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Electronic Supplementary Information

Affinity enhancement of polo-like kinase 1 polo box domain-binding ligands by a bivalent approach using a covalent kinase-binding component

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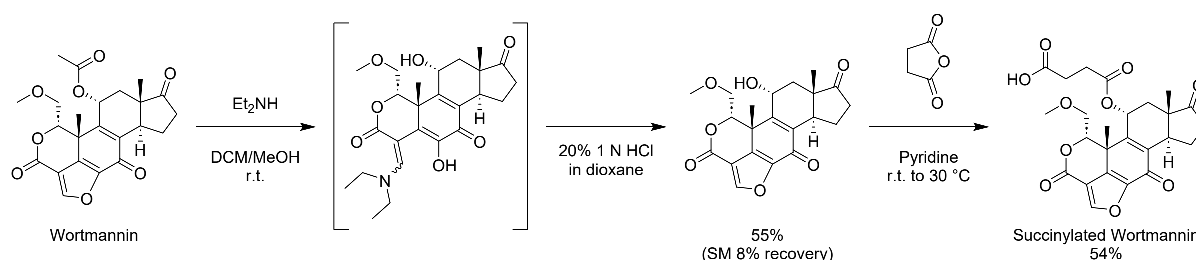
I. SYNTHETIC PROCEDURES

1. General procedure. All experiments involving moisture-sensitive compounds were conducted under anhydrous conditions (positive argon pressure) using standard syringe, cannula, and septa apparatus. Commercial reagents were purchased from Sigma, TCI America, Acros, Alfa Aesar, Chem-Impex, or Novabiochem. All solvents were purchased in anhydrous form (Aldrich) and used without further drying. HPLC-grade hexane, EtOAc, dichloromethane (DCM), and MeOH were used for chromatography. Silica gel column chromatography employed a Teledyne CombiFlash Rf 200 instrument with either EtOAc/hexane or MeOH/DCM gradients. Nuclear Magnetic Resonance (NMR) spectra were recorded using a Varian Inova 400 MHz or 500 MHz spectrometer. Preparative HPLC purification was performed using a Waters 2545 binary pump (0.1% trifluoroacetic acid (TFA) in MeCN/0.1% TFA in H_2O gradient) with a Phenomenex Gemini-C_{18} (5 µm, 250 x 21 mm) preparative column at a flow rate of 10 cm^3 min^{-1} with UV detection at 210 nm. Analytical HPLC analyses of purified peptides were performed using an Agilent 1200 series quaternary pump (0.1% TFA in MeCN/0.1% TFA in H_2O gradient) with a Phenomenex Gemini-C_{18} (5 µm, 250 x 4 mm) analytical column, 1 mL/min flow rate with UV detection at 210 nm.

2. General Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) protocols for the synthesis of bivalent ligands. NovaSyn® TGR resin (Novabiochem, 0.25 mmol/g) was pre-swollen in *N*-methylpyrrolidone (NMP) for 20 min with shaking. The following loading procedure was used where applicable. 9-Fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids (2.0 – 4.0 equivalents based on resin loading) were dissolved in NMP and pre-activated by the addition of 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 0.95 mole-equivalents relative to the amino acid) and *N*,*N*-

diisopropylethylamine (DIPEA, 2.0 mole-equivalents relative to the amino acid) with shaking (1 min). The resin was washed with NMP, and the HATU-activated amino acid solution was added to the washed resin. Coupling reactions were shaken at room temperature and allowed to proceed from 2 h to overnight, depending on the equivalents used and the steric bulk of each amino acid. Coupling reactions were routinely checked for completion using a Kaiser test.[1] Once completed, the resin was filtered and washed with NMP, followed by Fmoc-deprotection using 20% piperidine in DMF for 10 min with shaking. Deprotection of the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) group on the Lys ε-amine group was performed by treatment with 2% (v/v) hydrazine monohydrate in NMP (two times for 3 h to overnight each). The resin was subsequently coupled with succinylated Wortmannin (prepared as described below and shown in Scheme S1) (1.0 equivalents based on resin loading) using HATU (0.95 mole-equivalents relative to the amino acid) and DIPEA (2.0 mole-equivalents relative to the amino acid) at room temperature with shaking (from 3 h to overnight, twice). After completion of the coupling, the complete resins were washed with DCM and dried *in vacuo*. Cleavage from the finished resin with global deprotection was achieved using a cocktail of TFA/triisopropylsilane (TIPS)/H_2O = 95:2.5:2.5 (2.0 mL/50 mg resin, 2 h, twice). The mixture was filtered and concentrated under a stream of N_2. The resulting crude material was dissolved in 0.1% TFA containing MeCN and H_2O and subjected to preparative reverse-phase HPLC purification. Further purification was conducted using semi-preparative reverse-phase HPLC when needed.

3. Synthesis of succinylated Wortmannin.

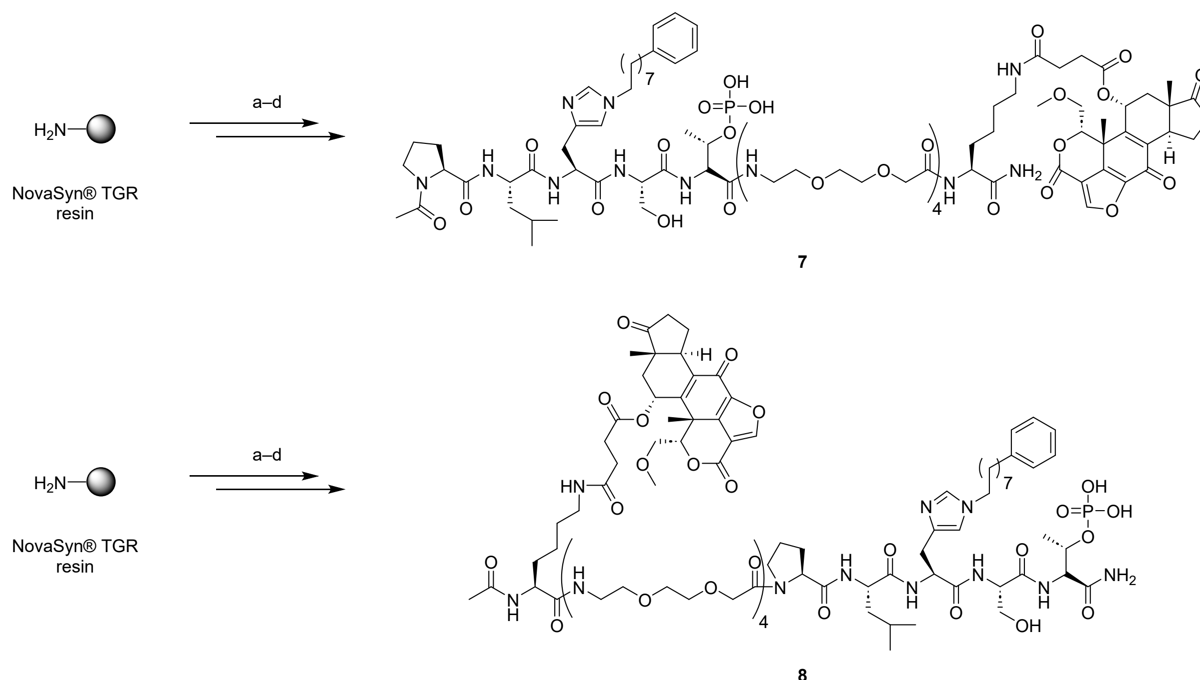


Scheme S1. Synthesis of succinylated Wortmannin.[2,3]

Wortmannin (100 mg, 0.233 mmol) and Et_2N (724 μL, 7.00 mmol) were dissolved in DCM (1.0 mL) and MeOH (0.30 mL). The mixture was stirred at r.t. for 7 d with additional MeOH (0.60 mL) and Et_2N (241 μL, 10 eq.). The resulting mixture was evapolated *in vacuo* to remove volatiles. The resulting crude material was treated with 20% 1 N HCl/dioxane (1 mL:4 mL) at r.t. for 1 d. The mixture was concentrated *in vacuo*, and then diluted with H_2O. The aqueous mixture was extracted with DCM and dried over Na_2SO_4. The crude material was purified by CombiFlash automatic silica gel column chromatography (0 to 50% hexane/EtoAc over 30 min) to yield (1*S*,6b*R*,9a*S*,11*R*,11b*R*)-11-hydroxy-1-(methoxymethyl)-9a,11b-dimethyl-1,6b,7,8,9a,10,11,11b-octahydro-3*H*-furo[4,3,2-de]indeno[4,5-h]isochromene-3,6,9-trione (49.3 mg, 55%) with Wortmannin (8.2 mg, 8.2%) recovery (Scheme S1).

The (1*S*,6b*R*,9a*S*,11*R*,11b*R*)-11-hydroxy-1-(methoxymethyl)-9a,11b-dimethyl-1,6b,7,8,9a,10,11,11b-octahydro-3*H*-furo[4,3,2-de]indeno[4,5-h]isochromene-3,6,9-trione (49.3 mg, 0.128 mmol) was dissolved in pyridine (2.6 mL) and the solution was treated with succinic anhydride (54.3 mg, 0.542 mmol). The mixture was stirred at r.t. for 3 d and at 30 °C for 3 d with additional succinic anhydride (4.25 eq., three times). The mixture was then evaporated, and the residue was purified by CombiFlash automatic silica gel column chromatography (0 to 80% hexane/EtOAc over 30 min) to yield the succinylated Wortmannin (33.7 mg, 54%) as brown oil (Scheme S1).

4. Synthesis of bivalent ligands 7 and 8.



Scheme S2. Synthesis of bivalent ligands **7** and **8**. (a) Fmoc-SPPS using Fmoc-Lys(ivDde)-OH; (b) 2% H_2NNH_2 in NMP; (b) succinylated Wortmannin, HATU, DIPEA, NMP; (c) TFA/TIPS/H_2O = 95:2.5:2.5.

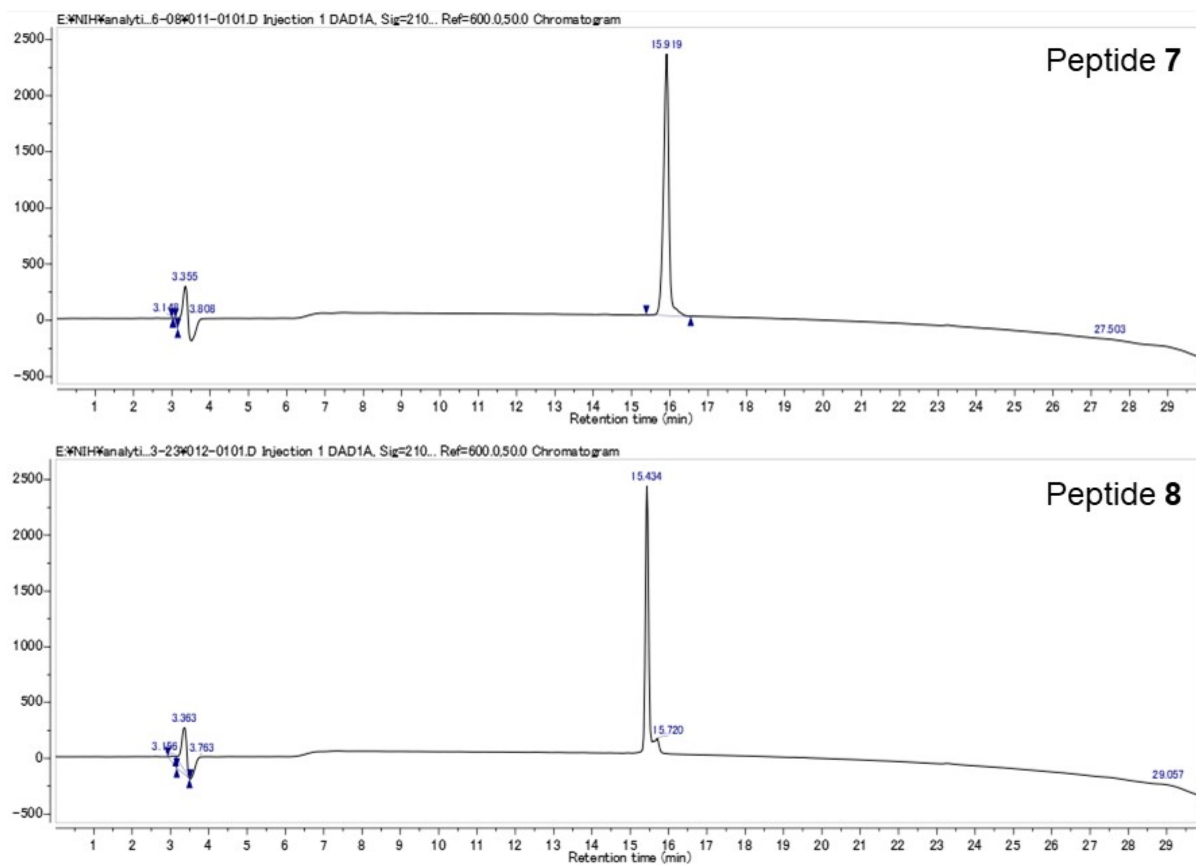
The synthesis of bivalent ligands **7** and **8** was achieved following the general Fmoc-SPPS protocols mentioned above and shown in Scheme S2. The characterized data of these synthesized peptides are shown in Table S1.

Table S1. Characterization data of synthesized peptides.

Compound number	**Calcd M.W.**	**Found**	**Preparative HPLC gradient**	**Analytical HPLC (10 to 100% of B in A over 30 min) Retention time (t_R, min)**
7	1020.5 $[M + 2H]^{2+}$	1020.6	0 to 70% of B in A over 30 min	15.9
8	1020.5 $[M + 2H]^{2+}$	1020.7	0 to 70% of B in A over 30 min	15.4

HPLC eluents were A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN.

5. Analytical HPLC data for peptides 7 and 8.



II. BIOLOGICAL EVALUATION

1. Expression and purification of full-length Polo-like kinase 1 (Plk1) for fluorescence polarization (FP) assays and fluorescence recovery assays. As previously reported,[4-7] a plasmid encoding myc-tagged full-length Plk1 (Plasmid #41160) was purchased from Addgene. ~20 M HEK-293T cells (2 x 15 cm plates) were transfected with the plasmid using TurboFect reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Following 48 h expression for full-length Plk1, cells were harvested, lysed in buffer [phosphate buffered saline (PBS, pH 7.4) containing 0.5% NP-40 and protease/phosphatase inhibitor (Pierce, Protease and Phosphatase Inhibitor Mini Tablets) cocktail] using freeze/thaw cycles (3x) and centrifuged at 12,500 x G for 10 min at 4 °C. The supernatant containing expressed protein was diluted into 8 mL of PBS (pH 7.4) containing protease/phosphatase inhibitor cocktail. This protein solution was added to a 1 mL bed of myc-agarose resin (Thermo Fisher Scientific) using a disposable 10 mL polypropylene columns (Thermo Fisher Scientific) and allowed to bind for 2 h at 4 °C with gentle rotation. The lysate was removed by filtration and the resin was washed 4x with HBST (HEPES buffered saline (HBS) containing 0.05% Tween-20, 1 mM DTT and 1 mM EDTA) for 10 min with gentle rotation. The bound myc-tagged Plk1 protein was then eluted with a 1 mg/mL solution of myc peptide (EQKLISEEDL) in HBS + 1 mM DTT and 1 mM EDTA. The purified myc-tagged Plk1 was dialyzed 5x with HBS + 1 mM DTT and 1 mM EDTA using a 10 kDa MWCO filter (Sigma, fixed angle rotor at 7,500 x G, 4 °C, 10 min). The concentration of the final protein solution was determined by absorbance at 280 nm and purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining using NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 12-well NuPAGE™ Sample Reducing Agent (10X), NuPAGE™ MOPS SDS Running Buffer (20X), SeeBlue™ Plus2 Pre-stained Protein Standard (Invitrogen), and GelCode™ Blue Safe Protein Stain (Thermo Scientific).

2. FP assays using purified full-length Plk1. Purified protein was diluted to a 2x working dilution in assay buffer (HEPES-buffered saline with 0.05% Tween-20, 1 mM DTT, and 1 mM EDTA) with the final protein concentration representing the approximate K_d values as determined for the probe **FITC-miniPEG-PLH*SpT**.[7] Inhibitors were serially diluted to generate 4x working dilutions in assay buffer. To each well of a 384-well plate was added 20 μL of 2x Plk1 solution (0% binding controls received 20 μL of assay buffer). A total of 10 μL of the 4x inhibitor solution (or assay buffer as blank) was added to corresponding wells and allowed to pre-incubate at room temperature for 30 min with shaking. Fluorescent probe **FITC-miniPEG-PLH*SpT** was diluted to 40 nM (4x) in assay buffer and then 10 μL was added to each well. The plate was allowed to equilibrate at room temperature for 30 min with shaking. The FP was read using a BioTek Synergy 2 plate reader with 485/20 excitation and 528/20 emission or a TECAN Spark plate reader with 485/15 excitation and 528/15 emission. The FP values were obtained in triplicate and normalized to 100% (no inhibitor) and 0% binding (no protein) controls. Normalized values were plotted versus concentration and analyzed using non-linear regression in GraphPad Prism 10 [log(inhibitor) vs response – variable slope (four parameter) model]. IC_{50} values represent average ± standard error of the mean (SEM).

3. Fluorescence recovery assays using purified full-length Plk1. Fluorescence recovery assay was performed as previously described.[8] Briefly, purified Plk1 was diluted to a 2x working dilution in assay buffer (HEPES-buffered saline with 0.05% Tween-20, 1 mM DTT, and 1 mM EDTA) with the final protein concentration representing the approximate K_d values as determined for the probe **FITC-miniPEG-BI2536**. Inhibitors were serially diluted to generate 4x working dilutions in assay buffer. To each well of a 384-well plate was added 20 μL of 2x Plk1 solution (0% binding controls received 20 μL of assay buffer). A total of 10 μL of the 4x inhibitor solution (or assay buffer as blank) was added to corresponding wells and

allowed to pre-incubate at room temperature for 30 min with shaking. Fluorescent probe **FITC-miniPEG-BI2536** were diluted to 80 nM (4x) in assay buffer and then 10 μL was added to each well. The plate was allowed to equilibrate at room temperature for 30 min with shaking. The fluorescence intensity was read using a BioTek Synergy 2 plate reader with 485/20 excitation and 528/20 emission. The fluorescence intensity values were obtained in triplicate and normalized to 100% (no inhibitor) and 0% binding (no protein) controls. Normalized values were plotted versus concentration and analyzed using non-linear regression in GraphPad Prism 10 [log(inhibitor) vs response – variable slope (four parameter) model]. IC_{50} values represent average ± standard error of the mean (SEM).

4. Cell proliferation assays using HeLa cells. Cell proliferation assays were performed as following procedure: HeLa cells (American Type Culture Collection (ATCC), Virginia, USA) were seeded in a 96-well plate (5×10^3 cells/well) with 100 μL of Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Gln, 100 μg/ml of penicillin, and 100 μg/ml of streptomycin for each wells. After 1 day incubation at 37 °C, the media were replaced with the serially diluted compounds containing media. The cells were incubated at 37 °C and cell proliferation was monitored for additional 3 days and analyzed using IncuCyte ZOOM 2015A (Sartorius AG, Göttingen, Germany).

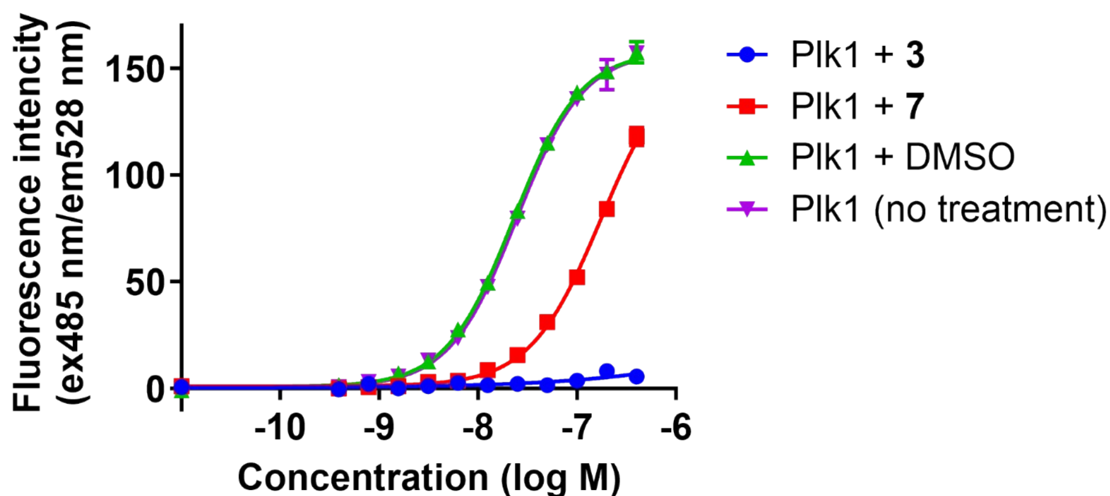


Figure S1. Results from fluorescence recovery assays to evaluated Plk1 KD binding of the probe (FITC-miniPEG-BI2536) with pre-treatment of Plk1 with the inhibitors (10 eq.) at 4 °C for 4 h and then the solutions were dialyzed with the assay buffer for 5 times using 10K Da filter. The K_d values of each conditions are Plk1 + **3** (not determined), Plk1 + **7** (180 ± 2.6 nM), Plk1 + DMSO (23 ± 0.24 nM), Plk1 (no treatment) (24 ± 0.13 nM). The X axis represents protein concentration (log M) and the Y axis represents fluorescence intensity (Ex: 485 nm, Em: 528 nm) related to the probe binging. Data points represent average ± SEM from two independent experiments and fit using non-linear regression in GraphPad Prism 10.

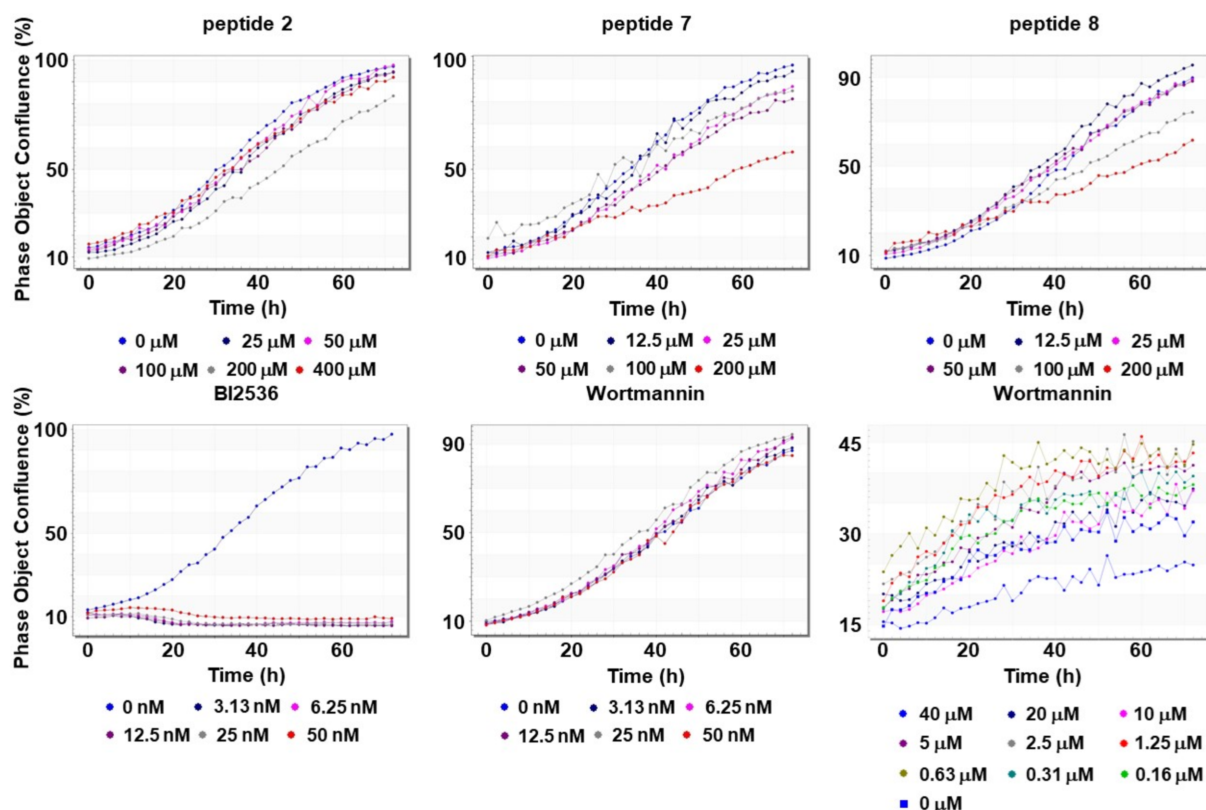


Figure S2. Results from cell proliferation assays using HeLa cells. The X axis represents incubation time from inhibitor addition (hours) and the Y axis represents phase object confluence (%). The cell proliferation was monitored and plotted using IncuCyte ZOOM 2015A.

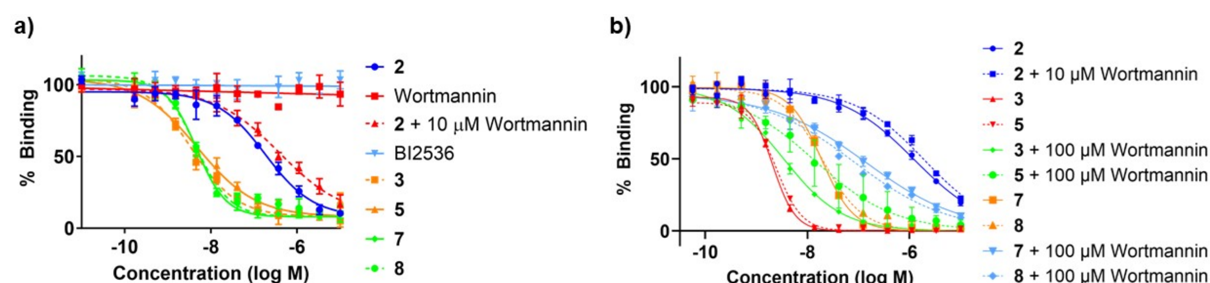


Figure S3. Results from fluorescence polarization (FP) assays in Table 1, which measured the ability of Plk1 inhibitors to compete with **FITC-miniPEG-PLH*SpT** for binding to full-length Plk1. The results of FP assays in Exp. 1 (a) and Exp. 2 (b) in Table 1 are shown. The X axis represents inhibitor concentration (log M) and the Y axis represents relative probe binging based on the FP (Ex: 485 nm, Em: 528 nm) of no inhibitor (100%) and blank (no protein, 0%).

Data points represent average ± SEM from three independent experiments and fit using non-linear regression in GraphPad Prism 10.

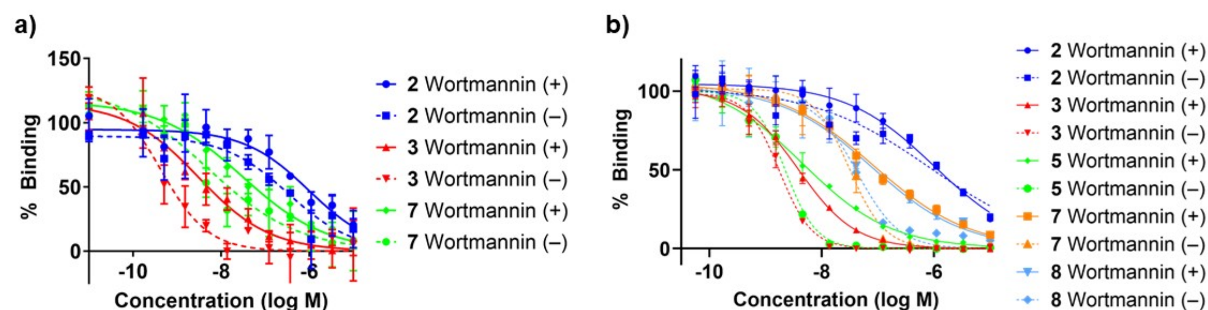


Figure S4. Results from fluorescence polarization (FP) assays in Table 2, which measured the ability of Plk1 inhibitors to compete with **FITC-miniPEG-PLH*SpT** for binding to full-length Plk1. The results of FP assays in Exp. 1 (a) and Exp. 2 (b) in Table 2 are shown. Wortmannin (+): Plk1 was treated with Wortmannin (10 eq.) at 4 °C for 2 h, and then the protein solution was dialyzed with assay buffer for 5 times using 10K Da filter; Wortmannin (–): Plk1 solution was dialyzed with assay buffer for 5 times using 10K Da filter. The X axis represents inhibitor concentration (log M) and the Y axis represents relative probe binging based on the FP (Ex: 485 nm, Em: 528 nm) of no inhibitor (100%) and blank (no protein, 0%). Data points represent average ± SEM from three independent experiments and fit using non-linear regression in GraphPad Prism 10.

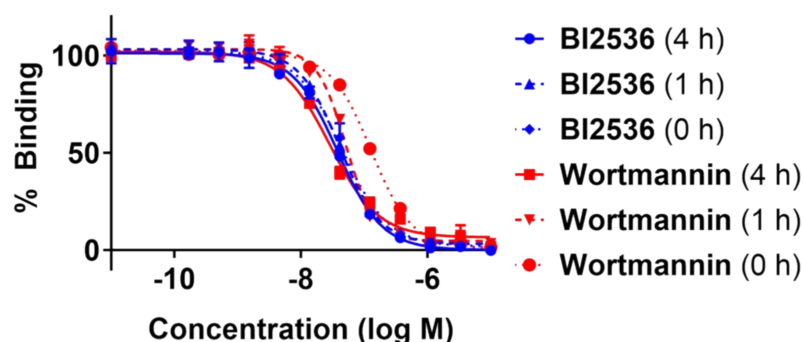


Figure S5. Results from fluorescence recovery assays in Table 3, which measured the ability of Plk1 inhibitors to compete with the probe (**FITC-miniPEG-BI2536**) for binding to full-length Plk1. Plk1 was incubated with various concentrations of BI2536 or Wortmannin at r.t. for 0, 2, or 4 h in a 384-well plate. The plate was then added the probe solution and allowed to equilibrate at room temperature for 30 min with shaking to measure the fluorescence intensity. The X axis represents inhibitor concentration (log M) and the Y axis represents relative probe binging based on the fluorescence intensity (Ex: 485 nm, Em: 528 nm) of no inhibitor (100%) and blank (no protein, 0%). Data points represent average ± SEM from three independent experiments and fit using non-linear regression in GraphPad Prism 10.

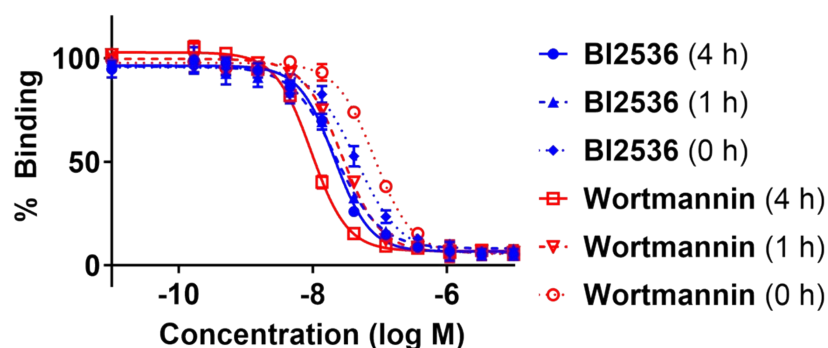


Figure S6. Results from fluorescence recovery assays in Table 3, which measured the ability of Plk1 inhibitors to compete with the probe (**FITC-miniPEG-BI2536**) for binding to full-length Plk1. Plk1 was incubated with various concentrations of BI2536 or Wortmannin at 4 °C for 0, 2, or 4 h in a 384-well plate. The plate was then added the probe solution and allowed to equilibrate at room temperature for 30 min with shaking to measure the fluorescence intensity. The X axis represents inhibitor concentration (log M) and the Y axis represents relative probe binging based on the fluorescence intensity (Ex: 485 nm, Em: 528 nm) of no inhibitor (100%) and blank (no protein, 0%). Data points represent average ± SEM from three independent experiments and fit using non-linear regression in GraphPad Prism 10.

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