Structure of the human autophagy initiating kinase ULK1 in complex with potent inhibitors

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Supplementary Information

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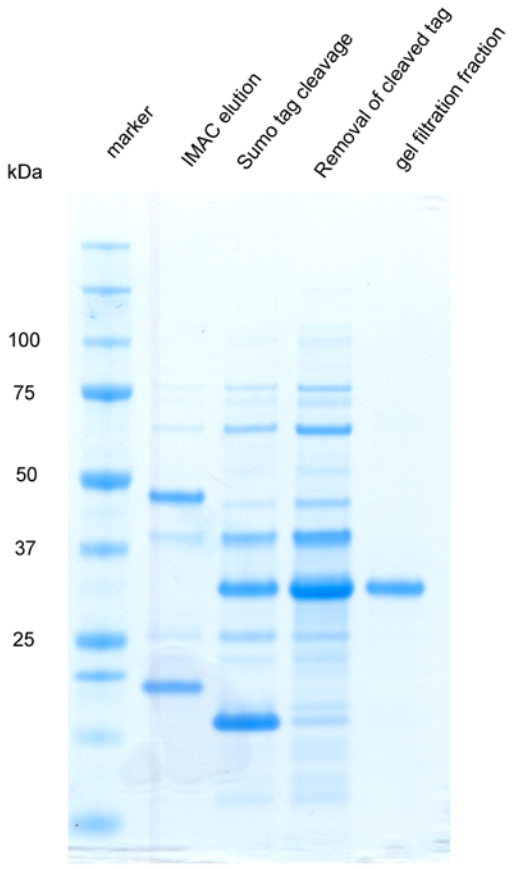
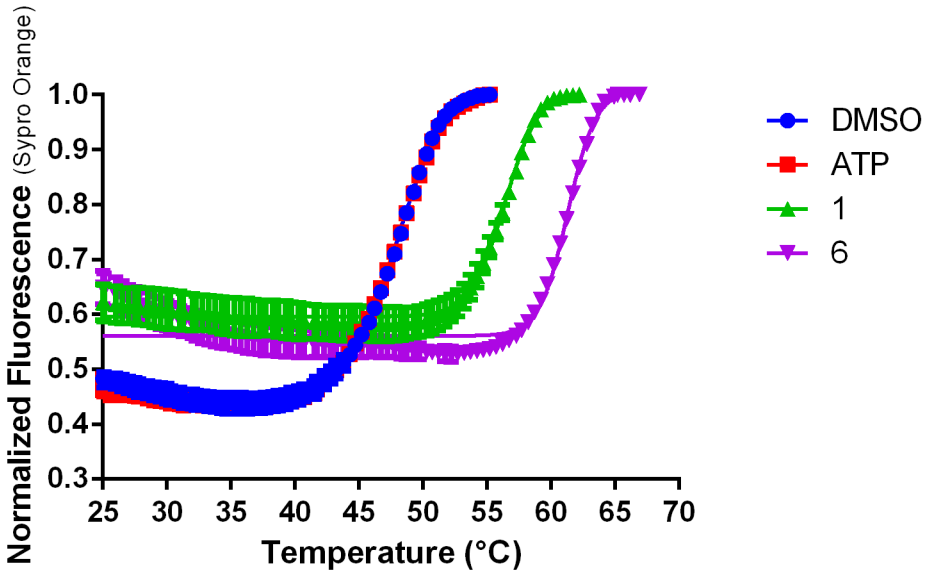
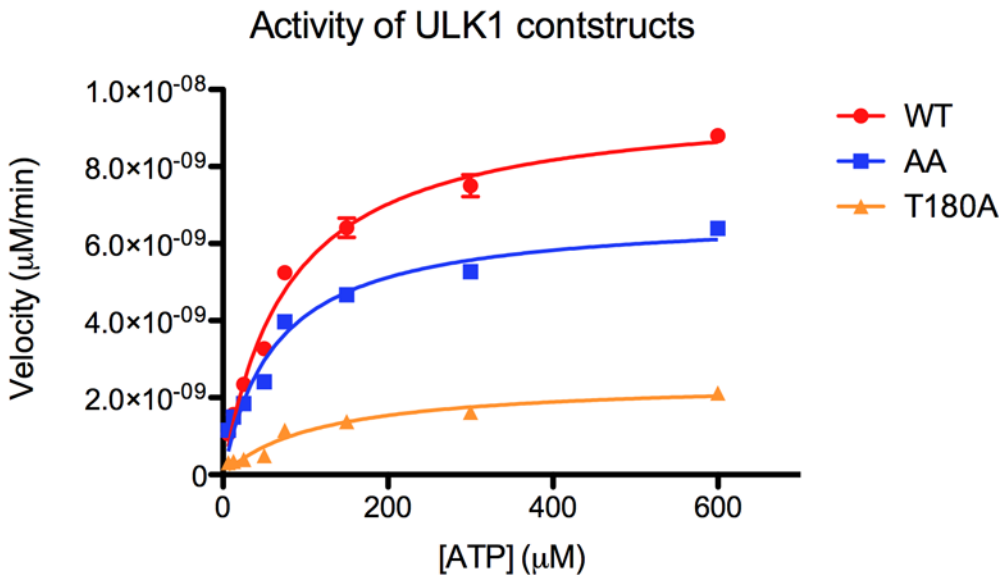


Supplementary Figure 1. Purification of ULK1. Coomassie-stained gel shows the different stages of the purification of ULK1. The final purified sample on the rightmost lane was used for crystallization.



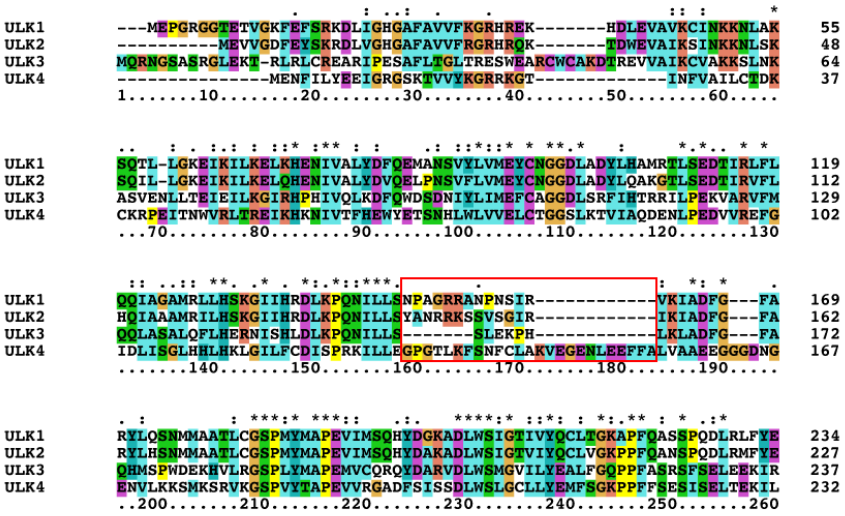
	DMSO	ATP	1	6
T_m	48.03	47.91	56.41	61.29
ΔT_m	0	-0.12	8.38	13.26

Supplementary Figure 2. ULK1 Stabilization by compounds 1 and 6. The melting point of the ULK1 kinase domain in the presence of DMSO, ATP, or inhibitor were determined by monitoring the fluorescent signal from Sypro Orange in a differential scanning fluorimetry assay.

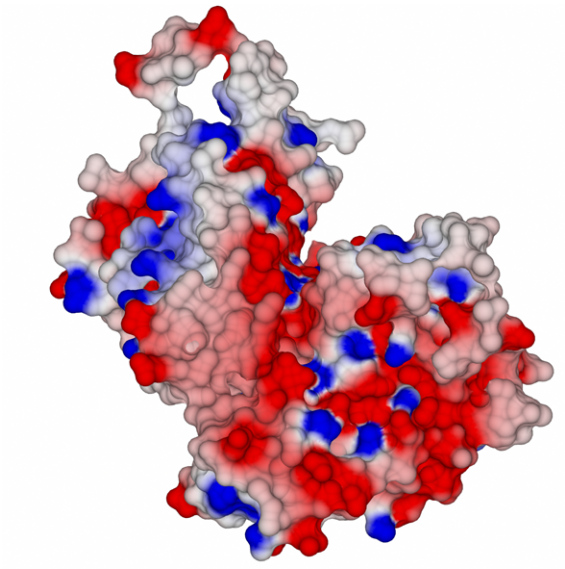


	K_m (ATP) µM	k_{cat} min^{-1}	k_{cat} / K_m $min^{-1} M^{-1}$
WT ULK1	78 ± 7	0.98 ± 0.03	1.3×10^4
AA ULK1	64 ± 7	0.70 ± 0.03	1.1×10^4
T180A ULK1	120 ± 20	0.31 ± 0.02	0.3×10^4

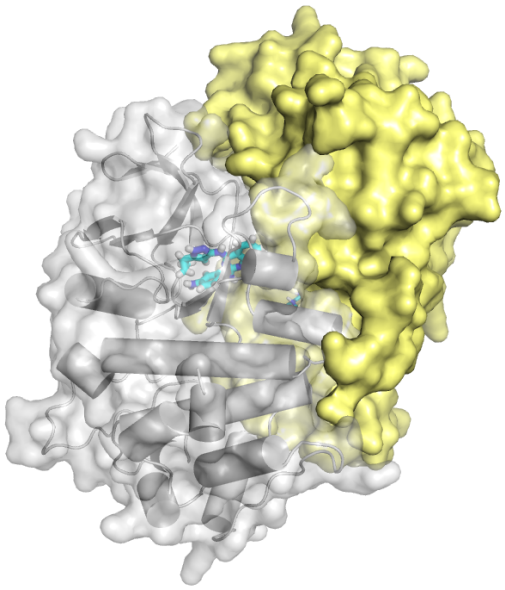
Supplementary Figure 3. Kinetics of ULK1 kinase domain constructs. Kinetic constants for ULK1 were calculated for the wild-type kinase (WT ULK1), for the crystallography construct (AA ULK1), and for a mutant without a phosphorylatable threonine in the activation loop (T180A ULK1), using a radioactive kinase assay with MBP as the substrate. Assay was performed in triplicate with error bars indicating standard error of the mean.



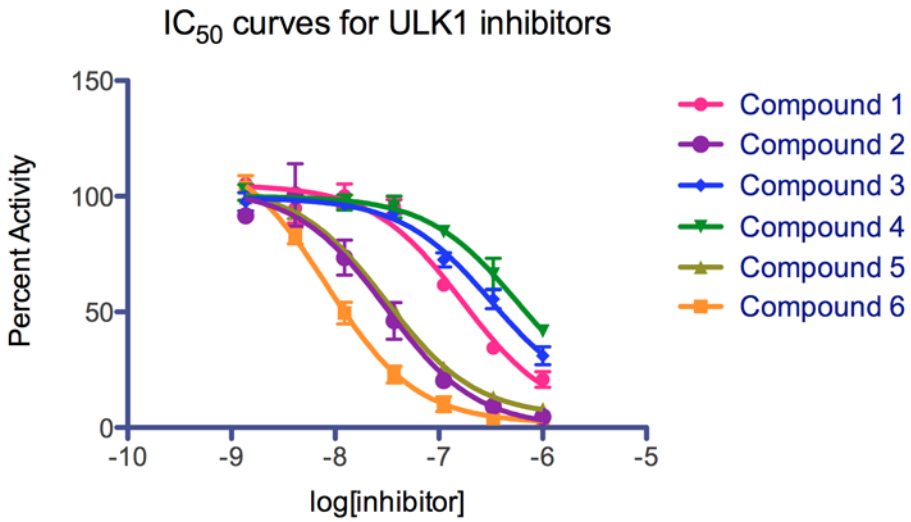
Supplementary Figure 4. Alignment of ULK1, ULK2, ULK3, and ULK4 kinase domains. The large interlobe loop that is unique to ULK1, ULK2, and ULK4 is highlighted with a red box. The kinase domains were aligned using ClustalX[1].



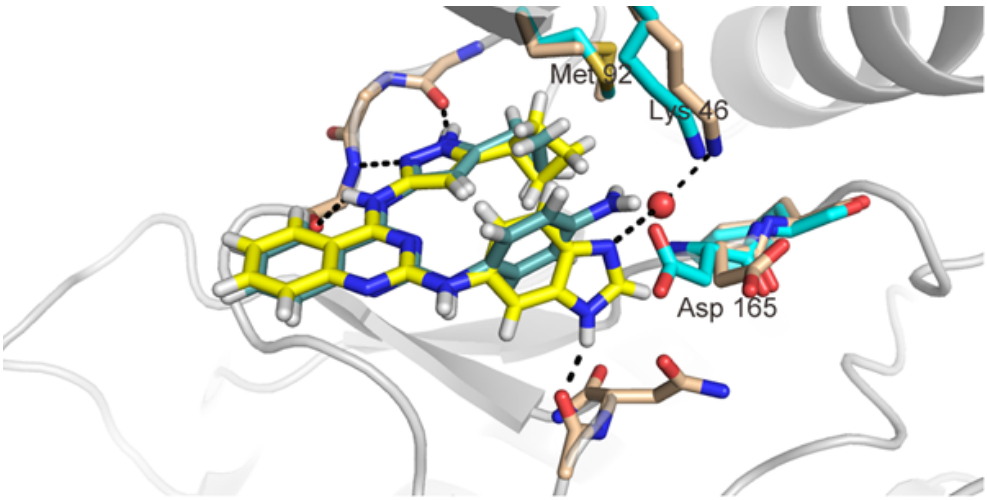
Supplementary Figure 5. Electrostatic surface rendering of the ATG13-ATG1 complex. The recently solved structure of ATG13 bound to the C-terminal domain of ATG1, the yeast ortholog of ULK1, shows strong negative charge on the surface. The structure is from PDB code 4P1N[2].



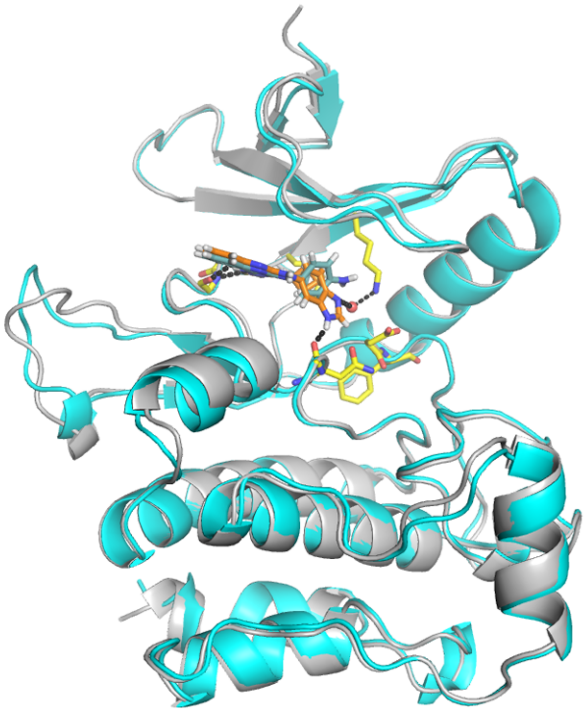
Supplementary Figure 6. Dimer interface of ULK1. This is the interface between the ULK1:**1** complex and a crystallographic symmetry mate. The surface area was calculated using the PISA server. The ΔG for this interaction was -1.9 kcal/mol, which suggests that this not strong enough to be an interaction in solution[3].



Supplementary Figure 7. IC_{50} curves for ULK1 inhibitors. Assay was performed in duplicate with error bars indicating standard error of the mean.



Supplementary Figure 8. Overlay of structures of Compound **1** and Compound **6** bound to ULK1. Compound **1** is shown in teal with corresponding sidechains shown in cyan. Compound **6** is shown in yellow with corresponding sidechains shown in beige. The major differences are the conformations of Asp165, which is forced to move by the bulkier benzimidiazole group of **6**. Additionally, smaller changes are seen in Lys46 and Met92.



Supplementary Figure 9. Overlay of ULK1 structures with compounds **1** and **6**, overall view. The structure with 1 is shown in cyan, with the **6** complex shown in gray. Compound **6** is shown in orange. The residues that interact with **6** are shown in yellow.

Supplementary Tables

Supplementary Table 1. Profiling of Compound **6** against representative kinases. Compound 6 was screened at 250 nM.

Kinase name	Activity Remaining with 250 nM Compound **6** (%)
MKK1	35
JNK1	95
p38a MAPK	98
RSK1	5
PDK1	56
PKBa	103
SGK1	6
S6K1	21
PKA	65
ROCK 2	75
PRK2	18
PKCa	92
PKD1	3
MSK1	21
CAMKKb	2
CAMK1	20
SmMLCK	39
CHK2	8
GSK3b	16
PLK1	65
Aurora B	4
LKB1	24
AMPK (hum)	1
MARK3	2
CK1δ	3
CK2	91
DYRK1A	1
NEK6	91
TBK1	12
PIM1	97
SRPK1	17
EF2K	99
HIPK2	62
PAK4	13
MST2	2
MLK3	3
TAK1	2
IRAK4	5
RIPK2	18
TTK	40
Src	4
Lck	1
BTK	14
JAK2	2
SYK	15
EPH-A2	22
HER4	32
IGF-1R	4
TrkA	1
VEG-FR	2

Supplementary Table 2. Primers used in this study

Primer Name	Sequence
MBL247N	5′-TAAAGAGCTCGCTCTGGTGCCACG-3′
MBL247C	5′-AGAACCACCACCAATCTGTTCTCTGTGAGC-3′
MBL248N	5′-CACAGAGAACAGATTGGTGGTGGTTCTATGGAGCCCGGCCGCGGCG-3′
MBL248C	5′-CGTGGCACCAGAGCGAGCTCTTTACGAGGGGCTGGCATCGAGG-3′
MBL275N	5′-CGCGCAGCACACGATTTGGAGGTCGCCGTCAAG-3′
MBL275C	5′-GTGTGCTGCGCGGTGGCGGCCCTTGAAGACCAC-3′
MBL317N	5′-GGCCGCACTCTGCGGCTCCCCCATGTACATG-3′
MBL317C	5′-AGAGTGCGGCCGCCATCATGTTGCTCTGGAGG-3′

Supplementary Methods

Purification of ULK1

The kinase domain of ULK1 (Residues 1 – 283) was cloned from a plasmid containing ULK1 cDNA (DNASU) into a modified pET 47b vector (EMD) with an N-terminal Sumo fusion tag instead of the HRV3C cleavage site. The plasmid was transformed into KRX cells along with a plasmid containing lambda phosphatase in a pCDF Duet vector. (Promega). An overnight culture was used to inoculate (1:500) 1 L of LB medium containing 50 mg/ml kanacmycin, 50 mg/ml streptamycin, and grown at 37 °C until the cells reached an OD600 of 1.0 to 1.3, at which point they were transferred to 16 °C and induced overnight with 0.2 mM IPTG and 0.1% rhamnose. The next day, cells were harvested and resuspended in TBS (pH 8.0, 250 mM NaCl) and lysed with a microfluidizer. After pelleting the cell debris, the lysate was loaded onto a column containing Ni-NTA (Qiagen) for IMAC purification. The column was equilibrated with TBS supplemented with 40 mM imidazole pH 8.0, washed with TBS with 50 mM imidazole, and eluted with 4 ml of TBS pH 7.4 with 250 mM Imidazole in 20% glycerol. The eluate was then supplemented with 0.5 mM THP (EMD) and concentrated with Amicon centrifugal filters (Millipore). The tag was then cleaved with Sumo protease (Life Sensors) overnight at 4 °C. The protein was then diluted in TBS with 20% glycerol to reduce the imidazole to 20 mM and then was incubated with Ni-NTA resin for an hour to bind the protease and uncleaved protein. The flowthrough was collected and concentrated and purified further on a Superdex200 gel filtration column (GE Lifescience) with TBS pH 7.4 with 150 mM NaCl and 20% glycerol. Finally, the protein was concentrated to 20 mg/ml.

Crystallization of ULK1

The protein was first diluted 1:1 in water so the final concentrations of glycerol and salt were 10% and 75 mM, respectively. After initial screening of the ULK1 kinase domain: Compound 1 complex yielded no diffracting crystals we mutated two residues at the surface, after analysis by the Surface Entropy Reduction server. Lysine 37 and Glutamate 38 were both mutated to alanine, to create ULK1AA. This crystallization construct generated crystals in multiple conditions. The best crystals were obtained by the hanging drop vapor diffusion method by incubating 1 μL ULK1AA:Compound 1 complex at 10 mg/ml with 1 μl reservoir solution consisting of 1.55 M sodium malonate pH 7.0 and 0.35 M sodium malonate pH 5.0. After 3 to 4 days, tetragonal crystals appeared. The crystals were then soaked in cryoprotectant

consisting of 1.85 M sodium malonate pH 7.0, 0.40 M sodium malonate pH 5.0, and 16% glycerol and flash frozen in liquid nitrogen for collection.

Data Collection and Structure Determination

All datasets were collected at Beamline 8.2.2 at the Advanced Light Source. Datasets were indexed and integrated with iMosflm[4], and scaled with Aimless[5] and Scala[6] in the CCP4 software suite[7]. The initial structure, of the ULK1AA:Compound 1 complex was solved by molecular replacement. Using Phenix MRage, a series of truncated models were tested as molecular replacement solutions, and the model based on the polo-like kinase 3 structure (PDB 4B6L) gave the best solution. This solution was then used as a starting model for Phenix Autobuild[8]. The Autobuild solution was then further refined with manual building in Coot and multiple rounds of refinement in Phenix[9] with simulated annealing, ADP (B factor) refinement, TLS (using parameters obtained from the TLSMD server[10]) and positional refinement, interspersed with manual adjustments in Coot[11]. The inhibitor geometrical restraints were obtained with Phenix Elbow[12]. The second structure with Compound 6 was determined using molecular replacement with PHASER,[13] using the Compound 1 structure as a search model. The structure was refined with Phenix and Coot as described for the first structure. All structural figures were made with Pymol except for figures with electrostatic surfaces, which were made with CCP4mg.

Kinase inhibitor screen

All reagents are from Sigma Aldrich unless otherwise specified. The kinase assays were set up with reaction buffer consisting of (final concentrations): 50 mM HEPES 7.5, 10 mM $MgCl_2$, 4 μM Myelin Basic Protein (EMD), 20 mM β-glycerophosphate, 10% glycerol, 0.02% Triton-X and 5 nM enzyme. The enzyme used for all assays had the sumo tag intact to help stabilize the protein. Next, inhibitors were added as 10% DMSO stocks to the reaction for a final concentration of 1% DMSO. Lastly, ATP was added to a final concentration of 50 mM cold ATP with 0.05 mCi/ml ^{32}P-γ-ATP (Perkin Elmer). The reactions were then incubated at room temperature and aliquots were removed at 15 minute or 30 minute time points and quenched by spotting onto nitrocellulose paper. The nitrocellulose paper was then washed with 1% phosphoric acid, dried, and exposed to a phosphor screen and imaged on a Typhoon 9000. The spots were quantified using the Spot program and for dose response curves, the IC_{50} values were calculated with GraphPad Prism. For kinetics, the assay was done the same except that the enzyme concentration was 10 nM and the cold ATP was varied from 6.25 μM to 600 μM, and the reactions were spotted at different timepoints. The K_m and k_{cat} values were then calculated from the ATP curves using Graphpad Prism.

Differential Scanning Fluorimetry

19 μL of a solution composed of 100 mM MOPS pH = 7.4, 200 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 0.1 mM DTT, 5x Sypro Orange (Invitrogen), and 2 μM of the ULK1 kinase domain (sumo tag removed) was added to the wells of a 96-well, low profile, white, PCR plate (USA scientific). 1 μL of 400 μM test compound in 40% DMSO was added to each well to give a final concentration of 20 μM compound and 2% DMSO per well. Each condition was tested in duplicate. The wells were mixed by pipetting, sealed with TempAssure clear PCR flat caps (USA Scientific), centrifuged at 500xg for 30s,

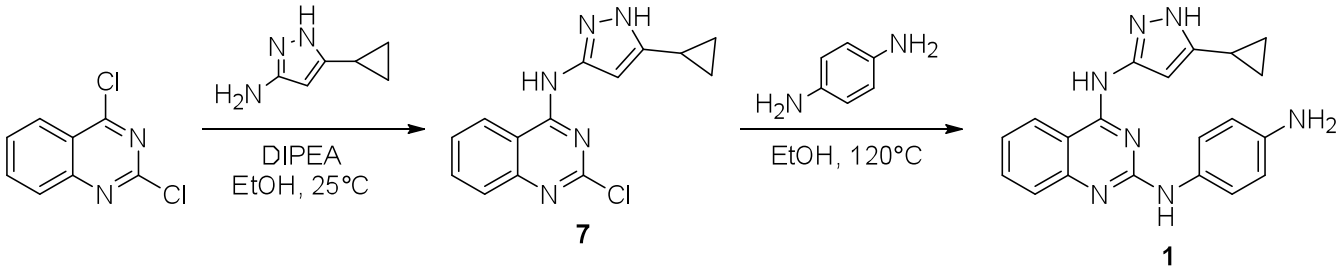
and heated in a Stratagene Mx3005P RT-PCR machine from 25°C to 95°C in 0.5°C increments every 30s after an initial incubation at 25°C for 10 min. Fluorescence was measured at the end of each 30s period with an excitation wavelength of 492nM and an emission wavelength of 610nM. To obtain the melting temperature, fluorescent signals were normalized to the maximum fluorescent signal for that well. Values after the well had reached a maximum signal were discarded and the signals were fit to the Boltzmann equation in Graphpad Prism. ΔT_m was calculated as the difference in melting temperature compared to the DMSO control.

Kinase Panel Profiling of compound 6

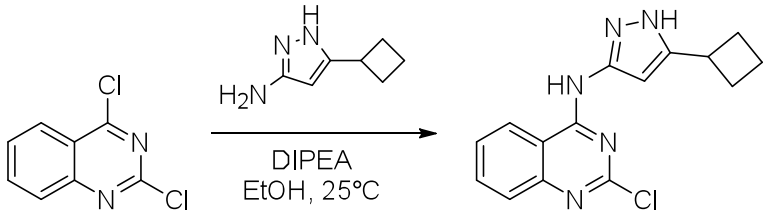
Compound 6 was profiled against 50 kinases using the International Centre for Protein Kinase Profiling in duplicate using a radioactive filter-binding assay.

Chemical Synthesis:

General Methods: Reactions were performed in sealed vials with magnetic stirring. ^{13}C NMR spectra were recorded on a Bruker Avance DRX500 spectrometer, ^{1}H NMR spectra were recorded on a Varian Innova 400 spectrometer and referenced to solvent peaks. Chemical shifts are reported in δ (ppm) as either s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Low resolution mass spectra (LC/ESI-MS) were recorded in positive mode on a Waters TQ detector with an Acquity UPLC equipped with a BEH C18 column. All commercial reagents were used without further purification. All RP-HPLC were performed with a Waters 2545 binary gradient module equipped with an XBridge prep C18 column using H2O + 0.1% formic acid and CH3CN + 0.1% formic acid (5-95% gradient) while monitoring at 254 nm.

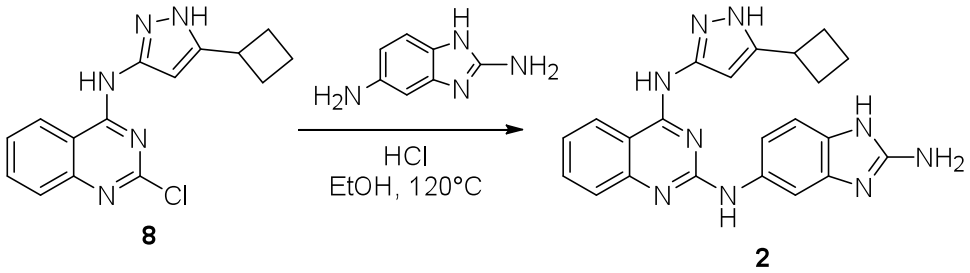


Compound 1: 2-chloro-N-(5-cyclopropyl-1H-pyrazol-3-yl)quinazolin-4-amine (**7**) was synthesized as previously described[14]. Briefly, DIPEA (0.426 mL, 2.39 mmol) was added to a suspension of 2,4-dichloroquinazoline(150 mg, .754 mmol) and 3-amino-5-cyclopropyl-1H-pyrazole (93mg, 0.754 mmol) in ethanol (3.5 mL). The suspension was stirred at 25 °C for 2h. The resulting precipitate was filtered and washed with ethanol to afford **7** (106.2 mg, 49% yield) as a white solid, which was used in the next step without further purification. MS (ES+) *m/z* 286.6 $(M + H)^{+1}$. A mixture of **7** (24.9mg, .087 mmol) and *p*-phenylenediamine (51.4 mg, 0.436 mmol) in ethanol (1.5 mL) was stirred at 120°C in a sealed vial for 30 minutes. The reaction was cooled and purified by RP-HPLC. 25.1 mg (80 % yield) of a yellow solid. MS (ES+) *m/z* 358.8 $(M + H)^{+1}$; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 0.72 (m, 2H), 0.91 (m, 2H), 1.88 (m, 1H), 6.21 (s, 1H), 6.58 (d, 2H, *J*=9.7 Hz), 7.17 (t, 1H, *J*=7.58 Hz), 7.39 (d, 1H, *J*=8.37 Hz), 7.45 (br, 2H), 7.60 (t, 1H, *J*=8,28 Hz), 8.12 (s 1H), 8.32 (d, 1H, *J*=8.1 Hz), 9.157 (s, 1H), 10.23 (br, 1H); ^{13}C NMR(DMSO-d6, 500 MHz) δ 8.26, 8.56, 100.93, 111.45, 114.52, 115.77, 121.82, 123.58, 133.42, 141.37, 150.76, 156.74, 163.97



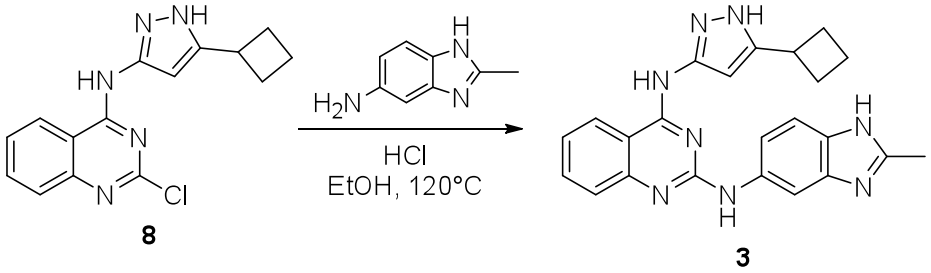
8

Compound 8: DIPEA (0.951 mL, 5.3 mmol) was added to a suspension of 2,4-dichloroquinazoline(370 mg, 1.85 mmol) and 3-amino-5-cyclobutyl-1H-pyrazole (250 mg, 0.754mmol) in ethanol (10 mL). The suspension was stirred at 25 °C for 10h. The resulting precipitate was filtered and washed with ethanol to afford **8** (401.5 mg, 74% yield) as a white solid. MS (ES+) *m/z* 300.8 $(M + H)^{+1}$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.85 (m, 1H), 1.97 (m, 1H), 2.16 (m, 2H), 2.30 (m, 2H), 3.54 (m, 1H), 6.62 (s, 1H), 7.56 (t, 1H, *J*=7.11 Hz), 7.67 (d, 1H, *J*=7.59 Hz), 7.83 (t, 1H, *J*=7.66 Hz), 8.64 (d, 1H, *J*=8.06 Hz), 10.78 (s, 1H), 12.38 (s, 1H);^{13}C NMR (DMSO-d6, 500 MHz) δ 18.56, 29.47, 31.58, 96.41, 113.98, 124.10, 127.01, 127.23, 134.38, 147.12, 147.70, 151.22, 156.85, 159.04.



8 2

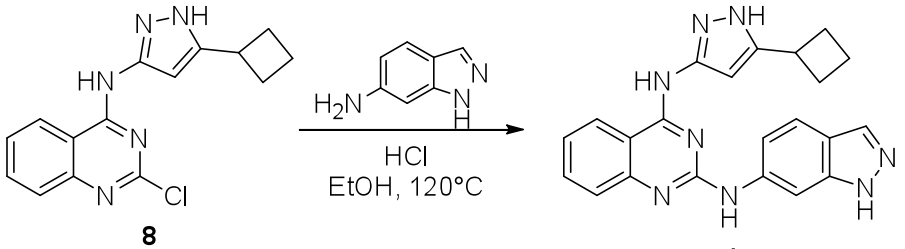
Compound 2: 1H-benzo[d]imidazole-2,5-diamine was synthesized as previously reported[15]. A mixture of **8** (25.0 mg, 0.083 mmol), and 1H-benzo[d]imidazole-2,5-diamine (64.2 mg, 0.433 mmol) was stirred in ethanol (2 mL). Concentrated acid was slowly added to adjust the pH of the solution to 5. The solution was then stirred at 120°C in a sealed vial for 1h. The reaction was cooled and purified by RP-HPLC. 15.8 mg (46 % yield) of a tan solid. MS (ES+) *m/z* 412.6 $(M + H)^{+1}$; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.84 (m, 1H), 1.94 (m, 1H), 2.13 (m, 2H), 2.23 (m, 2H), 3.45 (m, 1H), 6.404 (s, 1H), 6.82 (s, 2H), 7.05 (d, 1H, *J*=8.4 Hz), 7.17 (m, 1H), 7.27 (d, 1H, *J*=9.59 Hz), 7.41 (d, 1H, *J*=8.27 Hz), 7.6 (t, 1H, *J*=7.57 Hz), 7.87 (br, 1H), 8.25 (s, 1H), 8.35 (d, 1H, *J*=8.16 Hz), 9.20 (br, 1H), 10.21 (br, 1H); ^{13}C NMR (DMSO-d6, 500 MHz) δ 18.20, 28.99, 32.40, 98.58, 100.49, 110.90, 111.26, 114.34, 115.32, 121.46, 122.33, 123.15, 131.35, 132.88, 134.31, 134.97, 140.87, 151.64, 154.23, 156.73, 164.74.



8 3

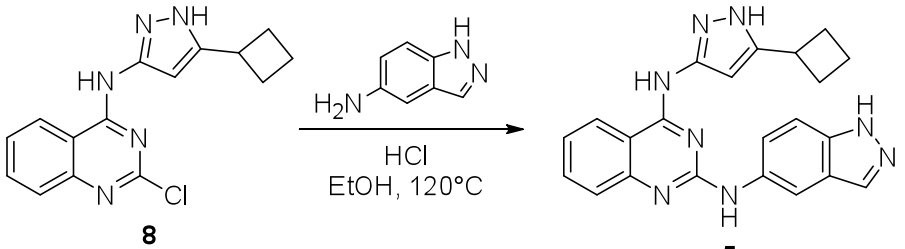
Compound 3: To a stirred suspension of **8** (25.5 mg, 0.085 mmol) and 2-methyl-1H-benzo[d]imidazol-5-amine (65.4 mg, 0.444 mmol) in ethanol (1.5 mL) at 25°C was added 5 µL of

concentrated HCl. The solution was then stirred at 120°C in a sealed vial for 3h. The reaction was cooled and purified by RP-HPLC. 18.3 mg (52 % yield) of a red/brown solid. MS (ES+) *m/z* 411.9 $(M + H)^{+1}$; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.83 (m, 1H), 1.96 (m, 1H), 2.15 (m, 2H), 2.25 (m, 2H), 2.46 (s, 3H), 3.47 (m, 1H), 6.44 (s, 1H), 7.21 (t, 1H, *J*=7.55 Hz), 7.41 (m, 3H), 7.64 (t, 1H, *J*=7.65 Hz), 8.19 (s, 1H), 8.38 (d, 1H, *J*=8.16 Hz), 9.3 (s, 1H), 10.24 (s, 1H); ^{13}C NMR (DMSO-d6, 500 MHz) δ 15.12, 18.67, 29.39, 32.89, 87.54, 99.03, 100.95, 101.90, 111.80, 114.98, 117.47, 123.61, 133.38, 135.69, 151.03, 152.09, 156.99, 163.98.



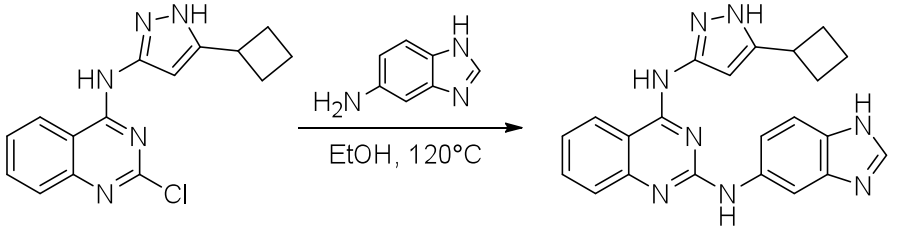
8 **4**

Compound 4: To a stirred suspension of **8** (24.9 mg, 0.083 mmol) and 6-Aminoindazole (56.3 mg, 0.422 mmol) in ethanol (1.5 mL) at 25°C was added 1 µL of concentrated HCl. The solution was then stirred at 120°C in a sealed vial for 1h. The reaction was cooled and purified by RP-HPLC. 29.8 mg (90 % yield) of an off white solid. MS (ES+) *m/z* 396.7 $(M + H)^{+1}$; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.86 (m, 1H), 1.95 (m, 1H), 2.18 (m, 2H), 2.26 (m, 2H), 3.5 (m, 1H), 6.5 (s, 1H), 7.28 (t, 1H, *J*=8.04 Hz), 7.35 (m, 1H), 7.53 (d, 1H, *J*=8.22 Hz), 7.64 (d, 1H, *J*=8.69 Hz), 7.70 (t, 1H, *J*=7.62 Hz), 7.93 (s, 1H), 8.18 (s, 1H), 8.43 (d, 1H, *J*=8.05 Hz), 8.55 (s, 1H), 9.57 (s, 1H), 10.33 (br, 1H); ^{13}C NMR (DMSO-d6, 500 MHz) δ 18.23, 28.96, 32.46, 97.84, 111.56, 114.88, 117.98, 120.12, 122.26, 125.33, 125.57, 128.22, 128.92, 133.11, 133.16, 139.32, 140.98, 151.29, 156.27, 156.85, 163.44



8 **5**

Compound 5: This compound was prepared from 23.4mg (.078 mmol) of **8**, and 56.7 mg (0.425 mmol) of 5-aminoindazole using the method described above for compound **4**. 22.6 mg (78% yield) of a tan solid.MS (ES+) *m/z* 396.9 $(M + H)^{+1}$; ^{1}H NMR (DMSO-d_6, 400 MHz) 1.84 (m, 1H), 1.93 (m, 1H), 2.13 (m, 2H), 2.24 (m, 2H), 3.46 (m, 1H), 6.44 (s, 1H),7.22 (t, 1H, *J*=8.01 Hz), 7.49 (m, 2H), 7.63 (m, 2H), 7.99 (s, 1H), 8.17 (s, 1H), 8.39 (d, 1H, *J*=8.17 Hz), 8.43 (br, 1H), 9.38 (s, 1H), 10.22 (br, 1H); ^{13}C NMR (DMSO-d6, 500 MHz) δ 18.19, 28.96, 32.27, 98.58, 106.69, 108.93, 111.33, 121.39, 121.69, 123.06, 125.33, 132.95, 134.02, 136.40, 149.73, 151.49, 156.68, 157.02, 163.38.



Compound 6: A mixture of **8** (24.3 mg, 0.081 mmol), and 5-Aminobenzimidazole (55.4 mg, 0.416 mmol) in ethanol (1.5 mL) was stirred at 120°C in a sealed vial for 1h. The reaction was cooled and purified by RP-HPLC. 28.5 mg (89 % yield) of an off white solid. MS (ES+) *m/z* 397.3 $(M + H)^{+1}$; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.84 (m, 1H), 1.94 (m, 1H), 2.14 (m, 2H), 2.24 (m, 2H), 3.47 (m, 1H), 6.46 (s, 1H), 7.23 (t, 1H, *J*=7.55 Hz), 7.51 (m, 3H), 7.66 (t, 1H, *J*=7.65 Hz), 8.15 (d, 2H, *J*=11.99 Hz), 8.37 (br, 1H), 8.40 (d, 1H, *J*=8.15 Hz), 9.46 (s, 1H), 10.29 (br, 1H);^{13}C NMR (DMSO-d6, 500 MHz) δ 18.23, 28.96, 32.40, 99.56, 111.39, 115.51, 121.84, 122.35, 123.22, 125.2, 126.98, 133.05, 134.99, 135.85, 140.89, 141.37, 151.26, 156.41, 156.83, 162.87, 163.32.

References

1. Larkin, M. A.; Blackshields, G.; Brown, N. P.; Chenna, R.; McGettigan, P. A.; McWilliam, H.; Valentin, F.; Wallace, I. M.; Wilm, A.; Lopez, R.; Thompson, J. D.; Gibson, T. J.; Higgins, D. G., Clustal W and Clustal X version 2.0. *Bioinformatics* **2007,** *23* (21), 2947-8.
2. Fujioka, Y.; Suzuki, S. W.; Yamamoto, H.; Kondo-Kakuta, C.; Kimura, Y.; Hirano, H.; Akada, R.; Inagaki, F.; Ohsumi, Y.; Noda, N. N., Structural basis of starvation-induced assembly of the autophagy initiation complex. *Nature structural & molecular biology* **2014,** *21* (6), 513-21.
3. Krissinel, E.; Henrick, K., Inference of macromolecular assemblies from crystalline state. *Journal of molecular biology* **2007,** *372* (3), 774-97.
4. Battye, T. G.; Kontogiannis, L.; Johnson, O.; Powell, H. R.; Leslie, A. G., iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. *Acta Crystallogr D Biol Crystallogr* **2011,** *67* (Pt 4), 271-81.
5. Evans, P. R.; Murshudov, G. N., How good are my data and what is the resolution? *Acta crystallographica. Section D, Biological crystallography* **2013,** *69* (Pt 7), 1204-14.
6. Evans, P., Scaling and assessment of data quality. *Acta Crystallogr D Biol Crystallogr* **2006,** *62* (Pt 1), 72-82.
7. The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* **1994,** *50* (Pt 5), 760-3.
8. Terwilliger, T. C.; Grosse-Kunstleve, R. W.; Afonine, P. V.; Moriarty, N. W.; Zwart, P. H.; Hung, L. W.; Read, R. J.; Adams, P. D., Iterative model building, structure refinement and density modification with the PHENIX AutoBuild wizard. *Acta crystallographica. Section D, Biological crystallography* **2008,** *64* (Pt 1), 61-9.
9. Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H., PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **2010,** *66* (Pt 2), 213-21.
10. (a) Painter, J.; Merritt, E. A., TLSMD web server for the generation of multi-group TLS models. *Journal of Applied Crystallography* **2006,** *39*, 109-111; (b) Painter, J.; Merritt, E. A., Optimal description of a protein structure in terms of multiple groups undergoing TLS motion. *Acta Crystallogr D Biol Crystallogr* **2006,** *62* (Pt 4), 439-50.
11. Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K., Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **2010,** *66* (Pt 4), 486-501.
12. Moriarty, N. W.; Grosse-Kunstleve, R. W.; Adams, P. D., electronic Ligand Builder and Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Crystallogr D Biol Crystallogr* **2009,** *65* (Pt 10), 1074-80.
13. McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J., Phaser crystallographic software. *J Appl Crystallogr* **2007,** *40* (Pt 4), 658-674.

14. Statsuk, A. V.; Maly, D. J.; Seeliger, M. A.; Fabian, M. A.; Biggs, W. H., III; Lockhart, D. J.; Zarrinkar, P. P.; Kuriyan, J.; Shokat, K. M., Tuning a Three-Component Reaction For Trapping Kinase Substrate Complexes. *Journal of the American Chemical Society* **2008,** *130* (51), 17568-17574.
15. Cao, X.; You, Q.-D.; Li, Z.-Y.; Liu, X.-R.; Xu, D.; Guo, Q.-L.; Shang, J.; Chern, J.-W.; Chen, M.-L., The design, synthesis and biological evaluation of 7-alkoxy-4-heteroarylamino-3-cyanoquinolines as dual inhibitors of c-Src and iNOS. *Bioorganic & Medicinal Chemistry Letters* **2008,** *18* (23), 6206-6209.