Supporting Information for Publication:

Title: Elucidation of GSK3α structure informs the design of novel, paralog-selective inhibitors

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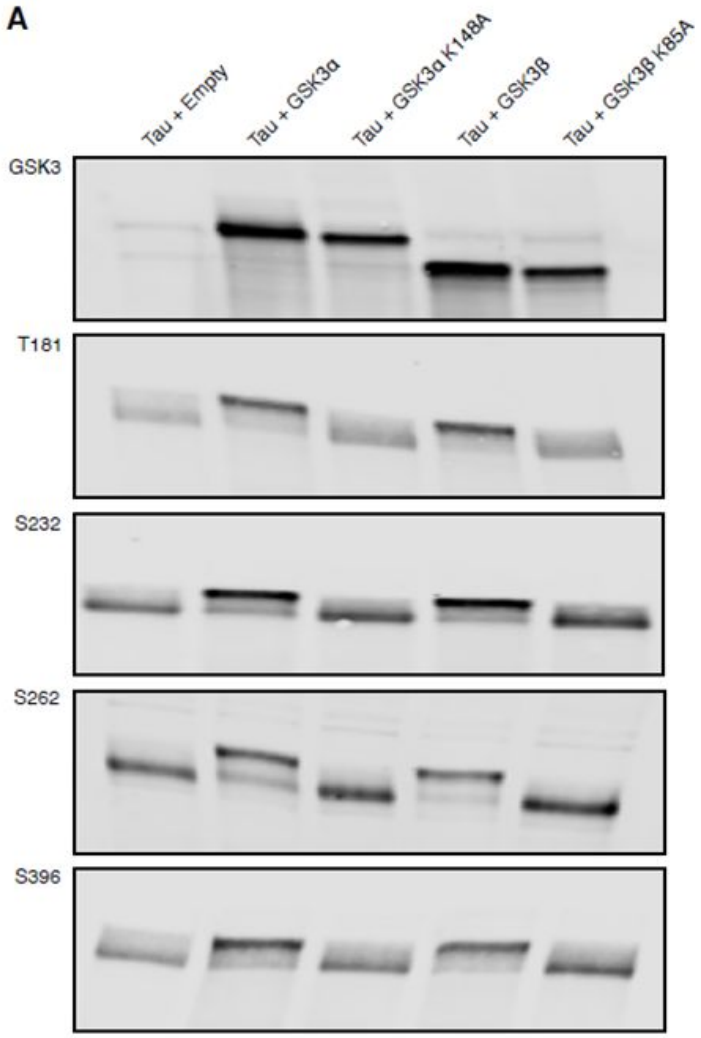
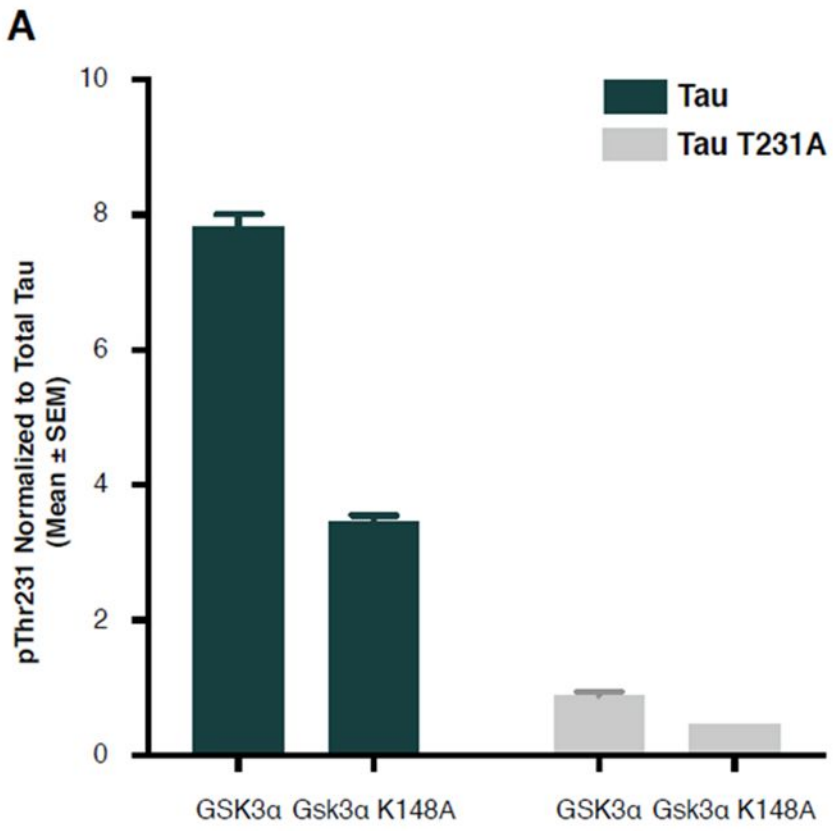
Figure S1: Phosphorylation of additional epitopes on tau by GSK3α and β



Supplemental Figure 1: Phosphorylation of additional epitopes on tau by GSK3 paralogs

HEK293 cells stably expressing human 2N4R tau (HEK-huTau) were transfected with plasmids expressing human GSK3α, human GSK3β, or kinase dead mutants in which the catalytic lysine of each kinase was mutated to an alanine. Twenty-four hours after transfection, cells were lysed. Cell lysates were run by SDS page to probe for phosphorylation of tau at different epitopes. Both GSK3α and GSK3β isoforms phosphorylate tau at multiple epitopes including T181, S232, S262, S396.

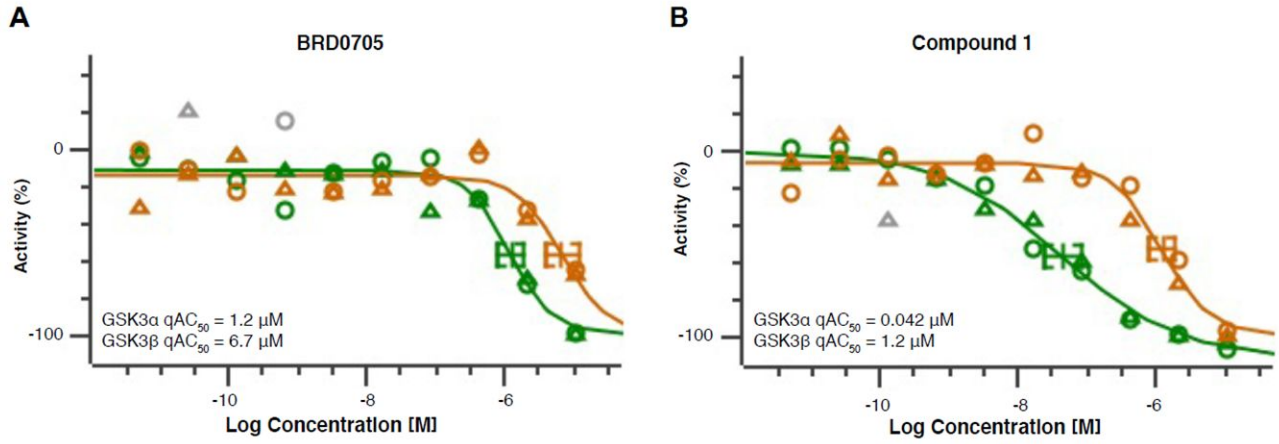
Figure S2: Specificity of Thr231 plate-based assay



Supplemental Figure 2: Specificity of Thr231 plate-based assay

HEK293 cells expressing either human 2N4R tau or human tau with a Tyrosine to Alanine substitution at amino acid position 231 were transfected with plasmids expressing active human GSK3α or a kinase-dead version GSK3α K148A. 24 hours after transfection cell lysates were taken to probe for phosphorylated and total tau. A plate-based assay was used to measure phosphorylation of Tau at Thr231. Results demonstrate that the assay signal increased with GSK3α kinase activity and the capture antibody was specific to phosphorylation at Thr231.

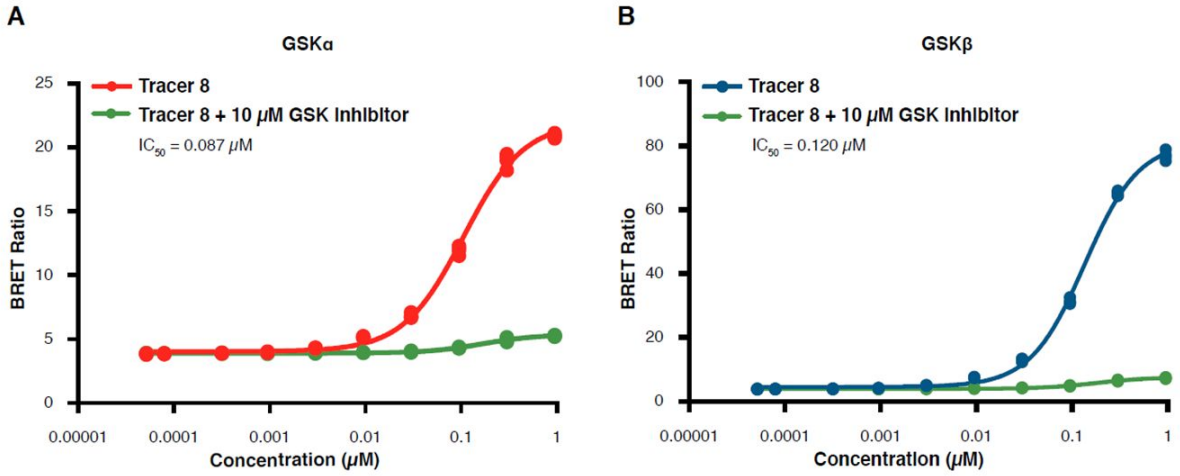
Figure S3: Effect of GSK3 inhibitors on tau phosphorylation at Ser202 and Thr205



Supplemental Figure 3: Effect of GSK3 inhibitors on tau phosphorylation at Ser202 and Thr205

HEK293 cells stably expressing human 2N4R tau (HEK-huTau) were transfected with plasmids expressing human GSK3α or human GSK3β. 24 hours after transfection, compounds were added at a 10-point dose response curve (range: 3nM-30μM) for 2 hours, and cell lysates were probed for total tau and tau phosphorylated at S202/T205 using FRET antibodies custom designed by Cisbio (S202/T205 Tb and total Tau D2 FRET antibodies). IC_{50} curves demonstrating cellular potency and GSK3 paralog selectivity of a previously identified GSK3α selective compound BRD0705 (A) and a novel GSK3α selective compound Compound 1 (B).

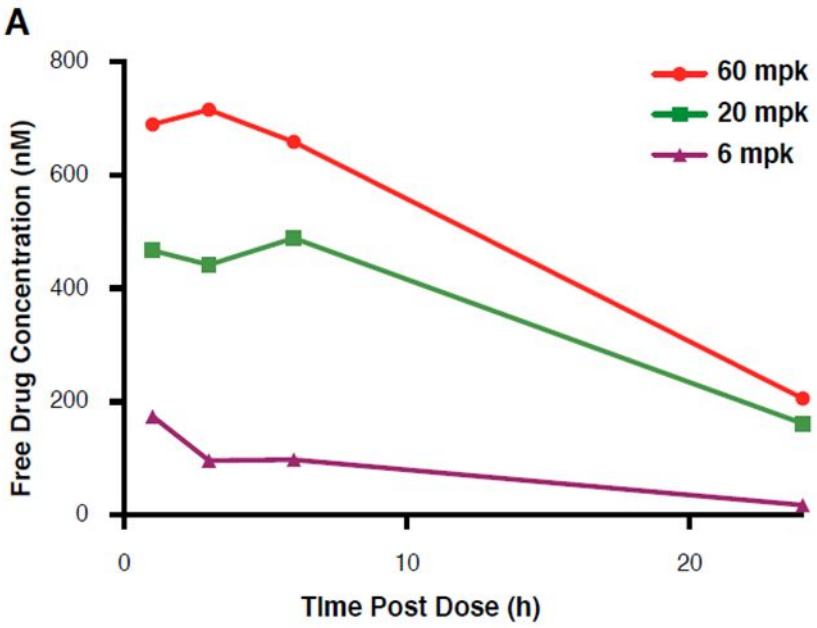
Figure S4: EC_{50} for Nanobret Tracer 8 at GSK3α and GSK3β



Supplemental Figure 4: EC_{50} for Nanobret Tracer 8 at GSK3α and GSK3β

HEK293T cells were transiently transfected with either NanoLuc-GSK3α or NanoLuc-GSK3β plasmids and the following day treated for 2 hours with NanoBRET Tracer-8 at a 10-point dose response curve (at a range of 0.1nM-1μM). Cells were incubated in the presence or absence of a saturating dose of Compound 1 at 10μM.

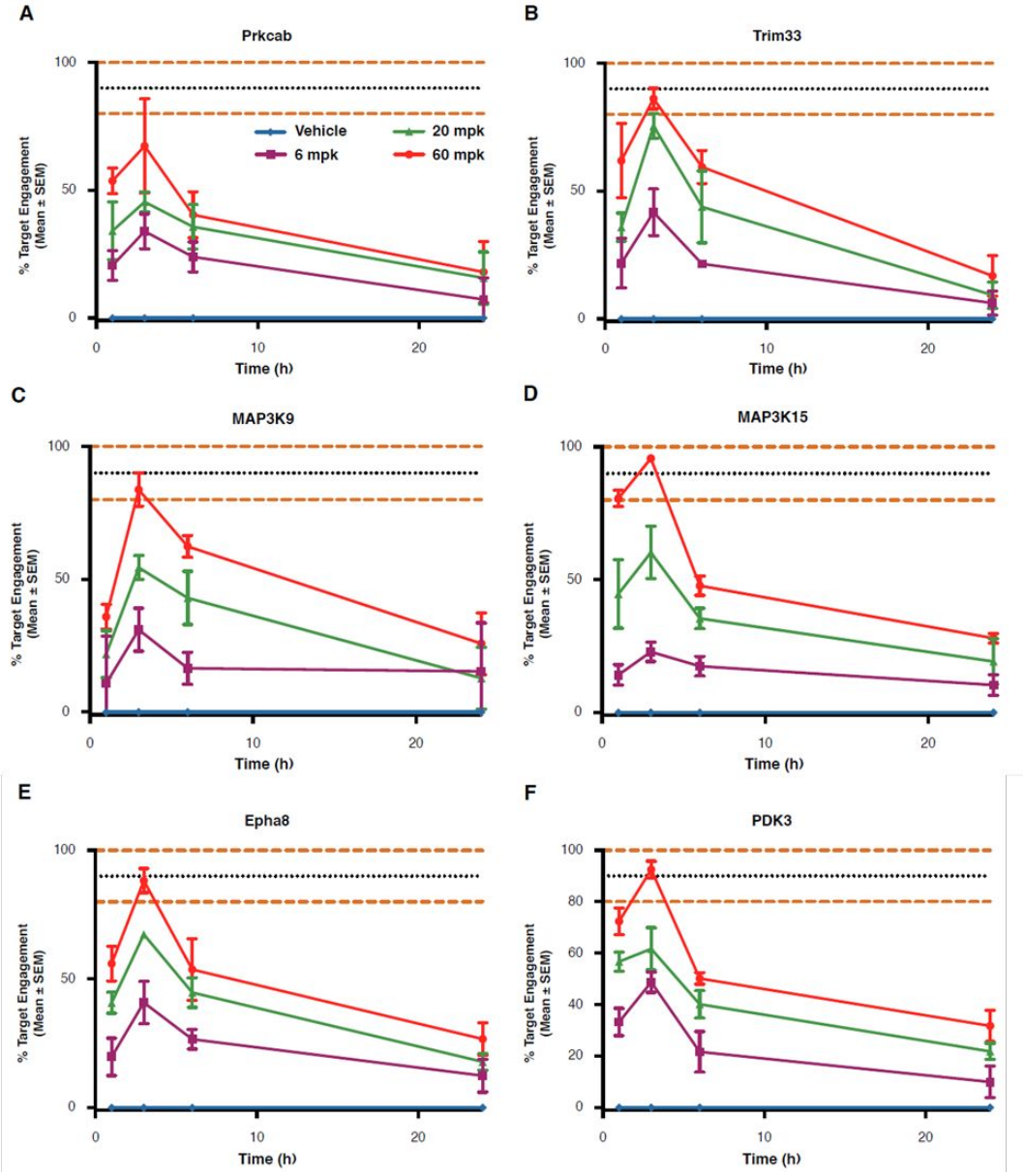
Figure S5: Kinetics of free drug concentration for Compound 1 after administration in P10 rats



Supplemental Figure 5: Kinetics of free drug concentration for Compound 1 after administration in P10 rats

P10 rats were injected with Compound 1 at 60, 20 and 6 mg/kg and sacrificed at several time points following treatment. Free drug concentration in the brain was estimated based on plasma protein binding data and demonstrates a Cmax of 715nM for the 60mpk dose, three hours after injection.

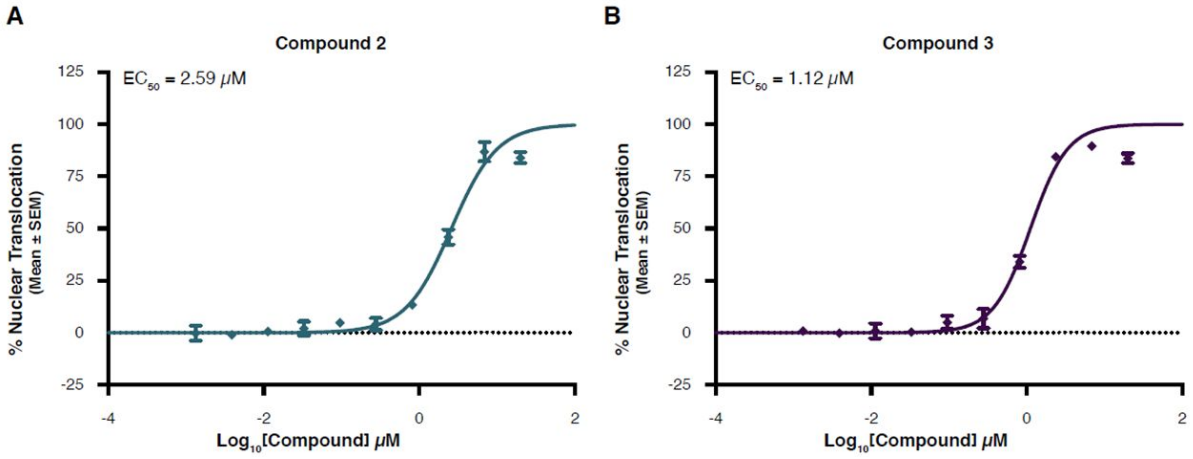
Figure S6: Target engagement of Compound 1 with identified off-target kinases demonstrating greater than 70% occupancy three hours post-dosing.



Supplemental Figure 6: Target engagement of Compound 1 with identified off-target kinases demonstrating greater than 70% occupancy three hours post-dosing.

To measure target engagement, brain lysates were incubated with non-selective kinase inhibitors coupled to sepharose beads (kinobeads; Bantscheff et.al.,2007). Enriched proteins were analyzed by liquid chromatography coupled with tandem mass spectrometry. Target engagement was then measured as competitive displacement of Compound 1 with the non-selective beads. Our results show that at the highest dose tested (60mg/kg) there were only 6 out of 251 unique detected kinases detected (other than GSK3α) which had a target engagement of greater than 70%.

Figure S7: Potency of Compound 2 and Compound 3 to activate β-catenin nuclear translocation.



Supplemental Figure 7: Potency of Compound 2 and Compound 3 to activate β-catenin nuclear translocation.

To examine β-catenin activation we looked for protein translocation to the nucleus in SH-SY5Y cells 6 hours after compound addition at a 9-point dose response curve (range of 20μM-20nM). IC_{50} curves demonstrating potencies of novel GSK3α selective inhibitors to cause β-catenin translocation to the nucleus.

Figure S8: None of the compounds evaluated in the current study showed cellular toxicity up to 30µM.

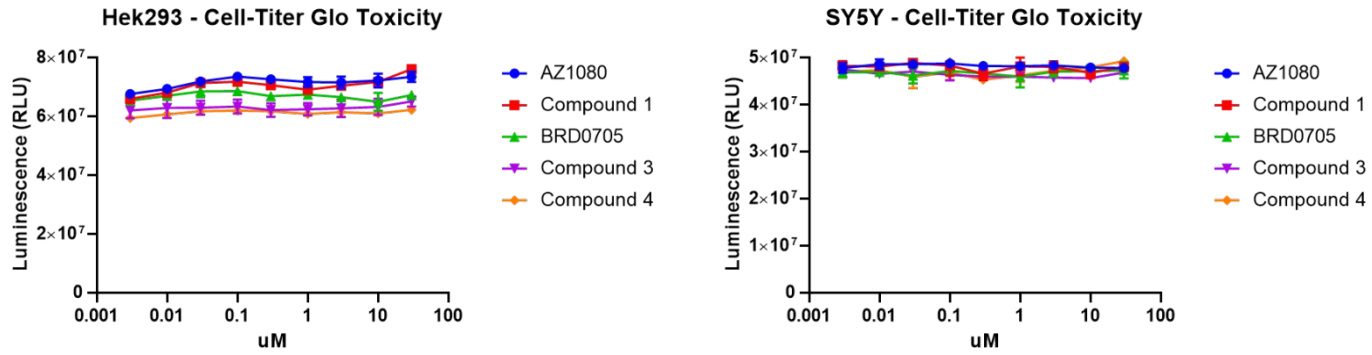
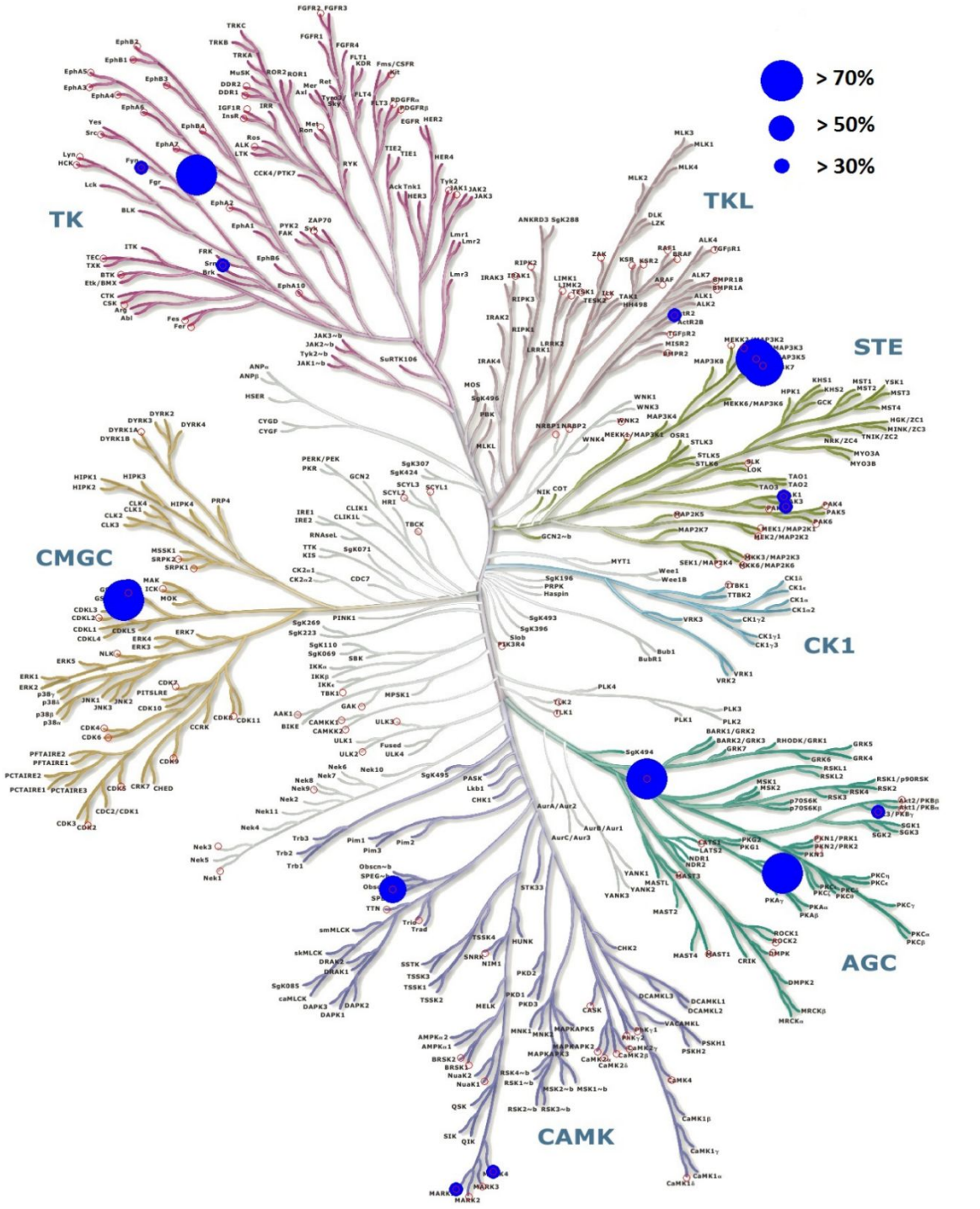


Figure S8: None of the compounds evaluated in the current study showed cellular toxicity up to 30µM.

HEK293 and SY5Y cells treated in 10 point dose response up to 30 µM. HEK293 cells were treated for 2 hrs and SY5Y cells for 6 hrs). 100 µl of Promega CellTiter-Glo 2.0 was added to each well of a 96 well culture plate. Plates were shaken for 2 mins at room temperature, allowed to incubate for an additional 10 mins, after which 150 ul of each sample was transferred to a white, 96-well Opti-plate and luminescence was read on an Envision plate reader (Molecular Devices).

Figure S9: High resolution image of Figure 4E - kinome tree indicating all kinases inhibited by Compound 1 at 60 mpk, 3 hours after administration.



Supplemental Table 1: ADME Properties of Compound 1

Assay	Compound 1
Multidrug resistant transporter P-glycoprotein (MDR1) P_{A-B}/P_{B-A} (efflux ratio)	0.9 / 38 (41)
LM intrinsic clearance (Cl_{int}) (rat, human)	56.1, 15.2 mL/min/kg
Hepatocyte Cl_{int} (rat, human)	7.5, 9.2 mL/min/kg
Cytochrome P450 (Cyp) 3A4T IC_{50}	1.8 µM
Cyp 3A4M IC_{50}	1.5 µM
Cyp 2D6 IC_{50}	>10 µM
Cyp 2C19 IC_{50}	>10 µM
Cyp 1A2 IC_{50}	>10 µM
Cyp 2C9 IC_{50}	9.75 µM
Cyp 2C8 IC_{50}	>10 µM
Solubility at pH = 6.8	22.6 µg/mL
Plasma protein binding fraction unbound (rat, human, mouse)	3.12, 1.97, 1.84 %
Human ether-a-go-go-related gene (hERG) IC50	>30 µM
Molecular weight (MW)	407.4
Total polar surface area (tPSA)	57.8
cLogP	3.5

Supplemental Table 2: Data collection and refinement statistics for crystallography

Data Collection/refinement statistics	BRD-0705 bound to GSK3alpha-axin complex	BRD0705 bound to GSK3beta-axin complex	Compound 3 bound to GSK3alpha-axin complex	Compound 3 bound to GSK3beta-axin complex
PDB Code	7SXF	7SXJ	7SXG	7SXH
Space Group	C 2	$P2_12_12_1$	C 2	$P2_12_12_1$
a, b, c (Å)	119, 55, 66	68, 73, 82	120, 55, 66	68, 72, 79
α, β, γ (°)	90, 97, 90	90, 90, 90	90, 97, 90	90, 90, 90
Resolution (Å)	1.94	50-1.85	2.4	2.1
R_{sym}[a]	0.03	0.03	0.06	0.06
I/σ	24.3	29.13	12.26	14.65
Multiplicity	3.5	6.6	3.4	6.5
Total / unique reflections	109474/31641	237493/36167	58008/16932	155896/23943
Completeness (%)	97.7 (91.06)	99.9 (99.9)	96 (98)	99.3 (95.0)
Rwork (Rfree)	20/23	17.9/20.2	23/26	21/25
$CC_{1/2}$	0.99 (0.97)	0.99 (0.72)	0.99 (0.54)	0.99 (0.6)
R.m.s.d. bond distance (Å)	0.006	0.01	0.003	0.003
R.m.s.d bond angle (deg)	0.89	1.00	0.55	0.97
Avg. protein B-value ($Å^2$)	61.6	47.83	72.99	57.8
Avg. solvent B-value ($Å^2$)	53.4	48.22	57.39	53.7
Ramachandran Values				
Preferred	97.6	97	97	98.2
Allowed	2.3	3	3	1.8
Disallowed	0	0	0	0

*The value in parentheses is for the highest resolution bin (approximate interval, 0.1 A), aRsym = ƒ°| ƒ§hkl.< ƒ§hkl >|/ ƒ° ƒ§hkl bRwork = ƒ°ƒ§hkl| |Fo . |Fc| | / ƒ°ƒ§hkl|Fo| for all data except 5% which is used for the Rfree calculation

Supplemental Table 3: LC-MS/MS conditions for the analysis of free drugs in the brain tissues.

HPLC Operating Conditions:

Column:	Phenomenex Kinetix C18, 50 X 3.0 mm, 2.6 µm
Mobile phase A:	0.1 % formic acid in water
Mobile Phase B:	0.1 % formic acid in acetonitrile
Injection Volume:	20 µL
Flow rate:	1000 µL/minute
Column Temperature:	Ambient
Autosampler Temperature:	5°C

LC chromatographic gradient conditions:

Duration (min)	*Flow Rate (µL/min)*	*Mobile Phase A (%)*	*Mobile Phase B (%)*
0.0	1000	95	5
0.5	1000	5	95
2.0	1000	95	5
2.5	1000	95	5

MRM parameters for Compound 1:

Analyte	*Q1 Mass (amu)*	*Q3 Mass (amu)*	*Dwell (msec)*	*Declustering Potential (V)*	*Collision Energy (V)*	*Collision Exit Potential (V)*
Compound 1	408.1	262.1	60	100	35	15

Data Analysis Calculations

Plasma protein binding (PPB) percent (%) unbound from the RED device was calculated as,

$$\%\text{Unbound} = \frac{\text{Average Conc of analyte in the buffer side}}{\text{Average Conc of analyte in the plasma side}} * 100$$

The stability of the protein binding outside the RED device was calculated as,

$$\%\ \text{Stability outside RED device} = \frac{\text{avg Conc of plasma at 4 h}}{\text{avg Conc of plasma at 0 h}} * 100$$

Brain homogenate protein binding (BrPB) percent (%) unbound from the RED device was calculated as,

$$f_u\ (\text{brain homogenate}) = \frac{1/Df}{((1/f_u\ (\text{hom} -1) + 1/Df}$$

f_u (hom) = measured fraction unbound in diluted brain homogenate; Df = dilution factor

Supplemental Table 4: Kinase selectivity data of BRD0705, Compound 1, Compound 2, and Compound 3 at 1 μM.

DiscoveRx Gene Symbol	Entrez Gene Symbol	% control with **BRD0705**	% control with **Compound 1**	% control with **Compound 2**	% control with **Compound 3**
AAK1	AAK1	90	88	97	87
ABL1(E255K)-phosphorylated	ABL1	78	65	77	79
ABL1(F317I)-nonphosphorylated	ABL1	98	100	100	81
ABL1(F317I)-phosphorylated	ABL1	98	84	88	63
ABL1(F317L)-nonphosphorylated	ABL1	100	100	100	87
ABL1(F317L)-phosphorylated	ABL1	100	79	98	70
ABL1(H396P)-nonphosphorylated	ABL1	75	48	88	91
ABL1(H396P)-phosphorylated	ABL1	81	72	80	100
ABL1(M351T)-phosphorylated	ABL1	100	93	100	73
ABL1(Q252H)-nonphosphorylated	ABL1	65	34	87	78
ABL1(Q252H)-phosphorylated	ABL1	66	60	64	96
ABL1(T315I)-nonphosphorylated	ABL1	100	100	95	90
ABL1(T315I)-phosphorylated	ABL1	100	100	99	77
ABL1(Y253F)-phosphorylated	ABL1	75	56	71	97
ABL1-nonphosphorylated	ABL1	61	33	67	100
ABL1-phosphorylated	ABL1	80	70	97	95
ABL2	ABL2	88	89	100	82
ACVR1	ACVR1	99	95	98	100
ACVR1B	ACVR1B	100	100	100	89
ACVR2A	ACVR2A	100	91	100	45
ACVR2B	ACVR2B	100	100	100	80
ACVRL1	ACVRL1	100	99	92	94
ADCK3	CABC1	100	100	100	100
ADCK4	ADCK4	96	100	100	100
AKT1	AKT1	100	100	100	90
AKT2	AKT2	100	100	100	100
AKT3	AKT3	100	100	92	99
ALK	ALK	79	73	84	81
ALK(C1156Y)	ALK	88	68	89	73

ALK(L1196M)	ALK	76	63	58	100
AMPK-alpha1	PRKAA1	100	100	96	78
AMPK-alpha2	PRKAA2	91	100	100	90
ANKK1	ANKK1	100	100	100	68
ARK5	NUAK1	80	91	98	100
ASK1	MAP3K5	100	100	95	68
ASK2	MAP3K6	100	100	100	66
AURKA	AURKA	100	100	100	65
AURKB	AURKB	89	90	65	62
AURKC	AURKC	99	100	100	61
AXL	AXL	100	89	100	100
BIKE	BMP2K	58	66	75	62
BLK	BLK	100	99	90	74
BMPR1A	BMPR1A	100	100	100	100
BMPR1B	BMPR1B	100	100	98	67
BMPR2	BMPR2	97	89	84	66
BMX	BMX	100	100	100	100
BRAF	BRAF	100	95	100	83
BRAF(V600E)	BRAF	100	98	100	84
BRK	PTK6	97	85	100	100
BRSK1	BRSK1	92	78	100	100
BRSK2	BRSK2	100	100	100	92
BTK	BTK	97	100	98	77
BUB1	BUB1	100	100	100	64
CAMK1	CAMK1	100	100	93	54
CAMK1B	PNCK	100	97	88	57
CAMK1D	CAMK1D	100	100	100	78
CAMK1G	CAMK1G	93	91	97	92
CAMK2A	CAMK2A	95	88	100	82
CAMK2B	CAMK2B	96	64	100	88
CAMK2D	CAMK2D	94	78	100	98
CAMK2G	CAMK2G	90	94	100	87
CAMK4	CAMK4	100	100	93	100
CAMKK1	CAMKK1	88	87	100	93
CAMKK2	CAMKK2	91	100	100	84
CASK	CASK	96	96	100	74
CDC2L1	CDK11B	100	100	100	100
CDC2L2	CDC2L2	100	100	100	100
CDC2L5	CDK13	77	92	75	70
CDK11	CDK19	100	100	100	71
CDK2	CDK2	91	97	100	2.2

CDK3	CDK3	99	97	100	2.6
CDK4	CDK4	92	79	71	44
CDK4-cyclinD1	CDK4	86	90	64	63
CDK4-cyclinD3	CDK4	100	98	100	86
CDK5	CDK5	100	100	96	70
CDK7	CDK7	100	95	100	86
CDK8	CDK8	100	100	100	100
CDK9	CDK9	100	100	100	100
CDKL1	CDKL1	73	61	89	94
CDKL2	CDKL2	100	100	100	90
CDKL3	CDKL3	100	100	100	100
CDKL5	CDKL5	100	100	100	82
CHEK1	CHEK1	91	97	83	75
CHEK2	CHEK2	100	100	100	88
CIT	CIT	100	99	100	96
CLK1	CLK1	100	93	100	84
CLK2	CLK2	100	91	100	53
CLK3	CLK3	97	94	100	73
CLK4	CLK4	96	100	100	99
CSF1R	CSF1R	100	100	100	96
CSF1R-autoinhibited	CSF1R	100	100	93	73
CSK	CSK	100	100	100	96
CSNK1A1	CSNK1A1	95	95	100	66
CSNK1A1L	CSNK1A1L	100	100	100	74
CSNK1D	CSNK1D	100	93	100	97
CSNK1E	CSNK1E	100	100	87	82
CSNK1G1	CSNK1G1	96	56	100	81
CSNK1G2	CSNK1G2	99	85	99	94
CSNK1G3	CSNK1G3	100	100	100	74
CSNK2A1	CSNK2A1	97	100	95	100
CSNK2A2	CSNK2A2	100	100	95	79
CTK	MATK	100	100	95	24
DAPK1	DAPK1	68	77	87	100
DAPK2	DAPK2	100	100	100	80
DAPK3	DAPK3	97	94	100	90
DCAMKL1	DCLK1	88	74	88	59
DCAMKL2	DCLK2	100	100	100	100
DCAMKL3	DCLK3	100	100	85	76
DDR1	DDR1	100	99	95	78
DDR2	DDR2	99	98	95	54
DLK	MAP3K12	100	100	100	58

DMPK	DMPK	96	77	85	77
DMPK2	CDC42BPG	94	99	91	100
DRAK1	STK17A	97	90	100	81
DRAK2	STK17B	99	99	100	80
DYRK1A	DYRK1A	95	71	69	64
DYRK1B	DYRK1B	86	100	93	100
DYRK2	DYRK2	100	100	82	55
EGFR	EGFR	100	97	100	100
EGFR(E746-A750del)	EGFR	97	100	100	86
EGFR(G719C)	EGFR	100	100	100	100
EGFR(G719S)	EGFR	100	100	100	88
EGFR(L747-E749del, A750P)	EGFR	100	65	100	100
EGFR(L747-S752del, P753S)	EGFR	79	82	64	89
EGFR(L747-T751del,Sins)	EGFR	88	68	89	93
EGFR(L858R)	EGFR	100	100	99	100
EGFR(L858R,T790M)	EGFR	100	95	100	76
EGFR(L861Q)	EGFR	100	100	100	100
EGFR(S752-I759del)	EGFR	100	73	99	92
EGFR(T790M)	EGFR	100	100	100	98
EIF2AK1	EIF2AK1	100	100	100	91
EPHA1	EPHA1	95	100	96	92
EPHA2	EPHA2	100	100	100	100
EPHA3	EPHA3	60	27	88	70
EPHA4	EPHA4	67	75	86	100
EPHA5	EPHA5	100	100	98	70
EPHA6	EPHA6	100	100	100	94
EPHA7	EPHA7	100	100	100	82
EPHA8	EPHA8	100	100	100	78
EPHB1	EPHB1	100	100	100	80
EPHB2	EPHB2	100	100	100	100
EPHB3	EPHB3	100	100	100	100
EPHB4	EPHB4	100	100	100	100
EPHB6	EPHB6	100	90	100	84
ERBB2	ERBB2	91	88	90	67
ERBB3	ERBB3	100	100	100	77
ERBB4	ERBB4	88	100	100	69
ERK1	MAPK3	100	88	93	93
ERK2	MAPK1	100	100	91	95
ERK3	MAPK6	100	98	100	100
ERK4	MAPK4	100	100	100	100

ERK5	MAPK7	100	100	100	100
ERK8	MAPK15	100	96	100	36
ERN1	ERN1	100	100	81	42
FAK	PTK2	100	96	100	86
FER	FER	100	100	100	100
FES	FES	100	99	100	100
FGFR1	FGFR1	93	100	100	96
FGFR2	FGFR2	100	100	100	100
FGFR3	FGFR3	100	100	100	100
FGFR3(G697C)	FGFR3	100	100	100	100
FGFR4	FGFR4	99	82	99	100
FGR	FGR	100	100	100	100
FLT1	FLT1	99	99	100	88
FLT3	FLT3	100	100	100	99
FLT3(D835H)	FLT3	100	100	100	77
FLT3(D835V)	FLT3	100	100	89	69
FLT3(D835Y)	FLT3	100	100	100	99
FLT3(ITD)	FLT3	100	100	100	93
FLT3(ITD,D835V)	FLT3	100	100	100	69
FLT3(ITD,F691L)	FLT3	92	100	100	65
FLT3(K663Q)	FLT3	80	68	94	94
FLT3(N841I)	FLT3	100	100	100	66
FLT3(R834Q)	FLT3	100	100	100	89
FLT3-autoinhibited	FLT3	100	97	100	78
FLT4	FLT4	100	100	100	91
FRK	FRK	100	75	100	100
FYN	FYN	100	92	100	100
GAK	GAK	100	65	100	95
GCN2(Kin.Dom.2,S808G)	EIF2AK4	85	83	95	100
GRK1	GRK1	82	56	100	79
GRK2	ADRBK1	100	100	94	54
GRK3	ADRBK2	51	65	51	100
GRK4	GRK4	99	100	100	83
GRK7	GRK7	100	95	93	92
GSK3A	GSK3A	8.5	0.55	0	0
GSK3B	GSK3B	54	4.5	1.1	0.5
HASPIN	GSG2	68	86	95	62
HCK	HCK	100	100	100	71
HIPK1	HIPK1	77	73	78	77
HIPK2	HIPK2	78	79	75	65
HIPK3	HIPK3	100	100	81	66

HIPK4	HIPK4	100	99	100	74
HPK1	MAP4K1	72	58	91	73
HUNK	HUNK	74	77	76	82
ICK	ICK	100	100	87	15
IGF1R	IGF1R	100	75	92	86
IKK-alpha	CHUK	100	100	90	85
IKK-beta	IKBKB	100	100	91	64
IKK-epsilon	IKBKE	100	100	100	93
INSR	INSR	100	100	100	80
INSRR	INSRR	100	100	100	90
IRAK1	IRAK1	97	95	85	100
IRAK3	IRAK3	96	82	82	73
IRAK4	IRAK4	98	95	99	84
ITK	ITK	100	100	100	79
JAK1(JH1domain-catalytic)	JAK1	87	100	100	100
JAK1(JH2domain-pseudokinase)	JAK1	99	100	95	74
JAK2(JH1domain-catalytic)	JAK2	76	77	97	82
JAK3(JH1domain-catalytic)	JAK3	100	100	100	64
JNK1	MAPK8	87	80	94	56
JNK2	MAPK9	100	73	100	59
JNK3	MAPK10	100	64	98	63
KIT	KIT	100	100	100	100
KIT(A829P)	KIT	100	100	97	56
KIT(D816H)	KIT	100	100	100	65
KIT(D816V)	KIT	100	100	100	100
KIT(L576P)	KIT	64	89	77	100
KIT(V559D)	KIT	100	100	100	100
KIT(V559D,T670I)	KIT	83	78	84	100
KIT(V559D,V654A)	KIT	93	97	98	89
KIT-autoinhibited	KIT	95	100	90	72
LATS1	LATS1	93	96	89	100
LATS2	LATS2	100	100	89	79
LCK	LCK	100	100	100	97
LIMK1	LIMK1	89	94	100	92
LIMK2	LIMK2	98	100	95	100
LKB1	STK11	71	52	93	100
LOK	STK10	100	100	99	77
LRRK2	LRRK2	70	79	83	56
LRRK2(G2019S)	LRRK2	77	81	92	74
LTK	LTK	100	100	100	70
LYN	LYN	100	100	100	100

LZK	MAP3K13	100	100	100	70
MAK	MAK	96	49	85	87
MAP3K1	MAP3K1	100	100	81	62
MAP3K15	MAP3K15	85	91	90	84
MAP3K2	MAP3K2	100	100	100	90
MAP3K3	MAP3K3	95	97	96	73
MAP3K4	MAP3K4	100	100	100	90
MAP4K2	MAP4K2	100	100	92	72
MAP4K3	MAP4K3	96	91	100	100
MAP4K4	MAP4K4	100	92	87	62
MAP4K5	MAP4K5	100	100	100	72
MAPKAPK2	MAPKAPK2	100	91	100	80
MAPKAPK5	MAPKAPK5	100	100	100	89
MARK1	MARK1	93	86	100	86
MARK2	MARK2	67	17	98	84
MARK3	MARK3	90	91	97	96
MARK4	MARK4	100	100	100	89
MAST1	MAST1	100	100	100	100
MEK1	MAP2K1	95	88	69	76
MEK2	MAP2K2	83	66	91	83
MEK3	MAP2K3	88	80	77	87
MEK4	MAP2K4	100	100	100	83
MEK5	MAP2K5	77	68	76	65
MEK6	MAP2K6	92	89	100	95
MELK	MELK	96	88	95	100
MERTK	MERTK	100	100	100	88
MET	MET	74	81	88	93
MET(M1250T)	MET	100	99	100	77
MET(Y1235D)	MET	100	100	100	75
MINK	MINK1	86	100	92	41
MKK7	MAP2K7	91	100	88	77
MKNK1	MKNK1	100	100	100	65
MKNK2	MKNK2	100	100	100	47
MLCK	MYLK3	89	100	100	83
MLK1	MAP3K9	100	92	100	93
MLK2	MAP3K10	81	91	100	100
MLK3	MAP3K11	100	100	100	92
MRCKA	CDC42BPA	95	100	100	100
MRCKB	CDC42BPB	100	100	100	100
MST1	STK4	100	100	95	81
MST1R	MST1R	66	85	68	99

MST2	STK3	100	100	96	100
MST3	STK24	91	88	100	100
MST4	MST4	100	100	100	59
MTOR	MTOR	67	45	57	100
MUSK	MUSK	100	97	100	93
MYLK	MYLK	100	100	100	92
MYLK2	MYLK2	100	100	100	84
MYLK4	MYLK4	97	96	100	100
MYO3A	MYO3A	100	100	100	75
MYO3B	MYO3B	100	100	96	100
NDR1	STK38	84	49	94	60
NDR2	STK38L	90	93	98	98
NEK1	NEK1	85	89	100	92
NEK10	NEK10	100	100	100	14
NEK11	NEK11	76	87	92	75
NEK2	NEK2	89	11	100	93
NEK3	NEK3	84	75	93	58
NEK4	NEK4	100	100	95	62
NEK5	NEK5	94	100	100	100
NEK6	NEK6	95	94	80	89
NEK7	NEK7	100	100	100	100
NEK9	NEK9	100	100	100	93
NIK	MAP3K14	100	100	100	60
NIM1	MGC42105	99	94	95	61
NLK	NLK	100	95	100	100
OSR1	OXSR1	100	100	100	80
p38-alpha	MAPK14	100	100	100	71
p38-beta	MAPK11	100	100	100	79
p38-delta	MAPK13	100	100	99	100
p38-gamma	MAPK12	99	100	100	70
PAK1	PAK1	100	100	100	95
PAK2	PAK2	100	100	100	79
PAK3	PAK3	100	100	100	72
PAK4	PAK4	100	100	100	100
PAK6	PAK6	100	100	100	99
PAK7	PAK7	88	93	66	96
PCTK1	CDK16	100	99	100	5.6
PCTK2	CDK17	86	72	100	18
PCTK3	CDK18	100	100	100	71
PDGFRA	PDGFRA	100	100	100	79
PDGFRB	PDGFRB	100	100	94	100

PDPK1	PDPK1	100	100	100	77
PFCDPK1(P.falciparum)	CDPK1	96	100	67	61
PFPK5(P.falciparum)	MAL13P1.279	100	100	100	43
PFTAIRE2	CDK15	100	100	100	96
PFTK1	CDK14	100	100	100	100
PHKG1	PHKG1	96	92	100	100
PHKG2	PHKG2	98	100	100	86
PIK3C2B	PIK3C2B	100	100	100	70
PIK3C2G	PIK3C2G	92	84	76	88
PIK3CA	PIK3CA	100	100	100	64
PIK3CA(C420R)	PIK3CA	76	78	85	88
PIK3CA(E542K)	PIK3CA	70	46	57	66
PIK3CA(E545A)	PIK3CA	92	88	96	72
PIK3CA(E545K)	PIK3CA	61	71	59	71
PIK3CA(H1047L)	PIK3CA	100	100	96	96
PIK3CA(H1047Y)	PIK3CA	100	100	100	100
PIK3CA(I800L)	PIK3CA	59	24	58	61
PIK3CA(M1043I)	PIK3CA	100	98	94	92
PIK3CA(Q546K)	PIK3CA	86	59	48	63
PIK3CB	PIK3CB	100	100	100	59
PIK3CD	PIK3CD	100	100	100	100
PIK3CG	PIK3CG	100	100	100	70
PIK4CB	PI4KB	94	100	100	66
PIKFYVE	PIKFYVE	100	97	93	81
PIM1	PIM1	100	100	100	100
PIM2	PIM2	100	100	100	98
PIM3	PIM3	100	100	100	100
PIP5K1A	PIP5K1A	98	81	96	59
PIP5K1C	PIP5K1C	65	60	100	100
PIP5K2B	PIP4K2B	100	100	100	100
PIP5K2C	PIP4K2C	91	79	90	100
PKAC-alpha	PRKACA	100	100	100	87
PKAC-beta	PRKACB	100	100	90	84
PKMYT1	PKMYT1	85	79	99	100
PKN1	PKN1	100	100	100	100
PKN2	PKN2	100	98	100	66
PKNB(M.tuberculosis)	pknB	100	100	100	75
PLK1	PLK1	100	99	100	56
PLK2	PLK2	85	81	77	60
PLK3	PLK3	100	95	83	60
PLK4	PLK4	100	100	95	74

PRKCD	PRKCD	100	100	100	100
PRKCE	PRKCE	93	100	100	69
PRKCH	PRKCH	100	100	100	91
PRKCI	PRKCI	100	91	100	78
PRKCQ	PRKCQ	73	80	73	100
PRKD1	PRKD1	100	90	99	88
PRKD2	PRKD2	76	71	66	100
PRKD3	PRKD3	95	79	77	76
PRKG1	PRKG1	100	100	100	88
PRKG2	PRKG2	100	95	100	85
PRKR	EIF2AK2	83	81	100	94
PRKX	PRKX	86	83	90	96
PRP4	PRPF4B	70	77	76	100
PYK2	PTK2B	100	100	100	74
QSK	KIAA0999	94	100	100	57
RAF1	RAF1	100	100	97	89
RET	RET	72	86	92	100
RET(M918T)	RET	100	99	100	78
RET(V804L)	RET	100	100	100	88
RET(V804M)	RET	98	100	89	100
RIOK1	RIOK1	100	100	100	59
RIOK2	RIOK2	100	92	84	80
RIOK3	RIOK3	100	100	95	70
RIPK1	RIPK1	98	100	100	100
RIPK2	RIPK2	100	91	100	100
RIPK4	RIPK4	97	78	74	66
RIPK5	DSTYK	92	75	86	61
ROCK1	ROCK1	91	93	84	76
ROCK2	ROCK2	77	97	76	57
ROS1	ROS1	100	98	81	78
RPS6KA4(Kin.Dom.1-N-terminal)	RPS6KA4	100	80	93	78
RPS6KA4(Kin.Dom.2-C-terminal)	RPS6KA4	100	100	100	74
RPS6KA5(Kin.Dom.1-N-terminal)	RPS6KA5	100	100	100	100
RPS6KA5(Kin.Dom.2-C-terminal)	RPS6KA5	86	90	100	98
RSK1(Kin.Dom.1-N-terminal)	RPS6KA1	96	92	99	100
RSK1(Kin.Dom.2-C-terminal)	RPS6KA1	85	86	97	100
RSK2(Kin.Dom.1-N-terminal)	RPS6KA3	100	100	100	82

RSK2(Kin.Dom.2-C-terminal)	RPS6KA3	55	55	59	68
RSK3(Kin.Dom.1-N-terminal)	RPS6KA2	100	100	100	100
RSK3(Kin.Dom.2-C-terminal)	RPS6KA2	78	73	99	100
RSK4(Kin.Dom.1-N-terminal)	RPS6KA6	100	100	100	84
RSK4(Kin.Dom.2-C-terminal)	RPS6KA6	98	91	100	98
S6K1	RPS6KB1	80	68	72	83
SBK1	SBK1	100	100	100	72
SGK	SGK1	95	92	97	90
SgK110	SgK110	100	100	100	100
SGK2	SGK2	96	100	100	100
SGK3	SGK3	100	100	100	68
SIK	SIK1	100	100	92	100
SIK2	SIK2	78	83	100	100
SLK	SLK	100	100	100	89
SNARK	NUAK2	100	100	100	66
SNRK	SNRK	86	67	95	89
SRC	SRC	100	100	100	94
SRMS	SRMS	100	96	88	69
SRPK1	SRPK1	95	99	86	81
SRPK2	SRPK2	95	91	88	87
SRPK3	SRPK3	100	100	100	84
STK16	STK16	91	90	94	78
STK33	STK33	98	100	96	100
STK35	STK35	98	98	100	100
STK36	STK36	100	100	100	100
STK39	STK39	100	99	88	79
SYK	SYK	100	100	100	98
TAK1	MAP3K7	81	65	37	31
TAOK1	TAOK1	100	100	100	61
TAOK2	TAOK2	92	69	93	79
TAOK3	TAOK3	100	97	96	71
TBK1	TBK1	98	61	100	90
TEC	TEC	98	100	100	88
TESK1	TESK1	100	100	100	100
TGFBR1	TGFBR1	98	80	100	97
TGFBR2	TGFBR2	100	100	100	100
TIE1	TIE1	100	100	100	80
TIE2	TEK	100	100	100	65

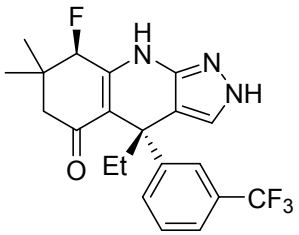
TLK1	TLK1	91	85	96	100
TLK2	TLK2	97	97	100	95
TNIK	TNIK	95	92	96	63
TNK1	TNK1	100	100	100	100
TNK2	TNK2	99	97	99	100
TNNI3K	TNNI3K	98	78	99	100
TRKA	NTRK1	95	88	100	64
TRKB	NTRK2	79	89	99	74
TRKC	NTRK3	83	75	77	88
TRPM6	TRPM6	100	100	97	77
TSSK1B	TSSK1B	95	85	90	95
TSSK3	TSSK3	98	100	100	87
TTK	TTK	100	100	100	80
TXK	TXK	100	100	100	89
TYK2(JH1domain-catalytic)	TYK2	100	100	100	66
TYK2(JH2domain-pseudokinase)	TYK2	100	82	47	45
TYRO3	TYRO3	100	100	99	100
ULK1	ULK1	100	98	100	67
ULK2	ULK2	100	95	77	72
ULK3	ULK3	84	69	91	48
VEGFR2	KDR	100	100	100	82
VPS34	PIK3C3	94	97	88	56
VRK2	VRK2	100	100	100	64
WEE1	WEE1	100	100	100	92
WEE2	WEE2	100	100	100	74
WNK1	WNK1	100	100	100	81
WNK2	WNK2	100	100	100	73
WNK3	WNK3	100	100	100	80
WNK4	WNK4	83	81	77	89
YANK1	STK32A	100	100	100	81
YANK2	STK32B	83	88	82	91
YANK3	STK32C	100	73	100	97
YES	YES1	100	95	100	100
YSK1	STK25	83	85	90	85
YSK4	MAP3K19	98	67	100	60
ZAK	ZAK	100	100	100	100
ZAP70	ZAP70	100	100	91	75

Supplemental Table 4: Competitive Ligand Binding Assay for (KINOMEscan Technology; Eurofins)

$$\text{\% Control Calculation} = \frac{\text{Test Compound Signal} - \text{Positive Control Signal}}{\text{Negative Control Signal} - \text{Positive Control Signal}}$$

Supplemental Methods: Novel compound synthesis and Characterization

Preparation of 4-ethyl-8-fluoro-7,7-dimethyl-4-(3-(trifluoromethyl)phenyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one:



Step 1: 1H-pyrazol-3-amine (164 mg, 1.98 mmol), 1-[3-(trifluoromethyl)phenyl]propan-1-one (400 mg, 1.98 mmol, 333 uL), and pTSA (37.7 mg, 198 µmol) were combined then toluene (4.00 mL) was added and the mixture was heated to 110°C for 16 hours. The reaction was then cooled to room temperature and concentrated and the crude residue was purified by column chromatography (0 to 30% MeOH in CH2Cl2) to give 4-[1-[3-(trifluoromethyl)phenyl]prop-1-enyl]-1H-pyrazol-3-amine (510 mg, 1.91 mmol, 96.4% yield). LCMS: Rt = 1.25 min, m/z 268.1.

Step 2: 4-fluoro-5,5-dimethyl-cyclohexane-1,3-dione (79.9 mg, 505 µmol) and 4-[(E)-1-[3-(trifluoromethyl)phenyl]prop-1-enyl]-1H-pyrazol-3-amine (135 mg, 505 µmol) were combined and dissolved in TFA (1.00 mL) and the mixture was heated to 110°C for 4 hours. The mixture was then cooled to room temperature and concentrated. The crude residue was purified with preparatory HPLC to give 4-ethyl-8-fluoro-7,7-dimethyl-4-[3-(trifluoromethyl)phenyl]-2,6,8,9-tetrahydropyrazolo[3,4-b]quinolin-5-one (37.0 mg, 90.8 µmol, 18% yield) as a mixture of isomers. LCMS: Rt = 1.59 min, m/z 408.2. The mixture of isomers was then purified by chiral SFC (CHIRALPAK IB 30x250mm, 5um, Method: 30% Methanol with 0.1% diethyl amine in CO2 [flow rate: 100mL/min, ABPR 120bar, MBPR 40psi]) to provide four stereoisomers (Rt: Peak 1: 2.22 min, Peak 2: 2.63, Peak 3: 3.05, Peak 4: 3.41). Compound 1 was isolated as peak 1 from the separation method.

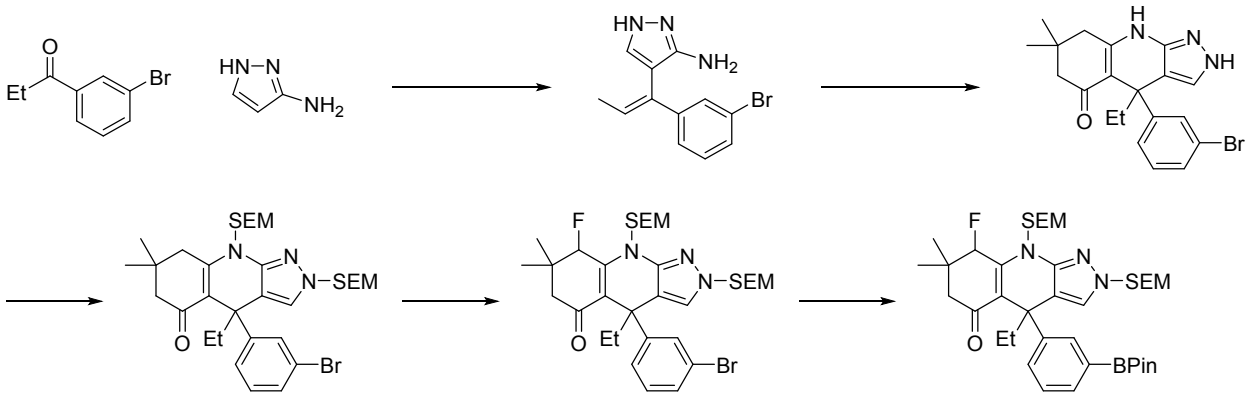
Peak 1: 40.4 mg (95% purity, 100% ee). LCMS: Rt = 1.76 min, m/z 408.2. 1H NMR (500 MHz, METHANOL-d4) δ 7.65 (s, 1H), 7.63 (br d, J=7.63 Hz, 1H), 7.42-7.31 (m, 2H), 6.96 (s, 1H), 4.47 (d, J=49.4 Hz, 1H), 3.08-2.98 (m, 1H), 2.85-2.78 (m, 1H), 2.63-2.54 (m, 1H), 2.15-2.05 (m, 1H), 1.22-1.17 (m, 3H), 1.14-1.09 (m, 3H), 0.86-0.78 (m, 3H). 13C NMR (151 MHz, METHANOL-d4) δ 190.3, 155.0, 152.6, 130.6, 129.2, 127.9, 126.0, 123.7, 122.9, 121.5, 110.7, 106.7, 97.1, 95.9, 45.0, 39.6, 36.3, 31.1, 25.0, 19.1, 9.0. 19F NMR (471 MHz, METHANOL-d4) δ -59.9 (s, 1F) -198.9 (br s, 1F).

Peak 2: 28.2 mg (95% purity, 100% ee). LCMS: Rt = 1.73 min, m/z 408.2. 1H NMR (400 MHz, CHLOROFORM-d) δ 7.60 (s, 2H), 7.36 (br. s., 2H), 6.90 (s, 1H), 4.26 - 4.56 (m, 1H), 2.93 - 3.16 (m, 1H), 2.41 - 2.59 (m, 2H), 1.95 - 2.14 (m, 1H), 1.23 (s, 3H), 1.08 (s, 3H), 0.77 (t, J = 7.40 Hz, 3H).

Peak 3: 13.3 mg (95% purity, 100% ee). LCMS: Rt = 1.73 min, m/z 408.2. 1H NMR (400 MHz, CHLOROFORM-d) δ 7.60 (s, 2H), 7.36 (br. s., 2H), 6.90 (s, 1H), 4.31 - 4.61 (m, 1H), 2.95 - 3.14 (m, 1H), 2.35 - 2.59 (m, 2H), 1.90 - 2.19 (m, 1H), 1.23 (s, 3H), 1.08 (s, 3H), 0.77 (t, J = 7.28 Hz, 3H).

Peak 4: 16.9 mg (95% purity, 100% ee). LCMS: Rt = 1.75 min, m/z 408.2. 1H NMR (400 MHz, CHLOROFORM-d) δ 7.60 (s, 2H), 7.36 (s, 2H), 6.86 (s, 1H), 4.18 - 4.52 (m, 1H), 2.98 - 3.26 (m, 1H), 2.34 - 2.60 (m, 2H), 1.93 - 2.14 (m, 1H), 1.20 (s, 3H), 1.17 (s, 3H), 0.82 (t, J = 7.28 Hz, 3H).

Intermediates were synthesized according to the following scheme.



Preparation of 4-(1-(3-bromophenyl)prop-1-en-1-yl)-1H-pyrazol-3-amine (mixture of E/Z isomers):

1H-pyrazol-3-amine (3.50 g, 42.1 mmol), 1-(3-bromophenyl)propan-1-one (8.98 g, 42.1 mmol) and 4-methylbenzenesulfonic acid hydrate (801 mg, 4.21 mmol) were combined then toluene (50.0 mL) was added. The mixture was stirred at 125°C for 18 hours under N2 atmosphere. The mixture was then concentrated and the residue was purified by flash chromatography (CH2Cl2:MeOH = 100:0 to 99:1 to 98:2) to supply 4-(1-(3-bromophenyl)prop-1-en-1-yl)-1H-pyrazol-3-amine (10.0 g, 35.95 mmol, 85% yield, 98% purity) as a mixture of isomers and as a pale-yellow solid. LCMS m/z = 279.9 (M+H)+.

Preparation of 4-(3-bromophenyl)-4-ethyl-7,7-dimethyl-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one:

4-(1-(3-bromophenyl)prop-1-en-1-yl)-1H-pyrazol-3-amine (10.0 g, 35.95 mmol) and 5,5-dimethylcyclohexane-1,3-dione (5.04 g, 35.95 mmol) were dissolved in trifluoroethanol (71.9 mL) and TFA (4.10 g, 35.95 mmol, 2.75 mL) was then added. The mixture was split into four microwave vials. The vials were then heated to 150°C in a microwave for 1.5 hours then cooled to room temperature. The samples were combined and the mixture was concentrated and purified by column chromatography (5-50% EtOAc/EtOH : Heptanes) to provide 4-(3-bromophenyl)-4-ethyl-7,7-dimethyl-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (4.11 g, 10.27 mmol, 29% yield). LCMS m/z = 402.1 (M+H)+.

Preparation of 4-(3-bromophenyl)-4-ethyl-7,7-dimethyl-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one:

A solution of 4-(3-bromophenyl)-4-ethyl-7,7-dimethyl-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (4.40 g, 10.99 mmol) in THF (110 mL) was cooled to 0°C and sodium hydride (923 mg, 38.5 mmol, 60% in mineral oil) was added portion wise. After the addition, the mixture was stirred at 0°C for 1 hour. SEM-chloride (7.33 g, 43.96 mmol, 7.80 mL) was then added dropwise and the resulting mixture was stirred at 0°C for 2 hour then warmed to room temperature and stirred overnight. The mixture was poured into 50 mL of ice-water slowly, then extracted with EtOAc (30 mL×2). The combined organic layers were dried over MgSO4 and concentrated. The residue was purified by column chromatography (5-20% EtOAc in heptane) to supply 4-(3-bromophenyl)-4-ethyl-7,7-dimethyl-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (5.40 g, 8.17 mmol, 74% yield) as pale-yellow gum. LCMS m/z = 662.3 (M+H)+.

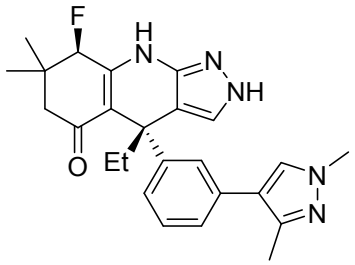
Preparation of 4-(3-bromophenyl)-4-ethyl-8-fluoro-7,7-dimethyl-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one:

4-(3-bromophenyl)-4-ethyl-7,7-dimethyl-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (1.52 g, 2.30 mmol) was dissolved in THF (23.0 mL) and cooled in a dry ice-acetone bath to -78°C. [bis(trimethylsilyl)amino]lithium (1 M in toluene, 2.19 mL) was then added and the mixture was stirred for 30 minutes then warmed to 0°C and stirred for an additional 30 minutes. The mixture was then cooled to -78°C and NFSI (798 mg, 2.53 mmol) was added. The mixture was allowed to warm to room temperature overnight. The mixture was then quenched upon addition of water and saturated, aqueous NH4Cl. The mixture was then extracted with ethyl acetate (50mL x 3) and the combined organic layers were dried over sodium sulfate, filtered, and concentrated. The crude material was then purified by column chromatography (5 to 25% EtOAc in heptanes) to provide 4-(3-bromophenyl)-4-ethyl-8-fluoro-7,7-dimethyl-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (1.15 g, 1.69 mmol, 74% yield) as a mixture of diastereomers. LCMS m/z = 680.7 (M+H)+.

Preparation of 4-ethyl-8-fluoro-7,7-dimethyl-4-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one:

4-(3-bromophenyl)-4-ethyl-8-fluoro-7,7-dimethyl-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (297 mg, 437 umol), 4,4,5,5-tetramethyl-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1,3,2-dioxaborolane (144 mg, 568 µmol), Pd(dppf)Cl2-CH2Cl2 (17.9 mg, 21.85 µmol) and KOAc (85.8 mg, 874 µmol) were combined in a vial and dissolved in dioxane (2.19 mL). Nitrogen was bubbled through the solution for 5 min then the vial was sealed and stirred at 100°C for 18 hours. The mixture was then concentrated and dissolved in EtOAc. The mixture was filtered and the filtrate was concentrated. The residue was purified by column chromatography (5 to 60% EtOAc in heptanes) to afford 4-ethyl-8-fluoro-7,7-dimethyl-4-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (231 mg, 318 µmol, 73% yield) as an orange oil. LCMS m/z = 726.9 (M+H)+.

Preparation of 4-(3-(1,3-dimethyl-1H-pyrazol-4-yl)phenyl)-4-ethyl-8-fluoro-7,7-dimethyl-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (**Compound 2**):



4-(3-bromophenyl)-4-ethyl-8-fluoro-7,7-dimethyl-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (200 mg, 295 µmol), 1,3-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyrazole (131 mg, 589 µmol), [2-(2-aminophenyl)phenyl]-methylsulfonyloxy-palladium;dicyclohexyl-[2-(2,4,6-triisopropylphenyl)phenyl]phosphane (12.5 mg, 14.7 µmol), and potassium phosphate tribasic (125 mg, 589 µmol) were added to a reaction vial. The vial was then sealed and degassed by evacuating and backfilling with nitrogen. Dioxane (786 uL) and water (393 uL) were then added and the mixture was stirred at 100°C for 24 hours. The mixture was then cooled to room temperature, concentrated, and used directly in the next step.

The crude mixture of 4-[3-(1,3-dimethylpyrazol-4-yl)phenyl]-4-ethyl-7,7-dimethyl-2,9-bis(2-trimethylsilylethoxymethyl)-6,8-dihydropyrazolo[3,4-b]quinolin-5-one (204.5 mg, 302.5 µmol) was dissolved in TFA (1.51 mL) and CH2Cl2 (4.54 mL) and stirred at room temperature for 3 hours under nitrogen. The mixture was then concentrated and purified by column chromatography (5-100% [3:1 EtOAc:EtOH] in heptanes). The residue obtained was then purified by chiral SFC (CHIRALPAK IB 30x250mm, 5um, Method: 30% MeOH w/ 0.1% DEA in CO2 [flow rate: 100mL/min, ABPR 120bar, MBPR 40psi, column temp 40 deg C]) to provide four stereoisomers (Rt: Peak 1: 2.36 min, Peak 2: 2.63, Peak 3: 2.89, Peak 4: 3.31). Compound 2 was isolated as peak 2 from the separation method.

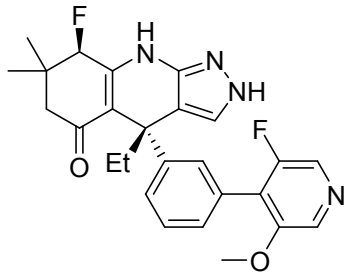
Peak 1: 11.3 mg (98.5% purity, 100% ee, 0.5 equiv Diethylamine solvate). LCMS m/z = 434.6 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 7.57-7.64 (m, 1H), 7.39 (t, J=1.83 Hz, 1H), 7.18-7.31 (m, 2H), 7.11 (d, J=7.33 Hz, 1H), 7.02 (s, 1H), 5.13-5.26 (m, 1H), 3.84 (s, 3H), 3.06-3.14 (m, 1H), 3.03 (q, J=7.33 Hz, 2H), 2.35 (dd, J=16.79, 4.58 Hz, 1H), 2.25-2.29 (m, 3H), 2.03-2.18 (m, 2H), 1.30 (t, J=7.33 Hz, 3H), 1.12-1.20 (m, 6H), 0.80-0.86 (m, 3H).

Peak 2: 14.0 mg (95.9% purity, 94.7% ee). LCMS m/z = 434.6 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 7.61 (s, 1H), 7.48-7.37 (m, 1H), 7.34-7.27 (m, 1H), 7.24 (t, J=7.63 Hz, 1H), 7.15-7.10 (m, 1H), 7.01 (s, 1H), 5.25-5.01 (m, 1H), 3.84 (s, 3H), 3.11-2.97 (m, 1H), 2.33-2.18 (m, 5H), 2.17-2.08 (m, 1H), 1.20 (s, 3H), 1.14 (d, J=1.22 Hz, 3H), 0.81 (t, J=7.33 Hz, 3H). 13C NMR (151 MHz, METHANOL-d4) δ 196.2, 155.9, 153.7, 146.7, 135.5, 133.9, 131.0, 129.1, 127.5, 127.3, 126.1, 125.5, 125.4, 122.9, 109.8, 94.5, 38.6, 37.4, 37.3, 32.1, 25.9, 22.6, 22.6, 13.2, 11.0. 19F NMR (471 MHz, METHANOL-d4) δ -193.25 (br s, 1 F).

Peak 3: 15.2 mg (96.7% purity, 84.3% ee). LCMS m/z = 434.6 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 7.61 (s, 1H), 7.41 (t, J=1.83 Hz, 1H), 7.28-7.34 (m, 1H), 7.24 (t, J=7.63 Hz, 1H), 7.12 (dt, J=7.63, 1.37 Hz, 1H), 7.01 (s, 1H), 5.08-5.23 (m, 1H), 3.84 (s, 3H), 3.01-3.10 (m, 1H), 2.19-2.33 (m, 5H), 2.07-2.16 (m, 1H), 1.20 (s, 3H), 1.14 (d, J=1.22 Hz, 3H), 0.81 (t, J=7.33 Hz, 3H).

Peak 4: 9.7 mg (98.4% purity, 83.8% ee). LCMS m/z = 434.6 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 7.62 (s, 1H), 7.37-7.42 (m, 1H), 7.24-7.29 (m, 1H), 7.20-7.24 (m, 1H), 7.09-7.13 (m, 1H), 7.02 (s, 1H), 5.08-5.26 (m, 1H), 3.84 (s, 3H), 3.05-3.13 (m, 1H), 2.31-2.39 (m, 1H), 2.28 (s, 3H), 2.06-2.18 (m, 2H), 1.10-1.19 (m, 6H), 0.83 (t, J=7.33 Hz, 3H).

Preparation of 4-ethyl-8-fluoro-4-(3-(3-fluoro-5-methoxypyridin-4-yl)phenyl)-7,7-dimethyl-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one:



4-ethyl-8-fluoro-7,7-dimethyl-4-(3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-2,9-bis((2-(trimethylsilyl)ethoxy)methyl)-2,4,6,7,8,9-hexahydro-5H-pyrazolo[3,4-b]quinolin-5-one (231 mg, 318 µmol), 4-bromo-3-fluoro-5-methoxy-pyridine (65.5 mg, 318 µmol), [2-(2-aminophenyl)phenyl]-methylsulfonyloxy-palladium;dicyclohexyl-[2-(2,4,6-triisopropylphenyl)phenyl]phosphane (13.5 mg, 15.9 µmol), and potassium phosphate tribasic (135 mg, 636 µmol) were added to a reaction vial. The vial was then sealed and degassed by evacuating and backfilling with nitrogen. Dioxane (848 uL) and water (424 uL) were then added and the mixture was stirred at 100°C for 24 hours. The mixture was then cooled to room temperature, concentrated, and used directly in the next step. LCMS m/z = 725.8 (M+H)+.

4-ethyl-8-fluoro-4-[3-(3-fluoro-5-methoxy-4-pyridyl)phenyl]-7,7-dimethyl-2,9-bis(2-trimethylsilylethoxymethyl)-6,8-dihydropyrazolo[3,4-b]quinolin-5-one (230 mg, 317 µmol) was dissolved in TFA (1.50 mL) and CH2Cl2 (4.50 mL) and stirred at room temperature for 3 hours under nitrogen. The mixture was then concentrated and purified by column chromatography (5-100% [3:1 EtOAc:EtOH] in heptanes). The residue obtained was then purified by chiral SFC (CHIRALPAK IB 30x250mm, 5um, Method: 30% MeOH w/ 0.1% DEA in CO2 [flow rate: 100mL/min, ABPR 120bar, MBPR 40psi, column temp 40 deg C]) to provide four stereoisomers (Rt: Peak 1: 2.44 min, Peak 2: 2.91, Peak 3: 3.30, Peak 4: 4.04). Compound 3 was isolated as peak 2 from the separation method.

Peak 1: 9.8 mg (95.7% purity, 100% ee). LCMS m/z = 465.5 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 8.22 (s, 1H), 8.17 (s, 1H), 7.46 (d, J=8.55 Hz, 1H), 7.39 (s, 1H), 7.31 (t, J=7.94 Hz, 1H), 7.14 (d,

J=6.71 Hz, 1H), 7.01 (s, 1H), 5.09-5.22 (m, 1H), 3.85 (s, 3H), 3.00-3.09 (m, 2H), 2.34 (dd, J=16.48, 4.88 Hz, 1H), 2.14 (d, J=16.48 Hz, 1H), 2.06-2.11 (m, 1H), 1.15 (d, J=8.55 Hz, 6H), 0.81 (t, J=7.33 Hz, 3H).

Compound 3: Peak 2: 13.1 mg (98% purity, 97.8% ee). LCMS m/z = 465.6 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 8.24 (s, 1H), 8.19 (s, 1H), 7.49 (d, J=7.94 Hz, 1H), 7.43 (d, J=1.83 Hz, 1H), 7.33 (t, J=7.63 Hz, 1H), 7.19-7.14 (m, 1H), 7.02 (s, 1H), 5.25-5.11 (m, 1H), 3.91-3.85 (m, 3H), 3.09-2.98 (m, 1H), 2.34-2.21 (m, 2H), 2.15-2.06 (m, 1H), 1.20 (s, 3H), 1.13 (d, J=1.22 Hz, 3H), 0.81 (t, J=7.33 Hz, 3H). 13C NMR (151 MHz, METHANOL-d4) δ 195.9, 159.2, 157.5, 155.6, 153.4, 132.4, 132.3, 131.7, 131.6, 130.5, 129.4, 128.7, 128.3, 128.0, 127.7, 113.3, 95.7, 94.5, 58.0, 49.9, 37.6, 32.4, 26.2, 22.5, 22.5, 11.1. 19F NMR (471 MHz, METHANOL-d4) δ -132.7 (s, 1F) -192.9 (br s, 1F).

Peak 3: 13.3 mg (96.8% purity, 95.1% ee). LCMS m/z = 465.6 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 8.22 (s, 1H), 8.18 (s, 1H), 7.48 (d, J=9.16 Hz, 1H), 7.42 (s, 1H), 7.32 (t, J=7.63 Hz, 1H), 7.15 (d, J=6.71 Hz, 1H), 7.00 (s, 1H), 5.09-5.23 (m, 1H), 3.86 (s, 3H), 3.02 (dd, J=12.82, 7.33 Hz, 1H), 2.18-2.31 (m, 2H), 2.09 (dd, J=12.82, 6.71 Hz, 1H), 1.18 (s, 3H), 1.11 (d, J=1.22 Hz, 3H), 0.79 (t, J=7.33 Hz, 3H).

Peak 4: 8.7 mg (97% purity, 100% ee). LCMS m/z = 465.6 (M+H)+. 1H NMR (500 MHz, METHANOL-d4) δ ppm 8.21 (s, 1H), 8.17 (s, 1H), 7.46 (d, J=8.55 Hz, 1H), 7.39 (s, 1H), 7.31 (t, J=7.63 Hz, 1H), 7.14 (d, J=7.33 Hz, 1H), 7.01 (s, 1H), 5.08-5.25 (m, 1H), 3.85 (s, 3H), 3.04 (dd, J=12.51, 7.63 Hz, 1H), 2.34 (dd, J=16.79, 4.58 Hz, 1H), 2.14 (d, J=17.09 Hz, 1H), 2.09 (dd, J=12.82, 7.33 Hz, 1H), 1.15 (d, J=9.77 Hz, 6H), 0.81 (t, J=7.33 Hz, 3H).