Supplementary Information

A small-molecule mimic of a peptide docking motif inhibits the protein kinase PDK1.

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SI Materials and Methods:

Reagents and Antibody Sources

GSK2334470 (>98% pure) was purchased from Sigma and used without further purification. Human IGF1 (#8917SC) and antibodies to phospho-S6 S235/6 (#4858) (1:2000) and phospho-AKT T308 (#13038) (1:1000) were purchased from Cell Signaling. The antibody to α-tubulin (T6199) (1:5000) was purchased from Sigma. The IR dye secondary antibodies Goat anti-Mouse 680RD (1:10000) and Donkey anti-Rabbit 800CW (1:10000) were purchased from Li-COR Biosciences.

Protein Expression and Purification

$PDK1_{50-359}$ (wild-type, Y288G Q292A, L155A, or L155E) and $S6K1_{24-421}$ T412E were expressed in Sf9 insect cells using the Invitrogen Bac-to-Bac protocol. Each kinase was cloned into the pFastBac HTB vector with an N-terminal 6xHis tag followed by a TEV protease cleavage site. During log-phase growth (~$2x10^6$ cells/mL), Sf9 cells were infected with baculovirus (MOI: 2) and grown for 72 hr. Cell pellets were lysed with a detergent-based *Lysis buffer* for 30 min on ice with intermittent swirling. Cell lysates were clarified by centrifugation and sterile filtration. Following purification by Ni-NTA affinity chromatography (GE Healthcare), 6xHis-TEV protease (1:40 w/w) was added and the sample was dialyzed overnight into *Gel filtration buffer.* Next, the sampled was spiked with 20 mM imidazole and passed through a Ni-NTA column. The cleaved kinase without its 6xHis tag was collected in the flow-through at >95% purity. Finally, the sample was concentrated to ~2 mL and run on a HiLoad 16/60 Superdex 200 (GE Healthcare) gel filtration column to isolate non-aggregated kinase. Protein identity and phosphorylation state was assessed by LC-MS of the intact protein using an LCT Premier mass spectrometer (Waters). All purified proteins were concentrated with ultrafiltration centrifugal filters (Millipore), snap frozen in liquid nitrogen, and stored at -80^{o}C.

Lysis buffer: 25 mM Tris, pH 7.5, 150 mM NaCl, 20 mM imidazole, 5 mM NaF, 1 mM Na_3VO_4 (activated), 1 mM $MgCl_2$, 5% v/v glycerol, and 0.5% v/v Igepal CA-630. Just before lysis, add 100 µg/mL DNAse I, 100 µg/mL RNAse A, and an EDTA-free protease inhibitor tablet (Roche).

Gel filtration buffer: 25 mM Tris, pH 7.5, 300 mM NaCl, 5% v/v glycerol, 2 mM DTT

High-Throughput Screen

The screening library consisted of 153,888 compounds purchased from ChemDiv, Chembridge, and Microsource Discovery Systems. The primary screen was conducted at a single dose (33 µM) using the FP competitive binding assay (**Fig. 1*A***) in a 15 µL volume in 384-well black plates (Corning #3676). PDK1 (200 nM) and the fluorescent PIFtide probe (50 nM) were combined in a buffer containing 25 mM Tris, pH 7.5, 125 mM NaCl, and 0.0625% v/v Pluronic F-68. Next, 50 nL of compound in DMSO was pin-transferred into each well using a BioMEK FX (Beckman Coulter). Following incubation for 1 hr, the fluorescence polarization value was read using the Analyst HT plate reader (LJL Biosystems; Ex: 530 nm; Em: 580 nm). Wells without PDK1 were used as positive controls and wells treated with DMSO were used as negative controls. This assay showed robust performance during the primary screen with an average Z' factor of 0.7.

Hits from the primary screen were selected using a statistical threshold of 3σ (1460 hits, 0.9% hit rate). Compounds that yielded a total fluorescence intensity (TFI) greater than 150% of the mean of the DMSO control wells were flagged as autofluorescent artifacts and excluded from further analysis. The remaining 1280 compounds were subjected to an 8-point dose response in the FP competitive binding assay. Based on potency and hill slope ($n < 2$), 100 hits were selected for validation by dose-response in a SPR assay that monitors displacement of PDK1 from immobilized PIFtide (1) using a Biacore T100 (GE Healthcare). Finally, the top 10 hits were repurchased or resynthesized and retested in the FP and SPR assays to confirm their chemical identity.

Crystallization of PDK1 and Soaking with Ligands

All crystals were obtained using a $PDK1_{50-359}$ double mutant (PDK1dm; Y288G Q292A), which was designed to disrupt a crystal contact that normally prevents ligands from binding to the PIF pocket(2). The two mutated residues are located in the αG helix in the C-terminal lobe of the kinase, which is more than 30 Å away from the PIF pocket. PDK1dm was concentrated to 28 mg/mL in a buffer containing 25 mM Tris, pH 7.5, 500 mM NaCl, and 1 mM DTT. Next, EDTA (16.6 mM) and ATP (9 mM, pH 7) were added resulting in a final protein concentration of 21 mg/mL. Hanging drops were set using a Mosquito Crystal (TTP Labtech) with 100 nL protein and 100 nL precipitant solution (0.1 M HEPES, pH 7.5, 1.2M sodium citrate) per drop. Crystals appeared within 3 days and were soaked with drug on day 5. Crystal-soaking solutions were made by adding drug to 1 mM in a mother liquor containing 90% of the contents of the dehydrated crystal drops (22.5 mM Tris pH 7.5, 450 mM NaCl, 0.9 mM DTT, 0.09 M HEPES, pH 7.5, 1.1M sodium citrate, 8 mM ATP, 15 mM EDTA). Crystal-soaking solution (1 µL) was added to each drop and allowed to soak overnight. Individual crystals were looped and transferred into a cryoprotectant solution (crystal-soaking solution with 25% v/v glycerol) to equilibrate for 5 min and were then flash frozen in liquid nitrogen.

Structure Solution and Refinement

Diffraction data was indexed and scaled using HKL-2000 (3). Structures were solved using molecular replacement with the structure of PDK1 bound to **PS210** (PDB ID 4AW1) as a search model in Phaser (4). Iterative model building and refinement was performed with Coot (5) and PHENIX (6), respectively. Structure validation was performed using MOLProbity (7).

Consensus Sequence for HM

The consensus hydrophobic motif (HM) sequence was determined from 27 kinases that are known or inferred to interact with PDK1 (AKT1,2,3; PKAα,β,γ; PKCα,β,γ,δ,ε,θ,η,ι,ζ; PKN1,2,3; ROCK1;

RSK1,2,3,4; S6K1,2; SGK1,2,3). Reference protein sequences were derived from Uniprot. Sequence logos were created using WebLogo 3.4 (8).

Monitoring the Activation of AKT and S6K1 in Cells

All drug-treatment experiments were conducted in 12-well tissue culture plates that were pre-treated for 1 hr at 37^{o}C with 50 µg/mL Poly-D-Lysine (MW 70K-150K; Sigma) dissolved in sterile water. HEK293 cells (200,000) were seeded into each well a 12-well plate and allowed to grow in complete media for 24 hours. The cells were then serum-starved overnight (16 hr) prior to drug treatment. All drug solutions were made at 1X concentration in serum-free media from concentrated DMSO stocks and the final DMSO concentration was fixed at 0.2%. Following serum-starvation, cells were exchanged into 1 mL of serum-free media with 1X drug. After 1 hr of drug treatment, cells were stimulated with 50 ng/mL IGF1 by the addition of 1 mL of serum-free media containing 1X drug and 2X IGF1. After 15 min of stimulation, media was aspirated, cells were washed once with ice-cold PBS, and then cells were lysed by the addition of 100 µL of M-PER lysis buffer (Thermo Scientific) containing protease inhibitor cocktail (Sigma), Tyr and Ser/Thr phosphatase inhibitor cocktails (Sigma), 2 mM Na_3VO_4 (activated), 1 mM PMSF, and 10 µM GSK2334470. Cells were scraped with a rubber policeman, lysates were transferred to microcentrifuge tubes, and cellular debris was pelleted by spinning at 20K x *g* on a tabletop centrifuge for 15 min at 4^{o}C. The clarified lysates were subsequently separated by SDS-PAGE and analyzed by IR Western Blot using an Odyssey Classic Imager (Li-COR Biosciences). All blots were scanned using the membrane present with a 700 Laser Intensity of 2.0 and an 800 Laser Intensity of 5.0. Western Blot band intensities were quantified using the ImageStudioLite package (Li-COR Biosciences) using left/right median background subtraction. The phospho-protein signal in each lane was normalized by the signal of the loading control α-tubulin. Next, the ratio of phospho-protein/α-tubulin for DMSO control lane was normalized to 1.0.

SI Figures and Tables:

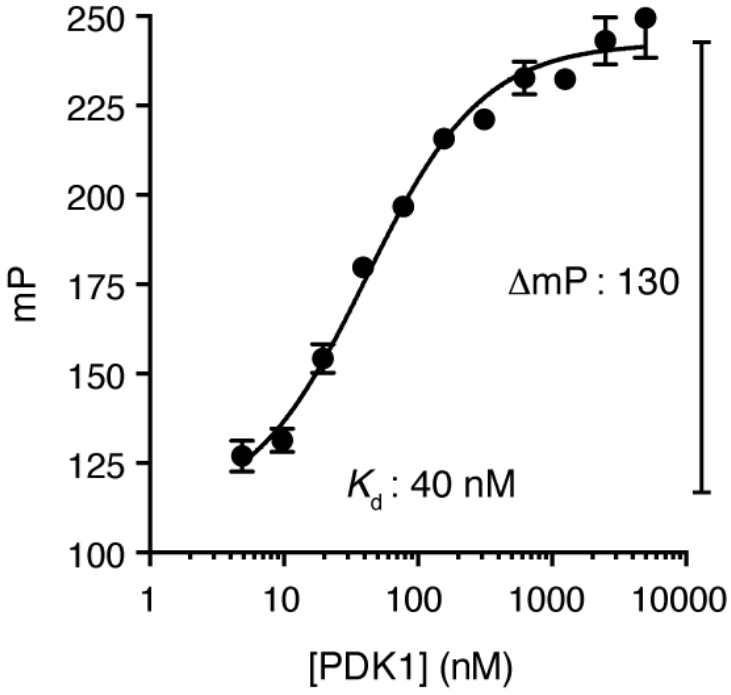


Figure S1. Optimized FP probe binds to PDK1 with high affinity and yields a large dynamic range. Increasing concentrations of PDK1 were incubated with 50 nM of the optimized FP probe until equilibrium binding was achieved. Error bars are ±SD (*n* = 2).

Probe sequence: 6-TAMRA-(2-bromo-Phe)-Arg-Asp-(3-bromo-Phe)-Asp-Trp-Ile-Ala-Asp-Trp-$CONH_2$.

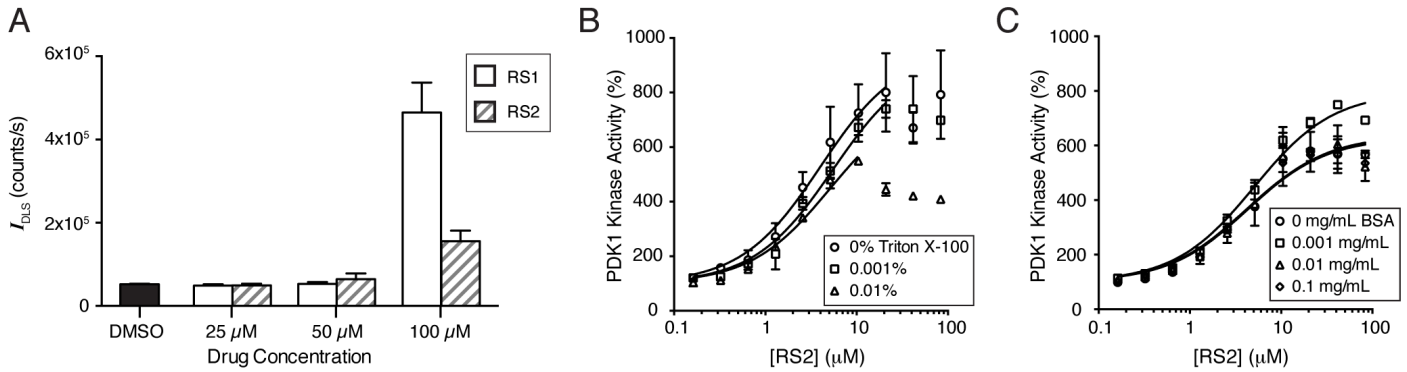


Figure S2. Diaryl sulfonamides modulate PDK1 through a specific interaction. (*A*) Dynamic light scattering measurements of increasing concentrations of RS1 or RS2 in PDK1 kinase activity assay buffer. DMSO in buffer is used as a control. Both RS1 and RS2 aggregated at 100 μM but not at concentrations up to 50μM. Error bars are ±SD (*n* = 3). (*B*) Effect of Triton X-100 on the enhancement of PDK1 activity by RS2. The activity of PDK1 towards the peptide substrate T308tide was monitored by a radiometric assay as a function of RS2 concentration in the presence or absence of Triton X-100 detergent. Error bars are ±SD (*n* = 2)**.** (*C*) Effect of BSA on the enhancement of PDK1 activity by RS2. Same as *B*, except BSA was used in the place of Triton X-100.

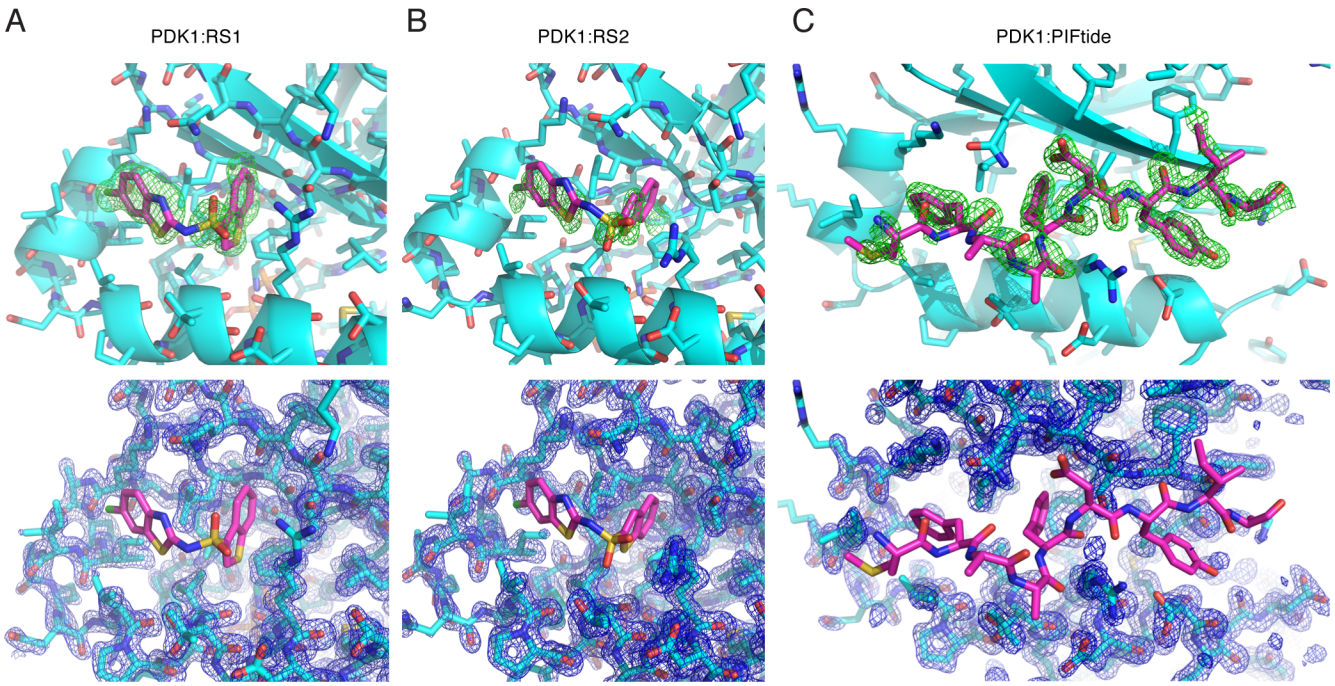


Figure S3. Density maps for PDK1-ligand complexes. Strong electron density was observed for the ligands (top panels F_o-F_c simulated annealing omit maps) and for the residues lining the PIF pocket (bottom panels; $2F_o$-F_c maps) in the (*A*) PDK1:RS1, (*B*) PDK1:RS2, and (*C*) PDK1:PIFtide complexes. F_o-F_c maps (green) were contoured to 3σ and $2F_o$-F_c maps (blue) were contoured to 1.25σ. PDK1 is colored cyan and the ligands are colored magenta.

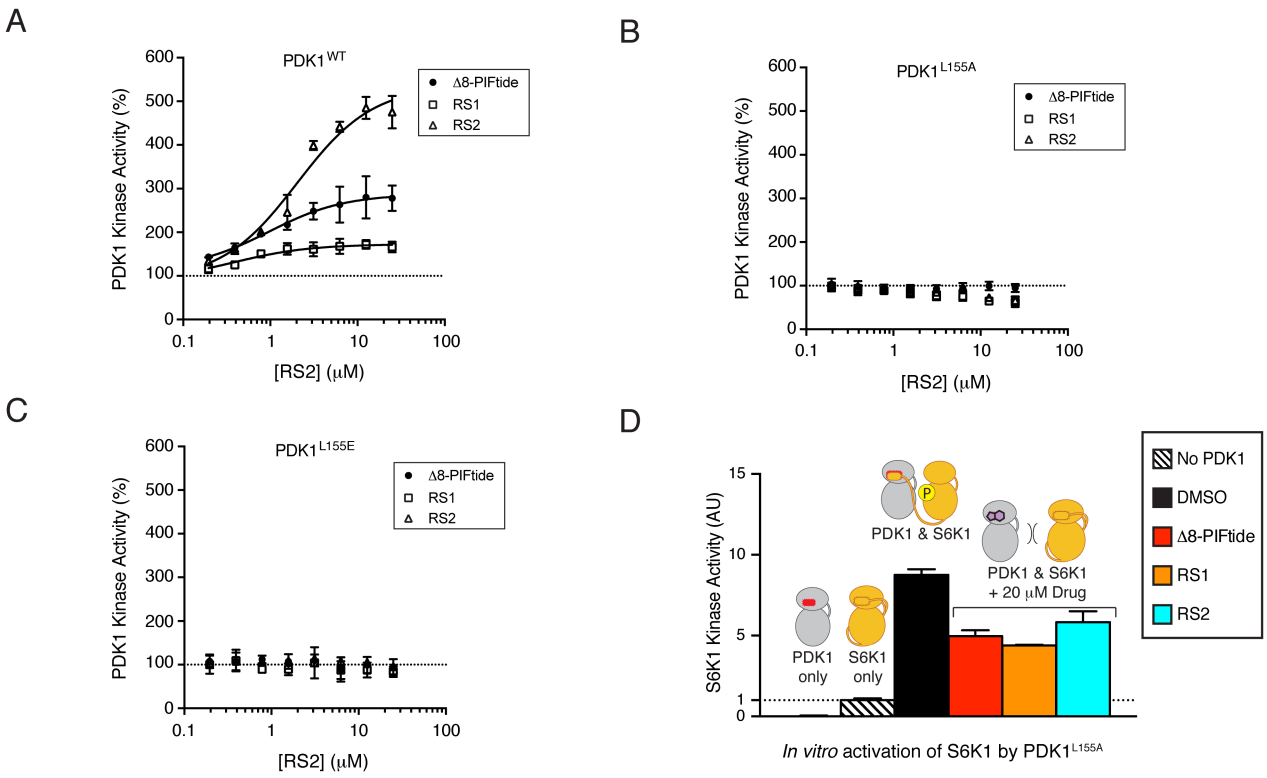


Figure S4. Mutation of Leu155 within the PIF pocket of PDK1 confers resistance to the diaryl sulfonamides. (*A*) Dose-response curves for PIFtide and the RS compounds in a radioactive kinase activity assay that monitors the phosphorylation of T308tide peptide substrate by wild-type PDK1. Error bars are ±SD (*n* = 2). (*B*) Same as *A*, except the mutant PDK1^{L155A} is used. (*C*) Same as *A*, except the mutant PDK1^{L155E} is used. (*D*) Effect of PIFtide and the RS compounds on the *in vitro* activation of S6K1 by PDK1^{L155A}. Following activation of S6K1 by PDK1 for 30 min, the kinase activity of S6K1 was determined by a radioactive kinase assay using Crosstide substrate. The activity of S6K1 alone was used for normalization (dotted line). Error bars are ±SD (*n*=3).

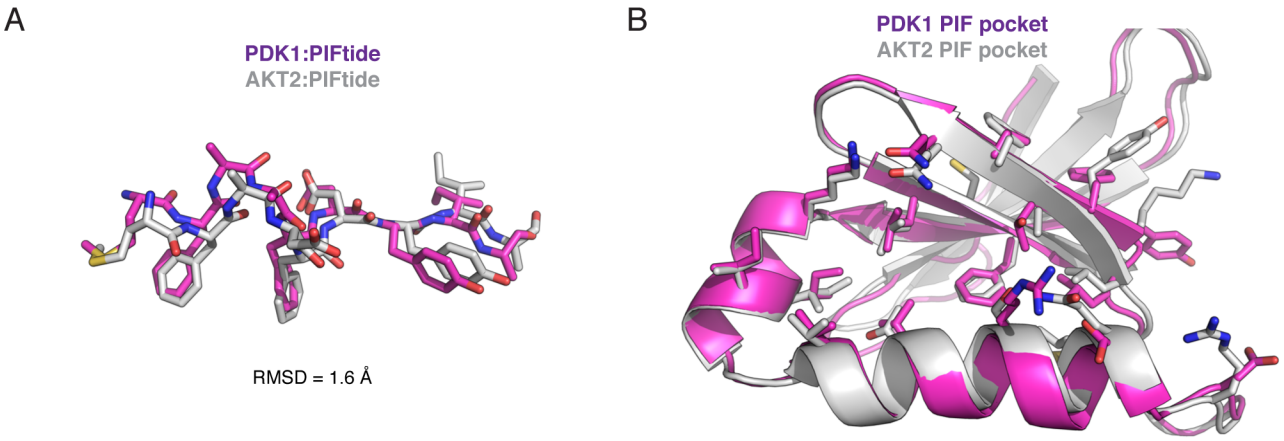


Figure S5. Structural comparison between the PDK1-PIFtide and AKT2-PIFtide (PDB: 1O6L) complexes. (*A*) Overlay of the PIFtide-binding poses of PDK1 (magenta) and AKT2 (grey). The root mean square deviation between these poses is 1.6 Å. (*B*) Overlay of the PIF pockets of PDK1 (magenta) and AKT2 (grey). Residues that contact PIFtide in one or both structures are shown as sticks. Only 50% of these residues are identical between the two structures.

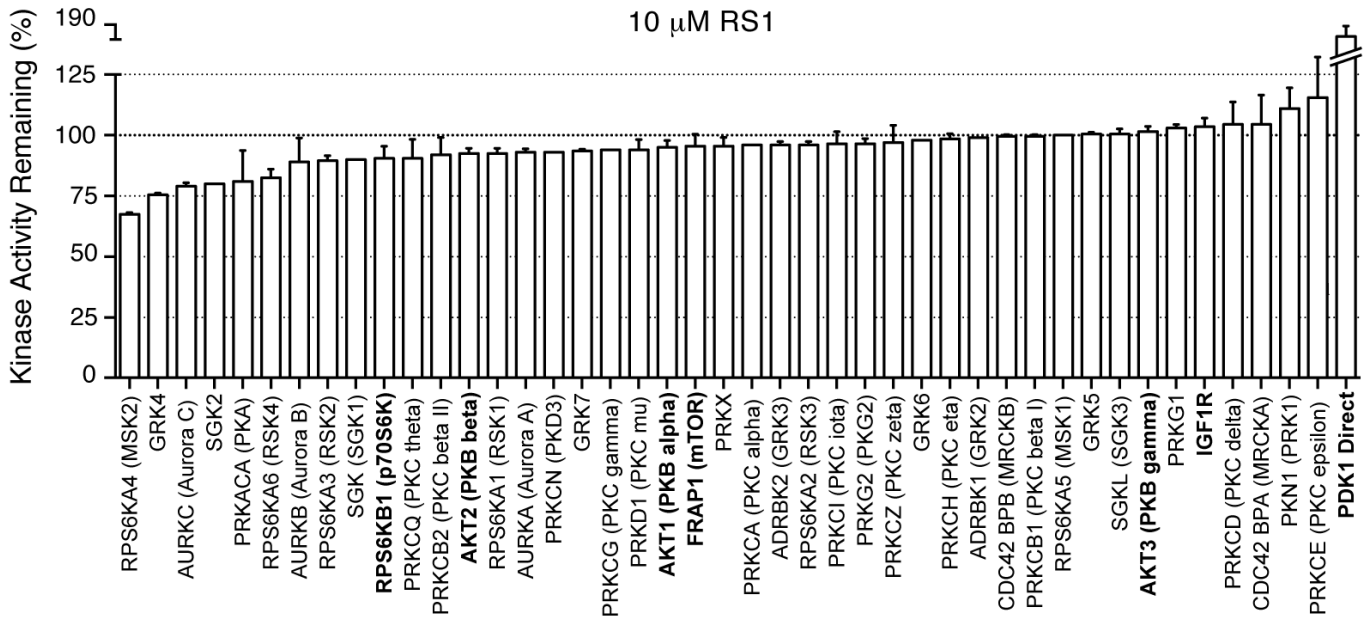


Figure S6. RS1 selectively modulates the activity of PDK1 but not closely related kinases. The effect of 10 μM RS1 on the catalytic activity of 44 protein kinases was determined using the Invitrogen SelectScreen service. Kinases named in bold are the subject of our cell-based experiments. Error bars are ±SD (*n* = 2).

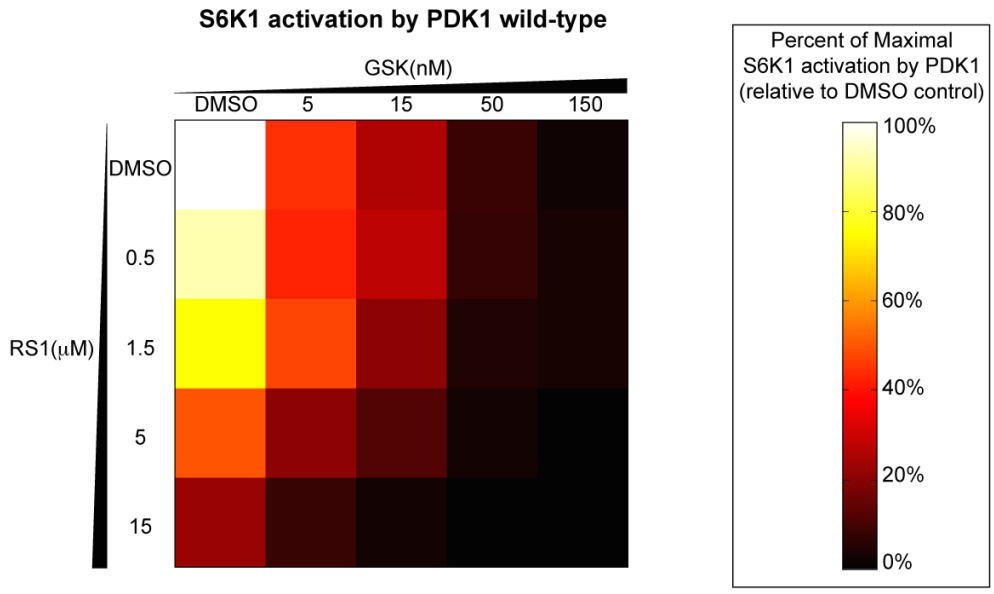


Figure S7. The PIF-pocket ligand **RS1** and the ATP-competitive inhibitor **GSK** combine additively to inhibit the activation of S6K1 *in vitro*. In this coupled assay, PDK1 first activates S6K1 for 15 min in the presence of drug or vehicle. Next the activity the S6K1 is measured by adding an S6K1-specific peptide substrate (Crosstide). The combined effects of a variety of doses of RS1 and GSK are displayed as a heat map above. The combination index (CI) for the two drugs at their respective IC50 values (CI_{50}) is 0.93, reflecting that this combination is additive, as opposed to synergistic ($CI_{50} < 1$) or antagonistic ($CI_{50} > 1$) (9).

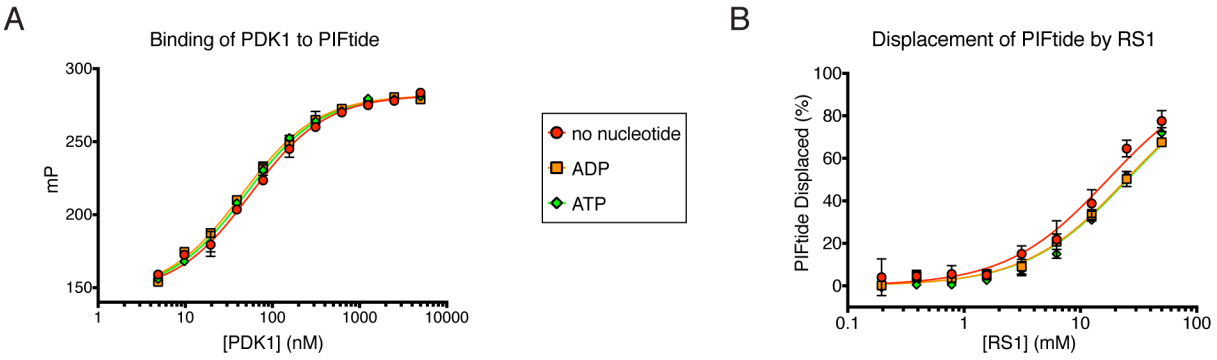


Figure S8. Binding of ligands at the ATP-binding pocket and the PIF pocket is not cooperative. (*A*) Titration of increasing concentrations of PDK1 against a fixed concentration of FP PIFtide probe. Addition of saturating nucleotide:Mg^{2+} (0.5 mM) does not affect the binding affinity of the PIFtide. (*B*) Titration of **RS1** against a fixed concentration of PDK1 and FP PIFtide probe. Addition of saturating nucleotide:Mg^{2+} does not significantly affect the apparent binding affinity of **RS1**.

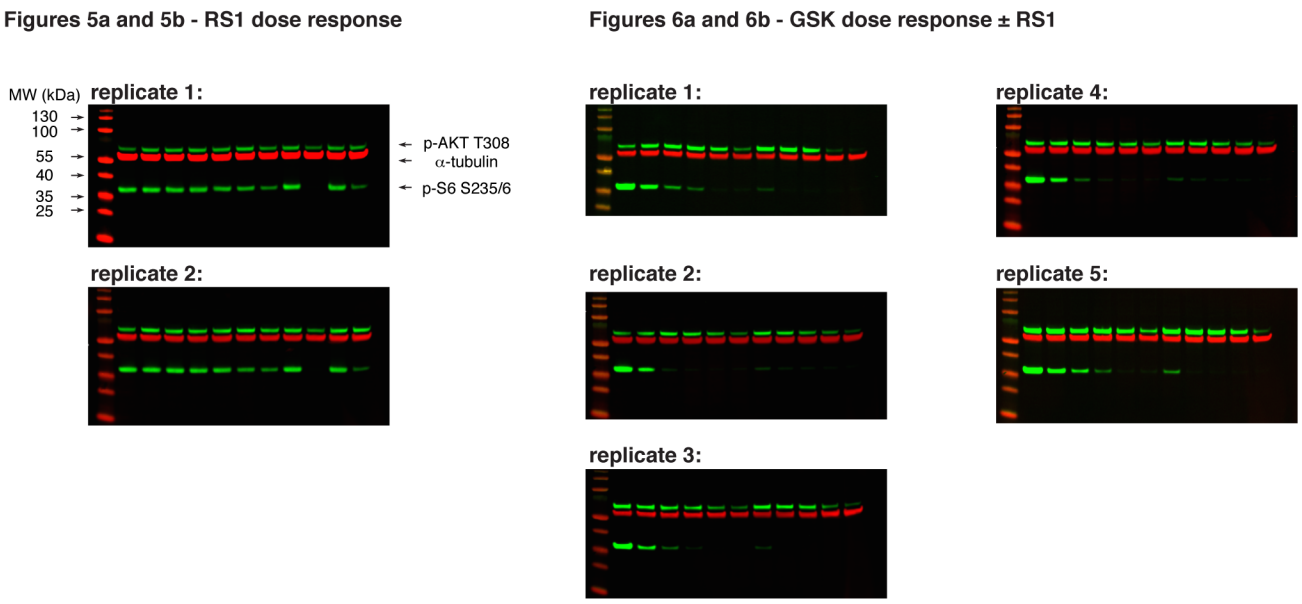


Figure S9. Original images of Western Blots. Phosphorylated AKT (T308) and S6 (S235-6) were detected using the Green (800) Li-COR secondary antibody, and α-tubulin was detected using the Red (700) Li-COR secondary antibody. The levels of the phospho-proteins and the loading control (α-tubulin) were measured simultaneously to reduce blot-to-blot variability.

Table S1. Data collection and refinement statistics (molecular replacement)

	PDK1+ATP+RS1 PDB ID: 4RQK	PDK1+ATP+RS2 PDB ID: 4RQV	PDK1+ATP+PIFtide PDB ID: 4RRV
Data collection[a]			
Space group	C2	C2	C2
Cell dimensions			
a, *b*, *c* (Å)	148.4, 44.2, 47.5	148.4, 44.2, 47.5	148.4, 44.2, 47.5
α, β, γ (°)	90, 100.6, 90	90, 100.6, 90	90, 100.6, 90
Resolution (Å)	50-1.55 (1.61-1.55)	50-1.50 (1.55-1.50)	50-1.41 (1.46-1.41)
R_{merge}	0.087 (0.918)	0.059 (0.556)	0.061 (0.832)
I / σ	14.7 (1.60)	18.5 (1.90)	18.9 (1.54)
Completeness (%)	99.6 (97.1)	94.6 (65.8)	98.8 (91.5)
Redundancy	3.4 (3.0)	3.5 (2.4)	3.5 (2.6)
Refinement			
Resolution (Å)	1.55 (1.61-1.55)	1.50 (1.55-1.50)	1.41 (1.46-1.41)
No. reflections	43970 (4160)	46184 (2900)	58386 (4501)
R_{work} / R_{free}	13.1 / 16.7	13.1 / 16.6	13.4 / 16.3
No. atoms	5170	5489	5414
Protein	4736	5013	5015
Ligand/ion	140	136	85
Water	294	340	314
Average *B* factors (Å^2):			
Protein	20.0	20.2	20.9
Ligand/ion	35.1	34.1	29.4
Water	32.9	31.8	33.2
R.m.s. deviations:			
Bond lengths (Å)	0.008	0.008	0.009
Bond angles (°)	1.234	1.292	1.326
Ramachandran statistics[b] (%):			
Favored	97.9	97.7	98.4
Allowed	2.1	2.3	1.6
Outliers	0	0	0

[a]Values in parentheses are for highest-resolution shell. [b]As calculated by Molprobity.

SI Note S1:

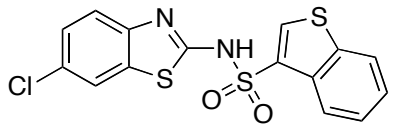
General Methods for Chemical Synthesis

All air or moisture sensitive reactions were performed under argon in oven-dried glassware. Chemical reagents and anhydrous solvents were obtained from commercial sources and used as-is. Flash chromatography purification was performed using a Biotage Isolera with prepacked silica columns (Silicycle). Reverse phase purification was performed on a Waters semi-preparative HPLC with C18 Xterra column (Waters). The mobile phase consisted of methanol and water (each containing 0.2% formic acid). LCMS data was acquired on a Waters 2795 Analytical HPLC equipped with a photodiode array detector, evaporative light scattering detector, and ZQ MS detector. LCMS analysis was performed using a gradient of 5-95% methanol in water (each containing 0.2% formic acid) over 8 minutes. ^{1}H and ^{13}C NMR data were collected on a Varian 400 MHz spectrometer in d_6-DMSO. Chemical shifts are reported relative to TMS. All of the small molecules used in this study were found to be greater than 95% pure based on LCMS and NMR analysis.

All peptides were synthesized using Fmoc chemistry on Rink Amide AM resin (EMD Biosciences) following standard procedures. Peptides were cleaved from the resin using a cocktail of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5) and precipitated in ice-cold ether. Peptides were purified by RP-HPLC using a C18 Xterra column (Waters) and then lyophilized. The identity of each peptide was confirmed by LC-MS and peptide purity exceeded 90% in all cases.

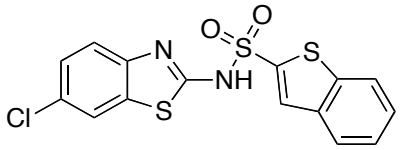
RS1 and **RS2** were synthesized by combining 1 equivalent amine (0.2 M) and 1.5 equivalent sulfonyl chlorides in pyridine and heating to 95^{o}C for 4 h. The pyridine was evaporated and then azeotroped with *n*-heptanes. The crude reaction mixture was dissolved in DMSO and purified by RP-HPLC. Fractions were analyzed by LC-MS, combined, and then lyophilized to yield to final products. Yields typically exceeded 80%. **PS210** and the diester **PS423** were synthesized and purified as described previously (10).

N-(6-chlorobenzo[d]thiazol-2-yl)benzo[b]thiophene-3-sulfonamide (RS1):



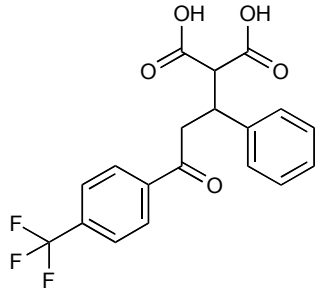
^{1}H NMR (400 MHz, d_6-DMSO): δ 13.44 (broad s, 1H), 8.54 (s, 1H), 8.14 (d, *J*=6.4 Hz, 1H), 8.10 (d, *J* = 6.4 Hz, 1H), 7.98 (d, *J* = 1.6 Hz, 1H), 7.53 (dt, *J* = 6.4, 0.8 Hz, 1H), 7.48 (td, *J* = 6.4, 0.8 Hz, 1H), 7.42 (dd, *J* = 7.2, 1.6 Hz, 1H), 7.27 (d, *J* = 6.4 Hz, 1H); ^{13}C NMR (125 MHz, d_6-DMSO): δ 167.2, 140.0, 135.4, 134.8, 133.7, 133.6, 127.7, 127.4, 126.8, 125.6, 125.5, 123.4, 123.2, 122.5, 114.3; LCMS (*m/z*): $[M+H]^+$ calcd., 380.96; found, 381.0

N-(6-chlorobenzo[d]thiazol-2-yl)benzo[b]thiophene-2-sulfonamide (RS2):



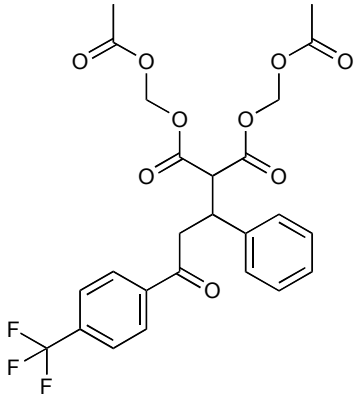
^{1}H NMR (400 MHz, d_6-DMSO): δ 8.05-8.0 (m, 4H), 7.49 (td, *J* = 7.2, 1.6 Hz, 2H), 7.44 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.33 (d, *J* = 8.4 Hz, 1H); ^{13}C NMR (125 MHz, d_6-DMSO): δ 168.0, 140.5, 137.4, 127.5, 127.4, 127.1, 125.7, 125.5, 123.0, 122.4, 114.7; LCMS (*m/z*): $[M+H]^+$ calcd., 380.96; found, 381.0

2-(3-oxo-1-phenyl-3-(4-(trifluoromethyl)phenyl)propyl)malonic acid (PS210):



^{1}H NMR (400 MHz, d_6-DMSO): δ 8.04 (d, *J* = 8.0 Hz, 2H), 7.86 (d, *J* = 7.6 Hz, 2H), 7.27 (d, *J* = 7.2 Hz, 2H), 7.20 (t, *J* = 7.6 Hz, 2H), 7.12 (t, *J* = 7.2 Hz, 1H), 3.87 (td, *J* = 10.8 Hz, 3.6 Hz, 1H), 3.74 (d, *J* = 10.8 Hz, 1H), 3.60 (dd, *J* = 17.2 Hz, 9.6 Hz, 1H), 3.42 (d, *J* = 17.2 Hz, 1H). LCMS (*m/z*): $[M+H]^+$ calcd., 380.09; found, 380.1

bis(acetoxymethyl) 2-(3-oxo-1-phenyl-3-(4-(trifluoromethyl)phenyl)propyl)malonate (PS423):



^{1}H NMR (400 MHz, d_6-DMSO): δ 8.03 (d, *J* = 10.0 Hz, 2H), 7.88 (d, *J* = 10.4 Hz, 2H), 7.28-7.34 (m, 2H), 7.23 (t, *J* = 9.2 Hz, 2H), 7.16 (t, *J* = 9.6 Hz, 1H), 5.72 (dd, *J* = 14.4, 6.0 Hz, 2H), 5.45 (q, *J* = 5.2 Hz, 2H), 4.19 (d, *J* = 10.4 Hz, 1H), 3.94 (td, *J* = 9.2, 4.0 Hz, 1H), 3.73 (dd, J = 17.6, 9.6 Hz, 1H), 3.44 (dd, J = 17.6, 4.0 Hz, 1H), 2.05 (s, 3H), 1.93 (s, 3H). LCMS (*m/z*): $[M+H]^+$ calcd., 525.13; found, 525.1

References Cited:

1. Engel M*, et al.* (2006) Allosteric activation of the protein kinase PDK1 with low molecular weight compounds. *EMBO J* 25(23):5469-5480.
2. Hindie V*, et al.* (2009) Structure and allosteric effects of low-molecular-weight activators on the protein kinase PDK1. *Nat Chem Biol* 5(10):758-764.
3. Otwinowski Z & Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Method Enzymol* 276:307-326.
4. McCoy AJ*, et al.* (2007) Phaser crystallographic software. *Journal of applied crystallography* 40(Pt 4):658-674.
5. Emsley P & Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D* 60:2126-2132.
6. Afonine PV*, et al.* (2012) Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr D* 68:352-367.
7. Chen VB*, et al.* (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D* 66:12-21.
8. Crooks GE, Hon G, Chandonia JM, & Brenner SE (2004) WebLogo: A sequence logo generator. *Genome Res* 14(6):1188-1190.
9. Chou TC & Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Advances in enzyme regulation* 22:27-55.
10. Busschots K*, et al.* (2012) Substrate-selective inhibition of protein kinase PDK1 by small compounds that bind to the PIF-pocket allosteric docking site. *Chemistry & biology* 19(9):1152-1163.