Published in final edited form as:

J Med Chem. 2015 August 13; 58(15): 6306–6312. doi:10.1021/acs.jmedchem.5b00411.

A Selective, Cell-Permeable Nonphosphorylated Bicyclic Peptidyl Inhibitor against Peptidyl-Prolyl Isomerase Pin1

Bisheng Jiang and Dehua Pei*

Department of Chemistry and Biochemistry, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43220

Abstract

Pin1 regulates the levels and functions of phosphoproteins by catalyzing phosphorylation-dependent cis/trans isomerization of peptidyl-prolyl bonds. Previous Pin1 inhibitors contained phosphoamino acids, which are metabolically unstable and have poor membrane permeability. In this work, we report a cell-permeable and metabolically stable nonphosphorylated bicyclic peptide as a potent and selective Pin1 inhibitor, which inhibited the intracellular Pin1 activity in cultured mammalian cells but had little effect on other isomerases such as Pin4, FKBP12, or cyclophilin A.

Keywords

Bicyclic peptide; Cell-penetrating peptide; Peptide library; Peptidyl-prolyl isomerism; Pin1 inhibitor

INTRODUCTION

Pin1 is a phosphorylation-dependent peptidyl-prolyl cis/trans isomerase (PPIase).^{1–3} It contains an N-terminal WW domain and a C-terminal catalytic domain, both of which recognize specific phosphoserine (pSer)/phosphothreonine (pThr)-Pro motifs in their protein substrates.^{4,5} Through cis-trans isomerization of specific pSer/pThr-Pro bonds, Pin1 regulates the levels, activities, as well as intracellular localization of a wide variety of phosphoproteins.⁶ For example, Pin1 controls the in vivo stability of cyclin D1^{7,8} and cyclin E⁹ and switches c-Jun,¹⁰ c-Fos,¹¹ and NF- κ B¹² between their inactive unstable forms and active stable forms. Isomerization by Pin1 also regulates the catalytic activity of numerous cell-cycle signaling proteins such as phosphatase CDC25C^{13,14} and kinase Wee1.¹⁵ Finally, Pin1-catalyzed conformational changes in β -catenin¹⁶ and NF- κ B¹² lead to subcellular translocation.

Given its critical roles in cell-cycle regulation and increased expression levels and activity in human cancers,¹⁷ Pin1 has been proposed as a potential target for the development of anticancer drugs.^{18,19} Pin1 is also implicated in neural degenerative diseases such as Alzheimer's disease.²⁰ Therefore, there have been significant interests in developing

*Corresponding Author: To whom correspondence should be addressed: pei.3@osu.edu; phone 614-688-4068.

Supporting Information. Experimental details and additional data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

specific inhibitors against Pin1. Small-molecule inhibitors such as Juglone,²¹ PiB,²² dipentamethylene thiauram monosulfide²³ and halogenated phenyl-isothiazolone (TME-001)²⁴ generally lack sufficient potency and/or specificity.²⁵ A number of potent peptidyl Pin1 inhibitors have been reported and are more selective than the small-molecule inhibitors.^{26–31} However, peptidyl inhibitors are generally impermeable to the cell membrane and therefore have limited utility as therapeutics or in vivo probes. We recently reported a cell-permeable bicyclic peptidyl inhibitor against Pin1, in which one ring (A ring) featured a Pin1-binding phosphopeptide motif [D-pThr-Pip-Nal, where Pip and Nal are (*R*)-piperidine-2-carboxylic acid and L-naphthylalanine, respectively] while the second ring (B ring) contained a cell-penetrating peptide, Phe-Nal-Arg-Arg-Arg-Arg (Figure 1, peptide 1).³² Although the bicyclic peptidyl inhibitor is potent ($K_D = 72$ nM) and active in cellular assays, we anticipated that its D-pThr moiety might be metabolically labile due to hydrolysis by nonspecific phosphatases. The negative charges of the phosphate group might also impede the cellular entry of the inhibitor. In this work, we discovered a nonphosphorylated bicyclic peptidyl inhibitor against Pin1 by screening a peptide library and hit optimization. The resulting bicyclic peptidyl inhibitor is potent and selective against Pin1 in vitro, cell-permeable, and metabolically stable in biological assays.

RESULTS AND DISCUSSION

Bicyclic Peptide Library Design, Synthesis, and Screening

We previously found that although removal of the phosphoryl group of peptide 1 significantly reduced its potency against Pin1, the nonphosphorylated peptide (Figure 1, peptide 2) was still a relatively potent Pin1 inhibitor ($K_D = 0.62$ μ M).³² We hypothesized that the potency of peptide 2 might be further improved by optimizing the sequences flanking the D-Thr-Pip-Nal motif. We therefore designed a second-generation bicyclic peptide library, bicyclo[Tm-(X¹X²X³-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]- β -Ala- β -Ala-Pra- β -Ala-Hmb- β -Ala- β -Ala-Met-resin (Figure 1, where Tm was trimesic acid, Dap was 2,3-diaminopropionic acid, β -Ala was β -alanine, Pra was L-propargylglycine, and Hmb was 4-hydroxymethyl benzoic acid), by randomizing the three N-terminal residues of peptide 2. X¹ and X² represented any of the 27 amino acid building blocks that included 12 proteinogenic L-amino acids [Arg, Asp, Gln, Gly, His, Ile, Lys, Pro, Ser, Thr, Trp, and Tyr], 5 nonproteinogenic α -L-amino acids [L-4-fluorophenylalanine (Fpa), L-norleucine (Nle), L-ornithine (Orn), L-phenylglycine (Phg), and L-Nal], 6 α -D-amino acids [D-Ala, D-Asn, D-Glu, D-Leu, D-Phe, and D-Val], and 4 N ^{α} -methylated L-amino acids [L-N ^{α} -methylalanine (Mal), L-N ^{α} -methyleucine (Mle), L-N ^{α} -methylphenylalanine (Mpa), and sarcosine (Sar)], while X³ was Asp, Glu, D-Asp, D-Glu, or D-Thr. Incorporation of these nonproteinogenic amino acids was expected to increase both the structural diversity and the proteolytic stability of the library peptides. The library had a theoretical diversity of $5 \times 27 \times 27$ or 3645 different bicyclic peptides, most (if not all) of which were expected to be cell-permeable. The library was synthesized on 500 mg of TentaGel microbeads (130 μ m, $\sim 7.8 \times 10^5$ beads/g, ~ 350 pmol peptides/bead). Peptide cyclization was achieved by forming three amide bonds between Tm and the N-terminal amine and the sidechain amines of the two Dap residues.³³ The β -Ala provides a flexible linker, while Pra serves as a handle for on-bead labeling of the bicyclic peptides with

fluorescent probes through click chemistry. The ester linkage of Hmb enables selective release of the bicyclic peptides from the resin for solution-phase binding analysis. Finally, the C-terminal Met allows peptide release from the resin by CNBr cleavage prior to MS analysis.

The library (100 mg of resin) was screened against a S16A/Y23A mutant Pin1, which has a defective WW domain.³⁴ The mutant Pin1 was produced as a maltose-binding protein (MBP) fusion at the N-terminus. During the first round of screening, Texas red-labeled MBP-Pin1 was incubated with the peptide library and fluorescent beads were removed from the library under a microscope. Three positive beads had substantially greater fluorescence intensities than the rest of hits and were directly subjected to peptide sequencing by partial Edman degradation mass spectroscopy (PED-MS)³⁵ (Figure S1 and Table 1). The other 13 fluorescent beads were subjected to a second round of screening, during which the bicyclic peptide on each bead was labeled with tetramethylrhodamine (TMR) azide at the Pra residue and released from the bead by treatment with a NaOH solution (Figure S2).

The released peptides were incubated with 5 μ M MBP-Pin1 and the increase of fluorescence anisotropy (FA) was measured. For bicyclic peptides that showed 50% FA increase (relative to the no-protein control), the corresponding beads (5 beads, which still contained the linear encoding peptides) were sequenced by PED-MS to give 4 additional complete sequences (Table 1). All 7 hit sequences contained a D-amino acid at the X³ position, consistent with the previous observation that Pin1 prefers D-pThr over pThr at this position.²⁷ There is a strong preference for hydrophobic especially aromatic hydrophobic residues at the X¹ position, but no obvious selectivity at the X² position.

Hit Optimization

The 6 hit sequences (hits 1 and 2 have the same sequence) were resynthesized with a Lys added to their C-termini, labeled with fluorescein isothiocyanate (FITC), and tested for binding to Pin1 by FA (Table 2, peptides **3-8**). All six peptides bound to Pin1 with moderate affinities ($K_D \sim 1 \mu$ M), but did not improve upon peptide **2** ($K_D = 0.62 \mu$ M). We next chose the relatively potent peptides **3** and **4** for structure-activity relationship analysis and optimization. Either expanding or contracting the size of the Pin1-binding ring (A ring) decreased the binding affinity (Table 2, peptides **9-16**). Replacement of the Ala residue of peptide **3** with amino acids containing side chains of different physicochemical properties including Arg, Asp, Ser, Tyr, and Val also failed to significantly improve the binding affinity (Table 2, peptide **17-21**). On the other hand, modification of the D-Ala residue revealed that substitution of a D-Phe at this position increases the Pin1 inhibitory activity by ~2-fold ($K_D = 0.48 \mu$ M for peptide **22**).

Peptide **4** was subjected to similar SAR studies. As observed for peptide **3**, modification of the Ala residue of peptide **4** (into Gly) had little effect (peptide **26**), but replacement of the D-Ala residue with D-Phe improved the binding affinity to Pin1 by ~2-fold (Table 2, $K_D = 0.27 \mu$ M for peptide **27**). Modifications of the Fpa residue at the X² position (e.g., replacement with other halogenated phenylalanine analogs) all decreased the inhibitor potency (peptides **28-32**). Likewise, removal of the aromatic side chain at the X¹ position

was detrimental to Pin1 binding (peptides **33** and **34**). However, substitution of halogenated D-Phe analogs improved the Pin1 binding activity (peptides **35-38**). In particular, replacement of D-Phe with D-4-fluorophenylalanine (D-Fpa)] resulted in the most potent Pin1 inhibitor of this series ($K_D = 0.12 \mu\text{M}$ for peptide **37**) (Figures 1 and 2a). Further attempts to modify the D-Thr residue or the CPP motif failed to improve the Pin1 activity (Table 2, peptides **39-45**).

Biological Evaluation

To determine whether peptide **37** binds to the catalytic site of Pin1, we first examined its ability to compete with peptide **1** for binding to Pin1 by FA analysis. Peptide **1** had previously been shown to bind to the Pin1 active site.³² As expected, peptide **37** inhibited the binding of peptide **1** to Pin1 with an IC_{50} value of 190 nM (Figure 2b). Next, we monitored the catalytic activity of Pin1 toward a peptide substrate, Suc-Ala-Glu-Pro-Phe-pNA,³⁶ in the presence of increasing concentrations of peptide **37**. Peptide **37** inhibited the Pin1 activity in a concentration-dependent manner, with an IC_{50} value of 170 nM (Figure 2c). These results demonstrate that peptide **37** binds at (or near) the active site of Pin1.

The selectivity of peptide **37** was assessed by two different tests. First, peptide **37** was tested for binding to a panel of arbitrarily selected proteins including bovine serum albumin (BSA), protein tyrosine phosphatases 1B, SHP1, and SHP2, the Grb2 SH2 domain, K-Ras, and tumor necrosis factor- α . Peptide **37** bound weakly to BSA ($K_D \sim 20 \mu\text{M}$), but not any of the other six proteins (Figure S3). We next tested peptide **37** for potential inhibition of Pin4, FKBP12, and cyclophilin A, the three other common human peptidyl-prolyl cis-trans isomerases.²⁵ Although Pin4 is structurally similar to Pin1 and has partially overlapping functions with Pin1, peptide **37** only slightly inhibited Pin4 ($\sim 15\%$ at $5 \mu\text{M}$ inhibitor), with an estimated IC_{50} value of $\sim 34 \mu\text{M}$ (Figure 2c). Peptide **37** had no effect on the catalytic activity of FKBP12 or cyclophilin A up to $5 \mu\text{M}$ concentration. These data suggest that peptide **37** is a highly specific inhibitor of Pin1.

The metabolic stability of peptide **37** was evaluated by incubating it in human serum for varying periods of time and analyzing the reaction mixtures by reversed-phase HPLC. The pThr-containing Pin1 inhibitor **1** was used as a control. After 6 h of incubation, 97% of peptide **37** remained intact, while $\sim 50\%$ of bicyclic peptide **1** was degraded after 3 h (Figure 2d). Loss of peptide **1** was accompanied by the concomitant appearance of a new peak in HPLC. Mass spectrometric analysis of the new species identified it as the dephosphorylation product of peptide **1** (peptide **2**). This result is in agreement with our previous observation that the structurally constrained bicyclic peptides are highly resistant to proteolytic degradation.³² As we had anticipated, the D-pThr moiety remains susceptible to hydrolysis by the nonspecific phosphatases in human serum.

The cellular uptake efficiency of peptide **37**, peptide **1**, and a previously reported membrane-impermeable monocyclic Pin1 inhibitor³¹ (Table 2, peptide **46**) was assessed by incubating HeLa cells with the FITC-labeled peptides ($5 \mu\text{M}$) for 2 h and quantifying the total intracellular fluorescence by flow cytometry analysis. As expected, untreated cells and cells treated with peptide **46** showed little cellular fluorescence, having mean fluorescence

intensity (MFI) values of 101 and 193, respectively (Figure 3a). By contrast, cells treated with peptides **1** and **37** gave MFI values of 2562 and 8792, respectively. Thus, peptide **37** is internalized by HeLa cells ~4-fold more efficiently than peptide **1**. Presumably, the negative charged phosphate group of peptide **1** interacted electrostatically with the positively charged CPP motif and reduced the cellular uptake efficiency of the latter.

Inhibition of Pin1 activity has previously been shown to decrease cell proliferation.³¹ We therefore examined the effect of peptide **37** on the growth of HeLa cells by using the MTT cell viability assay.³⁷ The membrane impermeable peptide **46** and a cell-permeable but inactive (defective in Pin1 binding) bicyclic peptide (Table 2, peptide **47**³²) were used as controls. Peptide **37** inhibited HeLa cell growth in a concentration-dependent manner, with an IC₅₀ value of 1.0 μ M (Figure 3b). As expected, neither peptide **46** nor **47** had any effect on cell growth. A time-course study also showed significant growth inhibition (>60%) after a 3-day treatment with 5 μ M peptide **37**, but not with peptide **46** or **47** (Figure S4). We previously reported an IC₅₀ value of 1.8 μ M for the phosphorylated bicyclic peptide **1** under similar testing conditions.³²

Finally, to ascertain that Pin1 is the molecular target of peptide **37** in vivo, we examined the intracellular protein level of a well-established Pin1 substrate, promyeloretinic leukemia protein (PML), by western blot analysis. Pin1 negatively regulates the PML level in a phosphorylation-dependent manner and inhibition of Pin1 activity is expected to stabilize PML and increase its intracellular level.³⁸ Indeed, treatment of HeLa cells with peptide **37** (0.2–5 μ M) resulted in concentration-dependent increases in the PML level (Figure 3c, d). The effect was already significant at 0.2 μ M inhibitor (1.8-fold increase in the PML level) and plateaued at ~1 μ M (3.3-fold increase). Again, bicyclic peptide **47** had no effect under the same conditions, while peptide **1** (the positive control, at 5 μ M) increased the PML level by 3.1-fold.

CONCLUSION

By screening a peptide library followed by conventional medicinal chemistry approaches, we have developed the first potent, selective, metabolically stable, and cell-permeable peptidyl inhibitor against human Pin1. Its high potency and selectivity should make it a useful chemical probe for exploring the cellular functions of Pin1. It may also serve as a lead compound for further development into therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was supported by the National Institutes of Health (Grants GM062820 and GM110208).

References

1. Yaffe MB, Schutkowski M, Shen MH, Zhou XZ, Stukenberg PT, Rahfeld JU, Xu J, Kuang J, Kirschner MW, Fischer G, Cantley LC, Lu KP. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science*. 1997; 278:1957–1960. [PubMed: 9395400]
2. Schutkowski M, Bernhardt A, Zhou XZ, Shen M, Reimer U, Rahfeld JU, Lu KP, Fischer G. Role of phosphorylation in determining the backbone dynamics of the serine/threonine-proline motif and Pin1 substrate recognition. *Biochemistry*. 1998; 37:5566–5575. [PubMed: 9548941]
3. Fischer G, Aumuller T. Regulation of peptide bond cis/trans isomerization by enzyme catalysis and its implication in physiological processes. *Rev Physiol Biochem Pharmacol*. 2003; 148:105–150. [PubMed: 12698322]
4. Lu PJ, Zhou XZ, Shen MH, Lu KP. Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science*. 1999; 283:1325–1328. [PubMed: 10037602]
5. Verdecia M, Bowman M, Lu KP, Hunter T, Noel JP. Structural basis for phosphoserine-proline recognition by group IV WW domains. *Nat Struct Biol*. 2000; 7:639–643. [PubMed: 10932246]
6. Lu KP, Zhou XZ. The prolyl isomerase Pin1: a pivotal new twist in phosphorylation signaling and disease. *Nat Rev Mol Cell Biol*. 2007; 8:904–916. [PubMed: 17878917]
7. Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, Fujimori F, Uchida T, Hunter T, Lu KP. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. *Proc Natl Acad Sci US A*. 2002; 99:1335–1340.
8. Yu Q, Geng Y, Sicinski P. Specific protection against breast cancers by cyclin D1 ablation. *Nature*. 2001; 411:1017–1021. [PubMed: 11429595]
9. Yeh ES, Lew BO, Means AR. The loss of PIN1 deregulates cyclin E and sensitizes mouse embryo fibroblasts to genomic instability. *J Biol Chem*. 2006; 281:241–251. [PubMed: 16223725]
10. Wulf GM, Ryo A, Wulf GG, Lee SW, Niu TH, Petkova V, Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. *EMBO J*. 2001; 20:3459–3472. [PubMed: 11432833]
11. Monje P, Hernandez-Losa J, Lyons RJ, Castellone MD, Gutkind JS. Regulation of the transcriptional activity of c-Fos by ERK: a novel role for the prolyl isomerase Pin1. *J Biol Chem*. 2005; 280:35081–35084. [PubMed: 16123044]
12. Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, Wulf G, Rottapel R, Yamaoka S, Lu KP. Regulation of NF- κ B signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol Cell*. 2003; 12:1413–1426. [PubMed: 14690596]
13. Crenshaw DG, Yang J, Means AR, Kornbluth S. The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1. *EMBO J*. 1998; 17:1315–1327. [PubMed: 9482729]
14. Stukenberg PT, Kirschner MW. Pin1 acts catalytically to promote a conformational change in Cdc25. *Mol Cell*. 2001; 7:1071–1083. [PubMed: 11389853]
15. Okamoto K, Sagata N. Mechanism for inactivation of the mitotic inhibitory kinase Wee1 at M phase. *Proc Natl Acad Sci US A*. 2007; 104:3753–3758.
16. Ryo A, Nakamura N, Wulf G, Liou YC, Lu KP. Pin1 regulates turnover and subcellular localization of β -catenin by inhibiting its interaction with APC. *Nat Cell Biol*. 2001; 3:793–801. [PubMed: 11533658]
17. Ryo A, Uemura H, Ishiguro H, Saitoh T, Yamaguchi A, Perrem K, Kubota Y, Lu KP, Aoki I. Stable Suppression of Tumorigenicity by Pin1-Targeted RNA Interference in Prostate Cancer. *Clin Cancer Res*. 2005; 11:7523–7531. [PubMed: 16243827]
18. Lu KP, Hanes SD, Hunter T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. *Nature*. 1996; 380:544–547. [PubMed: 8606777]
19. Winkler KE, Swenson KI, Kornbluth S, Means AR. Requirement of the prolyl isomerase Pin1 for the replication checkpoint. *Science*. 2000; 287:1644–1647. [PubMed: 10698738]
20. Driveer JA, Lu KP. Pin1: a new genetic link between Alzheimer's disease, cancer and aging. *Curr Aging Sci*. 2010; 3:158–165. [PubMed: 20735350]

21. Hennig L, Christner C, Kipping M, Schelbert B, Rucknagel KP, Grabley S, Kullertz G, Fischer G. Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone. *Biochemistry*. 1998; 37:5953–5960. [PubMed: 9558330]
22. Uchida T, Takamiya M, Takahashi M, Miyashita H, Ikeda H, Terada T, Matsuo Y, Shirouzu M, Yokoyama S, Fujimori F, Hunter T. Pin1 and Par14 peptidyl prolyl isomerase inhibitors block cell proliferation. *Chem Biol*. 2003; 10:15–24. [PubMed: 12573694]
23. Tataru Y, Lin YC, Bamba Y, Mori T, Uchida T. Dipentamethylene thiuram monosulfide is a novel inhibitor of Pin1. *Biochem Biophys Res Commun*. 2009; 384:394–398. [PubMed: 19422802]
24. Mori T, Hidaka M, Lin YC, Yoshizawa I, Okabe T, Egashira S, Kojima H, Nagano T, Koketsu M, Takamiya M, Uchida T. A high-throughput screen for inhibitors of the prolyl isomerase, Pin1, identifies a seaweed polyphenol that reduces adipose cell differentiation. *Biochem Biophys Res Commun*. 2011; 406:439–447. [PubMed: 21333629]
25. Moore J, Potter A. Pin1 inhibitors: Pitfalls, progress and cellular pharmacology. *Bioorg Med Chem Lett*. 2013; 23:4283–4291. [PubMed: 23796453]
26. Bayer E, Thutewohl M, Christner C, Tradler T, Osterkamp F, Waldmann H, Bayer P. Identification of hPin1 inhibitors that induce apoptosis in a mammalian Ras transformed cell line. *Chem Commun*. 2005:516–518.
27. Wildemann D, Erdmann F, Alvarez BH, Stoler G, Zhou XZ, Fanghanel J, Schutkowski M, Lu KP, Fischer G. Nanomolar inhibitors of the peptidyl prolyl cis/trans isomerase Pin1 from combinatorial peptide libraries. *J Med Chem*. 2006; 49:2147–2150. [PubMed: 16570909]
28. Guo C, Hou X, Dong L, Marakovits J, Greasley S, Dagostino E, Ferre R, Johnson MC, Humphreys PS, Li H, Parades GD, Piraino J, Kraynov E, Murray BW. Structure-based design of novel human Pin1 inhibitors (III): optimizing affinity beyond the phosphate recognition pocket. *Bioorg Med Chem Lett*. 2014; 24:4187–4191. [PubMed: 25091930]
29. Duncan KE, Dempsey BR, Killip LE, Adams J, Bailey ML, Lajoie GA, Litchfield DW, Brandl CJ, Shaw GS, Shilton BH. Discovery and characterization of a nonphosphorylated cyclic peptide inhibitor of the peptidylprolyl isomerase, Pin1. *J Med Chem*. 2011; 54:3854–3865. [PubMed: 21545152]
30. Xu GG, Slebodnick C, Etzkorn FA. Cyclohexyl Ketone Inhibitors of Pin1 Dock in a Trans-Diaxial Cyclohexane Conformation. *PLoS ONE*. 2012; 7:e44226. [PubMed: 23028504]
31. Liu T, Liu Y, Kao H, Pei D. Membrane Permeable Cyclic Peptidyl Inhibitors against Human Peptidylprolyl Isomerase Pin1. *J Med Chem*. 2010; 53:2494–2501. [PubMed: 20180533]
32. Lian W, Jiang B, Qian Z, Pei D. Cell-Permeable Bicyclic Peptide Inhibitors against Intracellular Proteins. *J Am Chem Soc*. 2014; 136:9830–9833. [PubMed: 24972263]
33. Lian WL, Upadhyaya P, Rhodes C, Liu Y, Pei D. Screening Bicyclic Peptide Libraries for Protein–Protein Interaction Inhibitors: Discovery of a Tumor Necrosis Factor- α Antagonist. *J Am Chem Soc*. 2013; 135:11990–11995. [PubMed: 23865589]
34. Shen M, Stukenburg PT, Kirschner MW, Lu KP. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. *Genes Dev*. 1998; 12:706–720. [PubMed: 9499405]
35. Joo SH, Xiao Q, Ling Y, Gopishetty B, Pei D. Highthroughput sequence determination of cyclic peptide library members by partial Edman degradation/mass spectrometry. *J Am Chem Soc*. 2006; 128:13000–13009. [PubMed: 17002397]
36. Kofron JL, Kuzmic P, Kishore V, Colonbonilla E, Rich DH. Determination of kinetic constants for peptidyl prolyl cis-trans isomerases by an improved spectrophotometric assay. *Biochemistry*. 1991; 30:6127–6134. [PubMed: 2059621]
37. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods*. 1989; 119:203–210. [PubMed: 2470825]
38. Reineke EL, Lam M, Liu O, Liu Y, Stanya KJ, Chang KS, Means AR, Kao HY. Degradation of the tumor suppressor PML by Pin1 contributes to the cancer phenotype of breast cancer MDA-MB-231 cells. *Mol Cell Biol*. 2008; 28:997–1006. [PubMed: 18039859]

Figure 1. Evolution of bicyclic peptide inhibitors against Pin1. The structural moieties derived from library screening are shown in red, while the changes made during optimization are shown in blue.

Figure 2.

Biochemical characterization of peptide 37. (a) Binding to FITC-labeled peptide 37 to Pin1 as analyzed by fluorescent anisotropy (FA). (b) Competition between peptide 37 and FITC-labeled peptide 1 (100 nM) for binding to Pin1 (400 nM) as monitored by FA. (c) Effect of peptide 37 on the cis-trans isomerase activity of Pin1, Pin4, FKBP12, and cyclophilin A using Suc-Ala-Glu-Pro-Phe-pNA as substrate. (d) Comparison of the serum stability of peptides 1 and 37.

Figure 3. Cellular activity of peptide 37. (a) Cellular uptake of peptides 1, 37, and 46 (5 μ M) by HeLa cells as analyzed by flow cytometry. MFI, mean fluorescence intensity; none, untreated cells (no peptide). (b) Anti-proliferative effect of peptides 37, 46, and 47 on HeLa cells as measured by MTT assay. (c) Western blots showing the effect of peptides 1, 37 and 47 on the protein level of PML in HeLa cells. β -Actin was used as loading control. (d) Quantification of western blot results from (c). Data reported were after background subtraction and represent the mean $\pm$ SD from 3 independent experiments.

Table 1Hit Sequences from Peptide Library Screening^a

hit	X ¹	X ²	X ³
1	Pro	Sar	D-Asp
2	Pro	Sar	D-Asp
3	D-Phe	Fpa	D-Thr
4	His	Phg	D-Thr
5	Mpa	Ile	D-Glu
6	Phg	His	D-Glu
7	Mpa	Gly	D-Thr

^aHits 1-3 were selected from 1st-round screening, whereas hits 4-7 were selected after 2nd-round screening.

Table 2

Sequences and Pin1 Binding Affinities of Peptides Used in This Work

<i>Peptide</i>	<i>Peptide sequence</i>	<i>K_D</i> (μM)
1	bicyclo[Tm-(D-Ala-Sar-D-pThr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.072 ± 0.021
2	bicyclo[Tm-(D-Ala-Sar-D-Thr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.62 ± 0.12
3	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.87 ± 0.17
4	bicyclo[Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.67 ± 0.12
5	bicyclo[Tm-(Mpa-Gly-D-Thr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.08 ± 0.12
6	bicyclo[Tm-(Phg-His-D-Glu-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.47 ± 0.19
7	bicyclo[Tm-(Mpa-Ile-D-Glu-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.25 ± 0.20
8	bicyclo[Tm-(His-Phg-D-Thr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.40 ± 0.24
9	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	2.59 ± 0.37
10	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	3.42 ± 0.61
11	bicyclo[Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.90 ± 0.25
12	bicyclo[Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	2.36 ± 0.48
13	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Ala-β-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	2.08 ± 0.31
14	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-β-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.75 ± 0.18
15	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-β-Ala-β-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	4.83 ± 0.96
16	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Ala-D-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	2.49 ± 0.57
17	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Tyr-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	2.17 ± 0.55
18	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Val-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.75 ± 0.24
19	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Arg-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.72 ± 0.09
20	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Asp-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	3.19 ± 0.50
21	bicyclo[Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg-Ser-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.57 ± 0.11
22	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Arg-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.48 ± 0.07
23	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Arg-D-Val)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.92 ± 0.19
24	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Arg-D-Arg)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.31 ± 0.10
25	bicyclo[Tm-(Pro-Sar-D-Asp-Pip-Nal-Arg-Arg-D-Asp)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	4.60 ± 1.42
26	bicyclo[Tm-(D-Phe-4-Fpa-D-Thr-Pip-Nal-Arg-Gly-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.74 ± 0.11
27	bicyclo[Tm-(D-Phe-4-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.27 ± 0.08
28	bicyclo[Tm-(D-Phe-Phe-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.26 ± 0.28
29	bicyclo[Tm-(D-Phe-3,4-diFpe-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.41 ± 0.10
30	bicyclo[Tm-(D-Phe-4-ClPhe-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.78 ± 0.05
31	bicyclo[Tm-(D-Phe-His-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.68 ± 0.17
32	bicyclo[Tm-(D-Phe-4-BrPhe-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.78 ± 0.42
33	bicyclo[Tm-(D-Ala-4-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.49 ± 0.11
34	bicyclo[Tm-(D-Val-4-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	1.07 ± 0.16
35	bicyclo[Tm-(D-2-Fpa-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.59 ± 0.05
36	bicyclo[Tm-(D-3-Fpa-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.39 ± 0.05
37	bicyclo[Tm-(D-4-Fpa-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.12 ± 0.03
38	bicyclo[Tm-(D-4-CyanoPhe-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.35 ± 0.04
39	bicyclo[Tm-(D-4-Phe-Fpa-D-Ile-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.46 ± 0.17

<i>Peptide</i>	<i>Peptide sequence</i>	<i>K_D</i> (μM)
40	<i>bicyclo</i> [Tm-(D-4-Phe-Fpa-D-Nle-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.71 ± 0.12
41	<i>bicyclo</i> [Tm-(D-4-Phe-Fpa-D-homoGlu-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	0.87 ± 0.08
42	<i>bicyclo</i> [Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Arg-Arg-Arg-Arg-Nal-Phe-Dap)]-Lys	0.98 ± 0.18
43	<i>bicyclo</i> [Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Arg-Arg-Nal-Phe-Arg-Arg-Dap)]-Lys	1.38 ± 0.16
44	<i>bicyclo</i> [Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(Arg-Nal-Arg-Phe-Arg-Arg-Dap)]-Lys	0.45 ± 0.05
45	<i>bicyclo</i> [Tm-(D-Phe-Fpa-D-Thr-Pip-Nal-Arg-Ala-D-Phe)-Dap-(D-Arg-Arg-D-Arg-Arg-Nal-D-Phe-Dap)]-Lys	3.10 ± 0.38
46	<i>cyclo</i> (D-Ala-Sar-D-pThr-Pip-Nal-Tyr-Gln)-Lys	0.24 ± 0.04
47	<i>bicyclo</i> [Tm-(D-Ala-Sar-D-Thr-D-Ala-Nal-Arg-Ala-D-Ala)-Dap-(Phe-Nal-Arg-Arg-Arg-Arg-Dap)]-Lys	No binding