# Cell-Permeable Bicyclic Peptide Inhibitors against Intracellular Proteins

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## Supporting information

**Materials.** Fmoc-protected amino acids were purchased from Advanced ChemTech (Louisville, KY), Peptides International (Louisville, KY), or Aapptec (Louisville, KY). Fmoc-$F_2$Pmp-OH was purchased from EMD Millipore (Darmstadt, Germany). Aminomethyl-ChemMatrix resin (0.66 mmol/g) was from SJPC (Quebec, Canada). Rink resin LS (100-200 mesh, 0.2 mmol/g) and N-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) were from Advanced ChemTech. O-Benzotriazole-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU), 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), 1-hydroxybenzotriazole hydrate (HOBt) were from Aapptec. Phenyl isothiocyanate in 1-mL sealed ampoules, fluorescein isothiocyanate (FITC), rhodamine B-labeled dextran (dextran$^{Rho}$) were purchased from Sigma-Aldrich. Cell culture media, fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin-EDTA, Dulbecco's phosphate-buffered saline (DPBS) (2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 137 mM sodium chloride, 8.06 mM sodium phosphate dibasic.), and anti-phospho-IR/IGF1R antibody were purchased from Invitrogen (Carlsbad, CA). Nuclear staining dye DRAQ5$^{TM}$ and anti-β-actin antibody were purchased from Thermo Scientific (Rockford, IL). Anti-pY antibody 4G10 was purchased from Millipore (Temecula, CA). Anti-PML antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). All solvents and other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) and were used without further purification.

**Cell Culture.** A549, HEK293, HeLa, and HepG2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS in a humidified incubator at 37 °C with 5% $CO_2$.

**Protein Expression, Purification and Labeling.** The gene coding for the catalytic domain of PTP1B (amino acids 1-321) was amplified by the polymerase chain reaction using PTP1B cDNA as template and oligonucleotides 5'-ggaattccatatggagatggaaaaggagttcgagcag-3' and 5'-gggatccgtcgacattgtgtggctccaggattcgtttgg-3' as primers. The resulting DNA fragment was digested with endonucleases *Nde* I and *Sal* I and inserted into prokaryotic vector pET-22b(+)-ybbR.[1] This cloning procedure resulted in the addition of a ybbR tag (VLDSLEFIASKL) to the N-terminus of PTP1B. Expression and purification of the ybbR-tagged PTP1B were carried out as previously described.[2] Texas Red labeling of PTP1B was carried out by treating the ybbR-tagged PTP1B protein (80 μM) in 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$ with Sfp phosphopantetheinyl transferase (1 μM) and Texas Red-CoA (100 μM) for 30 min at room temperature.[1] The reaction mixture was passed through a G-25 fast-desalting column equilibrated in 30 mM HEPES, pH 7.4, 150 mM NaCl to remove any free dye molecules. The full-length human S16A/Y23A mutant Pin1 was expressed and purified from *E. coli* as previously described.[3] All other PTPs were expressed and purified as previously described.[4]

**Library Synthesis.** The cyclic peptide library was synthesized on 1.35 g of aminomethyl-ChemMatrix resin (0.57 mmol/g). The library synthesis was performed at room temperature unless otherwise noted. The linker sequence (BBM) was synthesized using standard Fmoc

chemistry. The typical coupling reaction contained 5 equiv of Fmoc-amino acid, 5 equiv of HBTU and 10 equiv of diisopropylethylamine (DIPEA) and was allowed to proceed with mixing for 2 h. The Fmoc group was removed by treatment twice with 20% (v/v) piperidine in DMF (5 + 15 min), and the beads were exhaustively washed with DMF (6x). To spatially segregate the beads into outer and inner layers, the resin (after removal of N-terminal Fmoc group) was washed with DMF and water, and soaked in water overnight. The resin was quickly drained and suspended in a solution of Fmoc-Glu(δ-NHS)-OAll (0.10 equiv), Boc-Met-OSu (0.4 equiv) and N-methylmorpholine (2 equiv) in 20 mL of 1:1 (v/v) DCM/diethyl ether.[5] The mixture was incubated on a carousel shaker for 30 min. The beads were washed with 1:1 DCM/diethyl ether (3x) and DMF (8x). Next, the Fmoc group was removed by piperidine treatment. Then, Fmoc-Arg(Pbf)-OH (4x), Fmoc-Nal-OH, and Fmoc-Phe-OH were sequentially coupled by standard Fmoc chemistry to half of the resin. The other half was coupled with the same amino acids in the reverse sequence. The resin was combined and the random sequence was synthesized by the split-and-pool method using 5 equiv of Fmoc-amino acids, 5 equiv HATU and 10 equiv DIPEA as the coupling agent. The coupling reaction was repeated once to ensure complete coupling at each step. For random positions, a 24-amino acid set was selected based on their structural diversity, metabolic stability, and commercial availability, including 10 proteinogenic α-L-amino acids [Ala, Asp, Gln, Gly, His, Ile, Ser, Trp, Pro, and Tyr], 5 nonproteinogenic α-L-amino acids [L-4-fluorophenylalanine (Fpa), L-homoproline (Pip), L-norleucine (Nle), L-phenylglycine (Phg) and L-4-(phosphonodifluoromethyl)phenylalanine ($F_2$Pmp)], and nine α-D-amino acids [D-2-naphthylalanine (D-Nal), D-Ala, D-Asn, D-Glu, D-Leu, D-Phe, D-Pro, D-Thr, and D-Val]. To differentiate isobaric amino acids during PED-MS analysis, 4% (mol/mol) of $CD_3CO_2D$ was added to the coupling reactions of D-Ala, D-Leu, and D-Pro, while 4% $CH_3CD_2CO_2D$ was added to the Nle reactions. Fmoc-$F_2$Pmp-OH (0.06 equiv) and Fmoc-Tyr-OH (0.54 equiv) was placed in the middle of the random positions using HATU/DIPEA. After the entire sequence was synthesized, the allyl group on the C-terminal Glu residue was removed by treatment with a DCM solution containing tetrakis(triphenylphosphine)palladium [$Pd(PPh_3)_4$, 0.25 equiv] and phenylsilane (5 equiv) for 15 min (3x). The beads were sequentially washed with 0.5% (v/v) DIPEA in DMF, 0.5% (w/v) sodium dimethyldithiocarbamate hydrate in DMF, DMF (3x), DCM (3x), and DMF (3x). The Fmoc group on the N-terminal random residue was removed by piperidine as describe above. The beads were washed with DMF (6x), DCM (3x), and 1 M HOBt in DMF (3x). For peptide cyclization, a solution of PyBOP/HOBt/DIPEA (5, 5, 10 equiv, respectively) in DMF was mixed with the resin and the mixture was incubated on a carousel shaker for 3 h. The resin was washed with DMF (3x) and DCM (3x) and dried under vacuum for >1 h. Side-chain deprotection was carried out with a modified reagent K [78.5:7.5:5:5:2.5:1:1 (v/v) TFA/phenol/water/thioanisole/ethanedithiol/anisole/triisopropylsilane] for 3 h. The resin was washed with TFA and DCM and dried under vacuum before storage at -20 °C.

**Library Screening and Peptide Sequencing.** Library resin (100 mg, ~300,000 beads) was swollen in DCM, washed extensively with DMF, doubly distilled $H_2O$, and incubated in 1 mL of blocking buffer (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.4, 0.05% Tween 20 and 0.1% gelatin) containing 20 nM Texas red-labeled PTP1B at 4 °C for 3 h. The beads were examined under an Olympus SZX12 microscope equipped with a fluorescence illuminator (Olympus America, Center Valley, PA) and the most intensely fluorescent beads were manually collected as positive hits. Beads containing encoding linear peptides were individually sequenced by partial Edman degradation-mass spectrometry (PED-MS).[5]

**Individual Peptide Synthesis and Labeling.** The 42 hit sequences (Table S1) were separated into three classes based on their sequences: Class I hits were rich in D-amino acids, class II hits contained an aromatic residue at the -1 position, whereas the class III hits had small hydrophobic residues at the -1 position (relative to $F_2$Pmp which is designated as position 0).. One representative sequence from each class was chosen for resynthesis and further analysis. Monocyclic and bicyclic peptides were synthesized on Rink Resin LS (0.2 mmol/g) using standard Fmoc chemistry. For monocyclic peptides, after the last (N-terminal) residue was coupled, the allyl group on the C-terminal Glu residue was removed by treatment with $Pd(PPh_3)_4$ and phenylsilane (0.1 and 10 equiv, respectively) in anhydrous DCM (3 x 15 min). The N-terminal Fmoc group was removed by treatment with 20% (v/v) piperidine in DMF and the peptide was cyclized by treatment with PyBOP/HOBt/DIPEA (5, 5, and 10 equiv) in DMF for 3 h. For bicyclic peptides, the N-terminal Fmoc group was removed with piperidine and a trimesic acid was coupled on the N-terminal amine using HBTU as coupling agent. The allyloxycarbonyl groups on the side chains of two Dap residues were removed by treatment with $Pd(PPh_3)_4$ and phenylsilane (0.1 and 10 equiv, respectively) in anhydrous DCM for 2 h. The resulting peptide was cyclized with PyBOP as described above. The peptides were deprotected and released from the resin by treatment with 82.5:5:5:5:2.5 (v/v) TFA/thioanisole/water/phenol/ethanedithiol for 2 h. The peptides were triturated with cold ethyl ether (3x) and purified by reversed-phase HPLC on a $C_{18}$ column. The authenticity of each peptide was confirmed by MALDI-TOF mass spectrometry. Peptide labeling with FITC was performed by dissolving the purified peptide (~1 mg) in 300 µL of 1:1:1 (vol/vol) DMSO/DMF/150 mM sodium bicarbonate (pH 8.5) and mixing with 10 µL of FITC in DMSO (100 mg/mL). After 20 min at room temperature, the reaction mixture was subjected to reversed-phase HPLC on a $C_{18}$ column to isolate the FITC-labeled peptide.

**PTP Inhibition Assay.** PTP assays were performed in a quartz microcuvette (total volume 150 µL). The reaction mixture contains 100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM EDTA, 1 mM TCEP, 0-1 µM of PTP inhibitor, and 500 µM *para*-nitrophenyl phosphate (pNPP). The enzymatic reaction was initiated by the addition of PTP (final concentration 15-75 nM) and monitored continuously at 405 nm on a UV-VIS spectrophotometer. Initial rates were calculated from the reaction progress curves (typically <60 s). The half-maximal inhibition constant ($IC_{50}$) was defined as the concentration of an inhibitor that reduced the enzyme activity to 50% and was obtained by plotting the rates (V) against the inhibitor concentration [I] and fitting the data against the equation

$$V = V_0/(1 + [I]/IC_{50})$$

where $V_0$ is the enzymatic reaction rate in the absence of inhibitor. The inhibition constant ($K_i$) was determined by measuring the initial rates at fixed enzyme concentration (15 nM) but varying concentrations of pNPP (0-24 mM) and inhibitor (0-112 nM). The reaction rate (V) was plotted against the pNPP concentration ([S]) and fitted against the equation

$$1/V = K * 1/[S] + 1/V_{max}$$

to obtain the Michaelis constant *K*. The $K_i$ value was obtained by plotting the *K* values against the inhibitor concentration [I] and fitted to equation

$$K/K_0 = 1 + [I]/K_i$$

where $K_0$ is the Michaelis constant in the absence of inhibitor ([I] = 0).

**Confocal Microscopy.** Approximately 5 x $10^4$ A549 cells were seeded in 35-mm glass-bottomed microwell dish (MatTek) containing 1 mL of media and cultured for one day. A549 cells were gently washed with DPBS once and treated with the FITC-labeled PTP1B inhibitors (5 μM), dextran$^{Rho}$ (1 mg $mL^{-1}$) in growth media for 2 h at 37 °C in the presence of 5% $CO_2$. The peptide-containing media was removed and the cells were washed with DPBS three times and incubated for 10 min in 1 mL of DPBS containing 5 μM DRAQ5. The cells were again washed with DPBS twice. Then the cells were imaged on a Visitech Infinity 3 Hawk 2D-array live cell imaging confocal microscope (with a 60x oil immersion lens) at 37 °C in the presence of 5% $CO_2$. Live-cell confocal microscopic imaging of HEK293 cells after treatment with FITC-labeled Pin1 inhibitors were similarly conducted.

**Immunoblotting.** A549 cells were cultured in full growth media to reach 80% confluence. The cells were starved in serum free media for 3 h and treated with varying concentrations of PTP1B inhibitors for 2 h, followed by 30 min incubation in media supplemented with 1 mM sodium pervanadate. The solutions were removed and the cells were washed with cold DPBS twice. The cells were detached and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 10 mM sodium pyrophosphate, 5 mM iodoacetic acid, 10 mM NaF, 1 mM EDTA, 2 mM sodium pervanadate, 0.1 mg/mL phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 0.1 mg/mL trypsin inhibitor. After 30 min incubation on ice, the cell lysate was centrifuged at 15,000 rpm for 25 min in a microcentrifuge. The total cellular proteins were separated by SDS-PAGE and transferred electrophoretically to a PVDF membrane, which was immunoblotted using anti-phosphotyrosine antibody 4G10. The same samples were analyzed on a separate SDS-PAGE gel and stained by Coomassie brilliant blue to ascertain equal sample loading in all lanes.

To test the effect of inhibitor **4** on insulin signaling pathway, HepG2 cells were cultured to reach 80% confluence. The cells were starved for 4 h in serum free DMEM media before treated with PTP1B inhibitor (2 h), followed by stimulation with 100 nM insulin for 5 min. The samples were analyzed by SDS-PAGE as described above and immunoblotted using anti-phospho-IR/IGF1R antibody. The PVDF membrane was also probed by anti-β-actin antibody as the loading control.

To test the effect of Pin1 inhibitor on its substrate proteins, HeLa cells were grown in standard DMEM supplemented with 10% FBS and 1% penicillin/ streptomycin sulfate. The cells were treated with varying concentrations of Pin1 inhibitor **7** or DMSO for 48 h. The cells were lysed and the whole-cell lysates were separated by SDS-PAGE and immunoblotted using anti-PML antibody as described above. The nitrocellulose membrane was also probed by anti-β-actin antibody as the loading control.

**Serum Stability Test.** The stability tests were carried by modifying a previously reported procedure.[6] Diluted human serum (25%) was centrifuged at 15,000 rpm for 10 min, and the supernatant was collected. A peptide stock solution was diluted into the supernatant to a final

concentration of 5 μM and incubated at 37 °C. At various time points (0-24 h), 200-μL aliquots were withdrawn and mixed with 50 μL of 15% trichloroacetic acid and incubated at 4 ºC overnight. The final mixture was centrifuged at 15,000 rpm for 10 min in a microcentrifuge, and the supernatant was analyzed by reversed-phase HPLC equipped with a $C_{18}$ column. The amount of remaining peptide (%) was determined by integrating the area underneath the peptide peak (monitored at 214 nm) and comparing with that of the control reaction (no serum).

**Fluorescence Anisotropy.** FA experiments were carried out by incubating 100 nM FITC-labeled peptide with varying concentrations of protein in 20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM magnesium acetate, and 0.1% bovine serum albumin (BSA) for 2 h at room temperature. The FA values were measured on a Molecular Devices Spectramax M5 plate reader, with excitation and emission wavelengths at 485 and 525 nm, respectively. Equilibrium dissociation constants ($K_D$) were determined by plotting the FA values as a function of protein concentration and fitting the curve to the following equation:

$$Y = (A_{min} + (A_{max}*Q_b/Q_f - A_{min})*((L + x + K_D) - sqrt((L + x + K_D)\text{^}2 - 4*L*x))/2/L)/(1 + (Q_b/Q_f - 1)*((L + x + K_D) - sqrt((L + x + K_D)\text{^}2 - 4*L*x))/2/L)$$

where Y is the FA value at a given protein concentration x, L is the peptide concentration, $Q_b/Q_f$ is the correction factor for fluorophore-protein interaction, $A_{max}$ is the maximum FA value when all of the peptides are bound to protein, while $A_{min}$ is the minimum FA value when all of the peptides are free. FA competition assay was performed by incubating 100 nM FITC-labeled Pin1 inhibitor **5** with 1 μM Pin1, followed by the addition of 0-5 μM unlabeled inhibitor. The FA values were measured similarly on a pate reader. $IC_{50}$ values were obtained by plotting the FA values against the competitor concentration and curve fitting using the four-parameter dose-response inhibition equation (Prism 6, GraphPad).

**Flow Cytometry.** HeLa cells were cultured in six-well plates ($5 \times 10^5$ cells per well) for 24 h. On the day of experiment, the cells were incubated with 5 μM FITC-labeled peptides in phenol red-free DMEM supplemented with 1% FBS. After 2 h, the peptide solution was removed, and the cells were washed with DPBS, treated with 0.25% trypsin for 5 min and washed with DPBS again. Finally, the cells were resuspended in DPBS and analyzed by flow cytometry (BD FACS Aria III), with excitation at 535 nm.

**MTT Cell Proliferation Assay**: HeLa cells (100 μL/each well, $5 \times 10^4$ cells/mL) were seeded in a 96-well culture plate and allowed to grow overnight in DMEM supplemented with 10% FBS. Varying concentrations of Pin1 inhibitor (0-20 μM) were added to the wells and the cells were incubated at 37 °C with 5% $CO_2$ for 72 h. After that, 10 μL of a MTT stock solution (5 mg/mL) was added into each well. The plate was incubated at 37 °C for 4 h and 100 μL of SDS-HCl solubilizing solution was added into each well, followed by thorough mixing. The plate was incubated at 37 °C overnight and the absorbance of the formazan product was measured at 570 nm on a Molecular Devices Spectramax M5 plate reader.

**Table S1.** Peptide Sequences Selected from Cyclic Peptide Library against PTP1B[a]

| Bead No. | Sequence | Bead No. | Sequence |
|---|---|---|---|
| 1 | Pro-Pip-Gly-$F_2$Pmp-Tyr-Arg | 22 | D-Glu-Ala-Phg-$F_2$Pmp-D-Val-Arg |
| 2 | Ser-Pip-Ile-$F_2$Pmp-$F_2$Pmp-Arg | 23 | Ile-D-Val-Phg-$F_2$Pmp-Ala-Arg |
| 3 | Ile-His-Ile-$F_2$Pmp-Ile-Arg | 24 | Tyr-D-Thr-Phg-$F_2$Pmp-Ala-Arg |
| 4 | Ala-D-Ala-Ile-$F_2$Pmp-Pip-Arg | 25 | D-Asn-Pip-Phg-$F_2$Pmp-Ile-Arg |
| 5 | Fpa-Ser-Pip-$F_2$Pmp-D-Val-Arg | 26 | Pip-D-Asn-Trp-$F_2$Pmp-His-Arg |
| 6 | Pip-D-Asn-Pro-$F_2$Pmp-Ala-Arg | 27 | Tyr-Pip-D-Val-$F_2$Pmp-Ile-Arg |
| 7 | Tyr-Phg-Ala-$F_2$Pmp-Gly-Arg | 28 | D-Asn-Ser-D-Ala-$F_2$Pmp-Gly-Arg |
| 8 | Ala-His-Ile- $F_2$Pmp-D-Ala-Arg | 29* | D-Thr-D-Asn-D-Val-$F_2$Pmp-D-Ala-Arg |
| 9 | Gly-D-Asn-Gly-$F_2$Pmp-D-Pro-Arg | 30 | D-Asn-D-Thr-D-Val-$F_2$Pmp-D-Thr-Arg |
| 10 | D-Phe-Gln-Pip-$F_2$Pmp-Ile-Arg | 31 | Ser-Ile-D-Thr-$F_2$Pmp-Tyr-Arg |
| 11 | Ser-Pro-Gly-$F_2$Pmp-His-Arg | 32 | D-Asn-Fpa-D-Asn-$F_2$Pmp-D-Leu-Arg |
| 12 | Pip-Tyr-Ile-$F_2$Pmp-His-Arg | 33 | Tyr-D-Asn-D-Asn-$F_2$Pmp-Nle-Arg |
| 13* | Ser-D-Val-Pro-$F_2$Pmp-His-Arg | 34 | D-Asn-Tyr-D-Asn-$F_2$Pmp-Gly-Arg |
| 14 | Ala-Ile-Pro-$F_2$Pmp-D-Asn-Arg | 35 | Ala-Trp-D-Asn-$F_2$Pmp-Ala-Arg |
| 15 | Fpa-Ser-Ile-$F_2$Pmp-Gln-Phe | 36 | D-Val-D-Thr-His-$F_2$Pmp-Tyr-Arg |
| 16 | Ala-D-Ala-Phg-$F_2$Pmp-D-Phe-Arg | 37 | Pro-Phg-His-$F_2$Pmp-Pip-Arg |
| 17 | D-Asn-D-Thr-Phg-$F_2$Pmp-Phg-Arg | 38 | D-Asn-Phg-His-$F_2$Pmp-Gly-Arg |
| 18* | Ile-Pro-Phg-$F_2$Pmp-Nle-Arg | 39 | Pro-Ala-His-$F_2$Pmp-Gly-Arg |
| 19 | Gln-Pip-Fpa-$F_2$Pmp-Pip-Arg | 40 | Ala-Tyr-His-$F_2$Pmp-Ile-Arg |
| 20 | D-Asn-Ala-Fpa-$F_2$Pmp-Gly-Arg | 41 | D-Asn-Pip-D-Glu-$F_2$Pmp-Tyr-Arg |
| 21 | D-Asn-D-Thr-Tyr-$F_2$Pmp-Ala-Arg | 42 | D-Val-Ser-Ser-$F_2$Pmp-D-Thr-Arg |

[a]Fpa, L-4-fluorophenylalanine; Pip, L-homoproline; Nle, L-norleucine; Phg, L-phenylglycine; $F_2$Pmp, L-4-(phosphonodifluoromethyl)phenylalanine. *Sequences subjected to further analysis.

**Table S2.** Potency of Selected Monocyclic Peptide Inhibitors against PTP1B

| Monocyclic Inhibitor | Sequence | $IC_{50}$ (nM) |
|---|---|---|
| **1** | *cyclo*(D-Thr-D-Asn-D-Val-$F_2$Pmp-D-Ala-Arg-Arg-Arg-Arg-Nal-Phe-Gln) | 96 ± 42 |
| **2** | *cyclo*(Ser-D-Val-Pro-$F_2$Pmp-His-Arg-Arg-Arg-Arg-Nal-Phe-Gln) | 31 ± 3 |
| **3** | *cyclo*(Ile-Pro-Phg-$F_2$Pmp-Nle-Arg-Arg-Arg-Arg-Nal-Phe-Gln) | 720 ± 500 |
| $F_2$Pmp | $F_2$Pmp-$NH_2$ | 500,000[a] |

[a]Data from ref. 7. All measurements were performed in triplicates and the $IC_{50}$ values represent the mean ± SD of three data sets.

**Table S3**. Selectivity of Bicyclic Inhibitor **4** against Various PTPs[a]

| PTP | PTP1B | TCPTP | HePTP | PTPRC | SHP1 | PTPRO | PTPH1 |
|---|---|---|---|---|---|---|---|
| $IC_{50}$ (nM) | 30 ± 4 | 500 ± 250 | NA | NA | NA | NA | NA |

[a]NA, no significant inhibition at 1 μM inhibitor. All measurements were performed in triplicates and the $IC_{50}$ values represent the mean ± SD of three data sets.

**Table S4.** Comparison of Inhibitor **4** with Previously Reported PTP1B Inhibitors

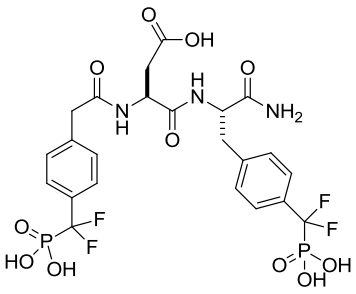
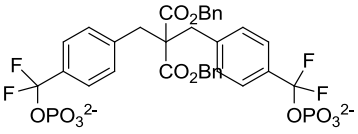
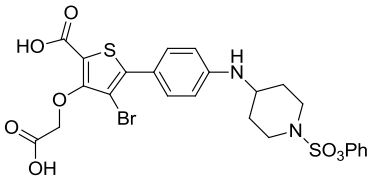
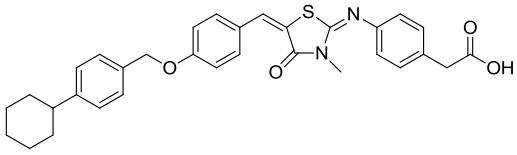
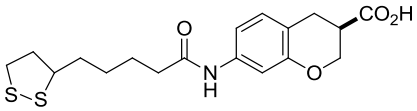
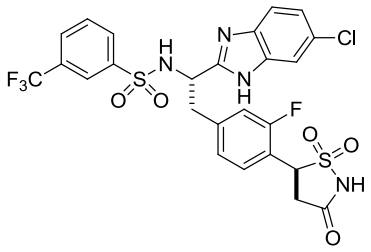
| Inhibitor | Potency against PTP1B | Selectivity over TCPTP | Cell permeability | Ref. |
|---|---|---|---|---|
| Inhibitor **4** | $K_I$ = 37 ± 4 nM | 17-fold | Yes | This work |
|  | $K_I$ = 2.4 nM | 10-fold | No | 8 |
|  | $IC_{50}$ = 60 nM | 1-fold (no selectivity) | Yes | 9 |
|  | $K_I$ = 4 nM | 1-fold | Yes | 10 |
|  | $IC_{50}$ = 240 nM | 41-fold | Yes | 11 |
|  | $IC_{50}$ = 52 nM | 30-fold | Yes | 12 |
|  | $IC_{50}$ = 10 nM | 1-fold | Yes | 13 |

**Table S5.** Dissociation Constants of Monocyclic and Bicyclic Peptides against Pin1 as Determined by FA Analysis

| Pin1 Inhibitor | Sequence[a] | $K_D$ (nM) |
|---|---|---|
| **5** | *cyclo*(D-Ala-Sar-D-pThr-Pip-Nal-Tyr-Gln)-Lys-$NH_2$ | 258 ± 65 |
| **6** | *bicyclo*[Tm(D-Ala-Sar-D-pThr-Pip-Nal-Arg-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys-$NH_2$ | 130 ± 44 |
| **7** | *bicyclo*[Tm(D-Ala-Sar-D-pThr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys-$NH_2$ | 72 ± 21 |
| **8** | *bicyclo*[Tm(D-Ala-Sar-D-Thr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys-$NH_2$ | 620 ± 120 |
| **9** | *bicyclo*[Tm(D-Ala-Sar-D-Thr-D-Ala-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys-$NH_2$ | ND |

[a]Dap, L-2,3-diaminopropionic acid; Nal, L-β-naphthylalanine; Pip, L-pipecolic acid; Sar, sarcosine; Tm, trimesic acid. For FA analysis, all peptides were labeled at the C-terminal lysine side-chain with FITC. ND, no detectable binding. All measurements were performed in triplicates and the $K_D$ values represent the mean ± SD of three data sets.

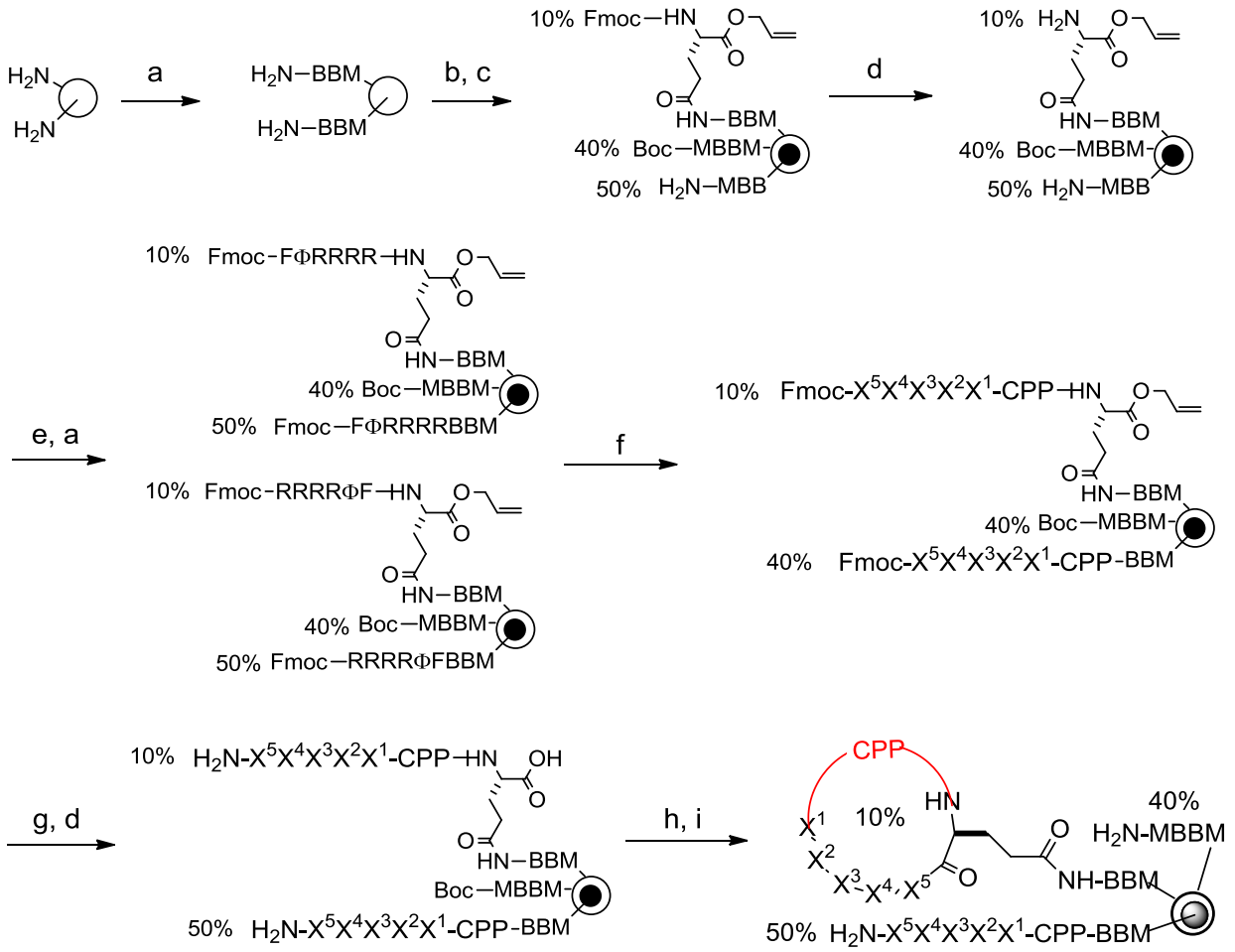



**Figure S1.** Design and synthesis of cyclic peptide library. Reagents and conditions: (a) standard Fmoc/HBTU chemistry; (b) soak in water; (c) 0.1 equiv Fmoc-Glu(δ-NHS)-OAll, 0.4 equiv Boc-Met-OH in $Et_2O/CH_2Cl_2$; (d) piperidine; (e) split into two parts; (f) split-and-pool synthesis by Fmoc/HATU chemistry; (g) $Pd(PPh_3)_4$; (h) PyBOP, HOBt; and (i) Reagent K. $X^2$, 10% $F_2$Pmp and 90% Tyr; $X^1$ and $X^3$-$X^5$, random positions; Φ, L-2-naphthylalanine; B, β-alanine; CPP, cell-penetrating motif $F\Phi R_4$ or $R_4\Phi F$.

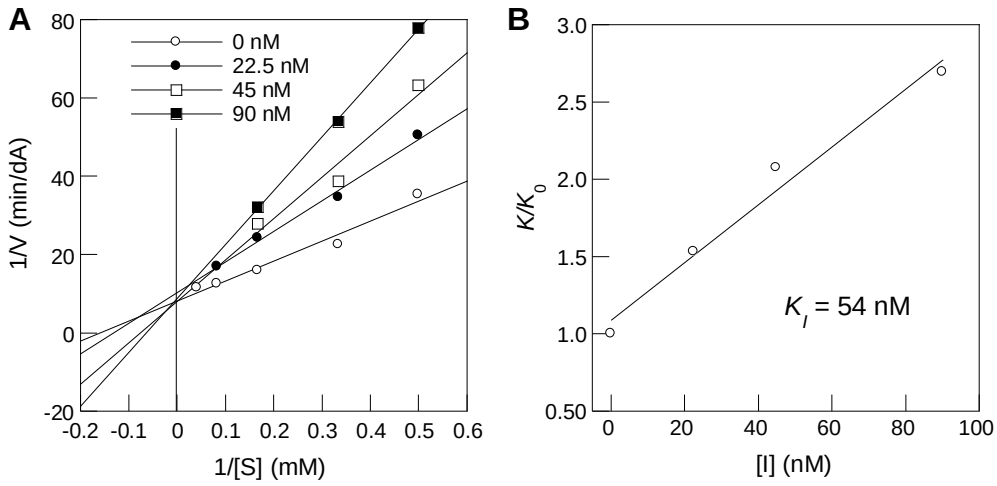



**Figure S2**. Competitive inhibition of PTP1B by monocyclic peptide inhibitor **2**. (A) Lineweaver-Burk plots for PTP1B-catalyzed hydrolysis of pNPP (0-24 mM) in the presence of varying concentrations of inhibitor **2** (0, 22.5, 45, and 90 nM). (B) Secondary plot of the Michaelis constant ratio ($K/K_0$) as a function of [I] (R = 0.9877). Data fitting gave a $K_I$ value of 54 ± 5 nM.

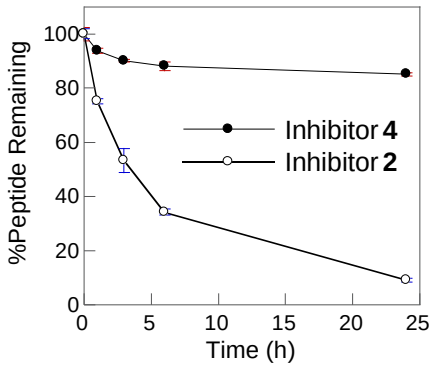



**Figure S3.** Comparison of the serum stability of monocyclic inhibitor **2** and bicyclic inhibitor **4**. Data presented are the mean ± SD of three independent experiments.

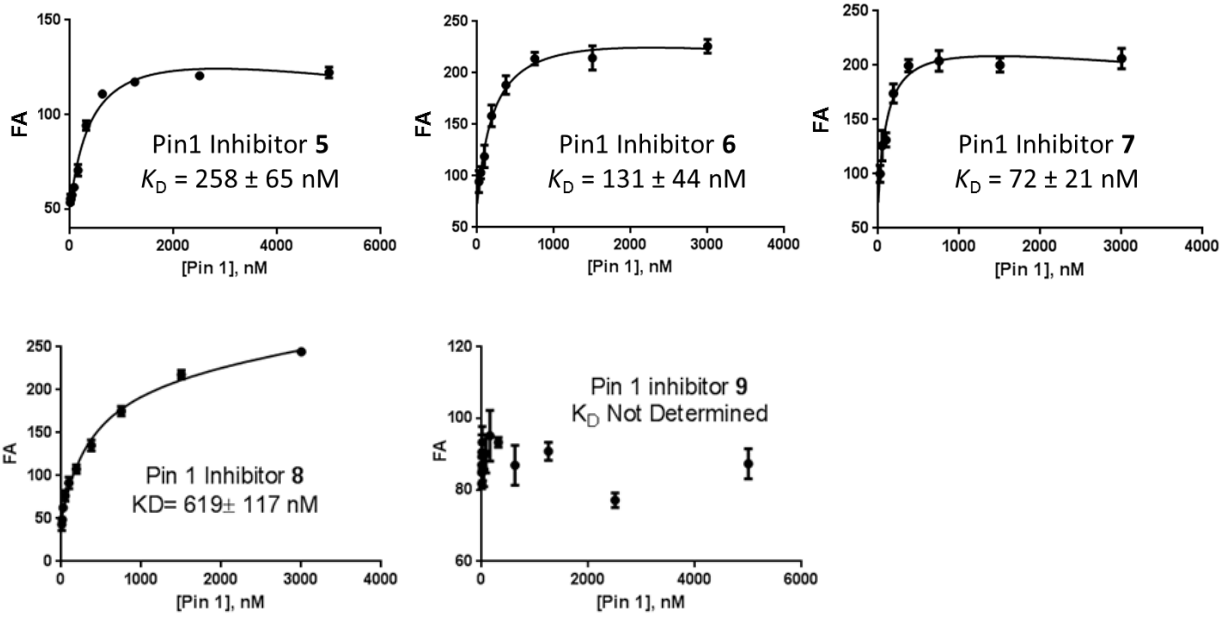



**Figure S4**. FA analysis of the binding of Pin1 inhibitor **5-9** to Pin1. All measurements were performed in triplicates and the $K_D$ values represent the mean ± SD of three data sets.

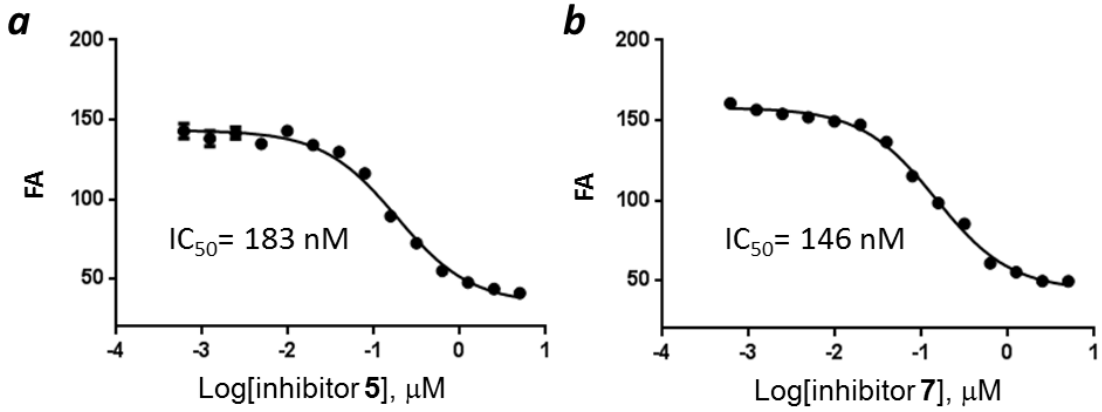



**Figure S5**. Competition for binding to Pin1 by inhibitors **5** and **7**. Each reaction contained 0.1 μM FITC-labeled inhibitor **5**, 1 μM Pin1, and 0-5 μM unlabeled inhibitor **5** (***a***) or inhibitor **7** (***b***) and the FA value was measured and plotted against the competitor concentration. All measurements were performed in triplicates.

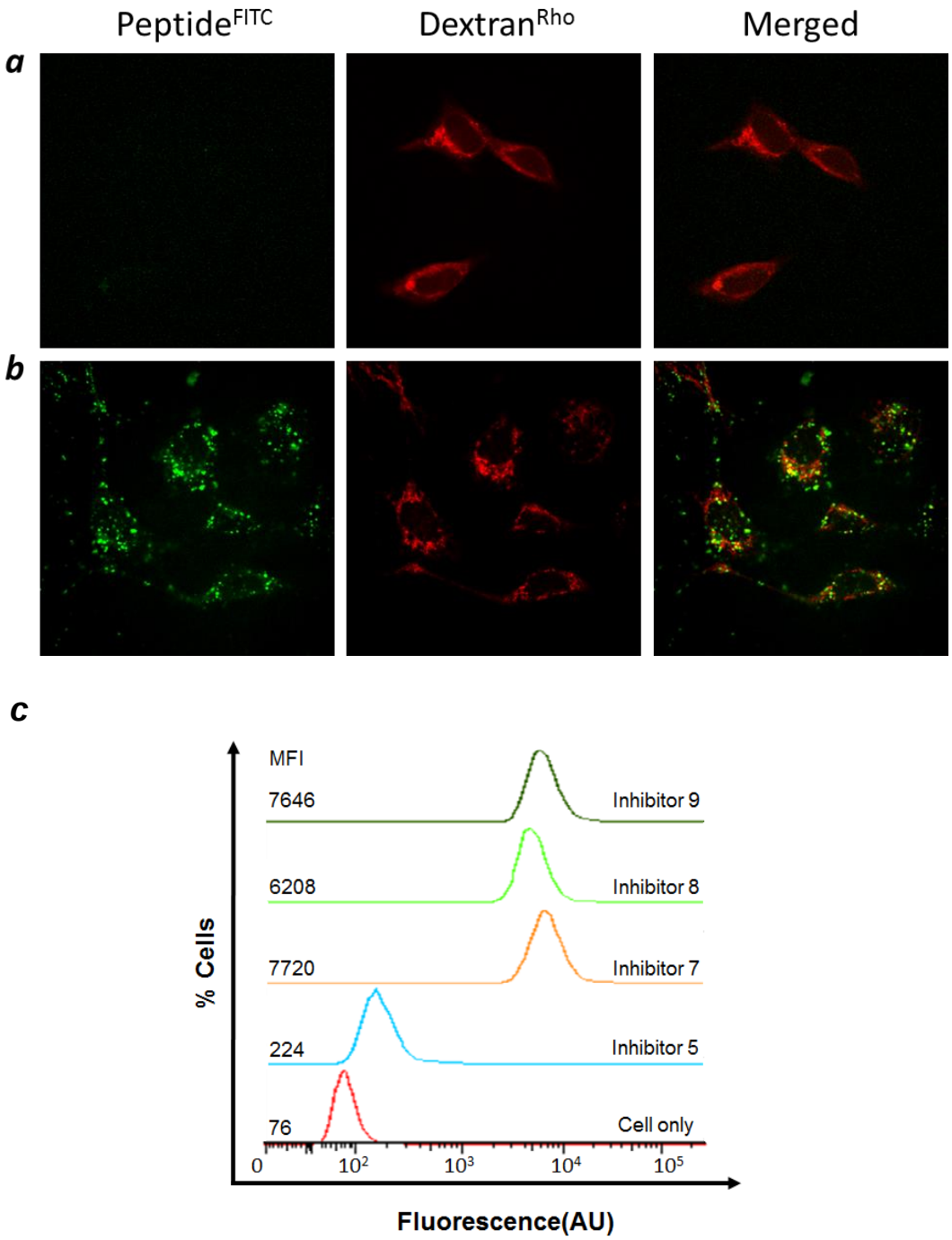



**Figure S6**. (***a, b***) Live-cell confocal microscopic images of HEK293 cells treated with 5 μM FITC-labeled Pin1 inhibitor **5** (***a***) or **7** (***b***) and 1 mg/mL endocytosis marker Dextran$^{Rho}$ for 2 h. All images were recorded at the same Z-section. (***c***) Flow cytometry of HeLa cells treated with 5 μM FITC-labeled peptides. MFI, mean fluorescence intensity. The data show that inhibitor **5** is impermeable to the cell whereas inhibitors **7-9** enter cells with similar efficiencies.

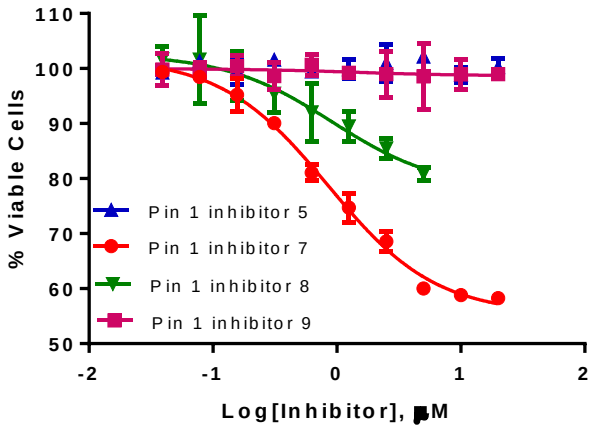



**Figure S7**. Effect of Pin1 inhibitors **5**, **7, 8** and **9** on HeLa cell proliferation as determined by MTT assay. Cells without treatment with peptide were used as control (100%). Data reported represent the mean ± SD of three independent experiments.

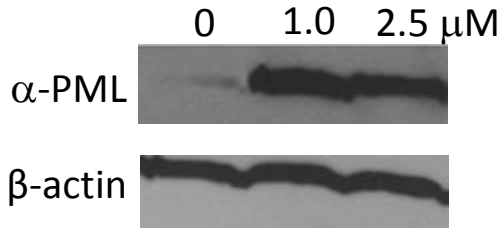



**Figure S8**. Western blots showing the protein levels of PML in HeLa cells in the absence and presence of Pin1 inhibitor **7** (0-2.5 μM). Immunoblotting with anti-β-actin antibodies showed equal protein loading in all lanes.

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