.64	79.15	94.43
30	229722	686761	240649	736344	239432	735694	123.88	88.09	106.01
40	182530	767280	224208	775342	244256	778429	88.10	77.94	102.20
50	178928	748120	181502	692046	161580	705574	88.58	70.69	74.59
60	202738	761042	225933	676351	190088	732573	98.66	90.04	84.52

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (PZ-II-029)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

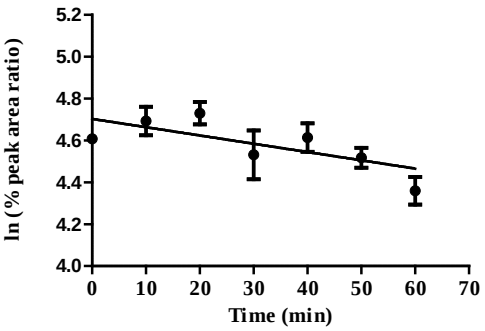
Day 1

Linear regression analysis:

Slope: -0.003961 ± 0.001458

At X= 60, Y = 4.465 ± 0.05385

R^2 = 0.2909



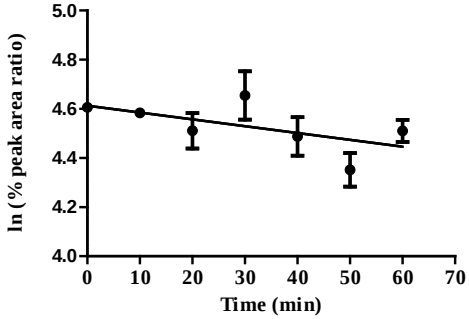
Day 2

Linear regression analysis:

Slope: -0.02768 ± 0.00138

At X = 60, Y = 4.446 ± 0.04892

R^2 = 0.1807



Metabolic Parameters:

Half-life: 175 min

V_d: 100 µL/mg

Intrinsic clearance: 0.4 µL/min/mg

Metabolic Rate: 8 nmol/min/mg

% remaining at 60 min: 87 ± 1.0 %

Metabolic Parameters:

Half-life: 250 min

V_d: 100 µL/mg

Intrinsic clearance: 0.277 µL/min/mg

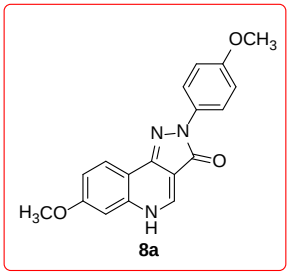
Metabolic Rate: 5.6 nmol/min/mg

% remaining at 60 min: 85.2 ± 1.0 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8a**
Concentration: 10 µM
Date: 04-08-2016
Facility: Milwaukee Institute for Drug Discovery



8a

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM Verapamil in Acetonitrile (ACN) as internal standard (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	447665	859377	462433	863304	455184	863057	99.98	99.93	100.07
10	432708	843709	436278	848682	438533	859089	98.43	95.90	96.86
20	414199	856316	434993	857592	430385	861791	92.84	94.63	94.76
30	397811	862960	404695	858362	401549	865308	88.48	87.96	88.05
40	383712	861536	396789	858415	389825	856243	85.48	86.23	86.39
50	364496	848736	391027	856027	379552	864618	82.42	85.22	83.29
60	375359	853833	383683	870545	376641	877200	84.37	82.22	81.47

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	PZ-II-029 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	505767	866531	461889	859715	473978	852607	99.94	100.04	99.98
10	485169	856481	449512	852390	461313	862061	96.99	98.20	96.24
20	455571	855136	424509	861092	444556	853068	91.22	91.80	93.72
30	434369	864280	404785	855574	412379	864451	86.05	88.10	85.79
40	423412	862610	394944	852059	407802	865784	84.05	86.31	84.71
50	394408	857542	382714	861341	396913	870869	78.75	82.74	81.97
60	403745	856301	375242	858103	397021	854640	80.73	81.43	83.55

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (PZ-II-029)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

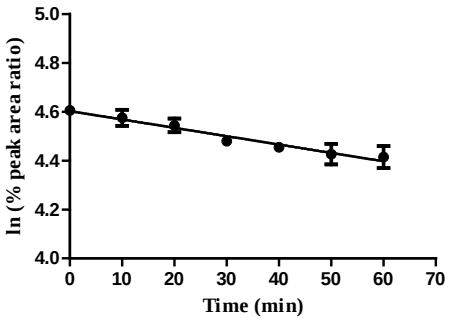
Day 1

Linear regression analysis:

Slope: -0.003424 ± 0.0001769

At X= 60, Y = 4.397 ± 0.006377

R^2 = 0.9517



Metabolic Parameters:

Half-life: 202.39 ± 10.45 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3424 µL/min/mg

Metabolic Rate: 6.848 nmol/min/mg

% remaining at 60 min: 81.20 ± 0.12 %

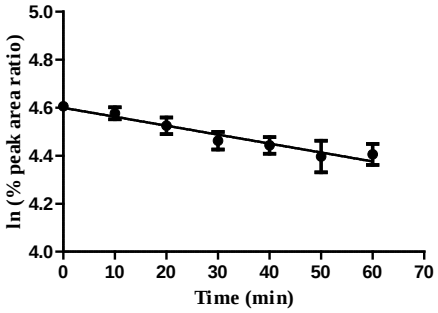
Day 2

Linear regression analysis:

Slope: -0.003723 ± 0.0002476

At X = 60, Y = 4.376 ± 0.008927

R^2 = 0.9225



Metabolic Parameters:

Half-life: 186.14 ± 12.37 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3723 µL/min/mg

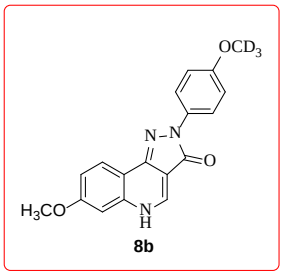
Metabolic Rate: 7.446 nmol/min/mg

% remaining at 60 min: 79.51 ± 0.16 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8b**
Concentration: 10 µM
Date: 05-04-2016
Facility: Milwaukee Institute for Drug Discovery



8b

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM 4,5 Diphenyl Imidazole ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	398212	2354869	384127	2302725	368877	2255486	100.06	99.88	99.72
10	361492	2153664	350612	2137443	364427	2307335	99.31	98.22	96.30
20	353751	2156111	351711	2207523	349893	2196606	97.08	95.40	97.12
30	353643	2216473	345716	2211492	331654	2156417	94.41	93.60	93.78
40	354234	2233525	343748	2144927	339505	2163298	93.84	95.96	95.69
50	336838	2133427	333079	2140599	328962	2168061	93.42	93.17	92.51
60	327874	2121028	353537	2272689	327292	2208601	91.46	93.14	90.36

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	387196	2270706	377106	2186412	386760	2248705	99.71	100.27	99.99
10	362459	2137087	356334	2114930	366413	2165630	99.18	97.95	98.36
20	364780	2211877	350046	2137002	370479	2212311	96.44	95.23	97.36
30	363646	2245148	349102	2151510	357194	2208806	94.71	94.33	94.02
40	340384	2162279	352923	2200550	350775	2182739	92.05	93.24	93.43
50	344590	2126528	347741	2190384	353240	2162260	94.76	92.30	94.98
60	349875	2221871	353066	2202566	336952	2140953	92.08	93.19	91.50

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-56-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

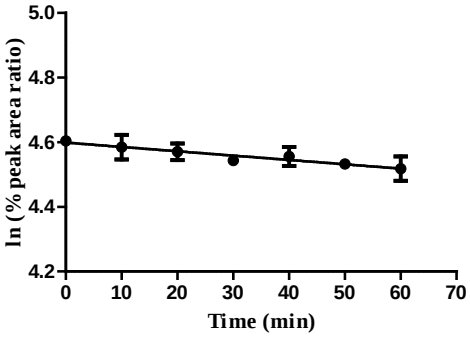
Day 1

Linear regression analysis:

Slope: -0.001342 ± 0.0001303

At X= 60, Y = 4.518 ± 0.004697

R^2 = 0.8481



Metabolic Parameters:

Half-life: 516.39 ± 50.1 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1342 µL/min/mg

Metabolic Rate: 2.684 nmol/min/mg

% remaining at 60 min: 91.65 ± 0.14 %

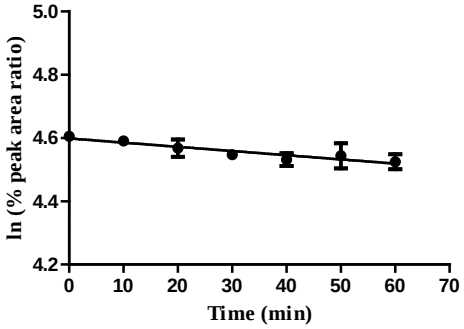
Day 2

Linear regression analysis:

Slope: -0.001326 ± 0.0001349

At X = 60, Y = 4.519 ± 0.004865

R^2 = 0.8357



Metabolic Parameters:

Half-life: 522.62 ± 53.16 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1326 µL/min/mg

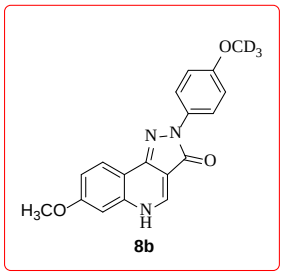
Metabolic Rate: 2.652 nmol/min/mg

% remaining at 60 min: 91.74 ± 0.10 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8b**
Concentration: 10 µM
Date: 05-04-2016
Facility: Milwaukee Institute for Drug Discovery



8b

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM 4,5 Diphenyl Imidazole ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	388928	2307698	391664	2332218	402950	2417074	99.72	99.96	99.82
10	379319	2277526	364953	2238315	377640	2272011	98.55	97.05	99.52
20	371987	2305299	350938	2182276	355730	2231217	95.48	95.72	95.46
30	360570	2221865	344748	2175396	367885	2328394	96.02	94.33	94.61
40	355823	2247913	351540	2227323	352866	2210036	93.66	93.94	95.60
50	342826	2151478	363128	2309151	328306	2119895	94.28	93.60	92.73
60	351429	2183246	346906	2233889	348682	2236280	95.24	92.43	93.36

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	DK-I-56-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	401832	2332034	399175	2395303	404444	2394973	100.18	99.79	99.92
10	398950	2326501	371579	2259018	381853	2286061	99.69	98.49	98.83
20	371128	2261581	372242	2319773	354429	2160499	95.40	96.08	97.07
30	365461	2192182	356359	2264013	354905	2197136	96.92	94.25	95.58
40	367394	2228779	341564	2141303	348008	2204773	95.83	95.51	93.39
50	355291	2187107	349208	2216275	354062	2198066	94.44	94.35	95.31
60	355799	2211839	350652	2298823	349085	2187612	93.52	91.33	94.42

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-56-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

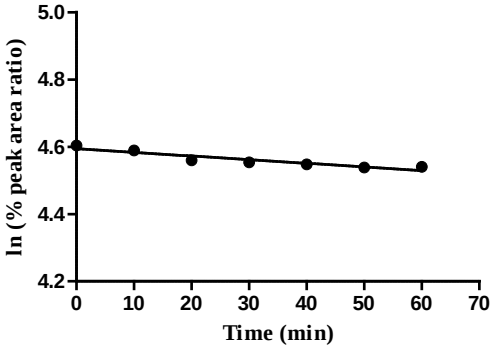
Day 1

Linear regression analysis:

Slope: -0.001081 ± 0.0001303

At X= 60, Y = 4.529 ± 0.004696

R^2 = 0.7838



Metabolic Parameters:

Half-life: 641.07 ± 77 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1081 µL/min/mg

Metabolic Rate: 2.162 nmol/min/mg

% remaining at 60 min: 92.66 ± 0.10 %

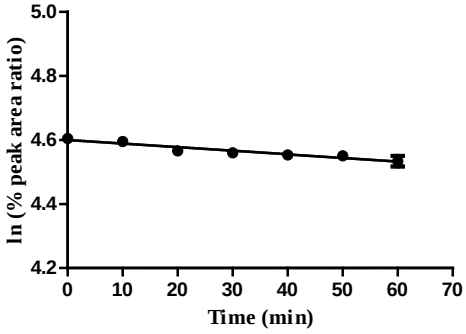
Day 2

Linear regression analysis:

Slope: -0.001124 ± 0.0001253

At X = 60, Y = 4.532 ± 0.004518

R^2 = 0.8089



Metabolic Parameters:

Half-life: 616.54 ± 69 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1124 µL/min/mg

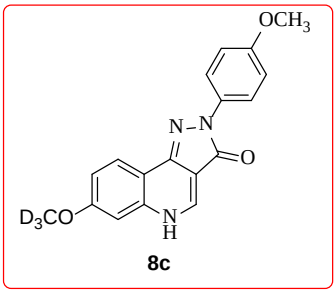
Metabolic Rate: 2.248 nmol/min/mg

% remaining at 60 min: 92.94 ± 0.09 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8c**
Concentration: 10 µM
Date: 06-17-2017
Facility: Milwaukee Institute for Drug Discovery



8c

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM 4,5 Diphenyl Imidazole in Acetonitrile as internal standard (ISTD) (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-3-1 Peak area	ISTD Peak area	DK-II-3-1 Peak area	ISTD Peak area	DK-II-3-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	236193	23908	240718	23550	242866	23587	100.00	99.99	99.99
10	232747	23689	228301	22980	236745	24005	99.45	97.19	95.77
20	220288	23974	235154	24176	220725	23502	93.01	95.15	91.20
30	229623	23893	230097	23822	231957	23406	97.28	94.49	96.24
40	226681	23894	228169	23790	232302	23693	96.03	93.82	95.21
50	226106	24008	224076	23499	222828	22836	95.33	93.28	94.76
60	209034	22893	224075	23243	222144	23009	92.42	94.31	93.76

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-3-1 Peak area	ISTD Peak area	DK-II-3-1 Peak area	ISTD Peak area	DK-II-3-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	240293	23322	245499	23714	238099	23446	100.00	100.00	100.00
10	237690	23842	236607	23170	240575	24206	96.76	98.64	97.87
20	231496	23497	224523	23336	233912	23968	95.62	92.94	96.10
30	224366	22966	240144	24028	228386	23461	94.82	96.54	95.86
40	227905	24075	229073	23639	210756	22679	91.88	93.61	91.51
50	228544	23641	230508	23626	221648	23188	93.83	94.24	94.12
60	218997	23816	228654	24088	219423	22930	89.24	91.69	94.23

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-3-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

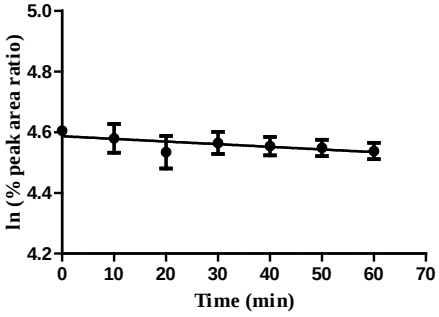
Day 1

Linear regression analysis:

Slope: -0.0008726 ± 0.0002206

At X= 60, Y = 4.534 ± 0.007955

R^2 = 0.4515



Metabolic Parameters:

Half-life: 794 ± 200 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08726 µL/min/mg

Metabolic Rate: 1.7452 nmol/min/mg

% remaining at 60 min: 93 ± 0.16 %

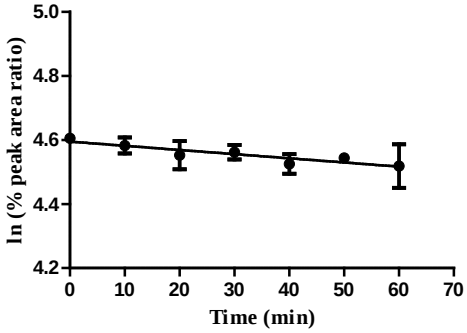
Day 2

Linear regression analysis:

Slope: -0.001302± 0.0001877

At X = 60, Y = 4.517 ± 0.006767

R^2 = 0.7171



Metabolic Parameters:

Half-life: 532 ± 76 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1302 µL/min/mg

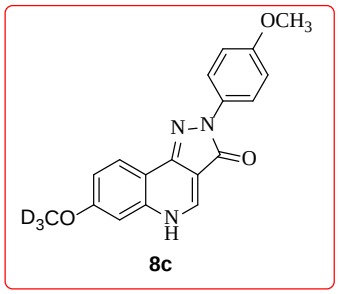
Metabolic Rate: 2.604 nmol/min/mg

% remaining at 60 min: 91 ± 0.13 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8c**
Concentration: 10 µM
Date: 04-08-2016
Facility: Milwaukee Institute for Drug Discovery



8c

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM Verapamil in Acetonitrile (ACN) as internal standard (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-3-1 Peak area	Verapamil Peak area	DK-II-3-1 Peak area	Verapamil Peak area	DK-II-3-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	111144	278595	98899	285987	104065	286966	99.98	99.94	99.90
10	107949	280252	95360	278895	99077	282663	96.53	98.82	96.56
20	106291	287540	92029	276919	96414	278874	92.64	96.05	95.24
30	104270	279360	91729	287346	97728	287461	93.54	92.26	93.65
40	101471	270388	93541	288645	96863	279779	94.05	93.66	95.37
50	105186	285058	92155	279402	94564	282182	92.48	95.32	92.31
60	106026	279377	95346	285213	97167	279799	95.11	96.61	95.66

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-3-1 Peak area	Verapamil Peak area	DK-II-3-1 Peak area	Verapamil Peak area	DK-II-3-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	92576	281935	98496	277116	100164	285161	100.11	100.12	100.07
10	92661	292879	95851	279035	96979	287881	96.45	96.76	95.97
20	89484	287644	97725	295691	83913	248471	94.84	93.09	96.21
30	91955	299743	96183	282336	95556	290799	93.53	95.96	93.61
40	90362	289887	94361	279974	97195	299641	95.03	94.93	92.41
50	87875	277434	92404	278043	93112	274697	96.56	93.61	96.57
60	88889	287296	92451	285458	92002	279777	94.32	91.23	93.68

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-3-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

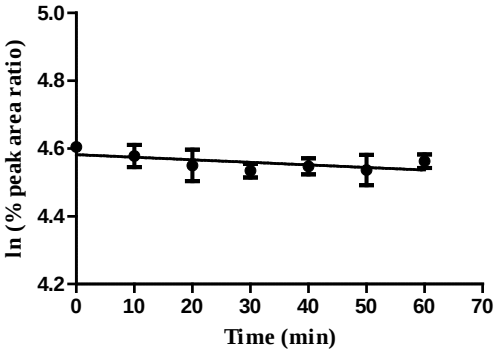
Day 1

Linear regression analysis:

Slope: -0.0007583 ± 0.0002330

At X= 60, Y = 4.536 ± 0.008400

R^2 = 0.3580



Metabolic Parameters:

Half-life: 913.88 ± 280.85 min

V_d: 100 µL/mg

Intrinsic clearance: 0.07583 µL/min/mg

Metabolic Rate: 1.5166 nmol/min/mg

% remaining at 60 min: 93.31 ± 0.17 %

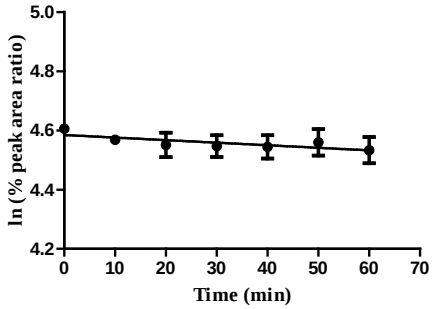
Day 2

Linear regression analysis:

Slope: -0.0008643± 0.0002043

At X = 60, Y = 4.533 ± 0.007365

R^2 = 0.4852



Metabolic Parameters:

Half-life: 801.80 ± 189.52 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08643 µL/min/mg

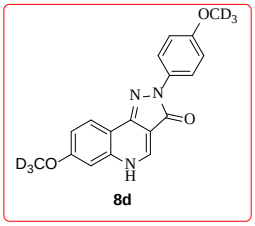
Metabolic Rate: 1.7286 nmol/min/mg

% remaining at 60 min: 93.03 ± 0.15 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8d**
Concentration: 10 µM
Date: 02-22-2016
Facility: Milwaukee Institute for Drug Discovery



8d

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM Verapamil in Acetonitrile (ACN) as internal standard (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	119623	751693	123300	762556	120046	764558	100.08	99.19	100.00
10	114114	768963	114322	764031	110461	740708	93.33	91.79	94.98
20	110327	769408	115777	753029	106689	743037	90.18	94.32	91.45
30	112935	756232	115004	757564	112745	750648	93.92	93.13	95.66
40	110428	744917	111041	740898	108647	736750	93.23	91.94	93.92
50	109616	758076	116028	753982	106766	745883	90.94	94.40	91.17
60	106932	741303	115257	745460	111099	754822	90.72	94.85	93.74

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	124924	767904	102412	749745	117099	759194	99.80	99.70	100.15
10	115805	748997	100631	765396	109568	740171	94.85	95.96	96.12
20	108862	713542	99251	769134	105247	741358	93.59	94.19	92.18
30	110702	750240	98189	771121	104484	736667	90.52	92.94	92.10
40	114315	765884	98401	763993	103824	742724	91.57	94.01	90.77
50	104617	699417	98970	776043	108827	751806	91.76	93.08	93.99
60	109088	730464	100918	777766	109146	763644	91.62	94.71	92.81

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-60-3)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

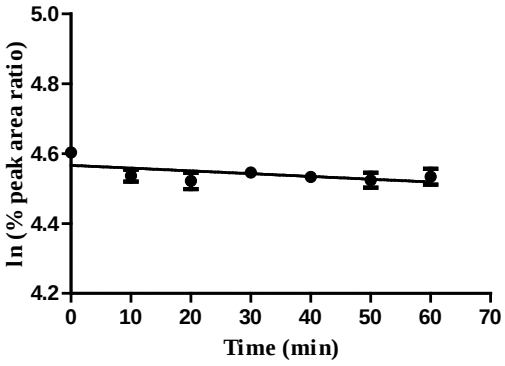
Day 1

Linear regression analysis:

Slope: -0.0007917 ± 0.0002839

At X= 60, Y = 4.519 ± 0.01023

R^2 = 0.2905



Metabolic Parameters:

Half-life: 875.33 ± 313 min

V_d: 100 µL/mg

Intrinsic clearance: 0.07917 µL/min/mg

Metabolic Rate: 1.5834 nmol/min/mg

% remaining at 60 min: 91.74 ± 0.20 %

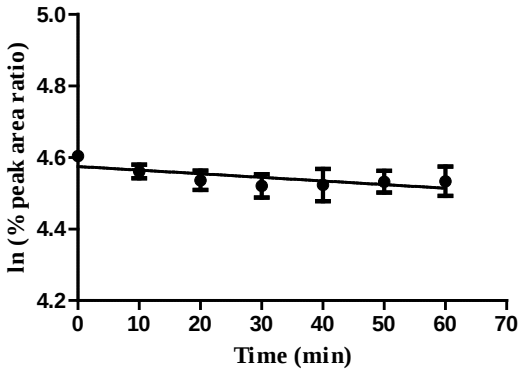
Day 2

Linear regression analysis:

Slope: -0.001010 ± 0.0002414

At X = 60, Y = 4.514 ± 0.008704

R^2 = 0.4793



Metabolic Parameters:

Half-life: 686.13 ± 164 min

V_d: 100 µL/mg

Intrinsic clearance: 0.101 µL/min/mg

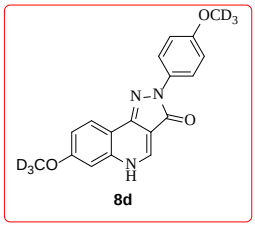
Metabolic Rate: 2.02 nmol/min/mg

% remaining at 60 min: 91.28 ± 0.17 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8d**
Concentration: 10 µM
Date: 02-22-2016
Facility: Milwaukee Institute for Drug Discovery



8d

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM Verapamil in Acetonitrile (ACN) as internal standard (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	106722	720638	112675	716355	108597	724437	100.06	100.18	99.93
10	104394	710505	111379	745295	109849	747161	99.27	95.18	98.01
20	98781	705588	108591	749281	107471	741115	94.59	92.31	96.67
30	103778	727932	109486	735798	99277	732657	96.32	94.77	90.33
40	102609	733376	105488	742147	109527	773078	94.53	90.53	94.45
50	101605	729328	112939	745040	106452	762456	94.13	96.55	93.07
60	102646	738921	107241	724588	109861	756321	93.86	94.26	96.83

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	DK-I-60-3 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	104490	754231	102920	732920	108522	731478	99.66	100.30	100.24
10	100281	749451	105884	760165	110207	781689	96.26	99.49	95.261
20	95278	760108	102711	770740	103552	746542	90.17	95.18	93.72
30	100096	750328	100660	761342	103925	755479	95.97	94.43	92.94
40	94133	731526	97701	748691	105962	734495	92.57	93.21	97.47
50	101839	774789	103090	781126	104050	752296	94.56	94.26	93.45
60	99525	754772	98905	752921	103084	739907	94.86	93.83	94.13

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-60-3)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

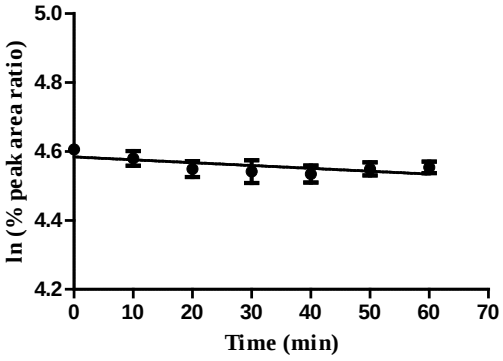
Day 1

Linear regression analysis:

Slope: -0.0008286 ± 0.0002774

At X= 60, Y = 4.534 ± 0.01000

R^2 = 0.3195



Metabolic Parameters:

Half-life: 836.35 ± 280 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08286 µL/min/mg

Metabolic Rate: 1.6572 nmol/min/mg

% remaining at 60 min: 93.13 ± 0.20 %

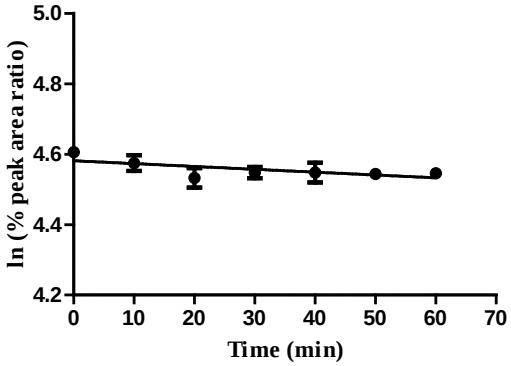
Day 2

Linear regression analysis:

Slope: -0.0008083 ± 0.0002580

At X = 60, Y = 4.533 ± 0.009301

R^2 = 0.3407



Metabolic Parameters:

Half-life: 857.35 ± 273 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08083 µL/min/mg

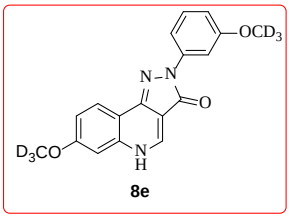
Metabolic Rate: 1.6166 nmol/min/mg

% remaining at 60 min: 93.03 ± 0.19 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8e**
Concentration: 10 µM
Date: 09-06-2017
Facility: Milwaukee Institute for Drug Discovery



8e

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Biosciences, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	99306	99262	103648	98877	108203	95577	100.04	100.02	100.00
10	98286	99764	97179	99045	99005	90106	98.51	93.62	97.06
20	89132	92094	88632	88730	96921	89394	96.78	95.31	95.77
30	85234	90864	82687	83885	100259	92635	93.80	94.05	95.61
40	83864	93281	95998	100515	107622	105755	89.90	91.13	89.89
50	81572	89315	95124	97589	95099	90297	91.33	93.01	93.03
60	83372	93197	87647	90647	89690	92014	89.45	92.26	86.10

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	106199	100912	107611	98271	105203	101265	100.03	100.00	99.98
10	97245	98407	106776	99495	99848	98640	93.93	98.00	97.42
20	97442	100104	94509	89958	97460	101350	92.52	95.94	92.55
30	79922	81324	90285	86895	95071	104982	93.41	94.88	87.16
40	95021	100662	101710	100862	89791	95078	89.73	92.09	90.89
50	98998	99580	88800	84984	98193	103362	94.50	95.42	91.43
60	98842	103127	100338	102233	89001	92949	91.10	89.63	92.15

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-94-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T = (T = 0, 10, 20,30,40,50 and 60)} \quad \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

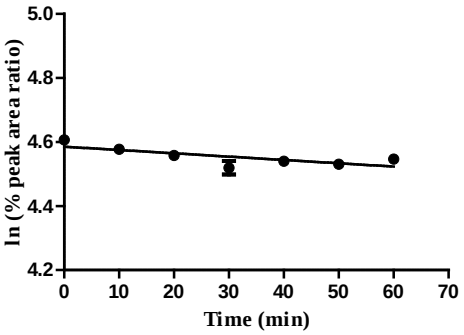
Day 1

Linear regression analysis:

Slope: -0.001729 ± 0.0002190

At X= 60, Y = 4.492 ± 0.007895

R^2 = 0.7664



Metabolic Parameters:

Half-life: 400 ± 50 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.1729 µL/min/mg

Metabolic Rate: 3.458 nmol/min/mg

% remaining at 60 min: 89 ± 0.15 %

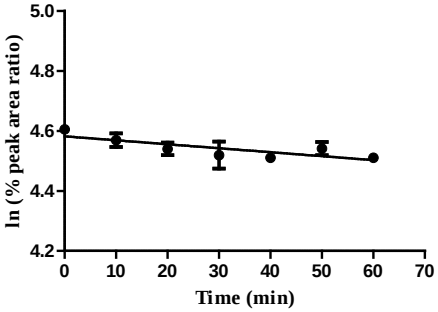
Day 2

Linear regression analysis:

Slope: -0.001321 ± 0.0003047

At X = 60, Y = 4.503 ± 0.01098

R^2 = 0.4975



Metabolic Parameters:

Half-life: 524 ± 121 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.1321 µL/min/mg

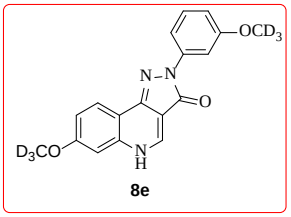
Metabolic Rate: 2.642 nmol/min/mg

% remaining at 60 min: 90 ± 0.22 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8e**
Concentration: 10 µM
Date: 09-06-2017
Facility: Milwaukee Institute for Drug Discovery



8e

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	104572	99604	112024	110770	108778	96978	99.98	100.03	99.97
10	91738	90948	88211	90287	100246	92692	96.06	96.63	96.39
20	91764	95841	90046	92865	98441	94038	91.18	95.90	93.30
30	86135	89117	87391	93992	93748	92059	92.05	91.96	90.76
40	100441	100218	93070	99075	93657	90546	95.45	92.91	92.18
50	85762	89440	85410	89605	101654	94108	91.32	94.28	96.27
60	92817	99307	87745	100069	93007	91710	89.01	86.73	90.38

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	DK-I-94-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	104699	1018323	110764	98225	108659	96253	99.82	99.96	99.99
10	98572	996723	96734	93146	93783	86232	96.01	92.06	96.33
20	97486	965623	107264	100826	96862	90872	98.01	94.31	94.41
30	100088	1025797	110260	106074	94846	92762	94.72	92.15	90.56
40	97817	985235	97958	92817	91545	85352	96.39	93.56	95.00
50	89664	956232	98009	89823	89496	84533	91.03	96.73	93.77
60	91032	927232	91730	87674	99959	100318	95.31	92.75	88.25

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-94-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T = (T = 0, 10, 20,30,40,50 and 60)} \quad \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

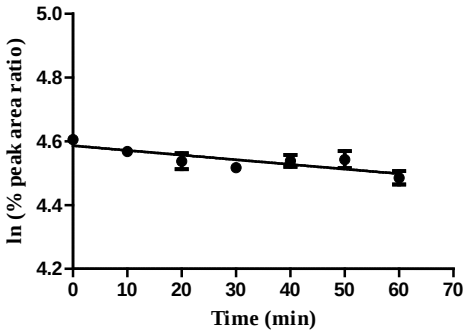
Day 1

Linear regression analysis:

Slope: -0.001462 ± 0.0002736

At X= 60, Y = 4.498 ± 0.009865

R^2 = 0.6004



Metabolic Parameters:

Half-life: 474 ± 88 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.1462 µL/min/mg

Metabolic Rate: 2.924 nmol/min/mg

% remaining at 60 min: 89 ± 0.2 %

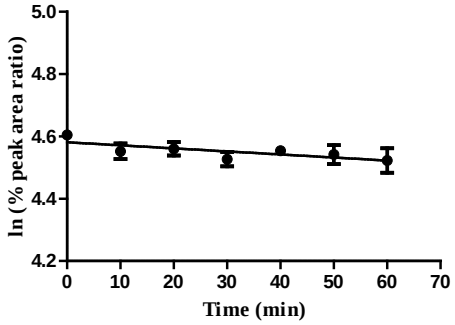
Day 2

Linear regression analysis:

Slope: -0.0009774 ± 0.0002948

At X = 60, Y = 4.522 ± 0.01063

R^2 = 0.4687



Metabolic Parameters:

Half-life: 709 ± 213 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.09774 µL/min/mg

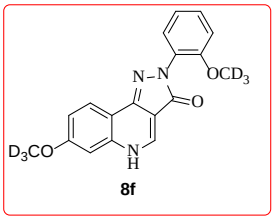
Metabolic Rate: 1.9548 nmol/min/mg

% remaining at 60 min: 92 ± 0.21 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8f**
Concentration: 10 µM
Date: 09-06-2017
Facility: Milwaukee Institute for Drug Discovery



8f

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Biosciences, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	236608	97337	238736	98664	232713	100728	99.99	99.98	100.01
10	221427	95464	225705	100063	238008	110325	95.41	93.20	93.39
20	217811	98301	237758	101900	210143	100442	91.14	96.41	90.57
30	209423	93592	211271	94675	200168	93236	92.04	92.21	92.93
40	217846	93678	228590	100491	253235	117263	95.65	93.99	93.48
50	218429	98402	227561	105740	219220	100623	91.31	88.92	94.31
60	194463	87967	218960	98077	194235	94232	90.93	92.25	89.23

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	238439	97456	225640	99282	232092	101664	99.98	99.98	99.99
10	221643	94344	214627	98997	219618	98274	96.00	95.38	97.88
20	213035	93895	201497	95083	213067	99571	92.72	93.23	93.73
30	199456	89662	198648	90822	231128	106927	90.90	96.22	94.68
40	226568	98362	199648	98765	210414	99083	94.13	88.93	93.01
50	197689	88901	227074	109028	245138	119022	90.87	91.62	90.21
60	244235	103839	190357	90631	233617	110390	96.12	92.40	92.69

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-90-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T = (T = 0, 10, 20,30,40,50 and 60)} \quad \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

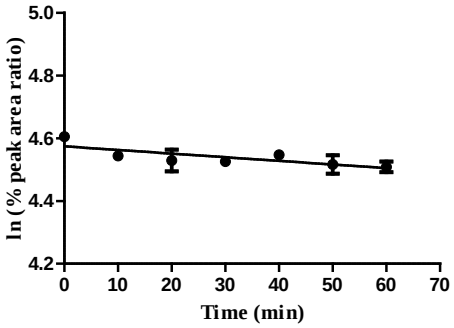
Day 1

Linear regression analysis:

Slope: -0.001160 ± 0.0002806

At X= 60, Y = 4.505 ± 0.01012

R^2 = 0.4734



Metabolic Parameters:

Half-life: 597 ± 114 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.116 µL/min/mg

Metabolic Rate: 2.32 nmol/min/mg

% remaining at 60 min: 90 ± 0.20 %

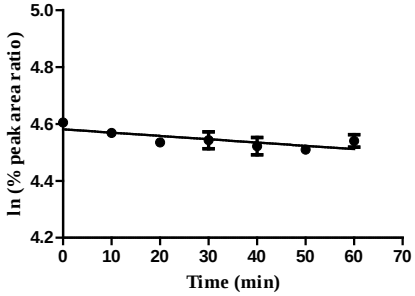
Day 2

Linear regression analysis:

Slope: -0.001161 ± 0.0002733

At X = 60, Y = 4.511 ± 0.009855

R^2 = 0.4869



Metabolic Parameters:

Half-life: 596 ± 140 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.1161 µL/min/mg

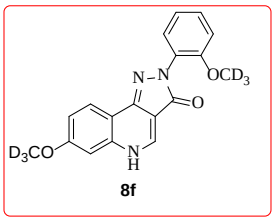
Metabolic Rate: 2.322 nmol/min/mg

% remaining at 60 min: 91 ± 0.19 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8f**
Concentration: 10 µM
Date: 09-06-2017
Facility: Milwaukee Institute for Drug Discovery



8f

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	241015	101158	232121	96514	237717	97118	99.98	100.00	99.98
10	219368	99282	221359	100502	229086	99088	92.72	91.58	94.44
20	184449	86883	200265	93708	225289	98323	89.08	88.86	93.59
30	214468	96017	205879	93595	239342	110661	93.73	91.46	88.35
40	201613	87721	224035	100785	232965	102764	96.44	92.42	92.60
50	229599	104225	232073	100739	199529	90294	92.44	95.78	90.26
60	212634	98920	212532	99063	234749	102678	90.20	89.20	93.39

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	DK-I-90-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	225863	99888	232550	100642	240713	99946	100.00	99.98	100.01
10	209150	95201	197790	90137	229251	101497	97.16	94.95	93.80
20	210837	96633	235261	96779	218061	95274	96.49	105.19	95.04
30	207752	96722	218201	96298	210809	92709	94.99	98.08	94.43
40	221146	106906	226707	102711	219082	98772	91.49	95.50	92.11
50	219038	106179	220071	97924	245723	105505	91.23	97.26	96.72
60	215862	99176	242613	110405	223572	98877	96.26	95.08	93.90

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-90-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T = (T = 0, 10, 20,30,40,50 and 60)} \quad \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

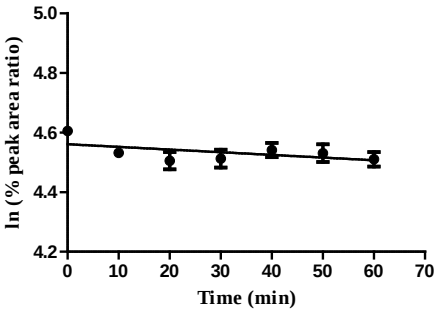
Day 1

Linear regression analysis:

Slope: -0.0008976 ± 0.0003729

At X= 60, Y = 4.507 ± 0.01345

R^2 = 0.2337



Metabolic Parameters:

Half-life: 772 ± 320 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.08976 µL/min/mg

Metabolic Rate: 1.7952 nmol/min/mg

% remaining at 60 min: 90 ± 0.26 %

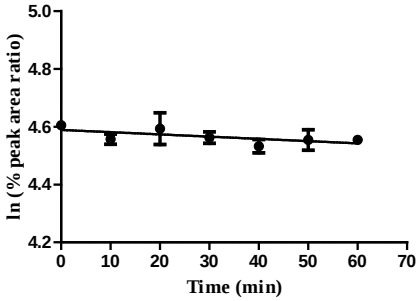
Day 2

Linear regression analysis:

Slope: -0.0007750 ± 0.0003317

At X = 60, Y = 4.542 ± 0.01196

R^2 = 0.2232



Metabolic Parameters:

Half-life: 894 ± 382 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.0775 µL/min/mg

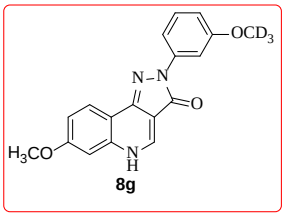
Metabolic Rate: 1.55 nmol/min/mg

% remaining at 60 min: 94 ± 0.25 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8g**
Concentration: 10 µM
Date: 02-28-2017
Facility: Milwaukee Institute for Drug Discovery



8g

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	146343	225342	139505	220893	152522	221744	100.06	99.92	99.97
10	133193	223873	132626	221883	149931	227834	91.67	94.57	95.65
20	115456	200892	128584	220983	135453	223843	88.55	92.06	87.95
30	116606	211893	122428	224909	133485	230781	84.79	86.13	84.07
40	116438	218734	120510	222874	135799	227843	82.02	85.55	86.63
50	120161	220183	117807	212843	126294	223847	84.08	87.57	82.00
60	119406	212672	113426	213974	124042	211893	86.51	83.87	85.08

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	145576	233083	151061	221893	153983	219878	99.93	99.96	100.04
10	142272	241983	147544	226132	144817	220613	94.07	95.81	93.77
20	128656	220834	140312	220633	138088	219723	93.21	93.38	89.78
30	121095	221089	130976	219734	131811	220782	87.63	87.52	85.28
40	119820	220891	124223	216734	133859	221067	86.79	84.16	86.50
50	116786	221373	121034	220874	128083	221074	84.40	80.46	82.76
60	116967	224090	119293	212894	126552	223097	83.51	82.28	81.03

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-69-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

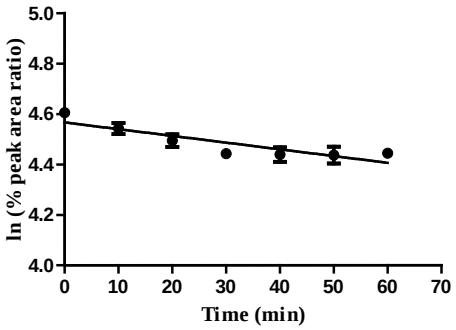
Day 1

Linear regression analysis:

Slope: -0.002671 ± 0.0003856

At X= 60, Y = 4.406 ± 0.01390

R^2 = 0.7164



Metabolic Parameters:

Half-life: 259 ± 37.4 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.2671 µL/min/mg

Metabolic Rate: 5.342 nmol/min/mg

% remaining at 60 min: 82 ± 0.26 %

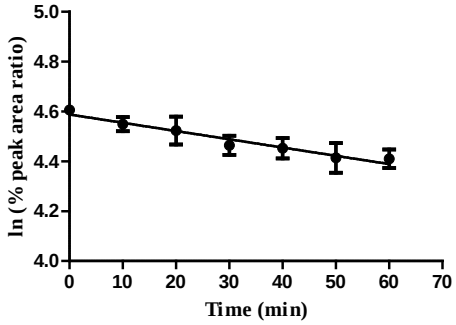
Day 2

Linear regression analysis:

Slope: -0.003312 ± 0.0002282

At X = 60, Y = 4.389 ± 0.008229

R^2 = 0.9172



Metabolic Parameters:

Half-life: 209 ± 14 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.3312 µL/min/mg

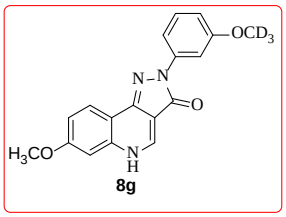
Metabolic Rate: 6.624 nmol/min/mg

% remaining at 60 min: 80.55 ± 0.15 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8g**
Concentration: 10 µM
Date: 02-28-2017
Facility: Milwaukee Institute for Drug Discovery



8g

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	153307	220362	161694	223187	148441	225613	99.95	100.06	99.99
10	149050	221782	156208	231089	139993	226741	96.56	93.36	93.83
20	142585	217634	149017	218634	138699	221387	94.13	94.14	95.21
30	146408	219723	149645	226164	136482	230012	95.73	91.39	90.17
40	139114	220782	154545	226383	134468	221634	90.53	94.29	92.20
50	143809	220173	155963	224613	132242	212421	93.84	95.90	94.61
60	145754	221723	154323	231083	138278	230781	94.45	92.24	91.06

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	DK-II-69-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	166184	221467	149474	221074	163260	220843	100.05	100.01	100.03
10	161409	230864	144162	222673	150466	221384	93.22	95.77	91.97
20	152157	221784	142196	221774	156739	220834	91.47	94.84	96.04
30	149888	221074	136979	220734	152815	221783	90.40	91.79	93.23
40	156102	223687	139293	230084	149011	224018	93.04	89.55	90.01
50	157420	217784	140291	221976	151630	223084	96.37	93.49	91.97
60	155484	218744	141615	220844	153046	220834	94.77	94.85	93.78

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-69-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

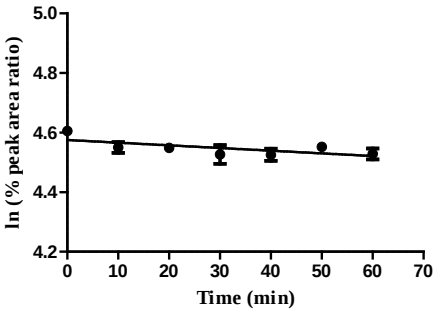
Day 1

Linear regression analysis:

Slope: -0.0008976 ± 0.0002707

At X= 60, Y = 4.521 ± 0.009762

R^2 = 0.3665



Metabolic Parameters:

Half-life: 772 ± 233 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.08976 µL/min/mg

Metabolic Rate: 1.8 nmol/min/mg

% remaining at 60 min: 91 ± 0.20 %

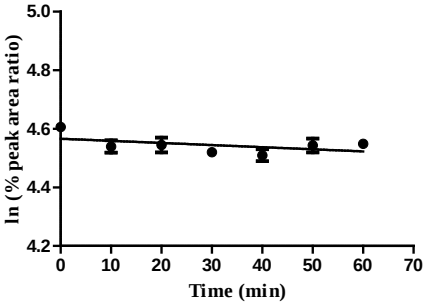
Day 2

Linear regression analysis:

Slope: -0.0007190 ± 0.0003285

At X = 60, Y = 4.523 ± 0.01184

R^2 = 0.2014



Metabolic Parameters:

Half-life: 963 ± 440 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.0719 µL/min/mg

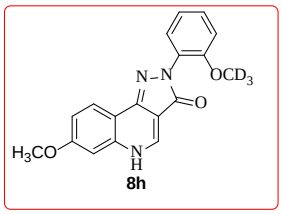
Metabolic Rate: 1.438 nmol/min/mg

% remaining at 60 min: 92.11 ± 0.42 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8h**
Concentration: 10 µM
Date: 08-15-2016
Facility: Milwaukee Institute for Drug Discovery



8h

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Biosciences, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	6000289	1978232	5748309	1978368	5972862	1976526	100.00	99.98	99.99
10	5901413	1976237	5536714	1962372	5691768	1967236	98.45	97.09	95.74
20	5359689	1986821	5121505	1956326	5328681	1972367	88.94	90.08	89.40
30	4963413	1896723	5038993	2089872	5116871	1962373	86.27	82.97	86.28
40	5041133	2004384	4914971	2000832	4926787	2000838	82.92	84.53	81.48
50	5063056	1978278	4897310	1977634	5029505	1962727	84.38	85.21	84.79
60	4821518	1976267	4696893	1967623	4942498	1978367	80.43	82.14	82.66

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	5779507	1987367	5999039	1987362	5972450	2000712	100.00	99.98	100.00
10	5360319	1978234	5686090	1978872	5660860	1997326	93.17	95.17	94.94
20	5169958	1936478	5426852	1978382	5350046	1972366	91.80	90.86	90.87
30	4896189	1965272	5125434	1923438	5107424	1978723	85.67	88.26	86.47
40	4817341	1976362	5036722	1942562	4940485	1982356	83.82	85.88	83.49
50	4729919	1976326	4850232	1897823	4826268	1972367	82.30	84.65	81.97
60	4835545	1956347	4921510	1987231	4795722	1956237	84.99	82.03	82.12

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-88-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

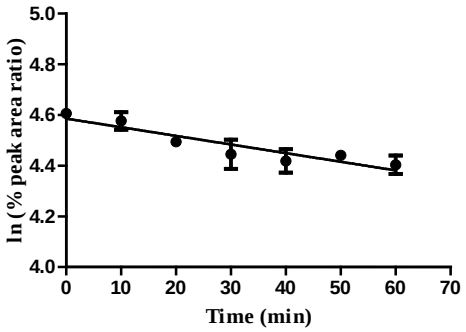
Day 1

Linear regression analysis:

Slope: -0.003402 ± 0.0003355

At X= 60, Y = 4.381 ± 0.01210

R^2 = 0.8440



Metabolic Parameters:

Half-life: 203.70 ± 20.88 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.3402 µL/min/mg

Metabolic Rate: 6.804 nmol/min/mg

% remaining at 60 min: 79.19 ± 0.22 %

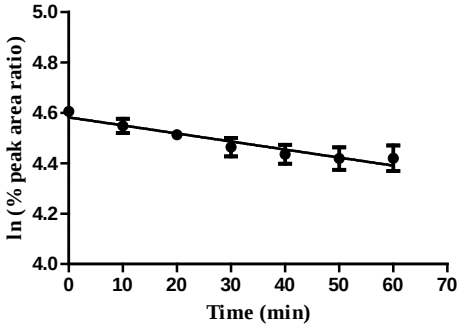
Day 2

Linear regression analysis:

Slope: -0.003190 ± 0.0002445

At X = 60, Y = 4.390 ± 0.008814

R^2 = 0.8997



Metabolic Parameters:

Half-life: 217.24 ± 16.65 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.319 µL/min/mg

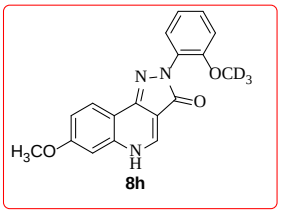
Metabolic Rate: 6.38 nmol/min/mg

% remaining at 60 min: 80.64 ± 0.16 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8h**
Concentration: 10 µM
Date: 08-15-2016
Facility: Milwaukee Institute for Drug Discovery



8h

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	5864208	1923424	5986197	1923456	6104405	1986723	99.99	100.00	99.98
10	5842143	2001663	5946613	2000102	5915367	1945623	95.72	95.53	98.93
20	5901471	1946432	5800697	1986746	5952081	2000103	99.44	93.82	96.84
30	5730111	1923513	5881350	1959021	5742564	1976326	97.70	96.47	94.55
40	5654542	1968542	5896694	1943252	5652491	1928635	94.21	97.50	95.37
50	5524788	1951543	5744072	1956723	5542147	1962538	92.85	94.33	91.89
60	5612563	1954739	5686981	1921344	5698561	1964251	94.17	95.11	94.40

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	DK-I-88-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	5984998	2034364	5968027	1998737	6002413	2098478	99.99	99.99	100.01
10	5824131	1987474	5806107	1962761	5701055	1998834	99.60	99.06	99.72
20	5507668	1923454	5702257	1978383	5563421	2001543	97.32	96.52	97.18
30	5502585	1978848	5750415	1957672	5417161	1976832	94.51	98.37	95.81
40	5287891	1923646	5658083	1998922	5306658	2008383	93.43	94.79	92.38
50	5558948	1996765	5642027	1956732	5300999	1988734	94.62	96.56	93.20
60	5652513	2087247	5532053	1981238	5234824	1978724	92.05	93.51	92.50

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-88-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T = (T = 0, 10, 20,30,40,50 and 60)} \quad \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

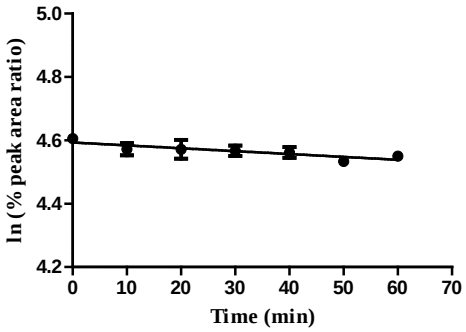
Day 1

Linear regression analysis:

Slope: -0.0009083 ± 0.0001941

At X= 60, Y = 4.538 ± 0.006998

R^2 = 0.5355



Metabolic Parameters:

Half-life: 762.96 ± 163.04 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.09083µL/min/mg

Metabolic Rate: 1.8166 nmol/min/mg

% remaining at 60 min: 93.50 ± 0.14 %

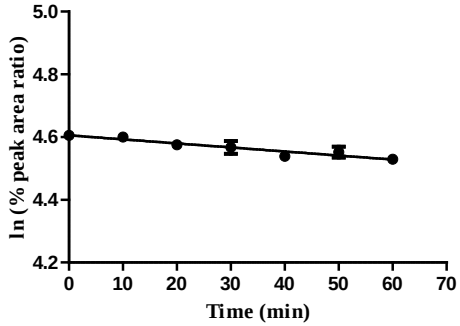
Day 2

Linear regression analysis:

Slope: -0.001288 ± 0.0001431

At X = 60, Y = 4.528 ± 0.005159

R^2 = 0.8101



Metabolic Parameters:

Half-life: 538.04 ± 59.97 min

V_d: 2000 µL/mg

Intrinsic clearance: 0.1288 µL/min/mg

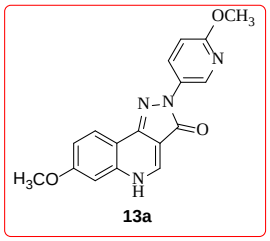
Metabolic Rate: 2.576 nmol/min/mg

% remaining at 60 min: 92.57 ± 0.10 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13a**
Concentration: 10 µM
Date: 04-04-2016
Facility: Milwaukee Institute for Drug Discovery



13a

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	1481304	2370706	1498648	2486412	1501602	2348705	99.97	99.95	100.05
10	1456174	2437087	1350839	2314930	1463439	2365630	95.60	96.77	96.81
20	1404302	2411877	1255093	2237002	1456023	2412311	93.15	93.04	94.45
30	1389377	2345148	1412423	2551510	1491949	2508806	94.79	91.80	93.06
40	1388276	2362279	1458393	2570550	1464132	2402739	94.03	94.08	95.36
50	1340079	2326528	1421667	2590384	1449469	2462260	92.16	91.01	92.12
60	1379227	2321871	1490076	2602566	1523329	2540953	95.04	94.94	93.82

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	1399330	2454869	1450558	2502725	1427070	2455486	100.00	99.93	100.03
10	1429964	2553664	1368760	2437443	1484773	2607335	98.24	96.82	98.01
20	1446883	2656111	1351638	2507523	1397386	2496606	95.56	92.93	96.33
30	1342793	2516473	1374490	2511492	1343836	2456417	93.61	94.35	94.16
40	1310764	2433525	1364299	2444927	1362123	2463298	94.49	96.20	95.17
50	1364772	2533427	1327910	2440599	1341635	2468061	94.51	93.80	93.56
60	1329779	2421028	1303521	2372689	1356927	2508601	96.36	94.72	93.10

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (RJ-02-71)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

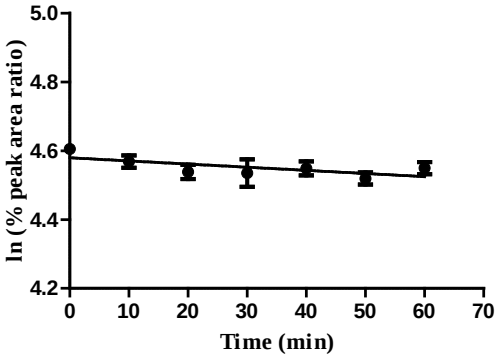
Day 1

Linear regression analysis:

Slope: -0.0009131 ± 0.0002255

At X= 60, Y = 4.525 ± 0.008131

R^2 = 0.4632



Metabolic Parameters:

Half-life: 759 ± 187.4 min

V_d: 100 µL/mg

Intrinsic clearance: 0.09131 µL/min/mg

Metabolic Rate: 1.8262 nmol/min/mg

% remaining at 60 min: 92.29 ± 0.16 %

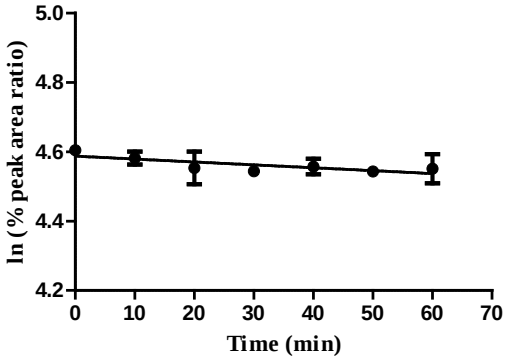
Day 2

Linear regression analysis:

Slope: -0.0008369 ± 0.0001796

At X = 60, Y = 4.537 ± 0.006474

R^2 = 0.5334



Metabolic Parameters:

Half-life: 828.05 ± 177.7 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08369 µL/min/mg

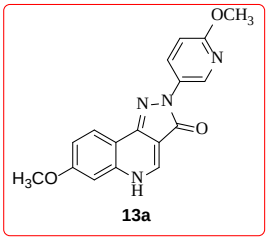
Metabolic Rate: 1.6738 nmol/min/mg

% remaining at 60 min: 93.41 ± 0.13 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13a**
Concentration: 10 µM
Date: 04-04-2016
Facility: Milwaukee Institute for Drug Discovery



13a

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	1304666	2507698	1412769	2632218	1438349	2617074	100.05	99.94	99.92
10	1342708	2677526	1390974	2638315	1388751	2572011	96.43	98.17	98.17
20	1273565	2705299	1352636	2882276	1344844	2631217	90.53	87.39	92.92
30	1164418	2621865	1218984	2675396	1302788	2728394	85.40	84.84	86.81
40	1152356	2747913	1225500	2827323	1155798	2510036	80.64	80.71	83.72
50	1097786	2651478	1185584	2809151	1084204	2519895	79.62	78.59	78.22
60	1059723	2683246	1109773	2833889	1065161	2636280	75.95	72.92	73.46

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	DK-II-13-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	1482791	2532034	1435822	2495303	1398346	2494973	99.934	100.07	100.08
10	1373230	2426501	1400615	2559018	1403527	2586061	96.575	95.18	96.91
20	1381544	2561581	1262111	2419773	1308674	2460499	92.036	90.71	94.97
30	1298371	2492182	1240184	2564013	1240839	2497136	88.904	84.12	88.73
40	1211580	2528779	1264080	2641303	1194202	2504773	81.761	83.23	85.13
50	1148195	2487107	1206014	2716275	1145296	2498066	78.781	77.21	81.87
60	1107205	2511839	1146511	2698823	1051518	2487612	75.221	73.88	75.48

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-13-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

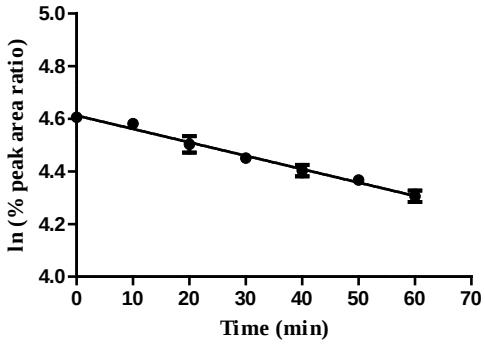
Day 1

Linear regression analysis:

Slope: -0.005095 ± 0.0002029

At X= 60, Y = 4.306 ± 0.007317

R^2 = 0.9707



Metabolic Parameters:

Half-life: 136.05 ± 5.41 min

V_d: 100 µL/mg

Intrinsic clearance: 0.5095 µL/min/mg

Metabolic Rate: 10.19 nmol/min/mg

% remaining at 60 min: 74.14 ± 0.12 %

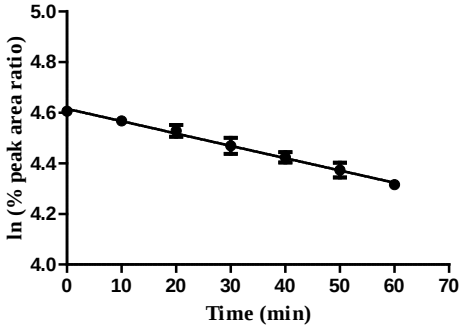
Day 2

Linear regression analysis:

Slope: -0.004868 ± 0.0002081

At X = 60, Y = 4.323 ± 0.007503

R^2 = 0.9664



Metabolic Parameters:

Half-life: 142.35 ± 6.08 min

V_d: 100 µL/mg

Intrinsic clearance: 0.4868 µL/min/mg

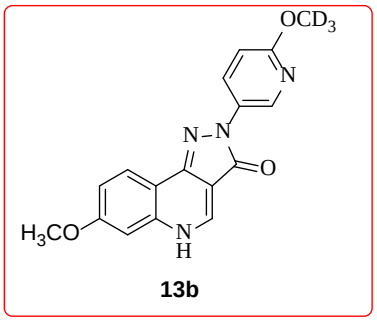
Metabolic Rate: 9.736 nmol/min/mg

% remaining at 60 min: 75.4 ± 0.13 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13b**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13b

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1756284	244343	1782966	254410	1795237	251121	99.99	98.87	99.99
10	1711624	243619	1744921	249823	1715529	246573	97.74	98.54	97.32
20	1649870	242568	1670054	246155	1650907	240772	94.62	95.71	95.91
30	1808744	268236	1658112	243636	1501247	221933	93.81	96.01	94.62
40	1774043	267372	1604211	236708	1582982	236575	92.30	95.61	93.59
50	1825221	271861	1605309	243519	1551608	232314	93.40	93.00	93.42
60	1875923	276783	1655851	247573	1565995	231980	94.29	94.36	94.42

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1739396	244473	1856188	260736	1851128	259070	99.99	100.00	100.00
10	1652649	235465	1752613	246751	1827173	261334	98.64	99.77	97.85
20	1607764	234543	1687805	248930	1731129	253844	96.34	95.24	95.44
30	1587724	240500	1630521	245489	1674483	247539	92.78	93.29	94.67
40	1588819	236967	1704883	257676	1624422	244069	94.23	92.94	93.15
50	1510170	221788	1619423	242663	1630685	248648	95.70	93.74	91.78
60	1487839	224359	1686917	247210	1562091	233734	93.20	95.85	93.53

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-60-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

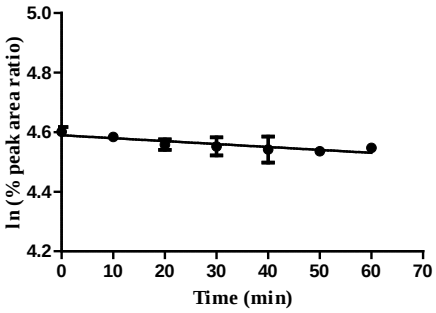
Day 1

Linear regression analysis:

Slope: -0.0009821 ± 0.0001453

At X= 60, Y = 4.530 ± 0.005238

R^2 = 0.7063



Metabolic Parameters:

Half-life: 554 ± 81.9 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1251 µL/min/mg

Metabolic Rate: 2.502 nmol/min/mg

% remaining at 60 min: 92.75 ± 0.10 %

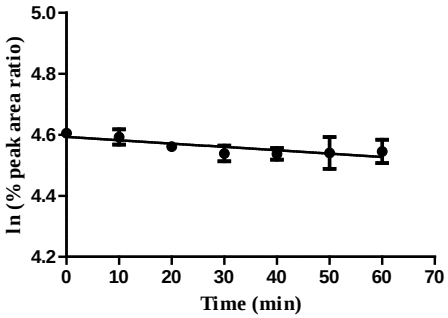
Day 2

Linear regression analysis:

Slope: -0.0001098 ± 0.0001911

At X = 60, Y = 4.527 ± 0.006892

R^2 = 0.6344



Metabolic Parameters:

Half-life: 631.14 ± 110 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1098 µL/min/mg

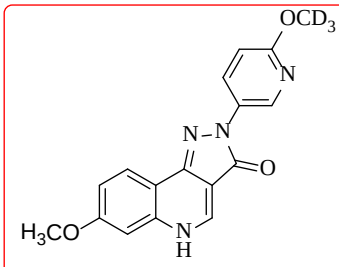
Metabolic Rate: 2.196 nmol/min/mg

% remaining at 60 min: 92.4 ± 0.14 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13b**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13b

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4, 5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1980897	286787	1989357	292666	2031686	294323	100.00	100.00	99.99
10	1928833	281364	1980914	301476	1981596	297128	99.25	96.67	96.61
20	1702400	252372	1803535	270907	2053633	308914	97.66	97.94	96.30
30	1593574	248948	1653173	256710	1946765	302009	92.67	94.74	93.38
40	1696744	257276	1647899	254711	1890234	290171	95.48	95.18	94.36
50	1666664	254754	1607831	256199	1846752	285585	94.71	92.33	93.67
60	1649310	253220	1848227	291005	1873190	292113	94.30	93.44	92.89

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	DK-II-60-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	2058936	337209	1947564	316544	2038726	335006	99.99	99.99	99.99
10	2096283	349902	1911365	323345	2002739	338162	98.11	96.07	97.31
20	2000575	343357	1982961	342398	1980098	347159	95.42	94.12	93.71
30	2047619	351527	1916376	339357	2019605	348138	95.39	91.77	95.32
40	1898193	330966	1938881	340193	1954188	344708	93.92	92.62	93.15
50	1784526	304689	1915426	337783	2096104	373344	95.92	92.16	92.25
60	1791485	318892	1994882	341903	2007358	355040	92.00	94.82	92.90

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-60-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

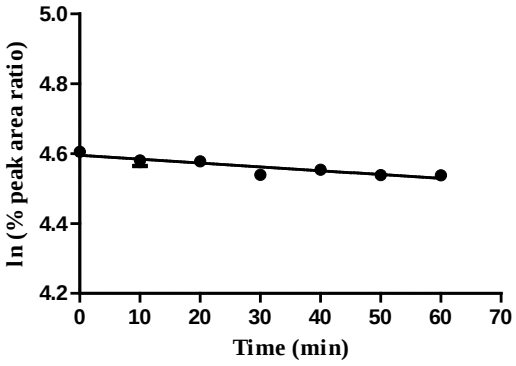
Day 1

Linear regression analysis:

Slope: -0.001099 ± 0.0001523

At X= 60, Y = 4.529 ± 0.005492

R^2 = 0.7325



Metabolic Parameters:

Half-life: 630.57 ± 87.38 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1099 µL/min/mg

Metabolic Rate: 2.198 nmol/min/mg

% remaining at 60 min: 92.66 ± 0.11 %

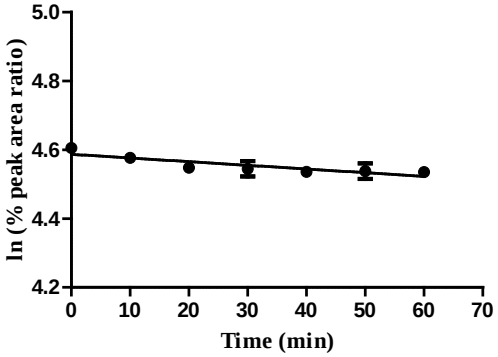
Day 2

Linear regression analysis:

Slope: -0.0001067 ± 0.0001939

At X = 60, Y = 4.523 ± 0.006992

R^2 = 0.6143



Metabolic Parameters:

Half-life: 649.48 ± 118 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1067 µL/min/mg

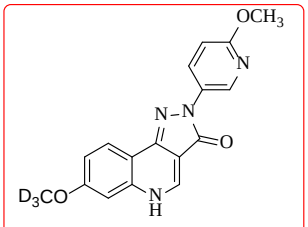
Metabolic Rate: 2.134 nmol/min/mg

% remaining at 60 min: 92.11 ± 0.14 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13c**
Concentration: 10 µM
Date: 02-24-2016
Facility: Milwaukee Institute for Drug Discovery



13c

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM Verapamil in Acetonitrile (ACN) as internal standard (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	792373	761693	787587	762552	759063	767398	100.02	99.98	100.01
10	775249	778963	759323	778215	722382	763900	95.69	94.45	95.61
20	747907	769408	720325	751835	705108	755157	93.46	92.74	94.41
30	752648	766232	732194	737114	704567	740283	94.44	96.15	96.23
40	745127	774917	729292	745328	715307	764064	92.45	94.72	94.66
50	748105	768076	736837	751091	714739	751444	93.65	94.96	96.17
60	742196	751303	716214	761424	695197	750039	94.98	91.05	93.71

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	792080	771654	755622	767152	750685	764993	100.04	99.99	100.03
10	763307	762869	718899	751779	725054	769289	97.52	97.08	96.07
20	739985	759373	712962	769369	684187	740762	94.97	94.08	94.15
30	732020	765124	701439	754561	700784	761722	93.24	94.37	93.78
40	731777	774078	696706	768514	704575	751634	92.14	92.03	95.55
50	713226	743504	690888	750265	694976	761536	93.49	93.48	93.02
60	713143	761196	697143	765448	701108	768530	91.31	92.46	92.99

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-86-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

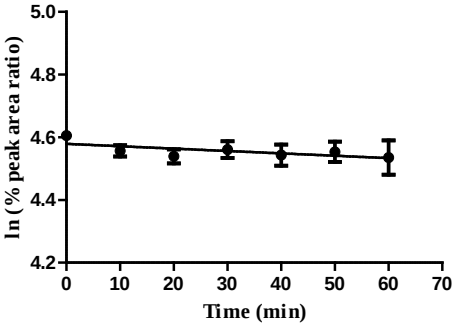
Day 1

Linear regression analysis:

Slope: -0.0007571 ± 0.0002152

At X= 60, Y = 4.533 ± 0.007758

R^2 = 0.3946



Metabolic Parameters:

Half-life: 915.33 ± 260 min

V_d: 100 µL/mg

Intrinsic clearance: 0.07571 µL/min/mg

Metabolic Rate: 1.5142 nmol/min/mg

% remaining at 60 min: 93.03 ± 0.16 %

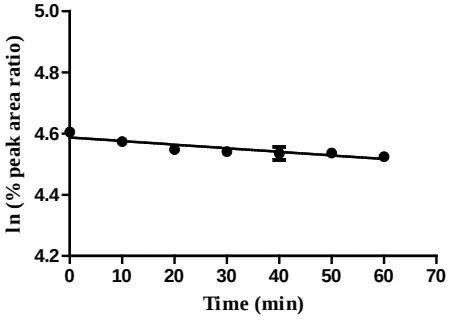
Day 2

Linear regression analysis:

Slope: -0.001176 ± 0.0001580

At X = 60, Y = 4.517 ± 0.005695

R^2 = 0.7448



Metabolic Parameters:

Half-life: 589.28 ± 261.93 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1176 µL/min/mg

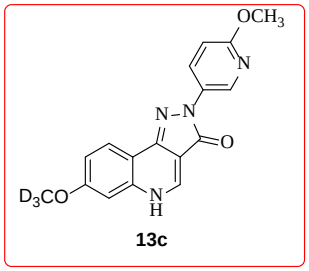
Metabolic Rate: 2.352 nmol/min/mg

% remaining at 60 min: 91.56 ± 0.11 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13c**
Concentration: 10 µM
Date: 02-24-2016
Facility: Milwaukee Institute for Drug Discovery



13c

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM Verapamil in Acetonitrile (ACN) as internal standard (store on ice).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	748286	763242	722465	767929	769758	742901	100.04	99.97	100.01
10	713998	749342	706222	755366	756894	758618	97.22	99.35	96.30
20	690434	750832	686823	767153	764830	773294	93.83	95.14	95.46
30	702462	771060	663671	745862	736979	755844	92.96	94.55	94.11
40	694160	769174	670892	771278	738418	765370	92.08	92.43	93.12
50	688116	768297	669515	747483	701337	740371	91.39	95.18	91.43
60	699208	755159	658858	761861	731836	752534	94.48	91.90	93.87

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	DK-I-86-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	703150	745961	719185	771365	719185	771365	99.95	100.03	100.04
10	685315	755803	699396	769657	699396	769657	96.15	97.50	97.81
20	668828	748912	653994	753512	653994	753512	94.70	93.12	95.01
30	672903	766432	670496	743115	670496	743115	93.10	96.81	93.92
40	675455	755264	664193	756441	664193	756441	94.83	94.21	92.71
50	671149	779998	656754	750123	656754	750123	91.24	93.94	92.52
60	652813	760945	643312	766695	643312	766695	90.97	90.02	93.06

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-86-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

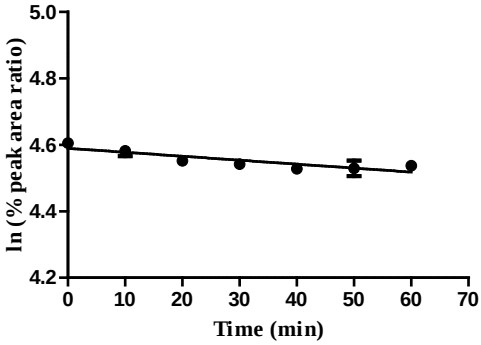
Day 1

Linear regression analysis:

Slope: -0.001192 ± 0.0001908

At X= 60, Y = 4.518 ± 0.006880

R^2 = 0.6724



Metabolic Parameters:

Half-life: 581.37 ± 93 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1192 µL/min/mg

Metabolic Rate: 2.384 nmol/min/mg

% remaining at 60 min: 91.65 ± 0.14 %

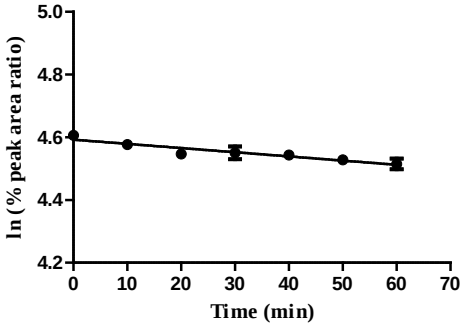
Day 2

Linear regression analysis:

Slope: -0.001333 ± 0.0001630

At X = 60, Y = 4.512 ± 0.005877

R^2 = 0.7789



Metabolic Parameters:

Half-life: 519.88 ± 63.57 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1333 µL/min/mg

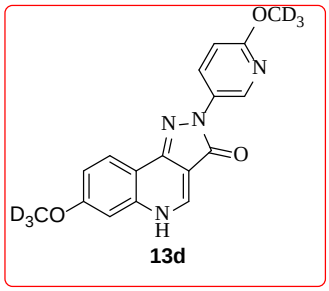
Metabolic Rate: 2.666 nmol/min/mg

% remaining at 60 min: 91.10 ± 0.11 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13d**
Concentration: 10 µM
Date: 02-28-2017
Facility: Milwaukee Institute for Drug Discovery



13d

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 3 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	153644	364063	140809	360823	147344	361083	100.00	100.06	100.01
10	144930	355973	134322	361783	140461	358982	96.47	95.19	95.90
20	145649	357844	131921	362018	135460	349781	96.45	93.43	94.91
30	136721	362984	135283	358972	133692	361193	89.25	96.63	90.72
40	141558	359723	126286	359981	131160	357892	93.25	89.95	89.82
50	133488	348756	128461	363874	136171	359671	90.70	90.52	92.79
60	134498	358623	128686	361211	136437	362084	88.87	91.34	92.35

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	148525	357823	147750	359789	151645	358973	100.01	99.91	100.10
10	143805	357791	140631	361882	146568	360078	96.84	94.55	96.45
20	141879	359721	133414	362874	143690	358612	95.04	89.45	94.94
30	140050	362073	138462	362991	143387	362543	93.20	92.81	93.72
40	134914	362734	133414	359834	140961	361834	89.62	90.21	92.31
50	140699	361764	140398	358976	141144	362893	93.71	95.16	92.16
60	140461	359722	136812	360823	146875	361383	94.08	92.25	96.30

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-III-6-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

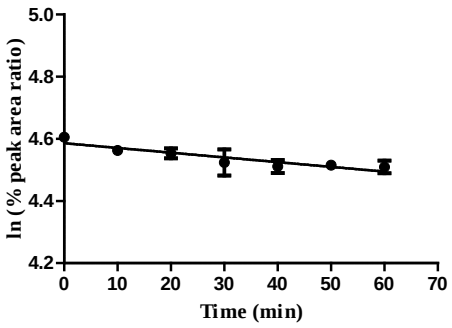
Day 1

Linear regression analysis:

Slope: -0.001523 ± 0.0002458

At X= 60, Y = 4.494 ± 0.008864

R^2 = 0.6688



Metabolic Parameters:

Half-life: 455 ± 73 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1523 µL/min/mg

Metabolic Rate: 3.046 nmol/min/mg

% remaining at 60 min: 89 ± 0.20 %

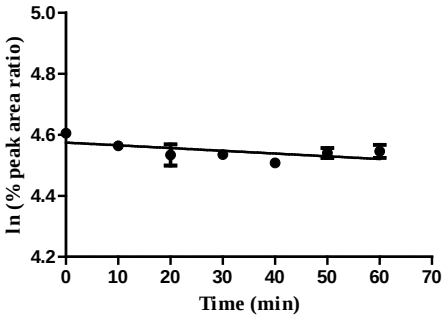
Day 2

Linear regression analysis:

Slope: -0.0008976 ± 0.0003018

At X = 60, Y = 4.520 ± 0.01088

R^2 = 0.3177



Metabolic Parameters:

Half-life: 772 ± 260 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08976 µL/min/mg

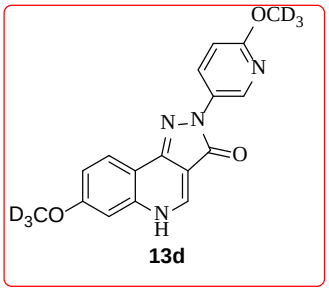
Metabolic Rate: 1.7952 nmol/min/mg

% remaining at 60 min: 91 ± 0.6 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13d**
Concentration: 10 µM
Date: 02-28-2017
Facility: Milwaukee Institute for Drug Discovery



13d

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 3 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	148490	360183	152920	361083	148522	359971	100.06	99.88	99.90
10	140281	359723	145884	358916	140207	358672	94.65	95.86	94.65
20	135278	354189	143711	358778	136552	356153	92.70	94.47	92.83
30	134096	339989	140660	356891	143925	371083	95.73	92.95	93.91
40	134133	360821	137701	360073	140962	360123	90.22	90.19	94.77
50	139839	358872	145090	361792	144050	359723	94.57	94.58	96.96
60	134525	357821	139905	360513	140084	356278	91.25	91.52	95.20

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	DK-III-6-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	150286	358974	145675	361284	148487	360083	99.91	100.05	100.09
10	143342	360173	141379	360083	142375	357982	94.98	97.42	96.53
20	140747	362894	138591	358673	138487	358901	92.56	95.88	93.65
30	136267	358513	140387	361008	141195	361008	90.71	96.49	94.93
40	143712	358971	133847	358900	142345	358978	95.54	92.54	96.24
50	140353	358921	137409	361287	139865	354793	93.32	94.37	95.68
60	139598	365723	139295	360078	140622	356872	91.09	95.99	95.64

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-III-6-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

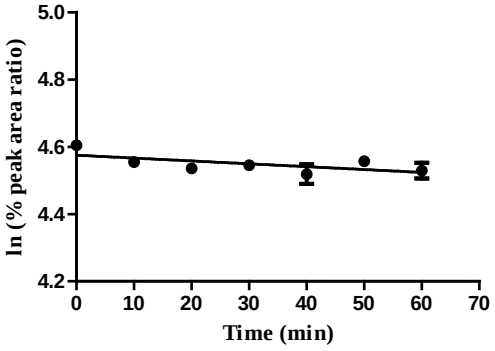
Day 1

Linear regression analysis:

Slope: -0.0008512 ± 0.0002741

At X= 60, Y = 4.524 ± 0.009883

R^2 = 0.3367



Metabolic Parameters:

Half-life: 814 ± 262 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08512 µL/min/mg

Metabolic Rate: 1.7024 nmol/min/mg

% remaining at 60 min: 92 ± 0.42 %

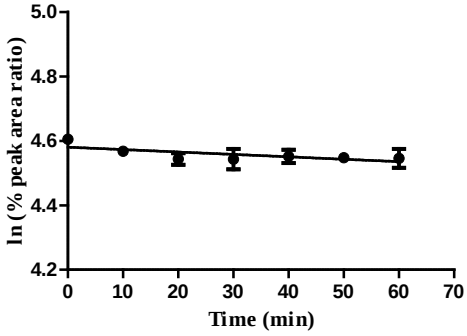
Day 2

Linear regression analysis:

Slope: -0.0007512 ± 0.0002533

At X = 60, Y = 4.535 ± 0.009133

R^2 = 0.3164



Metabolic Parameters:

Half-life: 922 ± 311 min

V_d: 100 µL/mg

Intrinsic clearance: 0.07512 µL/min/mg

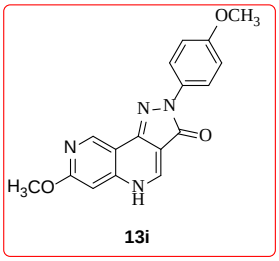
Metabolic Rate: 1.5024 nmol/min/mg

% remaining at 60 min: 93 ± 0.18 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13i**
Concentration: 10 µM
Date: 12-18-2017
Facility: Milwaukee Institute for Drug Discovery



13i

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

10. Preparation of solutions:
    c. 1 mM test compound in DMSO.
    d. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
11. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
    f. 282 µL of 18.2 mΩ of water.
    g. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
    h. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
    i. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
    j. 4 µL of test compound.
12. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Biosciences, Cat # 452156) on ice.
13. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
14. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
15. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
16. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
17. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
18. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	54463	122039	59964	120743	51713	120553	100.06	99.92	99.99
10	53675	119669	57444	119934	49323	119380	100.57	96.37	96.30
20	49025	118364	54634	120220	48219	119071	92.87	91.43	94.39
30	50811	120347	54972	119244	50374	122775	94.66	92.75	95.64
40	50209	117947	55783	118776	47867	119489	95.44	94.49	93.37
50	51373	119429	54310	120015	48485	120371	96.44	91.05	93.89
60	50053	121245	54343	117623	50031	119252	92.56	92.96	97.79

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	52360	118118	57747	120217	50843	121863	100.06	100.07	100.05
10	52744	120519	54744	118523	49417	118623	98.79	96.22	99.90
20	50870	122550	54007	118470	48444	120175	93.70	94.97	96.67
30	51424	121231	52223	120610	47045	123076	95.75	90.20	91.66
40	50503	124126	53668	119522	46736	120907	91.84	93.54	92.69
50	49277	118289	52233	117465	46091	121033	94.03	92.63	91.32
60	47525	116313	52483	119375	44808	119405	92.23	91.59	89.99

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (CW-03-030)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

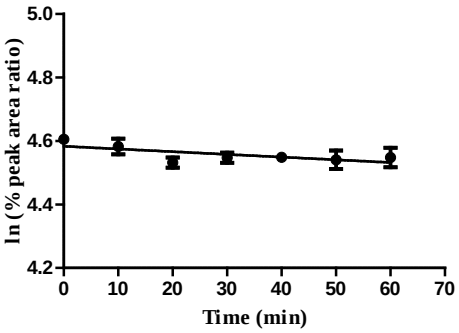
Day 1

Linear regression analysis:

Slope: -0.0008524 ± 0.0002775

At X= 60, Y = 4.532 ± 0.01001

R^2 = 0.3318



Metabolic Parameters:

Half-life: 813 ± 265 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08524 µL/min/mg

Metabolic Rate: 1.7048 nmol/min/mg

% remaining at 60 min: 93 ± 0.2 %

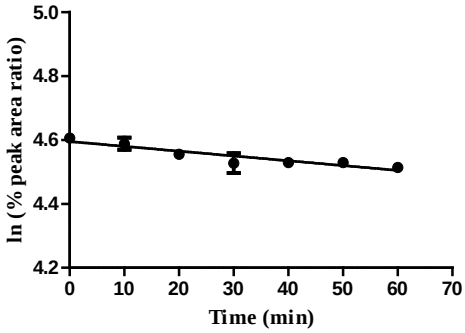
Day 2

Linear regression analysis:

Slope: -0.001504 ± 0.0002098

At X = 60, Y = 4.505 ± 0.007563

R^2 = 0.7300



Metabolic Parameters:

Half-life:460 ± 64 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1504 µL/min/mg

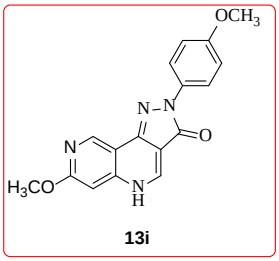
Metabolic Rate: 3.008 nmol/min/mg

% remaining at 60 min: 90 ± 0.15 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13i**
Concentration: 10 µM
Date: 12-18-2017
Facility: Milwaukee Institute for Drug Discovery



13i

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

19. Preparation of solutions:
    e. 1 mM test compound in DMSO.
    f. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
20. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
    k. 282 µL of 18.2 mΩ of water.
    l. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
    m. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
    n. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
    o. 4 µL of test compound.
21. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
22. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
23. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
24. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
25. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
26. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
27. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	54849	123424	56485	120211	55454	121817	100.08	99.97	100.04
10	54555	123689	54728	119879	54672	121646	99.33	97.13	98.77
20	51623	120410	52185	121097	53823	119279	96.56	91.68	99.17
30	50768	119304	53305	121049	50484	120090	95.84	93.69	92.39
40	51926	118435	51176	118460	53462	118334	98.74	91.91	99.29
50	53303	120736	51554	120332	51049	117545	99.43	91.15	95.44
60	50830	121128	50523	119293	50781	120366	94.51	90.11	92.72

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	CW03-030 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	53258	119305	56261	121550	57983	118109	100.09	99.97	99.98
10	52592	121231	53815	120470	53196	119751	97.26	96.48	90.47
20	51090	118281	52692	118234	54834	120263	96.84	96.25	92.86
30	51137	123924	51407	120551	52352	117266	92.52	92.10	90.92
40	52069	118562	51496	117290	55905	120825	98.46	94.82	94.23
50	50529	120743	52890	119520	53056	113142	93.83	95.57	95.50
60	47670	119553	51690	121416	54464	118362	89.40	91.95	93.71

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (CW-03-030)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T = (T = 0, 10, 20,30,40,50 and 60)} \quad \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

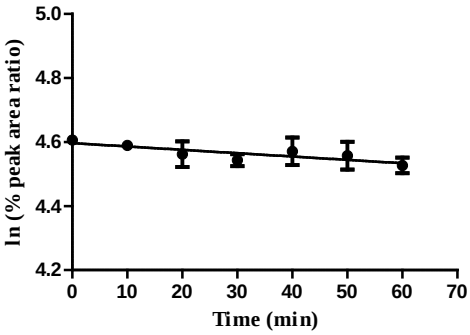
Day 1

Linear regression analysis:

Slope: -0.001045 ± 0.0003219

At X= 60, Y = 4.534 ± 0.01161

R^2 = 0.3569



Metabolic Parameters:

Half-life: 663 ± 204 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1045 µL/min/mg

Metabolic Rate: 2.09 nmol/min/mg

% remaining at 60 min: 93 ± 0.25 %

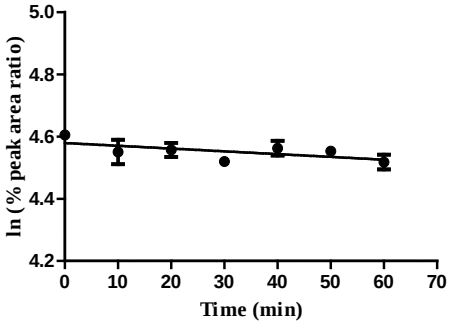
Day 2

Linear regression analysis:

Slope: -0.0008940 ± 0.0003124

At X = 60, Y = 4.526 ± 0.001126

R^2 = 0.3012



Metabolic Parameters:

Half-life:775 ± 270 min

V_d: 100 µL/mg

Intrinsic clearance: 0.0894 µL/min/mg

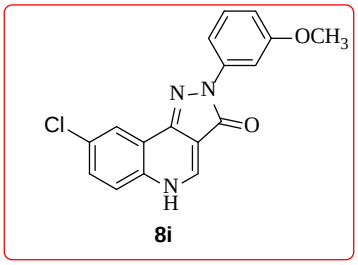
Metabolic Rate: 1.788 nmol/min/mg

% remaining at 60 min: 92 ± 0.3 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8i**
Concentration: 10 µM
Date: 04-06-2016
Facility: Milwaukee Institute for Drug Discovery



8i

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	80398	770706	79247	781412	81322	768705	100.30	100.41	99.80
10	78100	757087	75533	774930	79581	765630	99.19	96.50	98.05
20	76832	781877	73032	767002	76372	772311	94.48	94.27	93.29
30	71132	765148	68999	781510	73574	768806	89.38	87.41	90.28
40	68442	762279	65432	770550	67089	772739	86.33	84.07	81.90
50	68744	780528	68583	780384	68078	762260	84.68	87.01	84.25
60	65384	761871	66839	782566	66854	769953	82.52	84.56	81.91

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	83578	764869	81348	762725	82729	765486	100.24	99.67	100.06
10	81869	773664	79781	777443	80586	777335	97.08	95.90	95.99
20	77477	756111	76199	767523	78620	780606	94.00	92.78	93.25
30	76977	787473	73745	781492	76424	776417	89.68	88.19	91.14
40	70419	763525	68213	764927	71372	768298	84.61	83.34	86.01
50	69693	773427	66577	770599	70639	768061	82.66	80.74	85.15
60	71664	761028	68158	772689	69696	778601	86.39	82.43	82.88

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (LAU 159)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

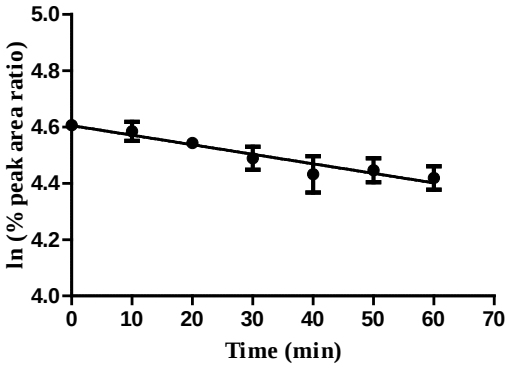
Day 1

Linear regression analysis:

Slope: -0.003394 ± 0.0002532

At X= 60, Y = 4.401 ± 0.009130

R^2 = 0.9044



Metabolic Parameters:

Half-life: 204.18 ± 15.23 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3394 µL/min/mg

Metabolic Rate: 6.788 nmol/min/mg

% remaining at 60 min: 81.53 ± 0.16 %

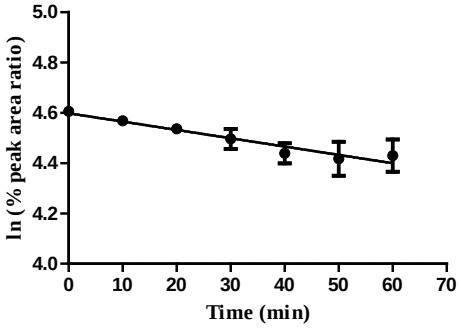
Day 2

Linear regression analysis:

Slope: -0.003310 ± 0.0002487

At X = 60, Y = 4.399 ± 0.008968

R^2 = 0.9031



Metabolic Parameters:

Half-life: 209.36 ± 15.73 min

V_d: 100 µL/mg

Intrinsic clearance: 0.331 µL/min/mg

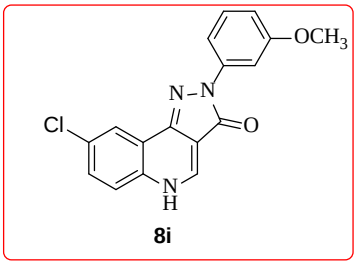
Metabolic Rate: 6.62 nmol/min/mg

% remaining at 60 min: 81.36 ± 0.16 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8i**
Concentration: 10 µM
Date: 04-06-2016
Facility: Milwaukee Institute for Drug Discovery



8i

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	86708	777698	85735	782218	85879	767074	100.44	99.64	99.96
10	76231	777526	74721	778315	78865	772011	88.32	87.27	91.21
20	66139	765299	68247	762276	70083	761217	77.85	81.39	82.20
30	59435	769865	60822	775396	66560	780394	69.55	71.30	76.15
40	57074	767913	58779	767323	62645	779036	66.95	69.63	71.79
50	55032	771478	57997	779151	55850	769895	64.26	67.66	64.77
60	53264	763246	54568	780889	53737	780280	62.87	63.52	61.49

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	LAU 159 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	86093	772034	86606	765303	84547	764973	99.56	100.14	99.57
10	75885	766501	74432	759018	77931	776061	88.39	86.78	90.46
20	67311	761581	67630	769773	67513	766499	78.91	77.75	79.35
30	61790	776182	61097	764013	63949	787136	71.07	70.76	73.19
40	58705	768779	57288	768303	58017	774773	68.18	65.98	67.46
50	56741	777107	55177	776275	53768	768066	65.19	62.90	63.06
60	55772	768839	54383	778823	53547	771612	64.76	61.79	62.51

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (LAU 159)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

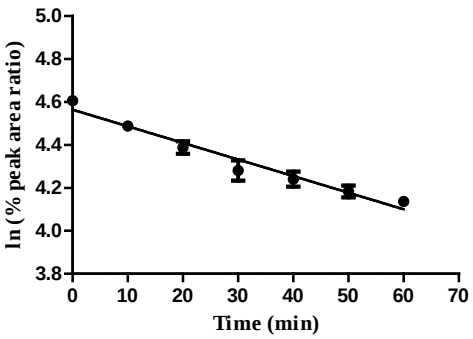
Day 1

Linear regression analysis:

Slope: -0.007724 ± 0.0004410

At X= 60, Y = 4.100 ± 0.01590

R^2 = 0.9417



Metabolic Parameters:

Half-life: 89.72 ± 5.12 min

V_d: 100 µL/mg

Intrinsic clearance: 0.7724 µL/min/mg

Metabolic Rate: 15.448 nmol/min/mg

% remaining at 60 min: 60.34 ± 0.23 %

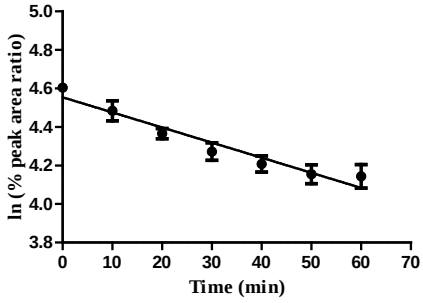
Day 2

Linear regression analysis:

Slope: -0.007837 ± 0.0004718

At X = 60, Y = 4.083 ± 0.01701

R^2 = 0.9356



Metabolic Parameters:

Half-life: 88.42 ± 5.32 min

V_d: 100 µL/mg

Intrinsic clearance: 0.7837 µL/min/mg

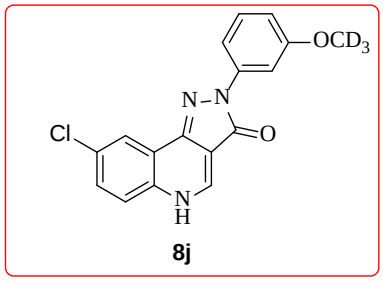
Metabolic Rate: 15.674 nmol/min/mg

% remaining at 60 min: 59.32 ± 0.24 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8j**
Concentration: 10 µM
Date: 05-06-2016
Facility: Milwaukee Institute for Drug Discovery



8j

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM 4,5 Diphenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	332661	2109886	331693	2088212	324161	2109905	99.79	99.90	99.76
10	321980	2089987	316452	2009030	317270	2100090	97.50	99.06	98.10
20	319442	2109877	321330	2087202	311732	2082911	95.82	96.82	97.18
30	300905	2009948	302080	1989310	299219	2008386	94.75	95.50	96.74
40	310592	2100979	313763	2099050	292364	2002890	93.56	94.01	94.78
50	319432	2193028	301923	2009884	288651	1968260	92.18	94.47	95.22
60	310961	2099871	295863	2008786	303022	2089233	93.72	92.63	94.18

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	331986	2092889	334435	2102273	318009	2083826	99.76	100.05	99.74
10	333335	2089284	320734	2028733	323313	2198835	100.34	99.43	96.10
20	321611	2109091	308978	2000783	305776	2089206	95.90	97.12	95.66
30	316422	2109273	326793	2163622	313245	2183287	94.34	94.99	93.77
40	313007	2098285	315920	2089327	303331	2078788	93.81	95.09	95.37
50	309075	2012827	316116	2109029	315823	2183281	96.57	94.26	94.54
60	311918	2091878	306049	2089289	300968	2082891	93.77	92.12	94.44

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-59-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

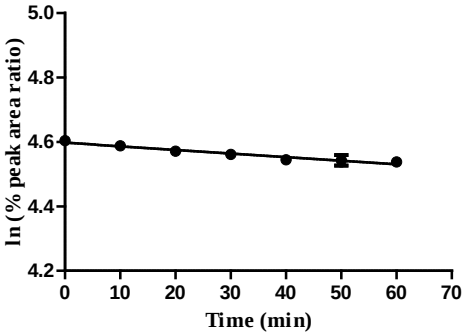
Day 1

Linear regression analysis:

Slope: -0.001113 ± 0.0001062

At X= 60, Y = 4.531 ± 0.003829

R^2 = 0.8526



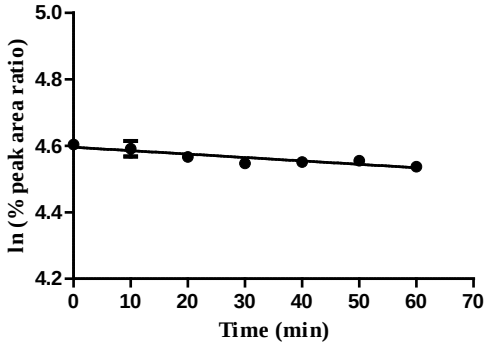
Day 2

Linear regression analysis:

Slope: -0.001026 ± 0.0001577

At X = 60, Y = 4.534 ± 0.005687

R^2 = 0.6902



Metabolic Parameters:

Half-life: 622.64 ± 60 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1113 µL/min/mg

Metabolic Rate: 2.226 nmol/min/mg

% remaining at 60 min: 92.85 ± 0.07 %

Metabolic Parameters:

Half-life: 675.43 ± 103 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1026 µL/min/mg

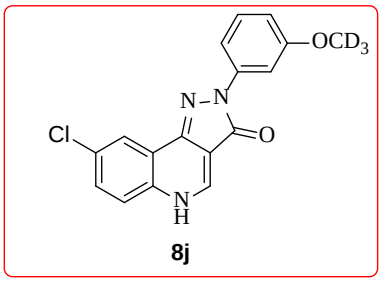
Metabolic Rate: 2.052 nmol/min/mg

% remaining at 60 min: 93.13 ± 0.11 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8j**
Concentration: 10 µM
Date: 05-06-2016
Facility: Milwaukee Institute for Drug Discovery



8j

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM 4,5 Diphenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	364668	2067368	384616	2097328	382943	2018928	100.22	100.21	99.83
10	334364	2087386	358462	2023892	372601	2100921	91.01	96.78	93.34
20	339956	2193899	337084	2083872	359689	2082817	88.04	88.39	90.89
30	313196	2027898	324275	2089289	353631	2120902	87.75	84.81	87.75
40	301354	2087323	319986	2172783	320297	2010256	82.03	80.47	83.85
50	285346	2000988	290054	2008751	320503	2178237	81.02	78.90	77.44
60	285294	2183826	301279	2153539	302164	2073280	74.22	76.44	76.70

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	DK-I-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	344482	1997834	344482	2009283	367481	1982372	100.24	100.26	100.20
10	335911	2082382	335911	2058389	360082	2072061	93.78	95.43	93.93
20	320196	2100381	320196	2089389	343194	2089499	88.63	89.61	88.78
30	307994	2081282	307994	2044013	317081	1997383	86.03	88.11	85.81
40	306138	2082919	286138	1968703	313906	2004773	85.45	84.99	84.63
50	306151	2182981	286151	2072378	300644	1998990	81.53	80.74	81.29
60	289745	2188239	279745	2109823	301367	2047582	76.98	77.53	79.55

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-59-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

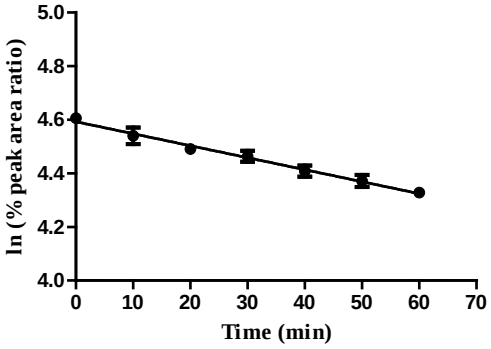
Day 1

Linear regression analysis:

Slope: -0.004473 ± 0.0002139

At X= 60, Y = 4.324 ± 0.007712

R^2 = 0.9584



Metabolic Parameters:

Half-life: 154.92 ± 7.4 min

V_d: 100 µL/mg

Intrinsic clearance: 0.4473 µL/min/mg

Metabolic Rate: 8.946 nmol/min/mg

% remaining at 60 min: 75.48 ± 0.13 %

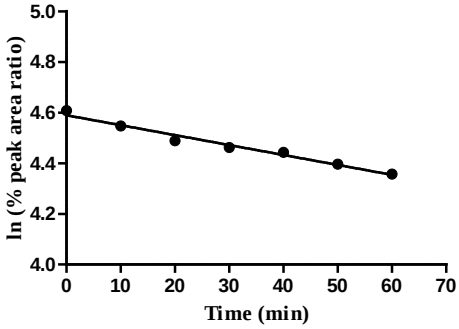
Day 2

Linear regression analysis:

Slope: -0.003925 ± 0.0001689

At X = 60, Y = 4.354 ± 0.006089

R^2 = 0.9660



Metabolic Parameters:

Half-life: 176.56 ± 8 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3925 µL/min/mg

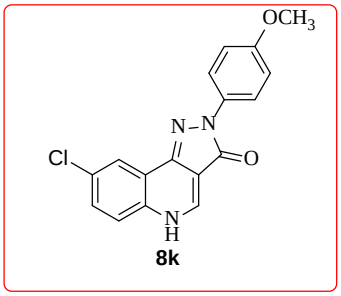
Metabolic Rate: 7.85 nmol/min/mg

% remaining at 60 min: 77.7 ± 0.10 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8k**
Concentration: 10 µM
Date: 05-11-2016
Facility: Milwaukee Institute for Drug Discovery



8k

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	324755	191542	346172	193172	339347	194350	100.02	100.00	100.00
10	303291	193874	334280	205037	314958	188485	92.29	90.97	95.70
20	279364	189575	312886	197462	294465	185685	86.94	88.42	90.82
30	262109	190556	281620	190809	269697	189934	81.15	82.36	81.32
40	261473	193926	266162	185650	252097	179750	79.54	80.00	80.32
50	236291	183965	260227	190263	263532	199428	75.77	76.32	75.68
60	224000	184270	238445	182098	249488	202810	71.71	73.07	70.45

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	340040	192439	331759	189954	345868	200640	100.00	99.97	99.99
10	318242	195404	324573	197827	312513	192301	92.17	93.91	94.26
20	305569	192981	299895	188781	299459	194073	89.61	90.93	89.50
30	298265	197605	286592	196819	274487	192160	85.42	83.35	82.85
40	285918	201639	280896	200397	262591	191811	80.24	80.23	79.40
50	261825	192246	264588	191591	253613	189168	77.07	79.05	77.76
60	247898	189899	259822	195517	241724	191307	73.87	76.06	73.29

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (PZ-II-028)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

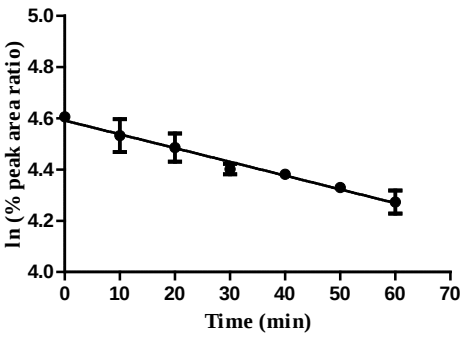
Day 1

Linear regression analysis:

Slope: -0.005374 ± 0.0002025

At X= 60, Y = 4.269 ± 0.007303

R^2 = 0.9737



Metabolic Parameters:

Half-life: 129 ± 4.85 min

V_d: 100 µL/mg

Intrinsic clearance: 0.5374 µL/min/mg

Metabolic Rate: 10.748 nmol/min/mg

% remaining at 60 min: 71.45 ± 0.12 %

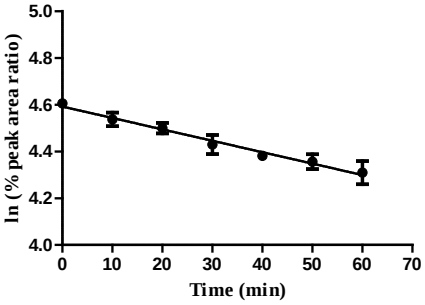
Day 2

Linear regression analysis:

Slope: -0.004882 ± 0.0001744

At X = 60, Y = 4.299 ± 0.006290

R^2 = 0.9763



Metabolic Parameters:

Half-life: 142 ± 5 min

V_d: 100 µL/mg

Intrinsic clearance: 0.4882 µL/min/mg

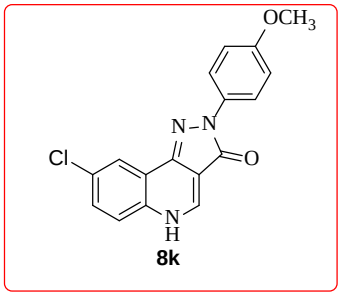
Metabolic Rate: 9.764 nmol/min/mg

% remaining at 60 min: 73.6 ± 0.10 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8k**
Concentration: 10 µM
Date: 05-11-2016
Facility: Milwaukee Institute for Drug Discovery



8k

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	366061	199827	336466	199039	337673	194171	99.99	100.02	100.00
10	354142	209737	307305	201713	319153	196427	92.16	90.14	93.43
20	304089	196538	280904	200559	304912	203823	84.45	82.87	86.02
30	289982	197643	269131	194897	289487	198575	80.08	81.70	83.83
40	272301	194366	254952	193436	270560	199235	76.47	77.98	78.09
50	261248	192467	244954	194893	259999	196266	74.09	74.37	76.17
60	243264	198621	224892	194818	226288	196987	66.85	68.30	66.05

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	PZ-II-028 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	343831	194879	339147	198969	346744	205015	100.01	99.97	100.01
10	323549	200214	312795	193433	313614	199614	91.61	94.84	92.91
20	308786	196656	297763	196507	289897	192823	89.01	88.87	88.90
30	294808	196426	288071	200701	288541	202020	85.08	84.18	84.46
40	281641	198577	271681	201071	264187	195683	80.40	79.24	79.83
50	270456	199404	264391	197514	257406	198739	76.88	78.51	76.59
60	237481	198225	230209	198359	220656	197443	67.91	68.06	66.08

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (PZ-II-028)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

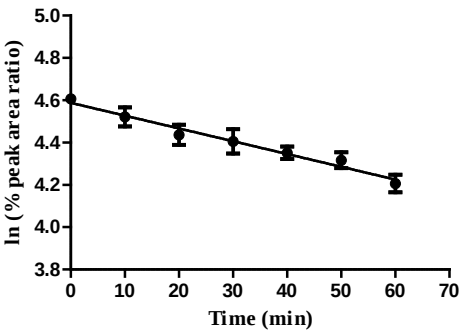
Day 1

Linear regression analysis:

Slope: -0.006044 ± 0.0002685

At X= 60, Y = 4.224 ± 0.009680

R^2 = 0.9639



Metabolic Parameters:

Half-life: 114.65 ± 5 min

V_d: 100 µL/mg

Intrinsic clearance: 0.6044 µL/min/mg

Metabolic Rate: 12.088 nmol/min/mg

% remaining at 60 min: 68.30 ± 0.15 %

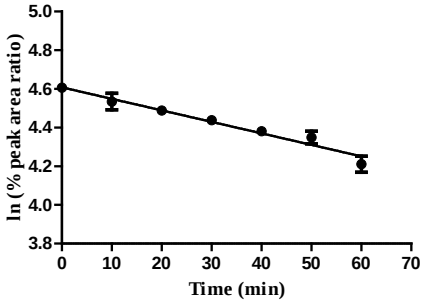
Day 2

Linear regression analysis:

Slope: -0.005946 ± 0.0002748

At X = 60, Y = 4.251 ± 0.009908

R^2 = 0.9610



Metabolic Parameters:

Half-life: 116.54 ± 5 min

V_d: 100 µL/mg

Intrinsic clearance: 0.5946 µL/min/mg

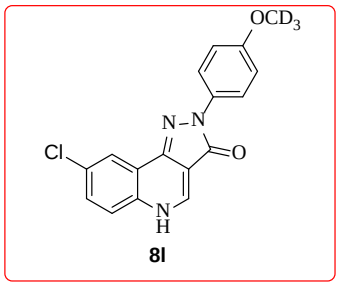
Metabolic Rate: 11.892 nmol/min/mg

% remaining at 60 min: 70.17 ± 0.16 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8l**
Concentration: 10 µM
Date: 05-11-2016
Facility: Milwaukee Institute for Drug Discovery



8l

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-93-1 Peak area	ISTD Peak area	DK-I-93-1 Peak area	ISTD Peak area	DK-I-93-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	250625	192387	237170	198327	234647	199878	99.97	99.98	99.99
10	242335	189302	224245	189872	225979	200083	98.24	98.74	96.20
20	238422	192987	232415	201838	222414	198723	94.81	96.27	95.33
30	238094	194382	225682	200982	220003	197823	94.00	93.88	94.72
40	223222	189899	217775	197267	216509	198727	90.21	92.30	92.80
50	227512	190923	213875	189283	206397	189982	91.45	94.47	92.53
60	243612	200837	217353	189231	213670	189238	93.09	96.03	96.17

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-93-1 Peak area	ISTD Peak area	DK-I-93-1 Peak area	ISTD Peak area	DK-I-93-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	232555	181934	254844	198536	235043	183806	100.01	99.97	99.98
10	231871	188134	242801	189616	239432	190946	96.43	99.72	98.03
20	230713	189672	241871	199585	235141	194722	95.17	94.38	94.41
30	229991	194398	231837	187871	217874	183237	92.57	96.10	92.96
40	217135	179986	229810	188630	229989	192164	94.39	94.88	93.57
50	214187	180515	236366	193786	230808	196736	92.84	94.99	91.72
60	239230	196288	209997	177296	220264	186986	95.36	92.24	92.10

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-93-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

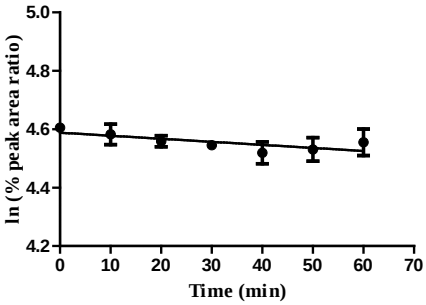
Day 1

Linear regression analysis:

Slope: -0.001044 ± 0.0002330

At X= 60, Y = 4.525 ± 0.008401

R^2 = 0.5138



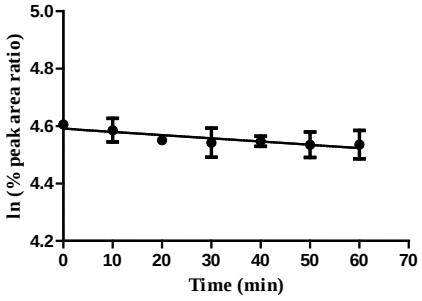
Day 2

Linear regression analysis:

Slope: -0.001125 ± 0.0001897

At X = 60, Y = 4.523 ± 0.006839

R^2 = 0.6493



Metabolic Parameters:

Half-life: 663.8 ± 148 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1044 µL/min/mg

Metabolic Rate: 2.088 nmol/min/mg

% remaining at 60 min: 92.29 ± 0.17 %

Metabolic Parameters:

Half-life: 616 ± 103.87 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1125 µL/min/mg

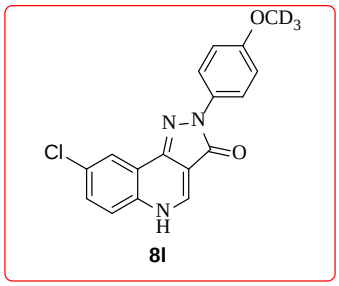
Metabolic Rate: 2.25 nmol/min/mg

% remaining at 60 min: 92.11 ± 0.13 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8l**
Concentration: 10 µM
Date: 05-11-2016
Facility: Milwaukee Institute for Drug Discovery



8l

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-93-1 Peak area	ISTD Peak area	DK-I-93-1 Peak area	ISTD Peak area	DK-I-93-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	247869	200832	242560	198728	240012	200383	100.01	99.96	99.98
10	241961	198938	229981	189972	228890	192038	98.56	99.14	99.49
20	219944	189239	219837	190398	227377	198723	94.18	94.56	95.50
30	219892	192783	223091	198289	222356	199873	92.43	92.14	92.86
40	215733	190283	223609	199089	202388	187327	91.87	91.98	90.18
50	217308	200038	210655	198289	201168	193989	88.03	87.00	86.56
60	210081	198238	199705	190098	197997	199723	85.87	86.03	82.75

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-93-1 Peak area	ISTD Peak area	DK-I-93-1 Peak area	ISTD Peak area	DK-II-93-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	223619	198287	256949	198289	230099	200812	99.97	99.98	99.98
10	216310	198732	253937	200198	222023	199128	96.49	97.87	97.29
20	214081	201832	243196	200928	208449	192983	94.03	93.39	94.25
30	211543	200783	238604	199289	210719	198997	93.40	92.38	92.40
40	207923	201838	235187	198181	198300	189877	91.32	91.56	91.13
50	197590	198328	224951	199823	201107	199887	88.32	86.86	87.79
60	203382	208278	213838	192566	203202	208787	86.56	85.68	84.926

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-93-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

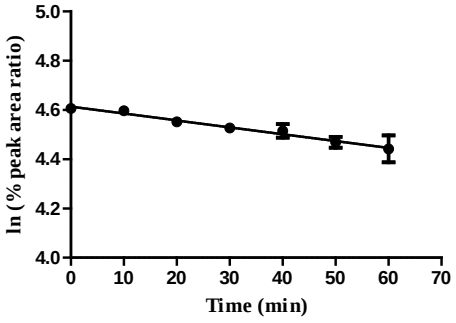
Day 1

Linear regression analysis:

Slope: -0.002795 ± 0.0001334

At X= 60, Y = 4.445 ± 0.004847

R^2 = 0.9579



Metabolic Parameters:

Half-life: 247.94 ± 11.92 min

V_d: 100 µL/mg

Intrinsic clearance: 0.2795 µL/min/mg

Metabolic Rate: 5.59 nmol/min/mg

% remaining at 60 min: 85.19 ± 0.09 %

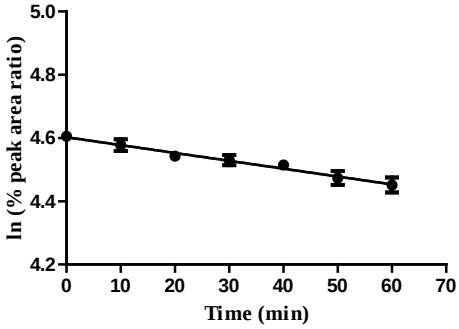
Day 2

Linear regression analysis:

Slope: -0.002487 ± 9.365e^{-}005

At X = 60, Y = 4.453 ± 0.003377

R^2 = 0.9738



Metabolic Parameters:

Half-life: 277.86 min

V_d: 100 µL/mg

Intrinsic clearance: 0.2494 µL/min/mg

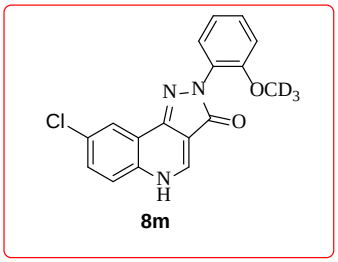
Metabolic Rate: 4.988 nmol/min/mg

% remaining at 60 min: 85.88 ± 0.06 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8m**
Concentration: 10 µM
Date: 05-31-2016
Facility: Milwaukee Institute for Drug Discovery



8m

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3152581	2574057	3090759	2547745	3041212	2506826	99.98	100.01	100.01
10	2808908	2335235	2912951	2501008	3038858	2554152	98.19	96.01	98.08
20	2866087	2400484	2847348	2427550	3040589	2629818	97.46	96.69	95.31
30	2972032	2516908	2927928	2523366	2843286	2411572	96.39	95.65	97.19
40	2710230	2301818	2876918	2476615	2874748	2490507	96.11	95.76	95.15
50	2957325	2548271	2899982	2513844	2742462	2389247	94.73	95.10	94.62
60	2886265	2480002	2892799	2482830	2789089	2399249	95.00	96.05	95.83

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3064533	2514855	3043938	2506906	3099337	2509389	99.96	100.01	100.00
10	3025303	2515223	2932365	2477887	3077022	2522920	98.67	97.48	98.75
20	2918654	2479369	2875835	2458655	3122423	2592134	96.56	96.34	97.53
30	2959314	2499725	2920979	2521593	2905454	2453499	97.11	95.41	95.88
40	2905799	2468160	2862172	2445543	3022306	2568476	96.58	96.40	95.27
50	2993719	2551914	2886596	2500423	2911271	2452557	96.23	95.09	96.11
60	2858457	2445646	2914618	2493893	2867156	2457617	95.88	96.26	94.46

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-87-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

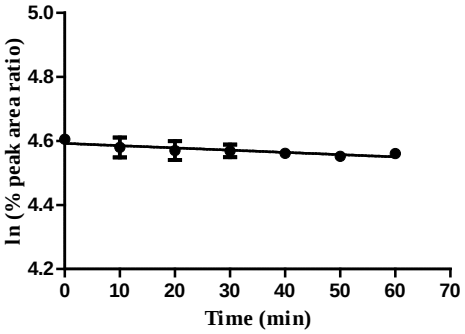
Day 1

Linear regression analysis:

Slope: -0.0007000 ± 0.0001150

At X= 60, Y = 4.550 ± 0.004145

R^2 = 0.6612



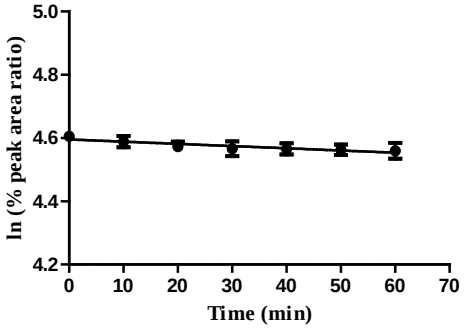
Day 2

Linear regression analysis:

Slope: -0.0006988 ± 0.0001008

At X = 60, Y = 4.553 ± 0.003633

R^2 = 0.7168



Metabolic Parameters:

Half-life: 990 ± 162.64 min

V_d: 100 µL/mg

Intrinsic clearance: 0.07 µL/min/mg

Metabolic Rate: 1.4 nmol/min/mg

% remaining at 60 min: 94.63 ± 0.08 %

Metabolic Parameters:

Half-life: 991.70 ± 143.05 min

V_d: 100 µL/mg

Intrinsic clearance: 0.06988 µL/min/mg

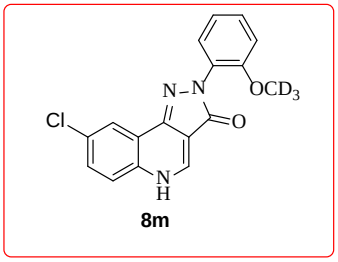
Metabolic Rate: 1.3976 nmol/min/mg

% remaining at 60 min: 94.91 ± 0.07 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8m**
Concentration: 10 µM
Date: 05-31-2016
Facility: Milwaukee Institute for Drug Discovery



8m

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3010880	2496127	3000020	2474890	3119712	2577903	100.01	100.01	100.01
10	2919613	2469905	3060634	2584737	2940036	2414532	98.01	97.70	100.63
20	2877808	2467796	2948704	2550083	2891272	2464767	96.69	95.40	96.94
30	2949345	2509812	2864981	2476848	2814761	2424509	97.44	95.43	95.94
40	2874816	2447825	2859953	2478746	2787443	2412483	97.38	95.19	95.49
50	2823464	2438940	2907401	2505846	2929879	2500532	95.99	95.73	96.83
60	2868479	2500485	2916626	2545748	2888867	2478648	95.12	94.52	96.32

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	DK-I-87-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3063855	2554326	3017647	2477860	3122426	2581928	100.04	99.98	100.02
10	3073622	2574381	3128942	2598728	2931678	2492897	99.57	98.85	97.27
20	2896000	2493902	3052088	2606005	2961703	2533688	96.85	96.15	96.68
30	2857230	2502888	2998628	2583360	3062090	2603003	95.21	95.29	97.30
40	2887437	2489385	2914665	2515120	2981544	2573374	96.73	95.14	95.83
50	2896283	2494688	2913298	2498493	2828265	2495987	96.82	95.73	93.72
60	2883458	2492776	2987679	2537837	2861939	2487628	96.47	96.65	95.15

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-87-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

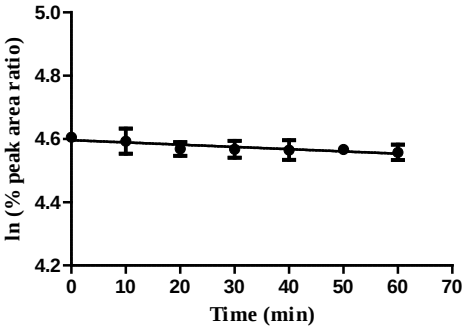
Day 1

Linear regression analysis:

Slope: -0.0007107 ± 0.0001294

At X= 60, Y = 4.553 ± 0.004665

R^2 = 0.6136



Metabolic Parameters:

Half-life: 975.09 ± 177.5 min

V_d: 100 µL/mg

Intrinsic clearance: 0.07107 µL/min/mg

Metabolic Rate: 1.4214 nmol/min/mg

% remaining at 60 min: 94.91± 0.10 %

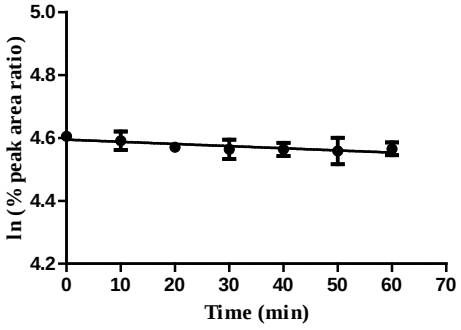
Day 2

Linear regression analysis:

Slope: -0.0006857 ± 0.0001368

At X = 60, Y = 4.553 ± 0.004934

R^2 = 0.5692



Metabolic Parameters:

Half-life: 1010.64 ± 201.62 min

V_d: 100 µL/mg

Intrinsic clearance: 0.06857 µL/min/mg

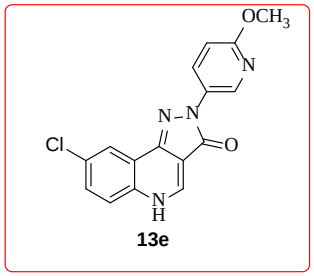
Metabolic Rate: 1.3714 nmol/min/mg

% remaining at 60 min: 94.91 ± 0.10 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13e**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13e

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	5644208	1919825	5875547	1981129	5841039	1934689	99.99	99.99	100.00
10	5456553	1899842	5706009	1973504	5797339	1962833	97.69	97.48	97.83
20	5328278	1918500	5552056	1938966	5512970	1884596	94.46	96.54	96.89
30	5168256	1864504	5636298	2016714	5444451	1881687	94.28	94.22	95.83
40	5385979	1966025	5540361	2025809	5515331	1975225	93.18	92.20	92.48
50	5440986	2009443	5449974	1939177	5358680	1895764	92.09	94.75	93.62
60	5504710	2003905	5206503	1888636	5485574	1981129	93.43	92.94	91.71

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	5710121	2003409	5613818	1965608	5604288	1995291	100.00	100.00	99.99
10	5452301	1929979	5571224	1958058	5522406	2000126	99.12	99.62	98.29
20	5400422	1918141	5301727	1920970	5297920	1926830	98.78	96.63	97.88
30	5182982	1899424	5302594	1936282	5227006	1955810	95.74	95.88	95.14
40	5159406	1912916	5164528	1949451	5194723	1988147	94.63	92.76	93.01
50	4880937	1855401	5027967	1935829	5150225	2000688	92.30	90.94	91.64
60	5141039	1926599	4959117	1880283	5016219	1915448	93.63	92.34	93.23

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-18-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

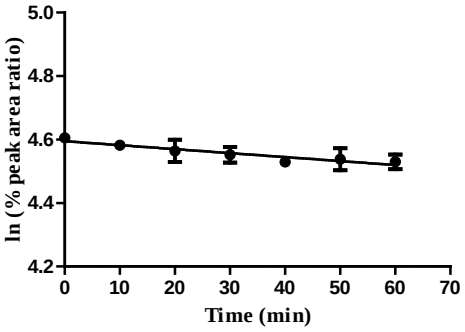
Day 1

Linear regression analysis:

Slope: -0.001251 ± 0.0001340

At X= 60, Y = 4.519 ± 0.004832

R^2 = 0.8210



Metabolic Parameters:

Half-life: 554 ± 48 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1251 µL/min/mg

Metabolic Rate: 2.502 nmol/min/mg

% remaining at 60 min: 91.74 ± 0.10%

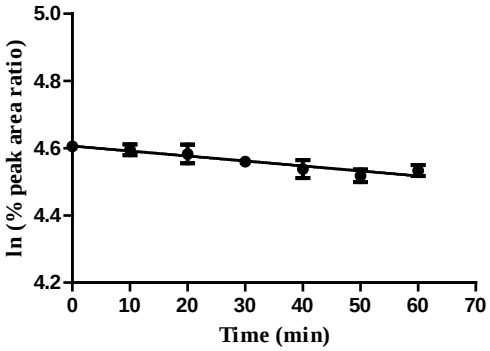
Day 2

Linear regression analysis:

Slope: -0.001481 ± 0.0001282

At X = 60, Y = 4.517 ± 0.004621

R^2 = 0.8754



Metabolic Parameters:

Half-life: 468 ± 40 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1481 µL/min/mg

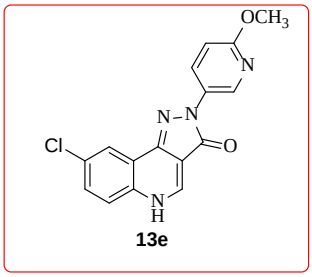
Metabolic Rate: 2.962 nmol/min/mg

% remaining at 60 min: 91.56 ± 0.10 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13e**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13e

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	4937563	1972562	5251403	1995612	5086726	1981323	100.00	100.01	100.01
10	4743029	2003783	5151998	2000212	5005602	1988213	94.56	97.89	98.07
20	4568830	1954232	5062891	2003111	4934856	2000123	93.40	96.06	96.11
30	4439613	1968634	4805231	1941987	4731044	1961422	90.09	94.04	93.96
40	4389231	1987724	4773953	1973222	4667774	1985162	88.22	91.95	91.59
50	4226640	1963123	4573238	1968173	4496211	1961134	86.01	88.31	89.31
60	4119508	1948631	4436071	1982333	4386336	1971123	84.46	85.05	86.68

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	DK-II-18-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	5010208	1974234	5302584	1989204	5058876	2000125	99.99	99.98	100.01
10	4862143	2001223	5170493	1956723	4837587	1939444	95.72	99.11	98.62
20	4641383	1962434	4986012	1922437	4623018	1914324	93.18	97.28	95.49
30	4706895	1962123	4947326	2004601	4676723	2000220	94.51	92.57	92.45
40	4539738	1981983	4819685	2000281	4537177	1921683	90.24	90.37	93.35
50	4412058	2002404	4444467	1945434	4381492	1963739	86.81	85.69	88.22
60	4214014	1934115	4422823	1981423	4265317	1944343	85.84	83.72	86.74

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-18-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

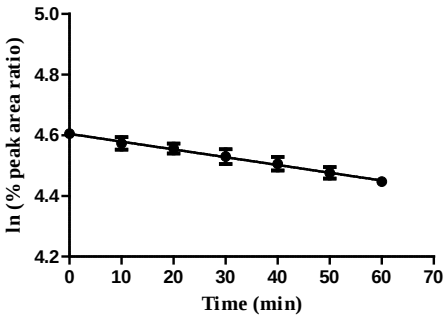
Day 1

Linear regression analysis:

Slope: -0.002564 ± 0.0001760

At X= 60, Y = 4.451 ± 0.006346

R^2 = 0.9178



Metabolic Parameters:

Half-life: 270.28 ± 18.55 min

V_d: 100 µL/mg

Intrinsic clearance: 0.2564 µL/min/mg

Metabolic Rate: 5.128 nmol/min/mg

% remaining at 60 min: 85.7 ± 0.12%

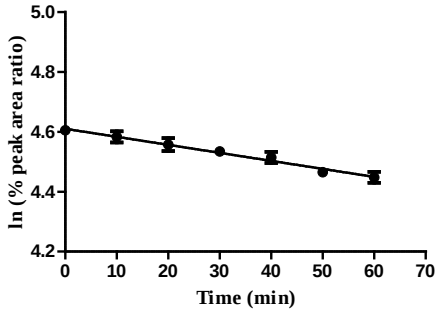
Day 2

Linear regression analysis:

Slope: -0.002679 ± 0.0001699

At X = 60, Y = 4.449 ± 0.006127

R^2 = 0.9290



Metabolic Parameters:

Half-life: 258.67 ± 16.4 min

V_d: 100 µL/mg

Intrinsic clearance: 0.2679 µL/min/mg

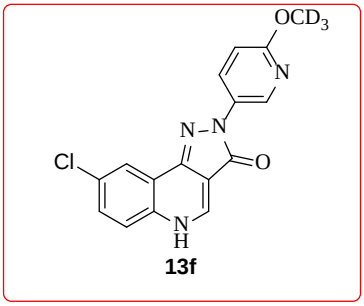
Metabolic Rate: 5.358 nmol/min/mg

% remaining at 60 min: 85.54 ± 0.11%

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13f**
Concentration: 10 µM
Date: 05-11-2016
Facility: Milwaukee Institute for Drug Discovery



13f

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3214207	196202	3239494	197689	3238583	200205	100.00	99.99	100.00
10	3173922	195337	3033035	189883	3108369	194321	99.18	97.47	98.88
20	3191550	200098	3261666	208782	3003935	190879	97.36	95.33	97.28
30	3099038	197935	3121234	200528	2905054	192940	95.57	94.98	93.08
40	2898276	189598	3008858	196575	2893897	189441	93.31	93.40	94.43
50	2855181	189062	3105323	199671	2992260	197581	92.18	94.90	93.62
60	3053840	200759	3071294	200786	2996742	194351	92.85	93.34	95.32

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3395328	205696	3206463	199836	3202311	199243	99.99	100.00	100.00
10	3197486	196263	3107429	195452	2971772	189697	98.69	99.08	97.47
20	2980275	190238	2969809	189676	3060284	199223	94.90	97.58	95.57
30	2995384	192622	3081130	201603	2942815	196349	94.20	95.25	93.25
40	2963889	191551	2906813	196822	3090401	201279	93.73	92.04	95.53
50	3237009	207483	2995395	199070	3007720	198190	94.51	93.78	94.42
60	3179593	200002	3092217	199044	2949833	192390	96.30	96.82	95.39

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-59-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

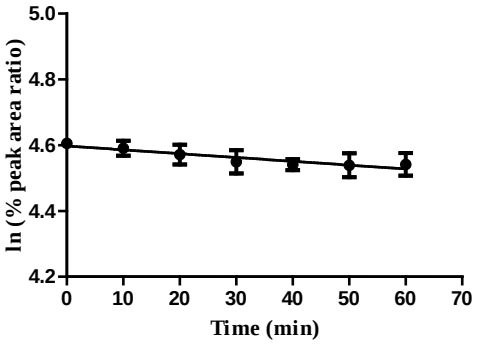
Day 1

Linear regression analysis:

Slope: -0.0001161 ± 0.0001473

At X= 60, Y = 4.527 ± 0.005309

R^2 = 0.7658



Metabolic Parameters:

Half-life: 596.89 ± 75.73 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1161 µL/min/mg

Metabolic Rate: 2.322 nmol/min/mg

% remaining at 60 min: 92.4 ± 0.10 %

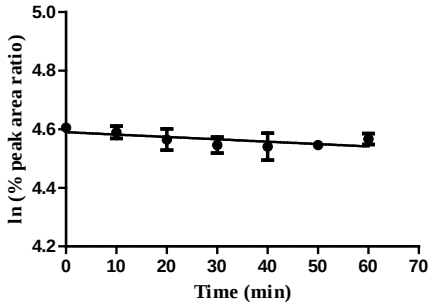
Day 2

Linear regression analysis:

Slope: -0.0008107 ± 0.0002054

At X = 60, Y = 4.541 ± 0.007406

R^2 = 0.4505



Metabolic Parameters:

Half-life: 854.81 ± 216.57 min

V_d: 100 µL/mg

Intrinsic clearance: 0.08107 µL/min/mg

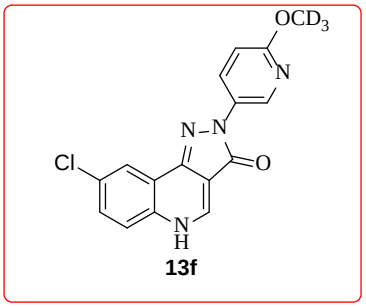
Metabolic Rate: 1.6214 nmol/min/mg

% remaining at 60 min: 93.78 ± 0.15 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13f**
Concentration: 10 µM
Date: 05-11-2016
Facility: Milwaukee Institute for Drug Discovery



13f

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3434185	199292	3403798	199962	3467119	198537	100.00	100.00	100.00
10	3261135	192712	3308124	200914	3316748	193077	98.20	96.73	98.37
20	3277305	198836	3252309	201825	3294910	194431	95.65	94.66	97.04
30	3195052	200928	3204258	202859	3226537	202460	92.27	92.79	91.26
40	3116114	198399	3109879	202925	3283568	207620	91.14	90.03	90.56
50	3075182	199859	2943205	199172	3189757	206428	89.29	86.81	88.48
60	2962666	201067	2891865	203629	2942467	196355	85.50	83.43	85.81

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	DK-II-59-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	3272456	203581	3398083	203758	3394417	199950	100.00	100.00	100.00
10	3194621	200636	3224902	198751	3152045	190949	99.05	97.29	97.23
20	3118089	199275	3116033	200838	3175198	198429	97.34	93.03	94.26
30	3030724	196565	3012793	200320	3136068	198920	95.92	90.18	92.86
40	2867077	195909	2931859	199978	3054711	193801	91.04	87.91	92.84
50	2812061	200539	2890102	197907	2933773	200393	87.23	87.56	86.24
60	2718349	203636	2806729	199949	2908503	200605	83.04	84.17	85.40

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-59-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

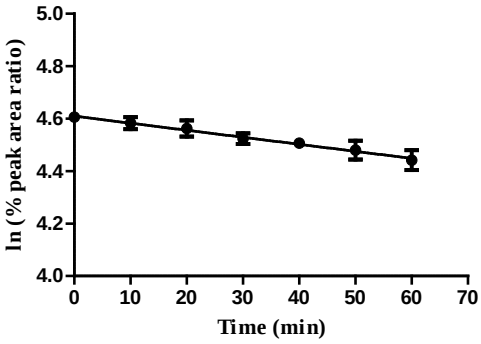
Day 1

Linear regression analysis:

Slope: -0.002686 ± 0.0001145

At X= 60, Y = 4.448 ± 0.004129

R^2 = 0.9666



Metabolic Parameters:

Half-life: 258.004 ± 10.99 min

V_d: 100 µL/mg

Intrinsic clearance: 0.2686 µL/min/mg

Metabolic Rate: 5.372 nmol/min/mg

% remaining at 60 min: 85.45 ± 0.07 %

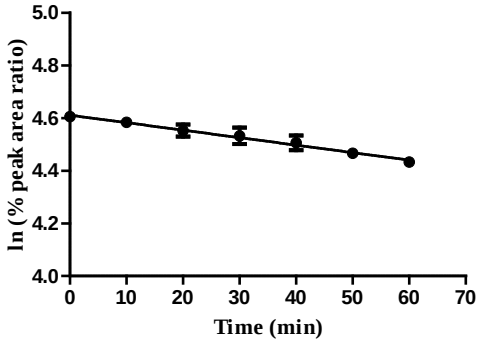
Day 2

Linear regression analysis:

Slope: -0.002849 ± 0.0001933

At X = 60, Y = 4.440 ± 0.006969

R^2 = 0.9196



Metabolic Parameters:

Half-life: 243.24 ± 16.5 min

V_d: 100 µL/mg

Intrinsic clearance: 0.2849 µL/min/mg

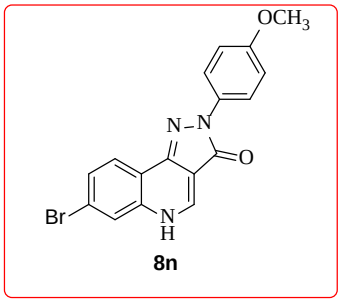
Metabolic Rate: 5.698 nmol/min/mg

% remaining at 60 min: 84.77 ± 0.13 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8n**
Concentration: 10 µM
Date: 04-13-2016
Facility: Milwaukee Institute for Drug Discovery



8n

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 0.5 µM 4,5 Diphenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	40017	110382	41858	119807	38420	110467	99.87	100.10	99.94
10	36405	108314	36156	110853	34332	107107	92.59	93.45	92.10
20	35068	111286	33804	108649	33885	114225	86.80	89.14	85.24
30	31839	109123	30803	108326	29669	103425	80.37	81.47	82.43
40	29562	109819	29855	110849	28562	110188	74.15	77.17	74.48
50	24886	99310	25961	103663	27453	109407	69.03	71.75	72.10
60	23335	100114	25309	115500	26763	116484	64.21	62.78	66.02

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	49475	117867	49987	119544	48124	110902	99.94	100.03	99.98
10	45683	118348	44098	114025	47589	117783	91.90	92.52	93.09
20	39116	104050	37792	102482	38666	103094	89.50	88.22	86.41
30	42354	116764	36851	107165	37992	110412	86.36	82.26	79.28
40	34884	102256	38809	117335	33961	102324	81.22	79.12	76.47
50	39590	126003	34528	108682	35448	110883	74.80	76.00	73.66
60	33816	121431	32387	118994	38044	127573	66.30	65.11	68.71

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (LAU 463)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

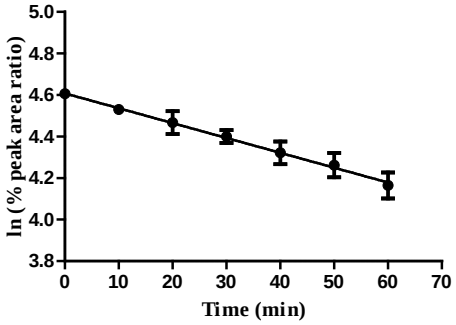
Day 1

Linear regression analysis:

Slope: -0.007155 ± 0.0001949

At X= 60, Y = 4.178 ± 0.007028

R^2 = 0.9861



Metabolic Parameters:

Half-life: 96.85 ± 2.63 min

V_d: 100 µL/mg

Intrinsic clearance: 0.7155 µL/min/mg

Metabolic Rate: 14.31 nmol/min/mg

% remaining at 60 min: 65.23 ± 0.10 %

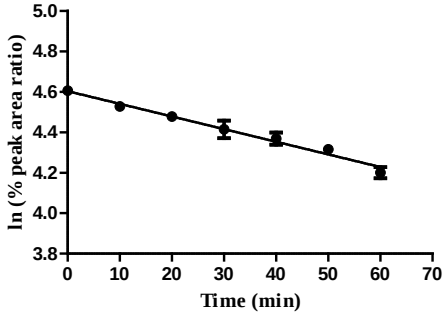
Day 2

Linear regression analysis:

Slope: -0.006250 ± 0.0002926

At X = 60, Y = 4.228 ± 0.01055

R^2 = 0.9600



Metabolic Parameters:

Half-life: 110.88 ± 5.19 min

V_d: 100 µL/mg

Intrinsic clearance: 0.625 µL/min/mg

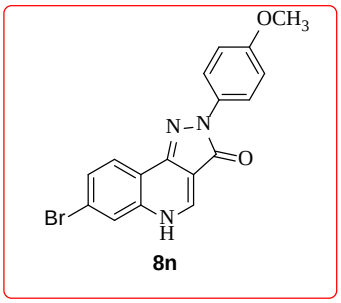
Metabolic Rate: 12.5 nmol/min/mg

% remaining at 60 min: 68.5 ± 0.17 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8n**
Concentration: 10 µM
Date: 04-13-2016
Facility: Milwaukee Institute for Drug Discovery



8n

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 0.5 µM 4,5 Diphenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	48890	112924	49279	113143	49215	113661	99.98	99.89	100.00
10	45153	112247	43675	107282	47056	117053	92.90	93.37	92.84
20	38035	103753	42468	116346	40403	109584	84.66	83.71	85.14
30	39080	108516	39403	111577	40013	113332	83.17	80.99	81.53
40	34991	101201	34999	104471	35979	112089	79.85	76.83	74.13
50	33923	105799	32017	103609	34231	110384	74.05	70.87	71.61
60	30997	104638	31893	109310	32070	112609	68.41	66.91	65.77

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	LAU 463 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	49868	114209	48673	113475	50609	115332	99.91	99.98	99.95
10	45918	111248	43511	106649	45190	108674	94.45	95.10	94.72
20	40638	108555	41931	111724	43760	113925	85.66	87.48	87.49
30	39591	107243	41207	112646	40629	107987	84.47	85.27	85.70
40	42615	117892	38437	108907	39788	108268	82.71	82.26	83.71
50	37730	112216	35424	110108	39287	115905	76.94	74.99	77.21
60	34666	113551	30020	105567	32646	109583	69.86	66.28	67.86

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (LAU 463)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

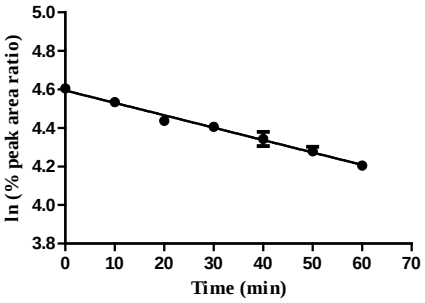
Day 1

Linear regression analysis:

Slope: -0.006435 ± 0.0002287

At X= 60, Y = 4.208 ± 0.008246

R^2 = 0.9766



Metabolic Parameters:

Half-life: 107.69 ± 3.82 min

V_d: 100 µL/mg

Intrinsic clearance: 0.6435 µL/min/mg

Metabolic Rate: 12.87 nmol/min/mg

% remaining at 60 min: 67.22 ± 0.13 %

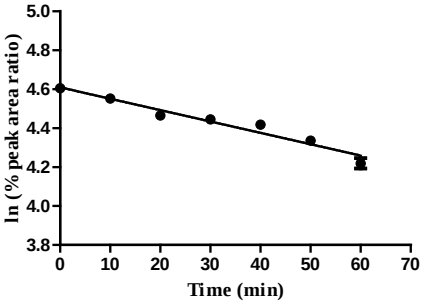
Day 2

Linear regression analysis:

Slope: -0.005840 ± 0.0003190

At X = 60, Y = 4.259 ± 0.01150

R^2 = 0.9464



Metabolic Parameters:

Half-life: 118.66 ± 6.48 min

V_d: 100 µL/mg

Intrinsic clearance: 0.584 µL/min/mg

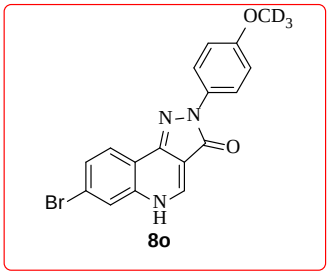
Metabolic Rate: 11.68 nmol/min/mg

% remaining at 60 min: 70.7 ± 0.19 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8o**
Concentration: 10 µM
Date: 05-05-2016
Facility: Milwaukee Institute for Drug Discovery



8o

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 0.5 µM 4,5 Diphenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	52579	105204	49506	99051	48659	98086	99.95	99.96	100.01
10	45825	93541	44451	93116	48471	98801	97.97	95.47	98.91
20	43583	94565	43623	95700	46503	99016	92.17	91.16	94.68
30	42546	96285	40594	92790	42711	96764	88.37	87.49	88.99
40	40999	96785	41454	96231	41457	96490	84.72	86.15	86.62
50	37334	91894	39270	94259	40259	95854	81.25	83.32	84.67
60	39122	95274	39560	97477	37942	93234	82.12	81.16	82.04

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	49813	99775	51634	107495	53015	106527	100.05	100.07	99.93
10	48894	100371	46330	100245	51318	103178	97.62	96.28	99.87
20	46014	97980	45490	99937	46137	100422	94.11	94.83	92.25
30	45730	101697	42043	99140	44245	99348	90.11	88.34	89.42
40	42831	97947	40003	95150	41764	97359	87.63	87.58	86.13
50	41858	98511	44806	109872	42381	101831	85.15	84.95	83.57
60	43517	106102	40987	101673	42905	101517	82.19	83.98	84.86

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-58-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

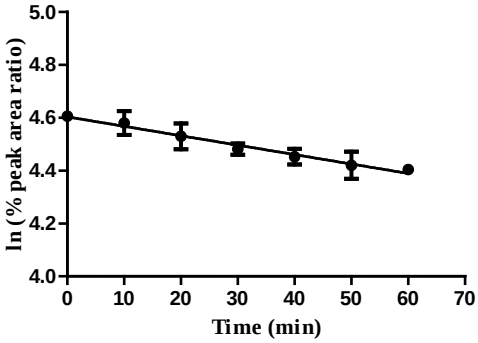
Day 1

Linear regression analysis:

Slope: -0.003567 ± 0.0001743

At X= 60, Y = 4.389 ± 0.006286

R^2 = 0.9566



Metabolic Parameters:

Half-life: 194.28 ± 9.48 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3567 µL/min/mg

Metabolic Rate: 7.134 nmol/min/mg

% remaining at 60 min: 80.55 ± 0.11 %

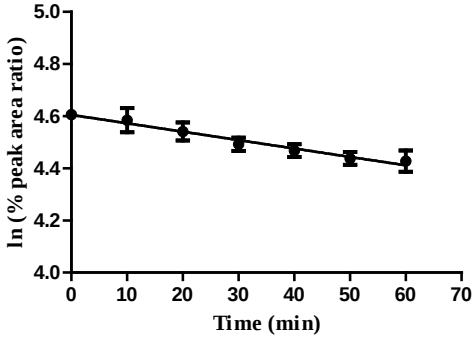
Day 2

Linear regression analysis:

Slope: -0.003224 ± 0.0001677

At X = 60, Y = 4.411 ± 0.006045

R^2 = 0.9511



Metabolic Parameters:

Half-life: 214.95 ± 11.18 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3224 µL/min/mg

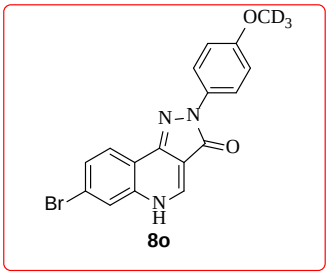
Metabolic Rate: 6.448 nmol/min/mg

% remaining at 60 min: 82.35 ± 0.11 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8o**
Concentration: 10 µM
Date: 05-05-2016
Facility: Milwaukee Institute for Drug Discovery



8o

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 0.5 µM 4,5 Diphenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	45407	99025	45778	109217	48351	111002	99.90	100.03	99.90
10	39689	88172	39750	98111	47817	112502	98.06	96.69	97.48
20	38346	95002	36676	101161	40927	111059	87.93	86.5	84.52
30	36091	94410	37944	107423	36126	98426	83.28	84.30	84.18
40	38332	101105	37964	110739	40143	115678	82.59	81.82	79.59
50	37879	103619	29626	91731	30557	93383	79.64	77.08	75.05
60	35899	101755	28546	92601	31989	100220	76.86	73.57	73.20

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	DK-I-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	47531	108669	49814	114293	53117	126614	100.09	99.96	99.88
10	41907	100372	46729	109931	47056	115838	95.54	97.49	96.72
20	39682	102754	44889	113608	43666	116211	88.37	90.62	89.46
30	36906	100187	42827	112938	41706	120148	84.29	86.97	82.64
40	32951	94121	40182	112083	36083	105504	80.11	82.22	81.43
50	32511	95049	36769	103352	35590	109154	78.27	81.59	77.63
60	31896	99642	34866	105232	34822	111570	73.25	75.99	74.31

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-58-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

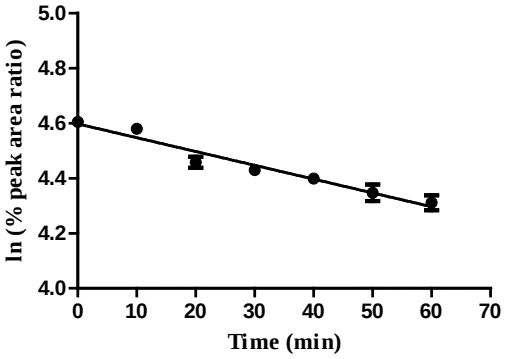
Day 1

Linear regression analysis:

Slope: -0.005018 ± 0.0003001

At X= 60, Y = 4.296 ± 0.01082

R^2 = 0.9364



Metabolic Parameters:

Half-life: 138.10 ± 8.25 min

V_d: 100 µL/mg

Intrinsic clearance: 0.5018 µL/min/mg

Metabolic Rate: 10.036 nmol/min/mg

% remaining at 60 min: 73.40 ± 0.18 %

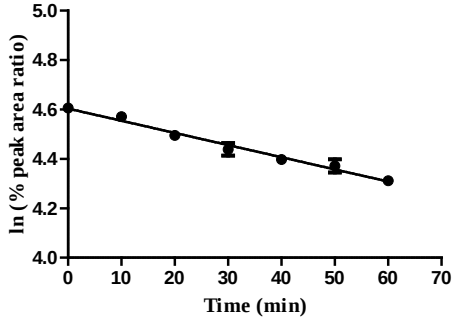
Day 2

Linear regression analysis:

Slope: -0.004920 ± 0.0002135

At X = 60, Y = 4.308 ± 0.007697

R^2 = 0.9511



Metabolic Parameters:

Half-life: 140.85 ± 6.11 min

V_d: 100 µL/mg

Intrinsic clearance: 0.492 µL/min/mg

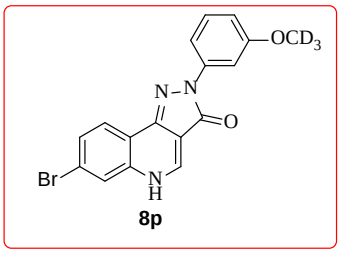
Metabolic Rate: 9.84 nmol/min/mg

% remaining at 60 min: 74.29 ± 0.13 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8p**
Concentration: 10 µM
Date: 02-28-2017
Facility: Milwaukee Institute for Drug Discovery



8p

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 3 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	59983	321908	58272	320874	62965	326832	100.18	99.78	99.82
10	50570	320891	51796	331636	59165	330127	84.72	85.81	92.86
20	48532	331288	48615	332097	50049	327548	78.76	80.43	79.17
30	45866	320891	42178	326711	45910	327644	76.84	70.93	72.60
40	42711	331008	40045	323974	41991	330194	69.37	67.91	65.89
50	39783	322898	43199	326744	43192	327634	66.24	72.64	68.30
60	40250	321356	41123	330100	45947	331073	67.33	68.44	71.90

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	58466	325834	57839	331063	61445	328646	100.24	99.83	99.98
10	52258	327543	50774	327534	52235	327167	89.13	88.58	85.37
20	43637	300183	44263	327846	46981	326348	81.21	77.14	76.98
30	44482	328643	41357	324543	43352	320087	75.61	72.81	72.42
40	41402	325673	39919	329634	40509	326849	71.02	69.20	66.27
50	39693	328964	40765	331208	41609	326183	67.40	70.33	68.21
60	39214	330132	39549	330974	42418	323491	66.35	68.28	70.12

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-92-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

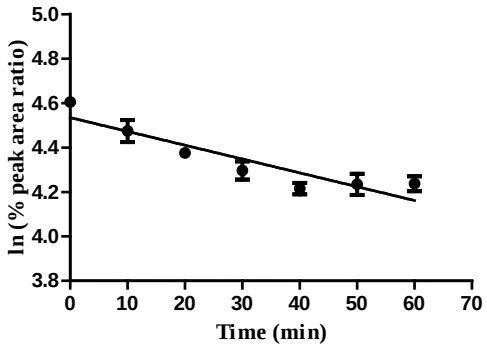
Day 1

Linear regression analysis:

Slope: -0.006219 ± 0.0006880

At X= 60, Y = 4.161 ± 0.02481

R^2 = 0.8114



Metabolic Parameters:

Half-life: 111 ± 12 min

V_d: 100 µL/mg

Intrinsic clearance: 0.6219 µL/min/mg

Metabolic Rate: 12.43 nmol/min/mg

% remaining at 60 min: 64 ± 0.32 %

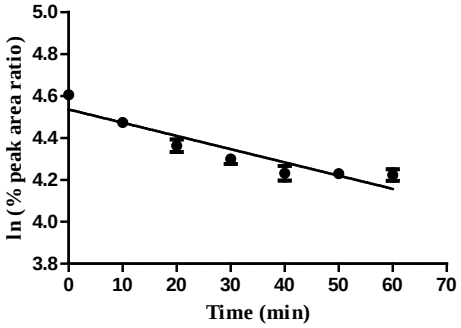
Day 2

Linear regression analysis:

Slope: -0.006312 ± 0.0006039

At X = 60, Y = 4.157 ± 0.02178

R^2 = 0.8518



Metabolic Parameters:

Half-life: 109 ± 10 min

V_d: 100 µL/mg

Intrinsic clearance: 0.6312 µL/min/mg

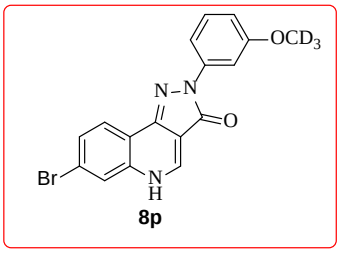
Metabolic Rate: 12.624 nmol/min/mg

% remaining at 60 min: 63 ± 0.33 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8p**
Concentration: 10 µM
Date: 02-28-2017
Facility: Milwaukee Institute for Drug Discovery



8p

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 3 µM Verapamil in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	56473	328643	54532	330183	56831	328124	99.90	100.09	100.11
10	52879	326447	52410	328634	53076	320843	94.17	96.65	95.62
20	49695	325843	49012	327634	49066	327843	88.67	90.66	86.51
30	49248	328643	47078	328634	46463	327563	87.12	86.82	81.99
40	47758	325766	44351	324451	44309	325684	85.23	82.84	78.64
50	46538	329766	42829	319874	46234	330198	82.04	81.14	80.93
60	43351	300784	44611	320073	46103	321843	83.79	84.47	82.80

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	DK-I-92-1 Peak area	Verapamil Peak area	Assay 1	Assay 2	Assay 3
0	64419	324783	61391	326673	62173	327893	100.17	99.96	99.79
10	63199	327912	58288	327684	58681	326874	97.33	94.61	94.48
20	58267	331287	52796	326943	54301	328743	88.82	85.89	86.93
30	55696	327564	50682	331084	52864	325873	85.87	81.42	85.38
40	52483	328634	51361	327439	51100	328964	80.65	83.43	81.75
50	53109	320734	52568	323289	52717	330023	83.62	86.49	84.07
60	54167	331674	51031	334087	52001	326484	82.48	81.24	83.82

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-92-1)}}{\text{Peak area of internal standard (Verapamil)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

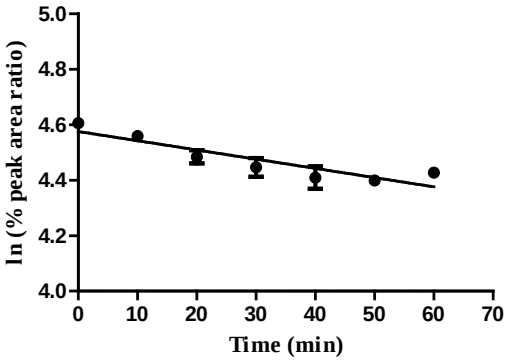
Day 1

Linear regression analysis:

Slope: -0.003323 ± 0.0004092

At X= 60, Y = 4.376 ± 0.01475

R^2 = 0.7763



Metabolic Parameters:

Half-life: 208 ± 25 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3323 µL/min/mg

Metabolic Rate: 6.646 nmol/min/mg

% remaining at 60 min: 79 ± 0.26 %

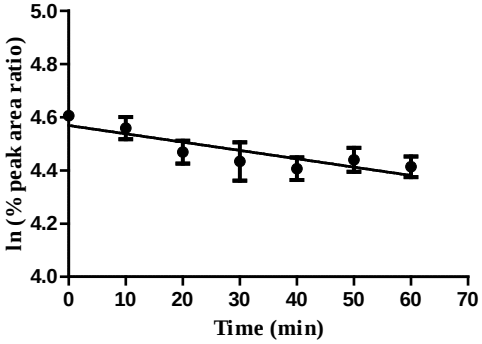
Day 2

Linear regression analysis:

Slope: -0.003129 ± 0.0004255

At X = 60, Y = 4.381 ± 0.01534

R^2 = 0.7399



Metabolic Parameters:

Half-life: 221 ± 30 min

V_d: 100 µL/mg

Intrinsic clearance: 0.3129 µL/min/mg

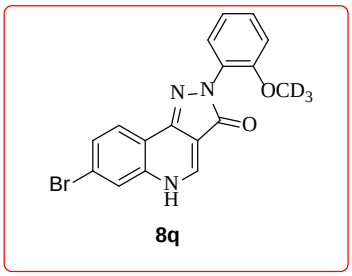
Metabolic Rate: 6.258 nmol/min/mg

% remaining at 60 min: 80 ± 0.30 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8q**
Concentration: 10 µM
Date: 08-15-2016
Facility: Milwaukee Institute for Drug Discovery



8q

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Biosciences, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	565063	1986723	588540	1967352	555706	1976123	100.14	100.05	100.07
10	543616	1967237	563545	1967235	547671	1976236	97.30	95.80	98.62
20	526501	1967236	539026	1896235	516591	1897823	94.23	95.07	96.86
30	504039	1967236	545481	2000823	500945	1897236	90.21	91.18	93.96
40	522804	1963562	562762	1987328	511914	1967263	93.75	94.70	92.60
50	525487	2000732	541596	1966237	524543	1987623	92.48	92.12	93.91
60	529045	1967235	542507	1956253	527483	1965232	94.69	92.74	95.51

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	542091	2008782	528526	1962632	563729	1989123	99.94	100.10	100.14
10	497942	1899972	498199	1967236	535488	2000126	97.06	94.14	94.60
20	508446	1972628	496390	1978236	528768	1988367	95.46	93.28	93.96
30	507591	1988316	500842	1956625	538342	1976213	94.55	95.15	96.25
40	511063	1987236	509942	1972637	515849	1963256	95.24	96.10	92.84
50	497689	1967262	497074	1976236	545138	2008362	93.69	93.50	95.91
60	506687	1972367	508595	1978236	516138	1967235	95.14	95.57	92.70

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-89-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T (T = 0, 10, 20,30,40,50 and 60)} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

DATA ANALYSIS:

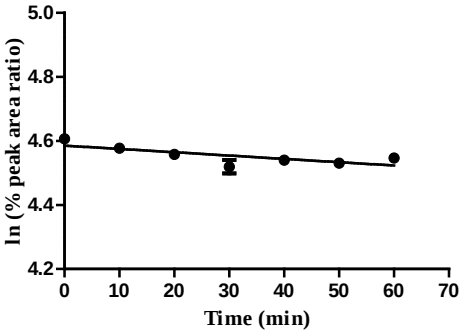
Day 1

Linear regression analysis:

Slope: -0.001035 ± 0.0002435

At X= 60, Y = 4.523 ± 0.0008781

R^2 = 0.4871



Metabolic Parameters:

Half-life: 669.5652 ± 157.52 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1035 µL/min/mg

Metabolic Rate: 2.07 nmol/min/mg

% remaining at 60 min: 92.11 ± 0.17 %

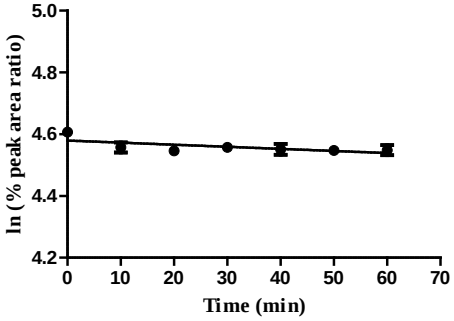
Day 2

Linear regression analysis:

Slope: -0.0006738 ± 0.0002075

At X = 60, Y = 4.539 ± 0.007480

R^2 = 0.3570



Metabolic Parameters:

Half-life: 1028.95 ± 316.72 min

V_d: 100 µL/mg

Intrinsic clearance: 0.06738 µL/min/mg

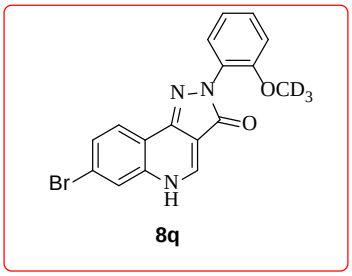
Metabolic Rate: 1.3476 nmol/min/mg

% remaining at 60 min: 93.59 ± 0.15 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **8q**
Concentration: 10 µM
Date: 08-15-2016
Facility: Milwaukee Institute for Drug Discovery



8q

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	576315	1967238	541302	1967322	566955	1967623	99.98	100.05	100.04
10	512948	1967625	464987	2007882	498225	1976723	88.97	84.21	87.51
20	451074	1956523	459273	1976235	481299	1897262	78.68	84.50	88.08
30	440035	2008287	424206	1965232	425241	1976232	74.78	78.49	74.71
40	413011	1942356	407167	1962362	401432	1987233	72.57	75.45	70.14
50	395228	1976233	356213	1976232	384691	1999733	68.25	65.54	66.79
60	366164	1972362	333006	1965253	347012	1978273	63.36	61.61	60.90

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	DK-I-89-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	549917	1976232	529475	1967253	575556	1976253	100.09	100.05	100.08
10	496314	1967232	459274	1956232	489236	1976232	90.75	87.27	85.07
20	458183	1972632	407367	1923762	459517	2000872	83.55	78.71	78.92
30	432253	1977623	391427	1952352	414530	1998762	78.62	74.53	71.26
40	395301	1955235	363066	2008273	416702	1962352	72.72	67.20	72.97
50	350019	1956232	359469	2000072	369384	1962533	64.36	66.81	64.68
60	338001	1997232	334995	1976232	363915	1976318	60.87	63.01	63.27

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-I-89-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

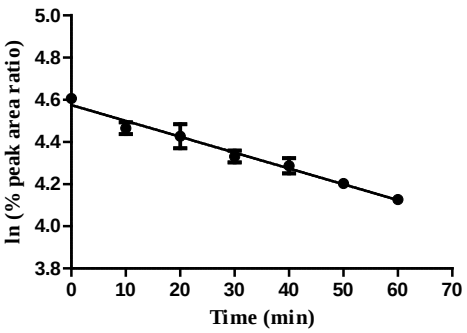
Day 1

Linear regression analysis:

Slope: -0.007506 ± 0.0003722

At X= 60, Y = 4.124 ± 0.01342

R^2 = 0.9554



Metabolic Parameters:

Half-life: 93.32 ± 4.577 min

V_d: 100 µL/mg

Intrinsic clearance: 0.7506 µL/min/mg

Metabolic Rate: 15.012 nmol/min/mg

% remaining at 60 min: 61.80 ± 0.2%

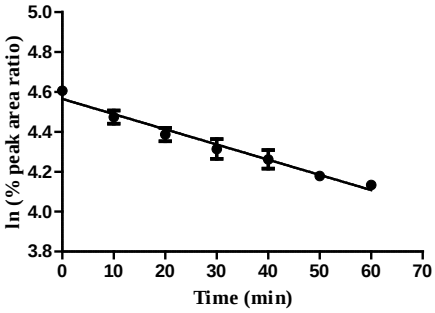
Day 2

Linear regression analysis:

Slope: -0.000738 ± 0.0002075

At X = 60, Y = 4.539 ± 0.007480

R^2 = 0.9480



Metabolic Parameters:

Half-life: 91.01 ± 4.89 min

V_d: 100 µL/mg

Intrinsic clearance: 0.7614 µL/min/mg

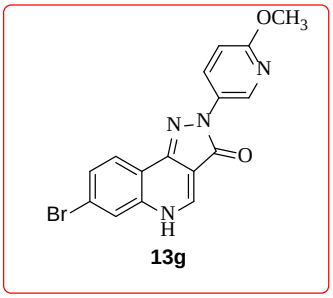
Metabolic Rate: 15.228 nmol/min/mg

% remaining at 60 min: 60.82 ± 0.21 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13g**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13g

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1239575	409876	1282331	408077	1272732	424404	100.00	100.01	99.99
10	1272583	421502	1278046	415028	1275562	432495	99.84	98.00	98.34
20	1254337	431729	1305269	428021	1229879	422496	96.07	97.05	97.06
30	1221632	426097	1270878	425579	1208114	431322	94.80	95.04	93.39
40	1213285	426869	1251740	426921	1237850	438598	93.99	93.31	94.10
50	1206569	425741	1245491	420860	1245919	441826	93.71	94.18	94.02
60	1193565	413673	1243063	414198	1229125	429212	95.41	95.51	95.48

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1370833	444955	1198733	408906	1220048	417681	99.99	99.98	100.00
10	1328181	436723	1164859	407987	1222948	422920	98.71	97.37	98.99
20	1336173	447939	1160980	415555	1180805	419134	96.81	95.28	96.44
30	1288471	435125	1118801	402193	1145140	415499	96.11	94.87	94.35
40	1303844	448160	1129419	413993	1175925	416876	94.42	93.04	96.57
50	1310818	441914	1141621	409483	1163535	424257	96.27	95.08	93.89
60	1272477	436169	1153092	414963	1182693	435717	94.69	94.77	92.92

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-48-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

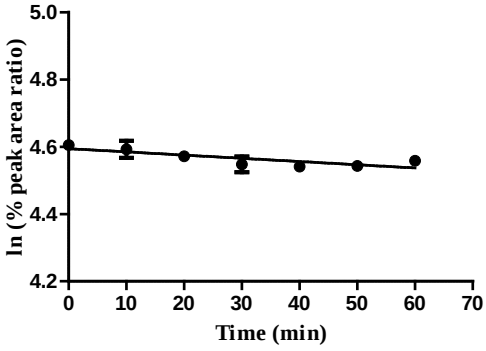
Day 1

Linear regression analysis:

Slope: -0.0009583 ± 0.0001613

At X= 60, Y = 4.537 ± 0.005817

R^2 = 0.6500



Metabolic Parameters:

Half-life: 723.15 ± 121 min

V_d: 100 µL/mg

Intrinsic clearance: 0.09583 µL/min/mg

Metabolic Rate: 1.9166 nmol/min/mg

% remaining at 60 min: 93.41 ± 0.12%

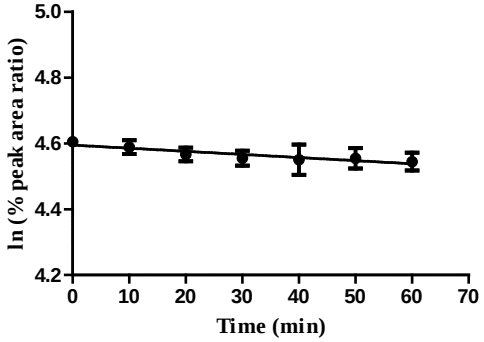
Day 2

Linear regression analysis:

Slope: -0.0009464 ± 0.0001399

At X = 60, Y = 4.538 ± 0.005045

R^2 = 0.7066



Metabolic Parameters:

Half-life: 732.24 ± 108.2 min

V_d: 100 µL/mg

Intrinsic clearance: 0.09464 µL/min/mg

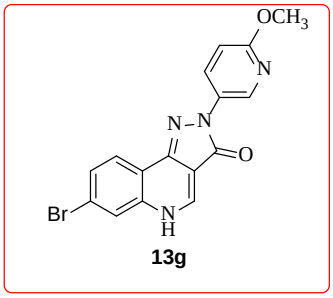
Metabolic Rate: 1.8928 nmol/min/mg

% remaining at 60 min: 93.5 ± 0.10 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13g**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13g

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 2 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # MSMC-PL) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1285705	419827	1242554	422780	1356651	447903	100.01	100.00	99.99
10	1284540	435905	1313352	447517	1327774	441532	96.23	99.85	99.28
20	1292275	437096	1264515	444110	1274051	435877	96.55	96.88	96.49
30	1287912	429522	1236947	446158	1274901	437129	97.92	94.33	96.28
40	1254958	444035	1226739	439257	1271572	442179	92.30	95.02	94.93
50	1237554	436140	1245217	450546	1280902	443536	92.66	94.03	95.34
60	1254241	438615	1282355	457098	1250474	439668	93.38	95.45	93.89

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	DK-II-48-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1307622	464746	1292157	462060	1295383	483598	99.98	99.98	99.98
10	1286526	464981	1272404	460828	1283952	495797	98.32	98.71	96.66
20	1243863	463202	1260023	460005	1173740	463148	95.42	97.93	94.59
30	1227268	468808	1222300	454360	1178574	460559	93.02	96.18	95.52
40	1232887	472905	1269061	482260	1113808	448574	92.64	94.08	92.68
50	1293819	482688	1280879	488503	1177897	471287	95.25	93.74	93.29
60	1236291	469676	1283362	474787	1150736	458568	93.54	96.64	93.67

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-48-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

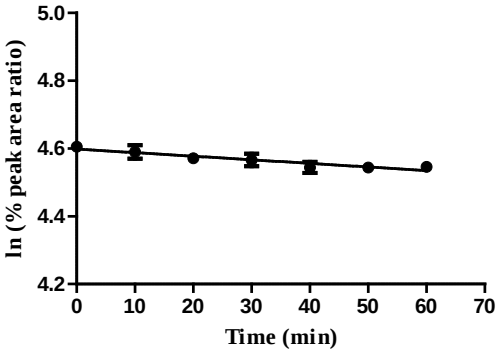
Day 1

Linear regression analysis:

Slope: -0.001060 ± 0.0001535

At X= 60, Y = 4.535 ± 0.005534

R^2 = 0.7150



Metabolic Parameters:

Half-life: 653.15 ± 94.58 min

V_d: 100 µL/mg

Intrinsic clearance: 0.1061 µL/min/mg

Metabolic Rate: 2.122 nmol/min/mg

% remaining at 60 min: 93.22 ± 0.11%

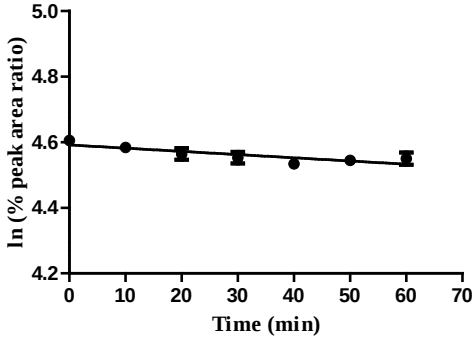
Day 2

Linear regression analysis:

Slope: -0.0009786 ± 0.0001849

At X = 60, Y = 4.533 ± 0.006666

R^2 = 0.5959



Metabolic Parameters:

Half-life: 708.15 ± 133.8 min

V_d: 100 µL/mg

Intrinsic clearance: 0.09786 µL/min/mg

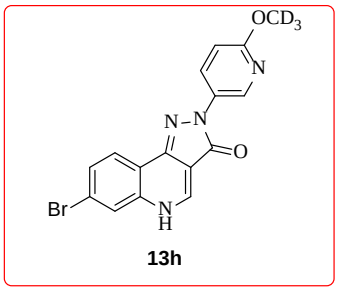
Metabolic Rate: 1.9572 nmol/min/mg

% remaining at 60 min: 93.03 ± 0.13 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Human Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13h**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13h

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (BD Gentest, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1421837	1942342	1419848	1962134	1417715	1912342	100.00	99.94	100.04
10	1380499	1892342	1403676	1955231	1459331	1961234	99.66	99.15	100.41
20	1378668	1912344	1392460	1942316	1386585	1891234	98.48	99.02	98.94
30	1368091	1923804	1423956	2000134	1363567	1886236	97.15	98.33	97.55
40	1380251	1923424	1423495	2012342	1449749	1978234	98.03	97.70	98.90
50	1411529	1979443	1414892	1965474	1408869	1933454	97.41	99.43	98.33
60	1423608	1983905	1398364	1962344	1456496	1991273	98.03	98.42	98.71

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1508498	2008329	1480681	1967456	1471597	1992344	100.01	99.94	99.94
10	1441371	1924324	1470155	1963452	1457755	2000126	99.73	99.43	98.62
20	1435215	1934535	1458718	1949042	1405631	1922344	98.78	99.39	98.94
30	1424084	1899991	1437127	1943214	1402880	1931234	99.80	98.21	98.29
40	1421610	1923434	1440579	1949987	1412381	1943244	98.41	98.10	98.35
50	1418164	1889474	1423051	1934324	1427904	2000688	99.94	97.70	96.57
60	1410739	1934543	1429296	1923444	1416949	1934324	97.10	98.68	99.12

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-58-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

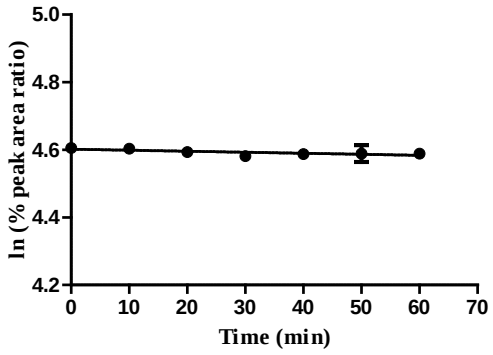
Day 1

Linear regression analysis:

Slope: -0.0003012 ± 8.169e^{-005}

At X= 60, Y = 4.583 ± 0.002945

R^2 = 0.4171



Metabolic Parameters:

Half-life: 2300.8 min

V_d: 100 µL/mg

Intrinsic clearance: 0.03012 µL/min/mg

Metabolic Rate: 0.6024 nmol/min/mg

% remaining at 60 min: 97.80 ± 0.06 %

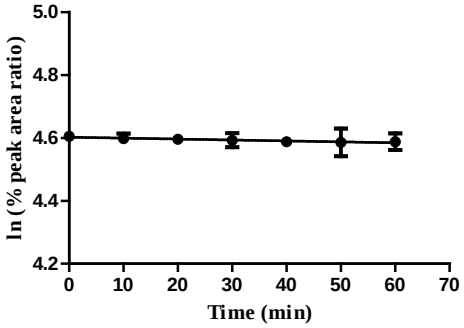
Day 2

Linear regression analysis:

Slope: -0.0002976 ± 8.722e^{-005}

At X = 60, Y = 4.584 ± 0.003145

R^2 = 0.3799



Metabolic Parameters:

Half-life: 2328.6 min

V_d: 100 µL/mg

Intrinsic clearance: 0.02976 µL/min/mg

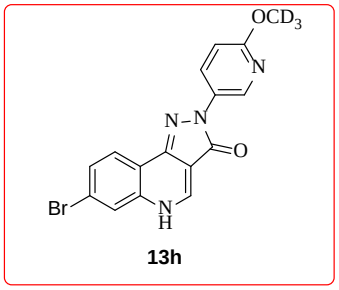
Metabolic Rate: 0.5952 nmol/min/mg

% remaining at 60 min: 97.90 ± 0.06 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.

Mouse Liver Microsomal Assay

Principal Investigator: Dr. Alexander Arnold
Operator: Revathi Kodali
Test Compound: **13h**
Concentration: 10 µM
Date: 05-26-2016
Facility: Milwaukee Institute for Drug Discovery



13h

Assay Protocol

Each evaluation included six independent assays carried out three at a time, on two different days

1. Preparation of solutions:
   a. 1 mM test compound in DMSO.
   b. 1 µM 4,5 Di Phenyl Imidazole in ice cold Acetonitrile (ISTD).
2. For a total volume of Microsomal Assay Mixture (MAM) 390 µL, sufficient for seven time points, combine the following
   a. 282 µL of 18.2 mΩ of water.
   b. 80 µL of 0.5 M potassium phosphate buffer (p^H 7.4)
   c. 20 µL of NADPH A. (Corning life sciences, Cat # 451220)
   d. 4 µL of NADPH B. (Corning life sciences, Cat # 451200)
   e. 4 µL of test compound.
3. Sonicate MAM for 5 min and meanwhile thaw microsomes (20 mg/mL) (Life technologies, Cat # 452156) on ice.
4. Aliquot 100 µL of ice cold ISTD into seven separate 1.5 mL conical vials and label them the time points for 0, 10, 20, 30, 40, 50, and 60 min.
5. Arrange the timer. Microsomes (Final concentration of 0.5 mg/mL) should be added to all time points except the zero time point.
6. Add 50 µL of the MAM solution to the conical vial labelled as zero time point. Place the remaining MAM solution in the incubator (37 ºC) for 5 min and initiate the reaction with addition of microsomes (8.8 µL) and record the time.
7. At the end of each time interval remove 50 µL and add to 100 µL ISTD in conical vial, sonicate for 10 sec and spin down at 10,000 rpm for 5 minutes.
8. Take 100 µL of supernatant and transfer to Spin-X HPLC filter tubes (Corning Incorporated, Cat # 8169) and centrifuge at 13,000 rpm for 5 minutes and take 5 µL from this solution and dilute in 495 µL of LCMS grade methanol (Fischer scientific, CAS # 67-56-1) in an 2mL glass auto sampler vial (Microsolv, Cat # 95025-WCV)
9. The samples are analyzed by LCMS-8040. (Shimadzu)

Data:

Day 1:

Time (min)	Assay 1		Assay 2		Assay 3		% remaining		
	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1435197	1954234	1423673	1973613	1424627	1943243	100.05	100.04	100.01
10	1443000	2001324	1412024	2000234	1404550	1928213	98.23	97.91	99.37
20	1409619	1932414	1417757	2003412	1435097	2000123	99.38	98.15	97.88
30	1386905	1910094	1380284	1956728	1427626	1971422	98.92	97.83	98.79
40	1388622	1952344	1392320	1931324	1413076	1935162	96.90	99.98	99.61
50	1415644	1952344	1365942	1924234	1407763	1942342	98.78	98.45	98.87
60	1400478	1928631	1362173	1934144	1405660	1962361	98.93	97.68	97.72

Table 1: Peak areas and % remaining values

Day 2:

Time (min)	Assay 1		Assay 2		Assay 3		(% remaining)		
	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	DK-II-58-1 Peak area	ISTD Peak area	Assay 1	Assay 2	Assay 3
0	1415534	1978364	1437757	2000783	1431126	1978362	99.93	99.94	100.05
10	1406743	1997364	1404661	1958912	1415235	1967532	98.36	99.73	99.48
20	1412266	1989874	1401648	1989783	1416480	2000732	99.12	97.97	97.92
30	1352676	1897634	1408788	1967562	1400171	1967623	99.55	99.58	98.42
40	1407009	1974777	1391135	1940782	1423223	1996723	99.51	99.69	98.58
50	1378771	1962365	1390888	1963265	1395581	1965356	98.12	98.53	98.21
60	1421844	2000837	1403716	1987673	1412645	1967236	99.24	98.22	99.32

Table 2: Peak areas and % remaining values

Calculation:

From the peak area, Calculate the following

$$\text{Peak area ratio} = \frac{\text{Peak area of test compound (DK-II-58-1)}}{\text{Peak area of internal standard (ISTD)}}$$

$$\text{\% remaining at time T} = \frac{\text{Peak area ratio at particular time T}}{\text{Peak area ratio at zero time point}} * 100$$

(T = 0, 10, 20,30,40,50 and 60)

DATA ANALYSIS:

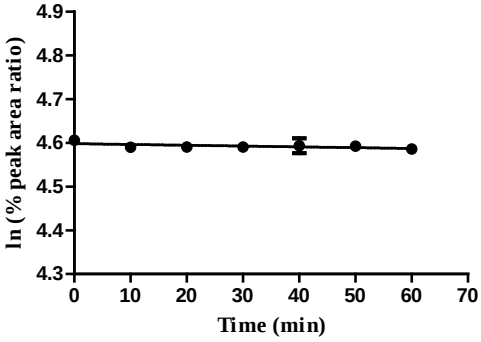
Day 1

Linear regression analysis:

Slope: -0.0001821 ± 9.454e^{-005}

At X= 60, Y = 4.587 ± 0.003409

R^2 = 0.1634



Metabolic Parameters:

Half-life: 3805.6 min

V_d: 100 µL/mg

Intrinsic clearance: 0.01821 µL/min/mg

Metabolic Rate: 0.3642 nmol/min/mg

% remaining at 60 min: 98.19 ± 0.07 %

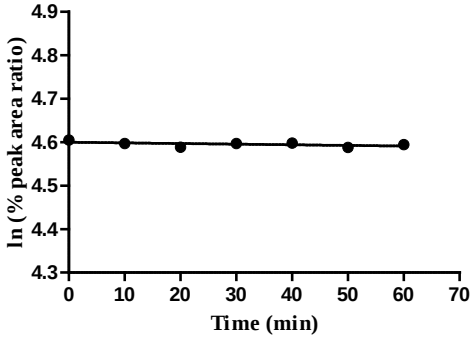
Day 2

Linear regression analysis:

Slope: -0.0001476 ± 7.506e^{-005}

At X = 60, Y = 4.591 ± 0.002706

R^2 = 0.1691



Metabolic Parameters:

Half-life: 4695.12 min

V_d: 100 µL/mg

Intrinsic clearance: 0.01476 µL/min/mg

Metabolic Rate: 0.2952 nmol/min/mg

% remaining at 60 min: 98.59 ± 0.05 %

Note: The peak area ratios and natural log values are calculated using Microsoft excel 2010 and linear regression analysis is calculated using GraphPad prism.