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multimedia online data

SUPPLEMENTARY ONLINE DATA

Characterization of WZ4003 and HTH-01-015 as selective inhibitors of the LKB1-tumour-suppressor-activated NUAK kinases

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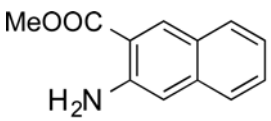
CHEMICAL SYNTHESIS

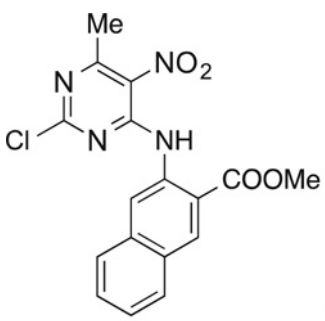
Methyl 3-amino-2-naphthoate



1

To a solution of 3-amino-2-naphthoic acid (562 mg, 3.0 mmol, 1.0 eq) in methanol/toluene (1:4, 10 ml) was added 2.0 M trimethylsilyldiazomethane solution in hexane (1.8 ml, 3.6 mmol, 1.2 eq) at 0 °C. The reaction was stirred overnight at room temperature (20 °C). Next day, the reaction was quenched with excess acetic acid until no bubbling was seen. The mixture was directly concentrated *in vacuo*. The residue was purified by silica-gel column chromatography with ethyl acetate and hexane (0–25 % gradient, v/v) to give compound **1** (500 mg, 83 %). ^{1}H-NMR (400 MHz, [^{2}H]methanol) δ 8.46 (s, 1 H), 7.70 (d, *J* 8.2 Hz, 1 H), 7.51 (d, *J* 8.2 Hz, 1 H), 7.37 (ddd, *J* 8.2, 6.8, 1.2 Hz, 1 H), 7.15 (ddd, *J* 8.2, 6.8, 1.2 Hz, 1 H), 7.05 (s, 1 H), 3.93 (s, 3 H). MS (ESI) calculated for $[C_{12}H_{12}NO_2]^+$, 202; found, 202.

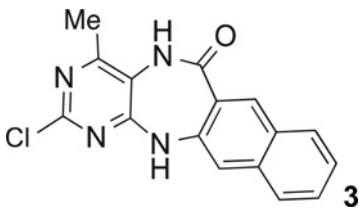
Methyl 3-[(2-chloro-6-methyl-5-nitropyrimidin-4-yl)amino]-2-naphthoate



2

A mixture of compound **1** (480 mg, 2.4 mmol, 1.0 eq), *N*,*N*-diisopropylethylamine (0.83 ml, 4.8 mmol, 2.0 eq) and 2,4-dichloro-6-methyl-5-nitropyrimidine (0.76 g, 3.6 mmol, 1.5 eq) in 2-propanol (43 ml) was stirred at room temperature overnight. The product crashed out of 2-propanol, and was collected by filtration and dried *in vacuo*. The crude compound **2** (0.79 g, 88 %) was used for the next step without further purification. ^{1}H-NMR (400 MHz, [^{2}H]chloroform) δ 12.04 (s, 1 H), 8.94 (s, 1 H), 8.68 (s, 1 H), 7.89 (d, *J* 8.2 Hz, 2 H), 7.63 (ddd, *J* 8.2, 7.0, 1.2 Hz, 1 H), 7.51 (ddd, *J* 8.2, 7.0, 1.2 Hz, 1 H), 4.05 (s, 3 H), 2.73 (s, 3 H). MS (ESI) calculated for $[C_{17}H_{14}ClN_4O_4]^+$, 373; found, 373.

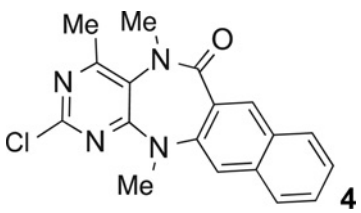
2-chloro-4-methyl-5,13-dihydro-6*H*-naphtho[2,3-*e*]pyrimido[5,4-*b*][1,4]diazepin-6-one



3

To a solution of compound **2** (0.79 g, 2.1 mmol, 1.0 eq) in acetic acid (90 ml) was added iron powder (1.7 g, 30.4 mmol, 14.5 eq). The reaction was stirred at 60 °C overnight. After the reaction was complete as monitored by reverse-phase analytical LC–MS, the solvent was removed *in vacuo*. The resulting residue was poured into ice-cold water and stirred, which resulted in a solid precipitate that was collected by filtration, washed with water and air-dried to give compound **3** (0.64 g, 98 %). ^{1}H-NMR (400 MHz, [^{2}H]chloroform) δ 8.63 (s, 1 H), 7.85 (d, *J* 8.2 Hz, 1 H), 7.68 (d, *J* 8.2 Hz, 1 H), 7.54 (ddd, *J* 8.2, 7.0, 1.4 Hz, 1 H), 7.42 (ddd, *J* 8.2, 7.0, 1.2 Hz, 1 H), 7.17 (s, 1 H), 7.15 (s, 1 H), 6.88 (s, 1 H), 2.52 (s, 3 H). MS (ESI) calculated for $[C_{16}H_{12}ClN_4O]^+$, 311; found, 311.

2-chloro-4,5,13-trimethyl-5,13-dihydro-6*H*-naphtho[2,3-*e*]pyrimido[5,4-*b*][1,4]diazepin-6-one



4

To a stirred suspension of compound **3** (0.64 g, 2.1 mmol, 1.0 eq) and methyl iodide (0.64 ml, 10.3 mmol, 5.0 eq) in dimethyl acetamide (20.0 ml) was added sodium hydride (300 mg, 60 % suspension in mineral oil, 3.6 eq) at 0 °C. After the reaction was complete as monitored by LC–MS, the solution was poured into ice-cold water, which resulted in a solid precipitate. The precipitate was collected by filtration, washed with water and air-dried to give the crude product. The crude product was purified by silica-gel column chromatography with ethyl acetate and hexane (0–80 % gradient, v/v) to give compound **4** (67 mg, 10 %). ^{1}H-NMR (400 MHz, [^{2}H]methanol) δ 8.30 (s, 1 H), 7.88 (d, *J* 8.2 Hz, 1 H), 7.84 (d, *J* 8.2 Hz, 1 H), 7.61 (s, 1 H), 7.54 (ddd, *J* 8.2, 7.0,

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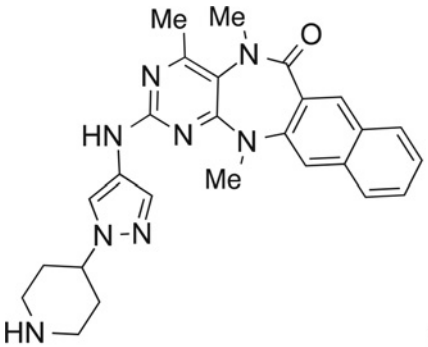
1.2 Hz, 1 H), 7.45 (ddd, *J* 8.2, 7.0, 1.2 Hz, 1 H), 3.51 (s, 3 H), 3.37 (s, 3 H), 2.48 (s, 3 H). MS (ESI) calculated for $[C_{18}H_{16}ClN_4O]^+$, 339; found, 339.

4,5,13-trimethyl-2-{[1-(piperidin-4-yl)-1*H*-pyrazol-4-yl]amino}-5,13-dihydro-6*H*-naphtho[2,3-*e*]pyrimido[5,4-*b*][1,4]diazepin-6-one



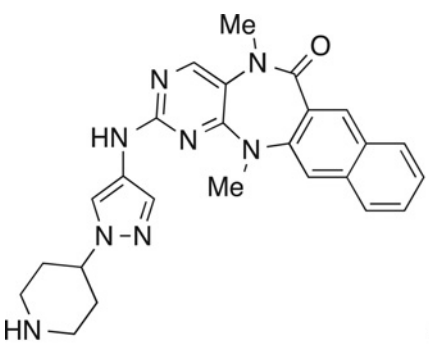
5, HTH-01-015

A mixture of compund **4** (34 mg, 0.1 mmol, 1.0 eq), t-butyl 4-(4-amino-1*H*-pyrazol-1-yl)piperidine-1-carboxylate (27 mg, 0.1 mmol, 1.0 eq), X-Phos (8.6 mg, 20 %), tris(dibenzylideneacetone)dipalladium(0) (11 mg, 10 %) and potassium carbonate (41.5 mg, 0.3 mmol) in 1.2 ml of t-butyl alcohol was heated at 85 °C in a sealed tube for 3.5 h. The reaction was then filtered through celite and eluted with dichloromethane. The dichloromethane was removed *in vacuo*. The resulting crude product was stirred with trifluoroacetic acid (0.38 ml, 5 mmol, 50 eq) in dichloromethane (2 ml) at room temperature overnight to afford Boc deprotection. The solvent was removed *in vacuo*. The residue was purified by reverse-phase prep-HPLC using a water (0.05 % trifuloroacetic acid)/methanol (0.05 % trifluoroacetic acid) gradient to afford the title compound HTH-01-015 as a trifluoroacetic acid salt (18 mg, yield 31 %). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.72–9.40 (br, 1 H), 8.74–8.61 (br, 1 H), 8.54–8.37 (br, 1 H), 8.29 (s, 1 H), 7.97 (d, *J* 8.2 Hz, 1 H), 7.92 (s, 1 H), 7.88 (d, *J* 8.2 Hz, 1 H), 7.67 (s, 1 H), 7.60 (s, 1 H), 7.56 (ddd, *J* 8.2, 7.0, 1.2 Hz, 1 H), 7.46 (ddd, *J* 8.2, 7.0, 1.2 Hz, 1 H), 4.54–4.41 (br, 1 H), 3.54–3.38 (br, 5 H), 3.27 (s, 3 H), 3.17–3.02 (br, 2 H), 2.33 (s, 3 H), 2.26–2.04 (br, 4 H). MS (ESI) calculated for $[C_{26}H_{29}N_8O^+]^+$, 469; found, 469.

XMD18-42 and XMD17-51

XMD18-42 and XMD17-51 were synthesized following similar strategies as shown in Scheme 1.

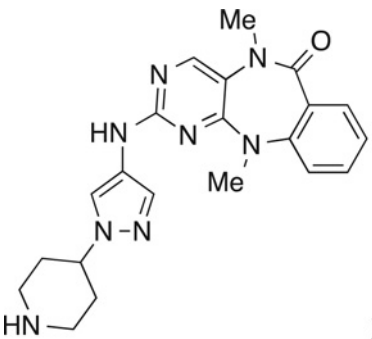
5,13-dimethyl-2-{[1-(piperidin-4-yl)-1*H*-pyrazol-4-yl]amino}-5,13-dihydro-6*H*-naphtho[2,3-*e*]pyrimido[5,4-*b*][1,4]diazepin-6-one



XMD18-42

^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.77–9.44 (br, 1 H), 9.18–8.96 (br, 2 H), 8.39 (s, 1 H), 8.33 (s, 1 H), 7.98 (d, *J* 8.2 Hz, 1 H), 7.95 (s, 1 H), 7.91 (d, *J* 8.2 Hz, 1 H), 7.75 (s, 1 H), 7.60 (s, 1 H), 7.57 (t, *J* 8.2 Hz, 1 H), 7.46 (t, *J* 8.2 Hz, 1 H), 4.56–4.43 (br, 1 H), 3.55–3.47 (br, 3 H), 3.45 (s, 3 H), 3.43–3.34 (br, 2 H), 3.13–2.99 (br, 2 H), 2.27–2.09 (br, 4 H). MS (ESI) calculated for $[C_{25}H_{27}N_8O^+]^+$, 455; found, 455.

5,11-dimethyl-2-{[1-(piperidin-4-yl)-1*H*-pyrazol-4-yl]amino}-5,11-dihydro-6*H*-benzo[*e*]pyrimido[5,4-*b*][1,4]diazepin-6-one



XMD17-51

^{1}H-NMR (400 MHz, DMSO-d_6) δ 9.77–9.60 (br, 1 H), 9.16–8.94 (br, 2 H), 8.35 (s, 1 H), 7.93 (s, 1 H), 7.68 (dd, *J* 7.9, 1.8 Hz, 1 H), 7.58 (s, 1 H), 7.51 (td, *J* 7.9, 1.8 Hz, 1 H), 7.28 (d, *J* 7.9 Hz, 1 H), 7.18 (t, *J* 7.9 Hz, 1 H), 4.52–4.44 (br, 1 H), 3.42–3.34 (br, 5 H), 3.38 (s, 3 H), 3.10–3.00 (br, 2 H), 2.21–2.12 (br, 4 H). MS (ESI) calculated for $[C_{21}H_{25}N_8O^+]^+$, 405; found, 405.

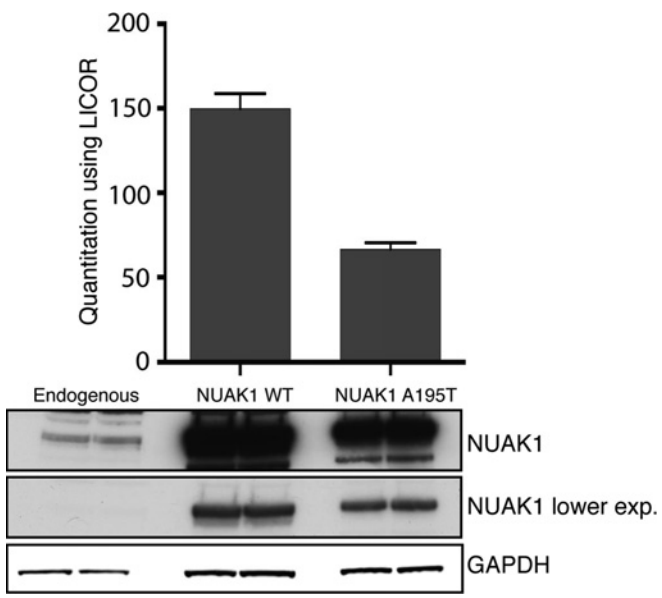
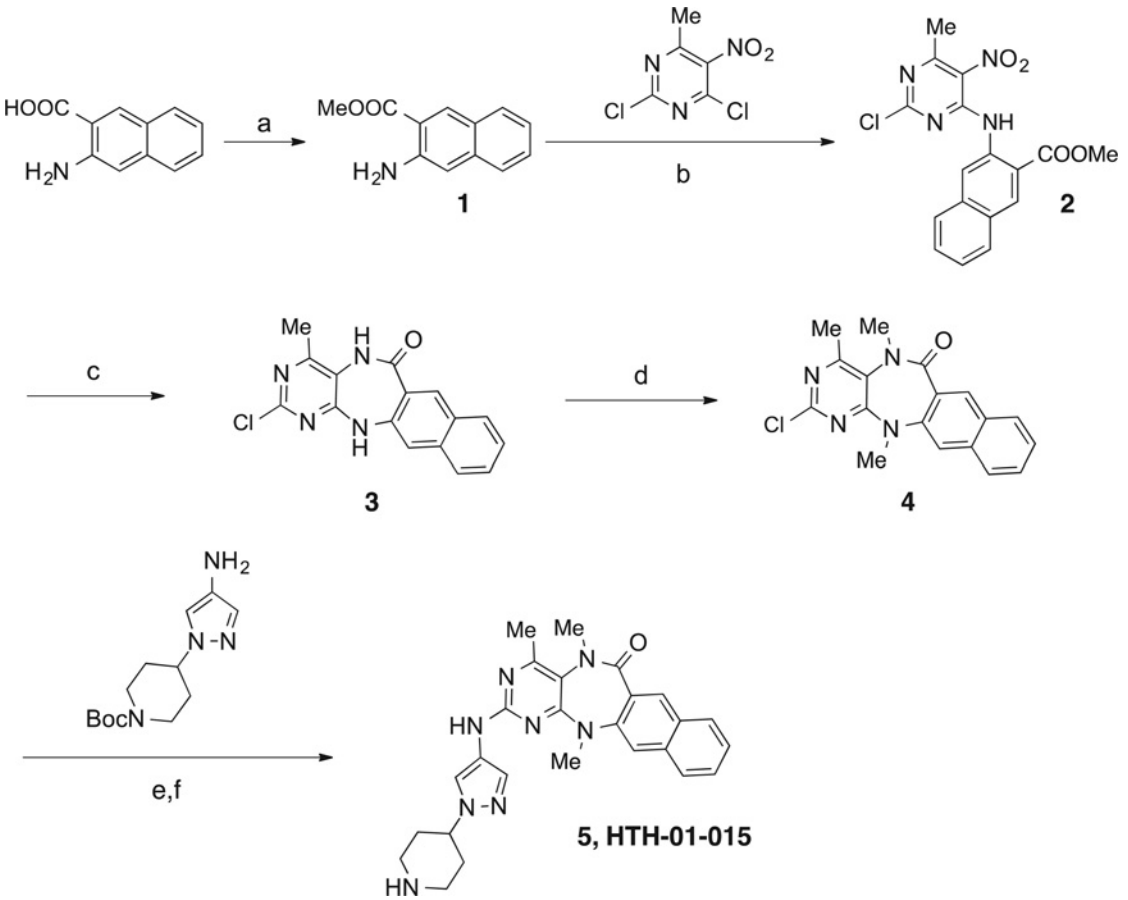


Figure S1 LI-COR quantification of the overexpression of wild-type (WT) and inhibitor-resistant NUAK1[A195T] mutant

The Western blot signals for endogenous and overexpressed NUAK1 in HEK-293 cells were quantified using LI-COR Odyssey technology. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control. The background signal was subtracted and then the band intensity of overexpressed NUAK1 was divided by the signal for the endogenous NUAK1 protein (in duplicate). Data are represented relative to the expression levels of the endogenous protein. lower exp., lower exposure.



Scheme S1 Synthesis of 4,5,13-trimethyl-2-{[1-(piperidin-4-yl)-1*H*-pyrazol-4-yl]amino}-5,13-dihydro-6*H*-naphtho[2,3-*e*]pyrimido[5,4-*b*][1,4]diazepin-6-one (HTH-01-015)

Reagents and conditions: (a) trimethylsilyldiazomethane (1.2 eq.), methanol/toluene (1:4), 0 °C; (b) *N*,*N*-diisopropylethylamine (2.0 eq), 2-propanol; (c) Fe (14.5 eq.), acetic acid, 60 °C; (d) methyl iodide (5.0 eq.), sodium hydride (3.6 eq.), dimethyl acetamide, 0 °C; (e) X-Phos (20 % mol), tris(dibenzylideneacetone)dipalladium(0) (10 % mol), potassium carbonate (3.0 eq.), t-butyl alcohol, 85 °C; (f) trifluoroacetic acid (50 eq), dichloromethane.

Table S1 Effect of the NUAK inhibitors upon the activity of 140 protein kinases

Results are presented as the percentage of kinase activity in DMSO control reactions. Protein kinases were assayed *in vitro* with 0.1 or 1 μM of the inhibitors as described previously [1], and the results are means ± S.D. for triplicate reactions. *Indicates AMPK-related kinase family members. Abbreviations are as follows: ABL, Abelson tyrosine-protein kinase 1; AMPK, AMP-activated protein kinase; ASK, apoptosis signal-regulating kinase; BRK, breast tumour kinase; BRSK, brain-specific kinase; BTK, Bruton"s tyrosine kinase; CaMK, calmodulin-dependent kinase; CaMKK, CaMK kinase; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; CK, casein kinase; CLK, CDC-like kinase; CSK, C-terminal Src kinase; DAPK, death-associated protein kinase; DDR, discoidin domain receptor; DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; EF2K, elongation-factor-2 kinase; EIF2AK, eukaryotic translation initiation factor 2-alpha kinase; EPH, ephrin; ERK, extracellular signal-regulated kinase; FGF-R, fibroblast growth factor receptor; GCK, germinal centre kinase; GSK, glycogen synthase kinase; HER, human epidermal growth factor receptor; HIPK, homeodomain-interacting protein kinase; IGF1R, IGF1 receptor; IKK, inhibitory κB kinase; IR, insulin receptor; IRAK, interleukin-1 receptor-associated kinase; IRR, insulin-related receptor; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; Lck, lymphocyte cell-specific protein tyrosine kinase; LKB1, liver kinase B1; MAPK, mitogen-activated protein kinase; MAPKAP-K, MAPK-activated protein kinase; MARK, microtubule-affinity-regulating kinase; MEKK, MAP kinase kinase kinase; MELK, maternal embryonic leucine-zipper kinase; MINK, misshapen/NIK-related kinase; MKK, MAPK kinase; MLK, mixed lineage kinase; MNK, MAPK-integrating protein kinase; MPSK, myristoylated and palmitoylated serine/threonine-protein kinase; MSK, mitogen- and stress-activated protein kinase; MST, mammalian homologue Ste20-like kinase; NEK, NIMA (never in mitosis in *Aspergillus nidulans*)-related kinase; NUAK, novel (NUA) family SnF1-like kinase; OSR, oxidative stress-responsive kinase; PAK, p21-activated protein kinase; PDGFRA, platelet-derived growth factor receptor-α; PDK, phosphoinositide-dependent kinase; PHK, phosphorylase kinase; PIM, provirus integration site for Moloney murine leukaemia virus; PINK (insect homologue), PTEN-induced kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PKD, protein kinase D; PLK, polo-like kinase; PRAK, p38-regulated activated kinase; PRK, protein kinase C-related kinase; RIPK, receptor-interacting protein kinase; ROCK, Rho-dependent protein kinase; RSK, ribosomal S6 kinase; S6K, p70 ribosomal S6 kinase; SGK, serum- and glucocorticoid-induced protein kinase; SIK, salt-induced kinase; smMLCK, smooth muscle myosin light-chain kinase; SRPK, serine/arginine protein kinase; STK, serine/threonine kinase; SYK, spleen tyrosine kinase; TAK, TGFβ-activated kinase; TAO, thousand and one amino acid; TBK1, TANK-binding kinase 1; TESK, testis-specific protein kinase; TGFBR, TGFβ receptor; TIE, tyrosine-protein kinase receptor; TLK, tousled-like kinase; TrkA, tropomyocin receptor kinase; TSSK, testis-specific serine/threonine-protein kinase; TTBK, tau-tubulin kinase; ULK, Unc-51-like kinase; VEGFR, vascular endothelial growth factor receptor; WNK, with no lysine; YES1, Yamaguchi sarcoma viral oncogene homologue 1; ZAP, ζ-chain-associated protein.

Kinase	HTH-01-015	WZ4003
NUAK1*	**11 ± 0**	**6 ± 0**
AMPK*	127 ± 9	89 ± 9
MARK1*	106 ± 4	76 ± 9
MARK2*	99 ± 4	85 ± 14
MARK3*	102 ± 1	51 ± 2
MARK4*	75 ± 2	79 ± 3
BRSK1*	130 ± 32	91 ± 1
BRSK2*	97 ± 3	75 ± 22
MELK*	99 ± 9	70 ± 2
SIK2*	112 ± 16	70 ± 0
SIK3*	109 ± 32	102 ± 9
LKB1	98 ± 10	87 ± 0
MKK1	113 ± 28	98 ± 14
MKK2	111 ± 10	98 ± 4
MKK6	87 ± 1	101 ± 4
ERK1	83 ± 1	94 ± 2
ERK2	125 ± 6	107 ± 6
ERK5	121 ± 4	79 ± 2
JNK1	100 ± 2	88 ± 4
JNK2	130 ± 9	97 ± 3
JNK3	101 ± 8	70 ± 2
p38α MAPK	118 ± 4	95 ± 4
p38β MAPK	112 ± 3	104 ± 6
p38γ MAPK	100 ± 0	91 ± 0
p38δ MAPK	113 ± 1	63 ± 10
ERK8	98 ± 7	90 ± 9
RSK1	93 ± 3	67 ± 5

Table S1 Continued

Kinase	HTH-01-015	WZ4003
RSK2	106 ± 15	76 ± 1
PDK1	102 ± 5	104 ± 10
PKBα	87 ± 15	98 ± 3
PKBβ	104 ± 2	114 ± 34
SGK1	97 ± 2	99 ± 7
S6K1	85 ± 3	83 ± 14
PKA	101 ± 0	84 ± 18
ROCK 2	110 ± 14	80 ± 2
PRK2	112 ± 8	99 ± 20
PKCα	117 ± 4	77 ± 8
PKCγ	107 ± 0	103 ± 15
PKCζ	98 ± 13	86 ± 15
PKD1	94 ± 8	57 ± 5
STK33	93 ± 2	33 ± 7
MSK1	111 ± 2	90 ± 3
MNK1	102 ± 9	104 ± 8
MNK2	106 ± 0	84 ± 16
MAPKAP-K2	118 ± 9	82 ± 10
MAPKAP-K3	96 ± 20	91 ± 5
PRAK	105 ± 10	94 ± 0
CAMKKβ	87 ± 12	44 ± 2
CAMK1	92 ± 0	81 ± 1
SmMLCK	77 ± 8	78 ± 2
PHK	104 ± 24	54 ± 11
DAPK1	100 ± 5	93 ± 12
CHK1	106 ± 6	58 ± 1
CHK2	105 ± 11	40 ± 0
GSK3β	119 ± 13	88 ± 0
CDK2-Cyclin A	96 ± 3	90 ± 32
CDK9-Cyc T1	68 ± 1	89 ± 2
PLK1	98 ± 5	84 ± 9
Aurora A	117 ± 1	75 ± 11
Aurora B	97 ± 13	72 ± 8
TLK1	106 ± 10	91 ± 7
TSSK1	95 ± 7	58 ± 11
CK1γ2	109 ± 11	102 ± 4
CK1δ	94 ± 5	97 ± 5
CK2	120 ± 11	76 ± 0
TTBK1	124 ± 19	78 ± 21
TTBK2	102 ± 4	89 ± 3
DYRK1A	89 ± 4	102 ± 2
DYRK2	113 ± 7	66 ± 8
DYRK3	98 ± 0	71 ± 9
NEK2α	103 ± 2	110 ± 3
NEK6	99 ± 13	79 ± 13
IKKβ	81 ± 9	72 ± 0
IKKε	85 ± 1	104 ± 0
TBK1	112 ± 8	84 ± 5
PIM1	92 ± 4	79 ± 7
PIM2	106 ± 6	101 ± 21
PIM3	97 ± 2	98 ± 1
SRPK1	103 ± 7	94 ± 1
EF2K	103 ± 8	91 ± 2
EIF2AK3	99 ± 13	76 ± 17
HIPK1	107 ± 7	103 ± 20
HIPK2	107 ± 6	86 ± 19
HIPK3	96 ± 13	91 ± 1
CLK2	54 ± 1	65 ± 7
PAK2	125 ± 13	92 ± 6
PAK4	110 ± 8	69 ± 6
PAK5	95 ± 5	89 ± 5
PAK6	95 ± 2	104 ± 1
MST2	118 ± 1	91 ± 2
MST3	111 ± 3	93 ± 8
MST4	119 ± 7	101 ± 0
GCK	126 ± 3	96 ± 4
MAP4K3	127 ± 5	111 ± 15
MAP4K5	106 ± 4	108 ± 2
MINK1	101 ± 1	120 ± 5
MEKK1	110 ± 14	91 ± 1

Table S1 Continued

Kinase	HTH-01-015	WZ4003
MLK1	102 ± 18	69 ± 0
MLK3	94 ± 7	73 ± 8
TESK1	106 ± 8	98 ± 12
TAO1	123 ± 10	101 ± 7
ASK1	105 ± 2	107 ± 7
TAK1	109 ± 7	85 ± 10
IRAK1	103 ± 2	88 ± 2
IRAK4	103 ± 11	92 ± 3
RIPK2	88 ± 4	105 ± 0
OSR1	107 ± 4	89 ± 1
TTK	118 ± 3	54 ± 17
MPSK1	103 ± 5	107 ± 9
WNK1	99 ± 7	95 ± 7
ULK1	132 ± 19	52 ± 2
ULK2	106 ± 13	37 ± 2
TGFBR1	102 ± 13	102 ± 7
Src	76 ± 8	109 ± 10
Lck	90 ± 3	99 ± 3
CSK	103 ± 5	105 ± 0
YES1	76 ± 7	89 ± 5
ABL	110 ± 3	104 ± 9
BTK	109 ± 4	114 ± 14
JAK2	101 ± 11	65 ± 2
SYK	125 ± 5	93 ± 4
ZAP70	103 ± 2	89 ± 6
TIE2	84 ± 12	94 ± 0
BRK	96 ± 0	79 ± 0
EPH-A2	84 ± 1	91 ± 13
EPH-A4	94 ± 8	106 ± 7
EPH-B1	97 ± 15	98 ± 3
EPH-B2	97 ± 15	107 ± 13
EPH-B3	120 ± 1	113 ± 39
EPH-B4	102 ± 4	83 ± 2
FGF-R1	105 ± 11	66 ± 2
HER4	102 ± 5	76 ± 22
IGF-1R	133 ± 26	33 ± 4
IR	105 ± 0	92 ± 12
IRR	106 ± 6	88 ± 2
TrkA	82 ± 1	85 ± 2
DDR2	91 ± 5	86 ± 21
VEG-FR	97 ± 12	74 ± 21
PDGFRA	109 ± 5	96 ± 2
PINK	99 ± 1	95 ± 7

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