Supporting Information

Histidine N(τ)-cyclized Macrocycles as a New Genre of Polo-like Kinase 1 Polo-box Domain-Binding Inhibitors

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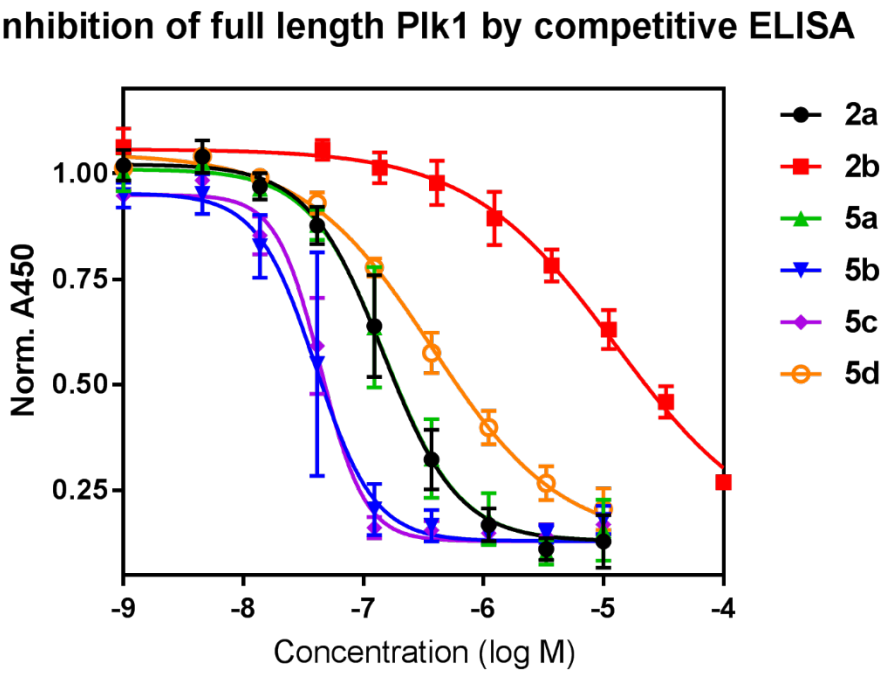


Figure S1. ELISA-based competitive inhibition of binding to full-length Plk1. Data points represent average ± SEM of normalized absorbance from six (for **2a** and **5d**) or three (for **2b**, **5a**, **5b** and **5c**) independent experiments and fit using non-linear regression in GraphPad Prism 7.

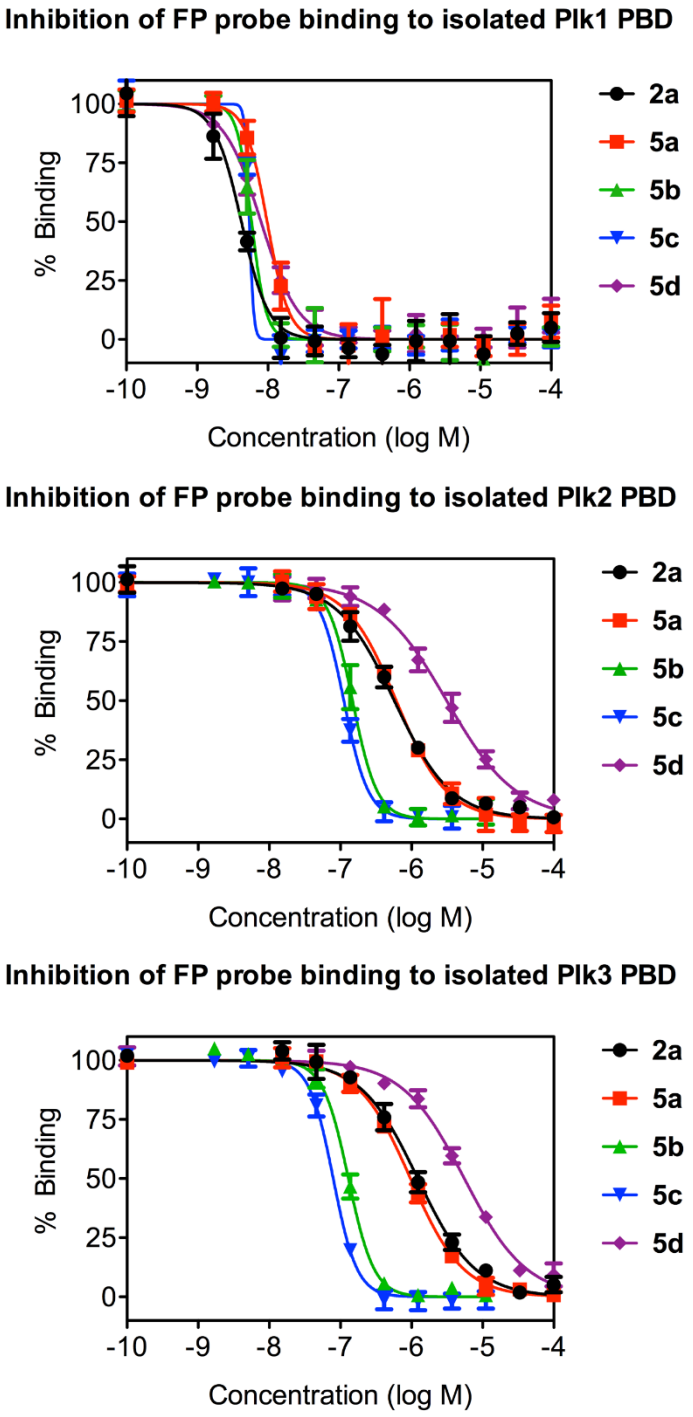


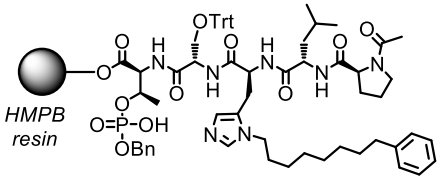
Figure S2. Fluorescence polarization assay for competitive inhibition of the isolated PBDs of Plks 1 – 3 by macrocyclic ligands. Data points represent average ± SEM of % binding (normalized FP signal) from triplicate data points and fit using non-linear regression in GraphPad Prism 7.

General Methods. 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, Aapptec or Chem-Impex. Fmoc-Ser(Trt)-OH, Fmoc-Ser(tBu), Fmoc-His(Trt)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH and Fmoc-Thr[PO(OH)((OBn)]-OH were purchased from Chem-Impex. The intermediates *tert*-butyl (4-iodobutyl)carbamate,[1] *tert*-butyl (5-iodopentyl)carbamate,[2] *tert*-butyl (6-iodohexyl)carbamate,[3] and Fmoc-His[N(π)-$(CH_2)_8$-Ph]-OH[4] were synthesized as previously described. The final peptide PLH*SpT (**2a**) was synthesized by SPPS as previously described to >95% purity.[5] All solvents were purchased in anhydrous form (Aldrich) and used directly. HPLC-grade hexanes, EtOAc, DCM, and methanol were used in chromatography. Analytical TLCs were performed using Analtech precoated plates (Uniplate, silica gel GHLF, 250 nm) containing a fluorescence indicator. Silica column chromatography employed a Telodyne CombiFlash Rf 200i instrument with either hexane/EtOAc or DCM/methanol gradients. Microwave reaction were conducted in a Biotage Initiator microwave synthesis apparatus. NMR spectra were recorded using a Varian Inova 400 MHz spectrometer. Coupling constants are reported in Hertz, and peak shifts are reported in δ (ppm) relative to $CDCl_3$ (^{1}H 7.26 ppm, ^{13}C 77.16 ppm). Low-resolution mass spectra (ESI) were measured with either an Agilent 260 1200 LC/MSD-SL system or a Shimadzu 2020 LC/MS system. High resolution mass spectra (HRMS) were obtained by positive ion, ESI analysis on a Thermo Scientific LTQ-XL Orbitrap mass spectrometer with HPLC sample introduction using a short narrow-bore C1s reversed-phase column with MeCN - H_2O gradients. Preparative HPLC of final peptides was performed using a Waters 2545 binary pump (MeCN/H_2O gradient) with a Phenomenex Gemini-C_{18} (5 μm, 250 x 21 mm) preparative column and UV detection at 210 nm. Semi-preparative HPLC purification was performed using an Agilent 1200 series quaternary pump (MeCN/H_2O gradient) with a Phenomenex Kinetix-C_{18} (5 μm, 250 x 4 mm) analytical column, 3 mL/min flow rate, and UV detection at 210 nm. Analytical HPLC of final peptides was performed using an Agilent 1200 series quaternary pump (MeCN/H_2O gradient) with a Phenomenex Gemini-C_{18} (5 μm, 250 x 4 mm) analytical column, 1 mL/min flow rate, and UV detection at 210 nm.

General Solid-Phase Peptide Synthesis (SPPS) procedure. SPPS resin is pre-swollen in DMF (4 mL) for 1 h with shaking. HMPB MBHA resin (Sigma or Chem-Impex) was utilized for certain peptides and the loading procedure is described where applicable. The resin is Fmoc-deprotected using 20% piperidine in DMF (4 mL) 2 times for 10 minutes each. Fmoc-protected amino acids (2 – 4 equivalents based on resin) are dissolved in DMF (3 – 4 mL) containing 4% DIEA and pre-activated by the addition of HATU (0.95 mole-equivalents relative to the amino acid) for 5 minutes with gentle agitation. The resin is washed 4 times with DMF (6 – 8 mL), and the HATU-activated amino acid solution is added to the washed resin. Coupling reactions are shaken at room temperature and allowed to proceed from 3 – 16 hours depending on the equivalents used and steric bulk of each amino acid. Coupling reactions are routinely checked for completion using a Kaiser test. Once completed, the resin is filtered and washed 4 times with DMF (6 – 8 mL), followed by Fmoc-deprotection using 20% piperidine in DMF (4 mL, 2 x 10 minutes each). Cleavage from Rink amide LL resin (NovaBioChem) and global deprotection is done using

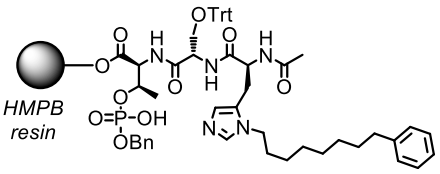
a cocktail of TFA/triisopropylsilane (TIPS)/H_2O (95/2.5/2.5) (4 mL x 2, 2 h each). Cleavage from HMPB MBHA resin (Sigma or Chem-Impex) is done using TFA/DCM (1:3) with 2% TIPS added. Crude peptides were purified using preparative reverse-phase HPLC with gradient elution (89.9/10/0.1 H_2O/MeCN/TFA to 99.9/0.1 MeCN/TFA over 30 minutes).

Acetyl-Prolyl-Leucyl-Histidinyl-[N(π)-8-phenyloctyl]-Seryl-(*O*-trityl)-Threonyl [mono-benzyl phosphoryl] HMPB MBHA resin (Resin 6a). HMPB MBHA resin (0.3 mmol scale, 0.7 mmol/g loading) was swelled in DCM for 1 h. Fmoc-Thr[PO(OH)((OBn)]-OH (2.5 equiv), HOBt (2.5 equiv), and DIEA (5 equiv) were dissolved into DCM (3 mL). The resin was washed 2 times with DCM then the amino acid solution was added. *N*,*N*'-Diisopropylcarbodiimide (DIC, 2.5 equiv) was added to the resin in solution, followed by DMAP (0.2 equiv). The resin loading was allowed to proceed for 8 h, then the resin was washed 2 times with DCM, and the coupling procedure repeated one additional time. The resin loading was quantified by the Fmoc-cleavage procedure to be 0.32 mmol/g. The remaining amino acids were coupled to the resin following the general SPPS procedure above to provide approximately 0.137 mmol of the desired material on-resin. NOTE: HPMB resin was chosen specifically for 2 reasons: 1) it provides C-terminal acids with mildly acidic cleavage, which preserves benzyl protection of the phosphothreonine residue and 2) it can withstand microwave conditions better than chlorotrityl resin, which prevents premature cleavage of the desired material.



Resin 6a

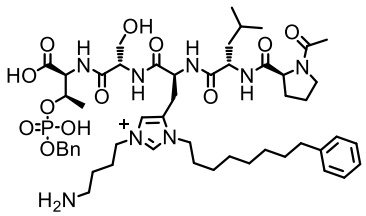
Acetyl-Histidinyl-[N(π)-8-phenyloctyl]-Seryl-(*O*-trityl)-Threonyl [mono-benzyl phosphoryl] HMPB MBHA resin (Resin 6b). HMPB MBHA resin (0.1 mmol scale, 0.7 mmol/g loading) was swelled in DCM for 1 h. Fmoc-Thr[PO(OH)((OBn)]-OH (2.5 equiv), HOBt (2.5 equiv), and DIEA (5 equiv) were dissolved into DCM (3 mL). The resin was washed 2 times with DCM then the amino acid solution was added. *N*,*N*'-Diisopropylcarbodiimide (DIC, 2.5 equiv) was added to the resin in solution, followed by DMAP (0.2 equiv). The resin loading was allowed to proceed for 8 h, then the resin was washed 2 times with DCM, and the coupling procedure repeated one additional time. The resin loading was quantified by the Fmoc-cleavage procedure to be 0.26 mmol/g. The remaining amino acids were coupled to the resin following the general SPPS procedure above to provide approximately 0.037 mmol of the desired material on-resin.



Resin 6b

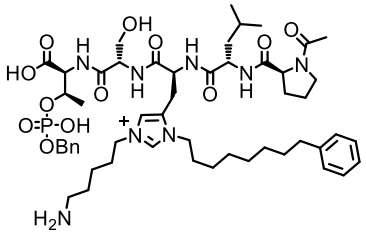
General Procedure A for On-resin Histidine N(τ)-alkylation. Resin **6** was swollen in DMF for 1 h and washed with additional DMF. The *N*-Boc protected iodoalkylamine (5 equiv) and DIEA (10 equiv) is dissolved in DMF (3 mL) and added to the resin in a microwave vial. The reaction was allowed to proceed for 18 h at 50 °C under microwave irradiation. Following the reaction, the resin was filtered and washed 4 times with DMF, 3 times with methanol, and 3 times with DCM to give resins **7a – 7d**. Cleavage and Boc/trityl deprotection was done using 25% TFA with 2% TIPS in DCM (2 x 4 mL, 30 min each). The crude material was purified by preparative reverse-phase HPLC with gradient elution [90/10 H_2O/MeCN to 100% MeCN (with 0.1% TFA added) over 30 minutes] to give peptides **8a – 8d**.

Acetyl-Prolyl-Leucyl-Histidinyl-[N(τ)-4-aminobutyl]-[N(π)-8-phenyloctyl]-Seryl-Threonine [mono-benzyl phosphoryl] (8a). Resin **6a** (0.045 mmol scale) was alkylated with *tert*-butyl (4-iodobutyl)carbamate according to General Procedure A to give resin **7a**. Cleavage and purification by preparative reverse-phase HPLC provided **8a** (18 mg, 39% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{51}H_{78}N_8O_{12}P^+$: 1025.5 [M+]; found: 1025.6.



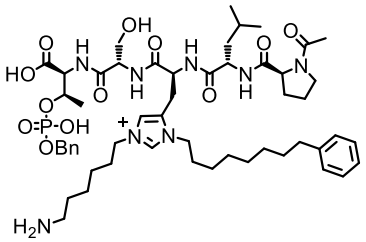
8a

Acetyl-Prolyl-Leucyl-Histidinyl-[N(τ)-5-aminopentyl]-[N(π)-8-phenyloctyl]-Seryl-Threonine [mono-benzyl phosphoryl] (8b). Resin **6a** (0.045 mmol scale) was alkylated with *tert*-butyl (5-iodopentyl)carbamate according to General Procedure A to give resin **7b**. Cleavage and purification by preparative reverse-phase HPLC provided **8b** (17 mg, 36% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{52}H_{80}N_8O_{12}P^+$: 1039.6 [M+]; found: 1039.7.



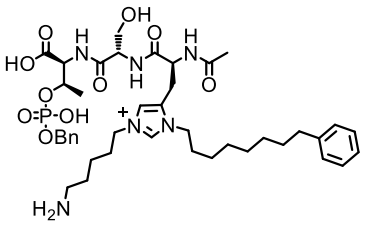
8b

Acetyl-Prolyl-Leucyl-Histidinyl-[N(τ)-6-aminohexyl]-[N(π)-8-phenyloctyl]-Seryl-Threonine [mono-benzyl phosphoryl] (8c). Resin **6a** (0.045 mmol scale) was alkylated with *tert*-butyl (6-iodohexyl)carbamate according to General Procedure A to give resin **7c**. Cleavage and purification by preparative reverse phase HPLC provided **8c** (19 mg, 40% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{53}H_{82}N_8O_{12}P^+$: 1053.6 [M+]; found: 1053.6.



8c

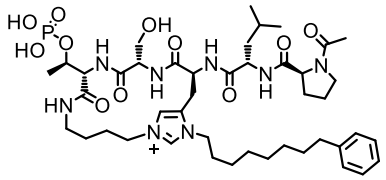
Acetyl-Histidinyl [N(τ)-5-aminopentyl]-[N(π)-8-phenyloctyl]-Seryl-Threonine [mono-benzyl phosphoryl] (8d). Resin **6b** (0.037 mmol scale) was alkylated with *tert*-butyl (5-iodopentyl)carbamate according to General Procedure A to give resin **7d**. Cleavage and purification by preparative reverse phase HPLC provided **8d** (11 mg, 36% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{41}H_{62}N_6O_{10}P^+$: 829.4 [M+]; found: 829.5.



8d

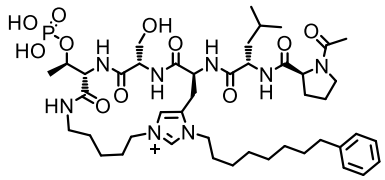
General Procedure B for Solution-phase Peptide Cyclization. The corresponding bis-alkyl histidine peptide **(8a – 8d)** was dissolved in DMF at concentration of 1 mM. PyBOP (1.5 equiv), HOBt (1.5 equiv), and DIEA (10 equiv) were added and the reaction was allowed to stir at room temperature overnight. Following complete (determined by LC/MS), the DMF was removed under a stream of nitrogen gas. The resulting residue was dissolved in methanol and degassed with bubbling argon gas for 15 minutes. Palladium on carbon (0.2 equiv, 10% w/w) was added and the reaction was placed under a balloon of hydrogen gas with vigorous stirring for 1 h. Following removal of the benzyl protecting group, the reaction was filtered through Celite, concentrated to dryness, and purified via semi-preparative reverse-phase HPLC (80/20 H_2O/MeCN to 30/70 H_2O/MeCN (with 0.1% TFA added) over 30 minutes).

Acetyl-Prolyl-Leucyl-cyclo[Histidinyl-[N(τ)-4-butyl]-[N(π)-8-phenyloctyl]-Seryl-(*O*-phospho)-Threonylamide] (5a). Compound **8a** (18 mg, 0.018 mmol) was cyclized and deprotected according to General Procedure B. Purification by semi-preparative reverse-phase HPLC provided **5a** (7 mg, 44% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{44}H_{70}N_8O_{11}P^+$: 917.5 [M+]; found: 917.5.



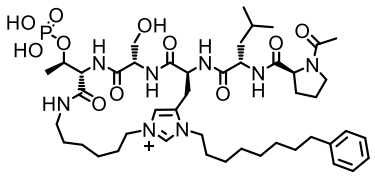
5a

Acetyl-Prolyl-Leucyl-cyclo[Histidinyl-[N(τ)-5-pentyl]-[N(π)-8-phenyloctyl]-Seryl-(*O*-phospho)-Threonylamide] (5b). Compound **8b** (17 mg, 0.016 mmol) was cyclized and deprotected according to General Procedure B. Purification by semi-preparative reverse-phase HPLC provided **5b** (7 mg, 46% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{45}H_{72}N_8O_{11}P^+$: 931.5 [M+]; found: 931.5.



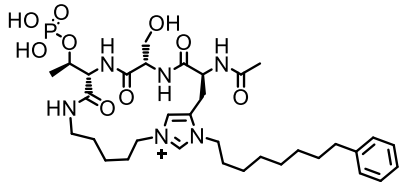
5b

Acetyl-Prolyl-Leucyl-cyclo[Histidinyl-[N(τ)-6-hexyl]-[N(π)-8-phenyloctyl]-Seryl-(*O*-phospho)-Threonylamide] (5c). Compound **8c** (19 mg, 0.018 mmol) was cyclized and deprotected according to General Procedure B. Purification by semi-preparative reverse-phase HPLC provided **5c** (8 mg, 47% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{46}H_{74}N_8O_{11}P^+$: 945.5 [M+]; found: 945.6.



5c

Acetyl-cyclo[Histidinyl-[N(τ)-5-pentyl]-[N(π)-8-phenyloctyl]-Seryl-(*O*-phospho)-Threonylamide] (5d). Compound **8d** (17 mg, 0.016 mmol) was cyclized and deprotected according to General Procedure B. Purification by semi-preparative reverse-phase HPLC provided **5d** (4 mg, 46% overall yield) as a lyophilized white powder. LR-MS (ESI+) calculated for $C_{34}H_{54}N_6O_9P^+$: 721.4 [M+]; found: 721.4.



5d

Biological Methods

ELISA inhibition versus full-length Plk1. ELISA assays to test for inhibitory potency versus full-length Plk1 were run as previously described.[6] Briefly, a biotinylated phosphopeptide (sequence: Biotin-Ahx-PMQS(pT)PLN-NH_2) was diluted into PBS (pH 7.4) to 1 μM (from a 2 mM DMSO stock solution) and loaded onto the wells of a 96-well Neutravidin-coated plate (Pierce Biotechnology) at 100 μL per well for 1 h. The wells were washed once with 150 μL PBST (PBS (pH 7.4) + 0.05% Tween-20), and then 100 μL of 1% BSA in PBS (pH 7.4) (blocking buffer) were added for 1 h. The cytosolic lysate containing myc-tagged protein was diluted to 300 μg/mL in PBS (pH 7.4) containing protease/phosphatase inhibitors (Pierce Biotechnology), mixed with competitive inhibitor (from a 10x stock in 0.5 or 3.3% DMSO/PBS), and allowed to pre-incubate for 1 h (100 μL per well in a 96-well plate, 30 μg total protein). The blocked ELISA plate was washed 2x with PBST (150 μL) and the pre-incubated lysates were added to the plate to incubate for 1 h. The wells were washed 4x with PBST (150 μL), then probed with anti-myc primary antibody (1:1,500 dilution, mouse monoclonal, Pierce Biotechnology) for 1 h. The wells were then washed 4x with PBST (150 μL), and incubated with rabbit anti-mouse HRP conjugate (1:3,000 dilution, Pierce Biotechnology) for 1 h. The wells were then washed 5x with PBST (150 μL) and incubated with Turbo TMB-ELISA solution (Pierce Biotechnology) until the desired absorbance is reached (5-10 minutes). The reaction was quenched by the addition of 2 M aqueous H_2SO_4 and the absorbance was measured at 450 nm using a BioTek Synergy 2 96-well plate reader. Absorbance was normalized to 1 (no inhibitor) and plotted versus concentration (log M) and fit to a non-linear regression using GraphPad Prism 7 software [model: log(inhibitor) vs. response -- Variable slope (four parameters)] to provide IC_{50} values. The IC_{50} values from multiple independent experiments were averaged to provide values ± standard error of the mean (SEM).

Selectivity screening versus isolated PBD*s*. Selectivity screening was performed as previously described.[6] Briefly, isolated PBD proteins from Plks 1 – 3 were diluted to a 2x working dilution in assay buffer (HEPES-buffered saline with 0.05% Tween-20, 1 mM dithiothreitol (DTT) and 1 mM EDTA). The following final protein concentrations were used: 100 nM for Plk1 PBD, 200 nM for Plk2 PBD, and 500 nM for Plk3 PBD. These concentrations represent the approximate K_d values determined for the respective fluorescence polarization (FP) probe sequences. Inhibitors were serially diluted to generate 4x working dilutions in assay buffer containing 4% DMSO. 20 μL of 2x PBD protein solution was added to each well of a 384-well plate. 10 μL of the 4x inhibitor solution (or DMSO blank) was added to corresponding wells (0% binding controls received 10 μL of assay buffer) and allowed to pre-incubated at room temperature for 30 minutes with shaking. The following sequences were utilized as fluorescent probes: 5CF-GPMQSpTPLNG-NH_2 for Plk1 PBD, 5CF-GPMQTSpTPKNG-NH_2 for Plk2 PBD, and 5CF-PLATSpTPKNG-NH_2 for Plk3 PBD.[7, 8] Fluorescent probes were diluted to 40 nM (4x) in assay buffer, then 10 μL was added to each well. The plate was allowed to equilibrate at room temperature for 30 minutes with shaking. Fluorescence polarization was read using a BioTek Synergy 2 plate reader with 485/20 excitation and 528/20 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 7 [model: log(inhibitor) vs. response -- Variable slope (four parameter)]. The IC_{50} values represent the average ± SEM.

X-ray crystallography

Crystallization of the PBD•5b complex. Plk1 PBD protein (residues 371-603), purified as previously described,[9] was provided by Dr. Dan Lim. Frozen PBD stock at 37 mg/mL in 10 mM Tris pH 8, 0.5M NaCl, 10 mM DTT was thawed and diluted to 10 mg/mL with the same buffer. A stock solution of the macrocycle at 100 mM in dimethylsulfoxide (DMSO) was added directly to the diluted protein to a final concentration of 1 mM. A 4 M ammonium acetate solution was then added to a final 0.4 M concentration. Crystals were grown by hanging drop vapor diffusion. The drops contained equal volumes of protein-macrocycle complex and well solution [0.2 M $CaCl_2$ and 12.5% polyethylene glycol (PEG)-3350]. Crystals were frozen in liquid nitrogen after being dipped in a solution of 37.5% ethylene glycol in well solution.

Structure solution and refinement. X-ray diffraction data were collected at the Advance Proton Source (APS) using the Q315 detector at the NE-CAT 24-ID-E beam line. Data were indexed, integrated and scaled to 1.45Å resolution using HKL2000.[10] The crystal's space group was $P2_1$ with a single PBD-macrocycle complex in the asymmetric unit. The structure was solved by molecular replacement using PHASER[11] with PDB entry 4DFW as the search model. COOT[12] was used for model building and PHENIX[13] for refinement. A refinement constraints file for the macrocycle ligand was generated by the GRADE web server (http://grade.globalphasing.org). Data collection and refinement statistics are shown in Table S1.

Table S1. Data Collection and Refinement Statistics

Space Group	$P12_11$
Unit Cell a	35.45 Å
β	51.29 Å
χ	58.03 Å
β	100.84°
Resolution	1.45 Å
(Highest shell)	1.48-1.45 Å
Completeness (%)	99.4 (99.1)
Redundancy	5.5 (4.6)
R_{sym}	0.066 (0.628)
R_{meas}	0.073 (0.709)
R_{pim}	0.031 (0.326)
CC1/2	(0.769)
R_{work}/R_{free}	0.1319/0.1656
RMSD bonds	0.009
RMSD angles	1.048
No. total atoms	4046
Solvent atoms	268
Hydrogens	1889
Ramachandran	
favored	97.7%
allowed	2.3%
outliers	0
Molprobity clash score	1.34

Analytical Traces for Peptide Ligands

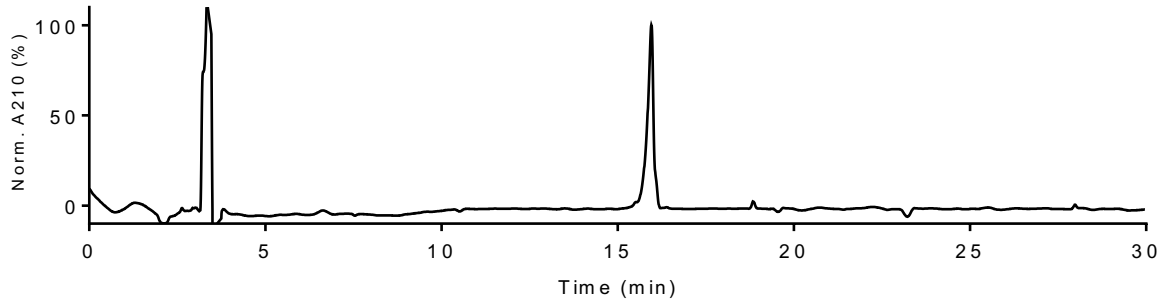


Figure S3. Analytical HPLC trace of **5a**.

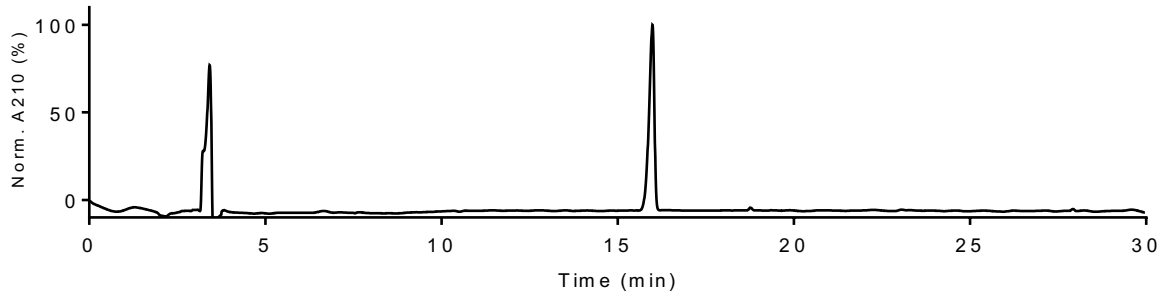


Figure S4. Analytical HPLC trace of **5b**.

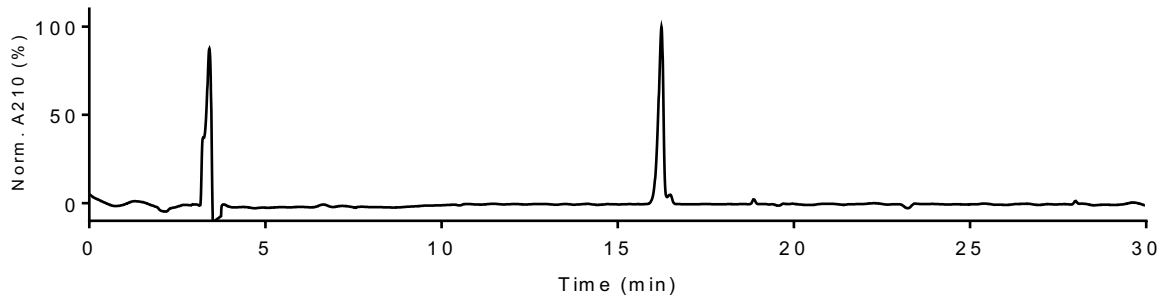


Figure S5. Analytical HPLC trace of **5c**.

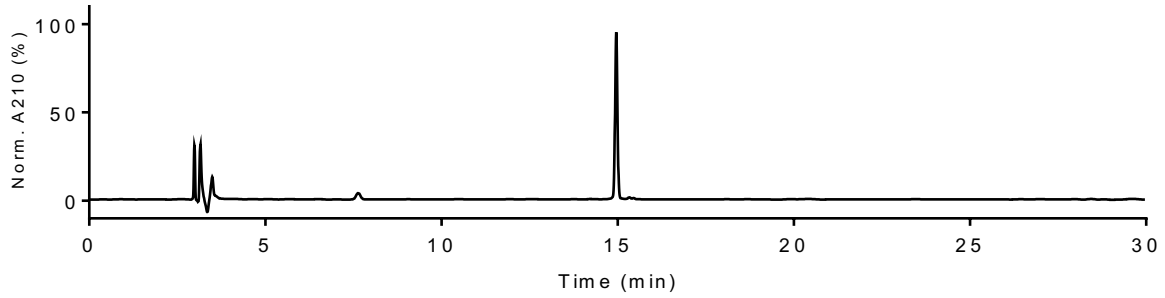


Figure S6. Analytical HPLC trace of **5d**.

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