Membrane Permeable Cyclic Peptidyl Inhibitors against Human Peptidylprolyl Isomerase Pin1

Tao Liu,[†] Yu Liu,[§] Hung-Ying Kao,[§] and Dehua Pei*,[†,‡]

[†]*Ohio State Biochemistry Program, and* [‡]*Department of Chemistry, The Ohio State University, Columbus, Ohio 43210, and*
[§]*Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106*

Peptidylprolyl isomerase Pin1 regulates the function and/or stability of phosphoproteins by altering the conformation of specific pSer/pThr-Pro peptide bonds. In this work, a cyclic peptide library was synthesized and screened against the catalytic domain of human Pin1. The selected inhibitors contained a consensus motif of D-pThr-Pip-Nal (where Pip is L-piperidine-2-carboxylic acid and Nal is L-2-naphthylalanine). Representative compounds were tested for binding to Pin1 by isothermal titration calorimetry and inhibition of Pin1 activity, and the most potent inhibitors had K_D (and K_I) values in the low nanomolar range. Treatment of breast cancer cells with the inhibitors, which were rendered membrane permeable by attachment of an octaarginine sequence, inhibited cell proliferation and increased the protein levels of two previously established Pin1 substrates, PML and SMRT. Finally, a second generation of cell permeable Pin1 inhibitors was designed by replacing the noncritical residues within the cyclic peptide ring with arginine residues and shown to have antiproliferative activity against the cancer cells.

Introduction

Pin1 is a member of the evolutionarily conserved peptidyl-prolyl isomerase (PPIase) family, which catalyzes the cis–trans isomerization of peptidylprolyl bonds in its substrate proteins.[1] In addition to the catalytic domain, Pin1 also contains an N-terminal WW domain, which mediates protein–protein interactions.[2] Both the catalytic domain and the WW domain of Pin1 recognize specific Ser/Thr-Pro motif(s) in its substrate proteins after the serine or threonine is phosphorylated.[3] Cis–trans isomerization by Pin1 can have a wide range of effects on its target proteins.[4] For example, Pin1-catalyzed cis–trans isomerization regulates the catalytic activity of cell-cycle phosphatase CDC25C[5–7] and kinase Wee1.[8] It has been shown to both increase and decrease the phosphorylation levels of proteins such as CDC25C,[7] RNA polymerase II,[9] and topoisomerase II.[10] Pin1 is known to modulate the in vivo stability of substrate proteins including cyclin D1,[11,12] cyclin E,[13] c-MYC,[14] p53,[15–17] and p73.[18] Isomerization by Pin1 enhances the transcriptional activity of c-Jun,[11] c-Fos,[19] and NF-κB.[20] Finally, Pin1 is capable of altering the subcellular localization and the protein–protein interaction of its substrate proteins (e.g., β-catenin).[21,22]

Since many of the Pin1 substrate proteins are important for cell-cycle regulation, Pin1 plays a key role in regulating the entry into mitosis and is required for the proper progression through mitosis.[23,24] Pin1 activity is tightly regulated at multiple levels, and its expression is generally correlated with cell proliferative potential in normal human tissues. Furthermore, Pin1 activity is up-regulated in many human tumors (e.g., breast, prost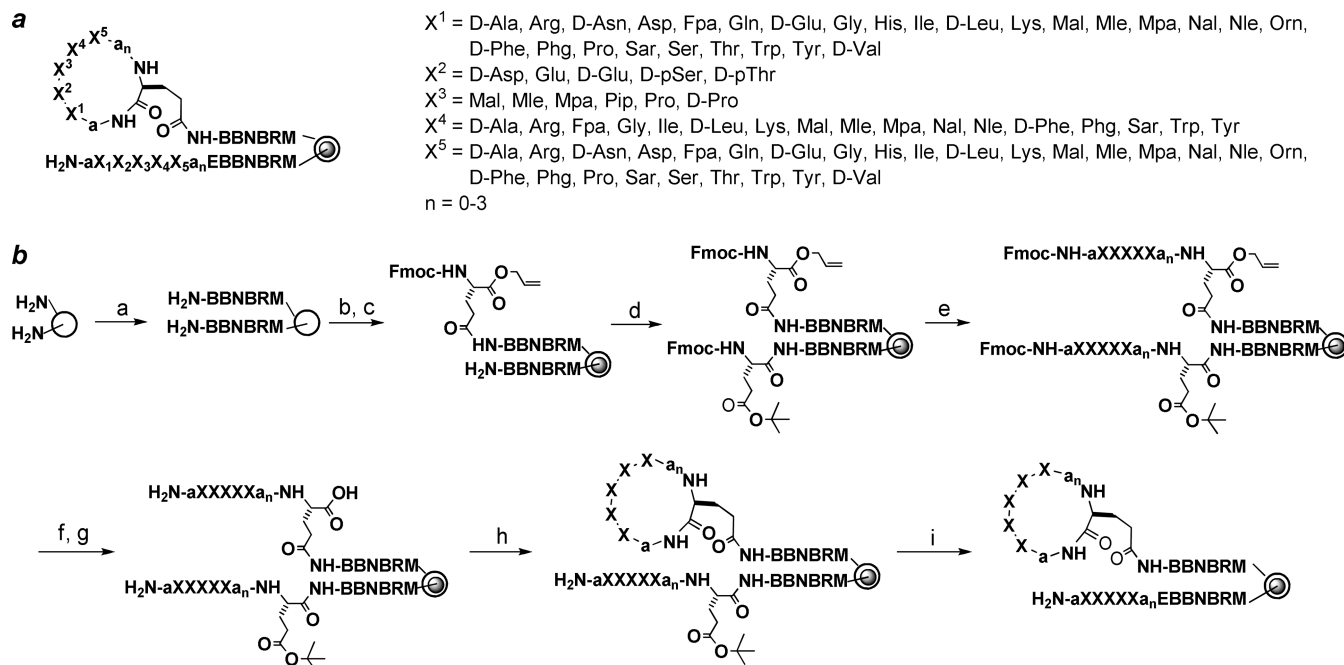ate, and lung cancers), and its overexpression correlates with tumor grade.[11,14] Depletion of Pin1 causes mitotic arrest and apoptosis in budding yeast and cancer cell lines.[23,25] It has been suggested that cancer cells expressing very high levels of Pin1 are more sensitive to Pin1 inhibitors.[26] These observations suggest that specific Pin1 inhibitors may provide a novel class of anticancer agents with low toxicity to the normal tissues.

Pin1 has already been subjected to extensive inhibitor design efforts. A number of small-molecule Pin1 inhibitors have been discovered through screening efforts as well as structure-based design, including juglone,[27] aryl indanyl ketones,[28] 3-benzofuranones,[29] dipentamethylene thiuram monosulfide (DTM),[30] and nonpeptidic pSer-Pro mimetics.[31] In general, these small molecules lack sufficient potency and/or selectivity for Pin1. Recently, a number of peptidyl Pin1 inhibitors have also been reported, some of which are highly potent and specific for Pin1.[32–35] However, the reported peptidyl inhibitors are susceptible to proteolytic degradation and impermeable to the cell membrane, limiting their potential applications as therapeutic agents or tools for in vivo studies. Cyclization of a peptide is a general strategy to improve its stability against proteolysis. In addition, a cyclic peptide may bind to its desired target with higher affinity and specificity than the linear peptide counterpart because of its reduced conformational freedom. In this work, we designed, synthesized, and screened a cyclic peptide library against the catalytic domain of Pin1 to identify a family of potent cyclic peptidyl inhibitors of Pin1. Subsequent modification of the cyclic peptidyl inhibitors through incorporation of arginine residues resulted in Pin1 inhibitors that are membrane permeable and active in cellular studies.

Results and Discussion

Design and Synthesis of Cyclic Peptide Library. Previous substrate/inhibitor specificity studies have revealed that the

---

*To whom correspondence should be addressed. Address: Department of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH 43210. Telephone: (614) 688-4068. Fax: (614) 292-1532. E-mail: pei.3@osu.edu.

X^1 = D-Ala, Arg, D-Asn, Asp, Fpa, Gln, D-Glu, Gly, His, Ile, D-Leu, Lys, Mal, Mle, Mpa, Nal, Nle, Orn, D-Phe, Phg, Pro, Sar, Ser, Thr, Trp, Tyr, D-Val

X^2 = D-Asp, Glu, D-Glu, D-pSer, D-pThr

X^3 = Mal, Mle, Mpa, Pip, Pro, D-Pro

X^4 = D-Ala, Arg, Fpa, Gly, Ile, D-Leu, Lys, Mal, Mle, Mpa, Nal, Nle, D-Phe, Phg, Sar, Trp, Tyr

X^5 = D-Ala, Arg, D-Asn, Asp, Fpa, Gln, D-Glu, Gly, His, Ile, D-Leu, Lys, Mal, Mle, Mpa, Nal, Nle, Orn, D-Phe, Phg, Pro, Sar, Ser, Thr, Trp, Tyr, D-Val

n = 0–3

Figure 1. (a) Pin1 inhibitor library design and the building blocks. (b) Synthesis of the Pin1 inhibitor library. Reagents: (a) standard Fmoc/HBTU chemistry; (b) soak in water; (c) 0.5 equiv of N^α-Fmoc-Glu(δ-NHS)-O-CH$_2$CH=CH$_2$ in Et$_2$O/CH$_2$Cl$_2$; (d) Fmoc-Glu(^{t}Bu)-OH, HBTU; (e) split-and-pool synthesis by Fmoc/HATU chemistry; (f) Pd(PPh$_3$)$_4$; (g) piperidine; (h) PyBOP, HOBt; (i) TFA.

active site of Pin1 prefers a pSer/pThr-Pro motif surrounded by aromatic or positively charged residues.[3,32,35] In a cocrystal structure of Pin1 bound to a peptidyl inhibitor, the D-pThr-Pip-Nal (where Pip is L-piperidine-2-carboxylic acid and Nal is L-2-naphthylalanine) tripeptide portion of the inhibitor makes intimate contacts with the catalytic site.[36] Moreover, the inhibitor adopts a β-turn conformation, suggesting that a cyclic peptide containing the pThr-Pip-Nal motif should be accommodated by the enzyme active site. We therefore designed a cyclic peptide library in the form of cyclo(aX^{1}X^{2}X^{3}X^{4}X^{5}a$_n$E)BBNBRM-resin (Figure 1), where X^1–X^5 represent random amino acids, a is D-Ala, and B^a is β-Ala. To increase the probability of identifying positive hits against Pin1, the building blocks at the most critical positions (X^2, X^3, and X^4) were judiciously selected on the basis of known Pin1 substrate sequences in the SWISS-PROT database, Pin1 substrate specificity,[3] and the structures of previously reported Pin1 inhibitors.[32,35] Specifically, the X^2 residue was biased toward D-pSer and D-pThr, which have previously been shown to be preferred by the Pin1 active site.[35] We also included Glu, D-Glu, and D-Asp at the X^2 position as potential pSer and pThr surrogates, hoping to obtain a Pin1 inhibitor that is free of pSer and pThr residues, which are metabolically unstable in vivo and impermeable to the cell membrane. At the X^3 position,

Pro, D-Pro, and its commonly used analogue, L-Pip, were selected. Three N^α-methylated amino acids, L-N^α-methylalanine (Mal), L-N^α-methylleucine (Mle), and L-N^α-methylphenylalanine (Mpa), were also included because they, like Pro and Pip, can exist in both cis and trans configurations. The X^4 position included 17 hydrophobic, aromatic, or positively charged residues known to be preferred by Pin1 at this position. At the less critical X^1 and X^5 positions, we incorporated 12 proteinogenic 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, used as a replacement of Met), L-ornithine (Orn), L-phenylglycine (Phg), and L-Nal], six α-D-amino acids [D-Ala, D-Asn, D-Glu, D-Leu, D-Phe, and D-Val], and four N^α-methylated L-amino acids [Mal, Mle, Mpa, and sarcosine (Sar)]. These nonproteinogenic amino acids, many of which are frequently found in naturally occurring nonribosomal peptides, were included to increase the structural diversity of the library and the stability of library members against proteolytic degradation. Other criteria used during the selection of the building blocks included minimization of mass degeneracy and commercial availability/cost. Our previous studies have shown that the ring size of a cyclic peptide can significantly influence its binding affinity to a protein target.[37] We thus incorporated zero to three D-alanine residues C-terminal to the random region to generate four different ring sizes (hepta-, octa-, nona-, and decapeptides). A D-alanine was added to the N-terminus of the random sequence to give more uniform peptide cyclization yields. The glutamate residue serves as a convenient anchor for attachment to the solid phase as well as the point of N-to-C peptide cyclization. The invariant BBNBRM sequence was chosen to provide a flexible linker between the cyclic peptide and the resin (β-Ala), facilitate peptide release by CNBr (Met), and provide a fixed positive charge for MALDI-MS analysis (Arg). The library has a theoretical diversity of $27 \times 5 \times 6 \times 17 \times 27 \times 4$ or 1.5×10^6 and was synthesized

---

^{a} Abbreviations: B, β-alanine; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DIEA, diisopropylethylamine; Fpa, L-4-fluorophenylalanine; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; ITC, isothermal titration calorimetry; Mal, L-N^α-methylalanine; MBP, maltose-binding protein; Mle, L-N^α-methylleucine; Mpa, L-N^α-methylphenylalanine; Nal, L-2-naphthylalanine; Nle, L-norleucine; NMM, *N*-methylmorpholine; Orn, L-ornithine; PED-MS, partial Edman degradation mass spectrometry; Phg, L-phenylglycine; Pip or HoPro, L-piperidine-2-carboxylic acid; pSer, phosphoserine; pThr, phosphothreonine; PyBop, benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; SA-AP, streptavidin-alkaline phosphatase; Sar, sarcosine.

on 1 g of TentaGel microbeads (90 μm, ~2.86 × 10^{6} beads/g, ~100 pmol peptides/bead) as described in Experimental Section.

Library Screening against Pin1 Catalytic Domain. Pin1 contains two functional domains, an N-terminal WW domain and a C-terminal catalytic domain, both of which are capable of binding to pSer/pThr-Pro sequences. To prevent the WW domain from binding to any potential library member, we used a S16A/Y23A mutant Pin1 protein for screening. These mutations do not affect the structure of Pin1 but abolish the ability of the WW domain to bind pSer/pThr-Pro ligands.[2,38] The mutant Pin1 was expressed in *Escherichia coli* as a fusion protein with the maltose-binding protein (MBP) at its N-terminus to facilitate its purification and chemical labeling. Screening of the peptide library against MBP-Pin1 was performed by two different methods. In method A, Texas-red labeled MBP-Pin1 was incubated with the peptide library and fluorescent beads were identified under a fluorescent microscope, removed from the library by a micropipet, and individually sequenced by partial Edman degradation mass spectrometry (PED-MS).[39] In method B, MBP-Pin1 was chemically labeled with a biotin molecule. Binding of the biotinylated protein to a positive bead recruits a streptavidin−alkaline phosphatase (SAAP) conjugate to the bead surface. Subsequent incubation with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) produced a turquoise color on the positive beads, which were isolated and similarly sequenced. Each method resulted in a large number of hits with similar color intensity, suggesting that they have similar affinities to the Pin1 active site. Altogether, 17 (method A) and 25 of the most colored beads (method B) were isolated from 50 mg of the library and their sequences are shown in Table 1. In addition, 50 mg of the library was first screened by method A and the positive beads were subjected to a second round of screening by method B, producing 9 positive beads (Table 1, peptides 43−51).

Inspection of the selected sequences revealed that Pin1 has the most stringent selectivity at the X^2 and X^3 positions, in agreement with the previous reports.[3,32,35] Out of the 51 sequences, 45 contained a D-pThr as the X^2 residue; only six sequences had D-pSer, Glu, or D-Asp at this position. The overwhelming preference for a D-pThr over D-pSer is surprising, since the reported Pin1 substrates contain L-pSer much more frequently than L-pThr. The crystal structure shows that the additional methyl group of D-pThr projects toward a hydrophobic surface in the Pin1 active site and may therefore provide additional binding energy through hydrophobic interactions.[36] At the X^3 position, Pin1 strongly prefers a Pip residue (36 sequences), followed by Pro (9 sequences), Mpa (4 sequences), and Mal (2 sequences). Note that none of the selected peptides contained a D-Pro at this position, indicating that an L-amino acid is required for proper conformation and/or binding to the Pin1 active site. The nature of the amino acids selected at the X^4 position somewhat depended on the screening method. All 17 peptides selected by method A contained a hydrophobic aromatic amino acid, predominantly Nal (Table 1), whereas method B selected predominantly positively charged residues (especially Arg) along with hydrophobic residues. The reason for this difference is not yet clear. However, previous studies have shown that both aromatic and positively charged residues are preferred by Pin1 at the pThr + 2 position.[3,32,35] Pin1 apparently has little selectivity at the pThr − 1 position, as

Table 1. Sequences of Pin1 Inhibitors Selected from the Peptide Library[a]

peptide no.	X^1	X^2	X^3	X^4	X^5	no. of D-Ala
1	D-Leu	D-pThr	Pip	Nal	Arg	0
2	His	D-pThr	Pip	Trp	Ile	0
3	Sar	D-pThr	Pip	Nal	Tyr	0
4	D-Phe	D-pThr	Pip	Nal	Lys	0
5	Gly	D-pThr	Pip	Nal	Orn	0
6	Tyr	D-pThr	Pip	Nal	Fpa	1
7	Mle	D-pThr	Pip	Nal	Trp	1
8	Trp	D-pThr	Pip	Nal	Orn	1
9	Trp	D-pThr	Pro	Mal	Nal	2
10	Mal	D-pThr	Pro	Nal	Phg	2
11	Tyr	D-pThr	Pip	Nal	Gln	2
12	Fpa	D-pThr	Pro	Trp	Nal	2
13	Gln	D-pThr	Pip	Nal	Phg	2
14	Mle	D-pThr	Pip	Nal	Asp	2
15	Fpa	D-pThr	Pro	Nal	Tyr	3
16	Nal	D-pThr	Pip	Tyr	Phg	3
17	Arg	D-pThr	Pip	Nal	Mpa	3
18	D-Asn	D-pThr	Pro	Arg	Arg	0
19	Tyr	D-pThr	Pip	Lys	Try	0
20	Ile	D-pThr	Pip	Arg	Phg	0
21	Arg	D-pThr	Pip	Arg	Phg	0
22	Gln	Glu	Mpa	Mal	Ile	0
23	Fpa	D-pThr	Pip	Arg	Lys	1
24	Pro	D-pThr	Pip	Arg	Ile	1
25	His	D-pThr	Mal	Nal	Orn	1
26	Gln	D-pThr	Pip	Arg	His	1
27	Lys	Glu	Mpa	Nle	D-Leu	1
28	Fpa	D-pThr	Pip	Tyr	Arg	1
29	Orn	D-pThr	Pip	Nal	Arg	1
30	Lys	D-Asp	Mpa	D-Ala	Arg	1
31	Lys	D-pThr	Pip	Arg	Arg	1
32	D-Phe	D-pThr	Pip	Arg	Fpa	1
33	Lys	D-pThr	Pip	Arg	Fpa	1
34	Nal	D-pThr	Mal	Nle	Arg	2
35	Mal	D-pThr	Pip	Arg	Phg	2
36	Lys	D-pThr	Pip	Arg	Lys	3
37	Thr	D-pThr	Pip	Nle	Arg	3
38	Ser	D-pThr	Pip	Lys	Arg	3
39	Phg	D-pThr	Pip	Fpa	Arg	3
40	Ile	D-pThr	Pip	Arg	Thr	3
41	Arg	D-pThr	Pip	Arg	Tyr	3
42	Arg	D-pSer	Pro	Nal	Arg	3
43	Tyr	D-pSer	Pro	Nal	Orn	0
44	Ile	D-pSer	Pro	Nal	Orn	0
45	Gln	D-pThr	Pip	Nal	Arg	1
46	Orn	D-pThr	Mal	Fpa	Arg	1
47	Trp	D-pThr	Pip	Lys	Tyr	1
48	Mle	D-pThr	Pip	Tyr	Lys	1
49	Mle	D-pThr	Pip	Tyr	Orn	3
50	Nal	D-pThr	Pip	Arg	Arg	3
51	Lys	D-pThr	Pro	Arg	Nle	3

[a] Numbers 1−17 were selected from Texas-Red based screening. Numbers 18−42 were selected from biotin-SAAP based screening. Numbers 43−51 were selected from a double screening, where the first round is biotin-SAAP based and the second round is Texas-Red based screening.

a wide range of amino acids were selected as the X^1 residue. This is in agreement with the structural studies, which showed no significant interaction between Pin1 and the pThr-1 residue.[36] A range of amino acids are also accepted at the pThr + 3 (or X^5) position. It is clear, however, that D-amino acids or N^α-methylated amino acids are not tolerated at this position. Out of the 51 sequences, only peptide 27 contained a D-Leu at the X^5 position; however, this peptide is

**Table 2.** Inhibition Constants of Cyclic Peptide Inhibitors against Pin1

Pin1 inhibitor	peptide sequence	IC$_{50}$ (μM)^a	K_D (μM)^b
A	cyclo(D-Ala-Sar-D-pThr-Pip-Nal-Tyr-Gln)	0.031 ± 0.003	0.047 ± 0.0022
B	cyclo(D-Ala-Gly-D-pThr-Pip-Nal-Orn-Gln)	0.043 ± 0.001	0.048 ± 0.004
C	cyclo(D-Ala-Ile-D-pSer-Pro-Nal-Orn-Gln)	1.1 ± 0.1	0.92 ± 0.06
D	cyclo(D-Ala-Gln-Glu-Mpa-Mal-Ile-Gln)	63 ± 15	ND
E	cyclo(D-Ala-Sar-D-pThr-Pip-Nal-Tyr-Gln)-Lys-SH	0.032 ± 0.010	ND
F	cyclo(D-Arg-D-Arg-D-pThr-Pip-Nal-Arg-Gln)	0.22 ± 0.04	0.033 ± 0.002
G	cyclo(D-Arg-D-Arg-D-pThr-Pip-Nal-Arg-D-Arg-D-Arg-D-Arg-Gln)	2.5 ± 0.4	0.17 ± 0.01
H	cyclo(Arg-Arg-Arg-D-pThr-Pip-Nal-Arg-Arg-Gln)	1.1 ± 0.2	0.98 ± 0.03
I	cyclo(D-Arg-D-Arg-D-Thr-Pip-Nal-Arg-Gln)	>300	ND
J	cyclo(D-Arg-D-Arg-D-Thr-Pip-Nal-Arg-D-Arg-D-Arg-D-Arg-Gln)	>300	ND
K	cyclo(Arg-Arg-Arg-D-Thr-Pip-Nal-Arg-Arg-Gln)	>300	ND

^{a} IC$_{50}$ values were determined by the chymotrypsin-coupled PPIase inhibition assay. ^{b} K_D values were determined by ITC. ND, not determined or could not be accurately determined.

unique in that it contains a Glu instead of D-pThr at the X^2 position. Finally, among the selected peptides, the four different ring sizes were essentially equally populated, suggesting that the actual ring size is not critical for binding to Pin1. This may be due to the fact that Pin1 binds to the end of a hairpin structure (the β-turn), and therefore, elongation of the stem region of the hairpin does not affect the interactions between Pin1 and the peptide inhibitor.

Analysis of Selected Inhibitors against Pin1 in Vitro. Four of the selected peptides (A−D, which correspond to peptides 3, 5, 44, and 22 in Table 1, respectively) were chosen for resynthesis and solution-phase inhibition assay against Pin1 (Table 2). Since Pin1 did not show any obvious preference for a particular ring size, we chose the four peptides that had the smallest ring size (cycloheptapeptide). Peptides A and B match closely to the major consensus sequence. Peptides C and D were selected to test the effectiveness of D-pSer-Pro and Glu-Mpa motifs as the pharmacophore, which were less frequently selected from the library compared to the D-pThr-Pip motif. The four peptides were synthesized on Rink amide resin, released, side chain deprotected, and purified by reversed-phase HPLC. Their ability to inhibit Pin1 catalytic activity was first assessed by a chymotrypsin-coupled assay using peptide Suc-Ala-Glu-Pro-Phe-pNA as the substrate.[33,40] As expected, peptides A and B showed potent inhibition of Pin1 activity, with IC$_{50}$ values of 0.031 and 0.043 μM, respectively (Table 2). Peptide C was ~20-fold less potent, with an IC$_{50}$ of 1.1 μM, confirming that the D-pSer-Pro motif is less effective than the D-pThr-Pip motif. Peptide D showed only weak inhibition against Pin1 (IC$_{50}$ = 63 μM). Next, isothermal titration calorimetry (ITC) was employed to determine the accurate binding affinity of the four peptides against Pin1, and dissociation constants (K_D) of 0.047, 0.048, and 0.92 μM were obtained for peptides A, B, and C, respectively, in good agreement with the IC$_{50}$ values derived from the enzyme inhibition assays (Figure 2). The K_D value for peptide D was too high to be reliably determined by ITC.

Synthesis of Cell Permeable Pin1 Inhibitors and Inhibition of Pin1 Function in Vivo. Initial cell proliferation assays showed that peptides A−C had no obvious activity against whole cells. We suspected that the negatively charged phosphopeptides might not be able to cross the cell membrane to reach the intended Pin1 target. We therefore attached an octaarginine sequence to peptide A though the invariant glutamate side chain via a disulfide bond (Figure 3a, peptide E). We have previously used the octaarginine sequence to transport other peptides into eukaryotic cells.[37,41] Upon entry into the cells, the
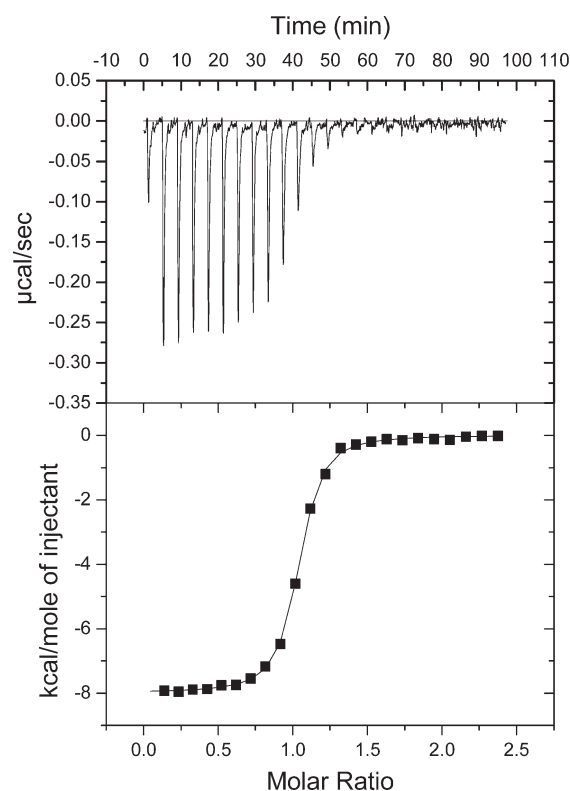
Figure 2. Isothermal titration calorimetry of the binding interaction between Pin1 catalytic domain and peptide A (K_D = 47 nM).

reducing environment in the cytoplasm would reduce the disulfide bond to release the cyclic peptide. In vitro Pin1 activity assay indicated that the released cyclic peptide had the same potency as peptide A (IC$_{50}$ = 0.032 μM) (Table 2). Human breast cancer cells were grown in the presence of cyclic peptide E (1 μM). As expected, peptide E showed significant inhibition of cancer cell proliferation at day 6 (Figure 3b). To ascertain that Pin1 is inhibited by peptide E, we examined the intracellular protein levels of two known Pin1 substrates, promyelocytic leukemia protein (PML) and silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), by Western blot analysis (Figure 3c). It has previously been established that the stability of these two proteins is negatively regulated by Pin1 in a phosphorylation-dependent manner; i.e., a reduction of Pin1 activity increases the intracellular level of both proteins.[42,43] Treatment of two different human cancer cell lines (HeLa and BT-474) with increasing concentrations of peptide E (0.1−1 μM) resulted in significantly elevated protein
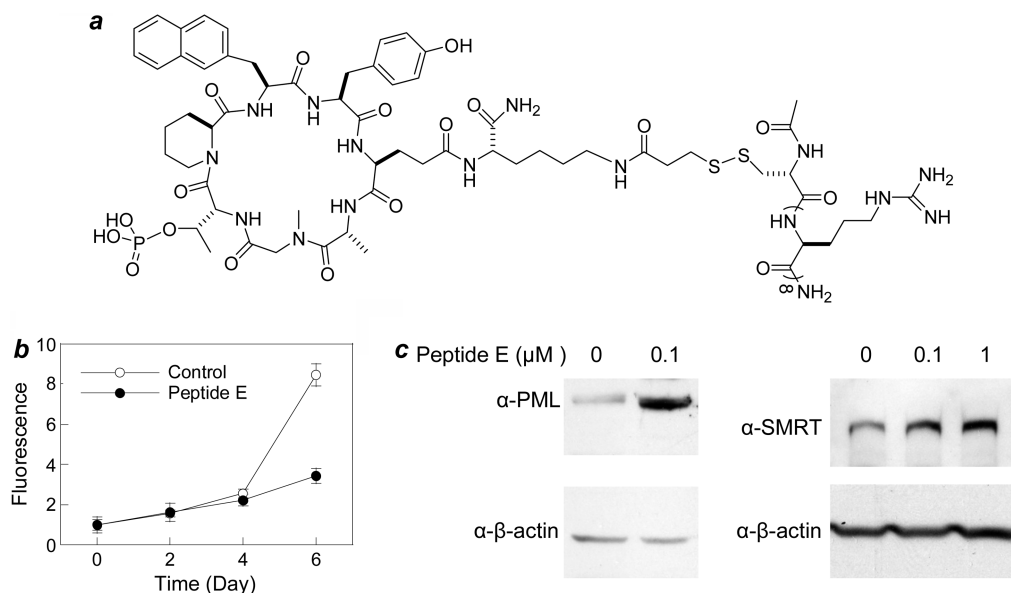
Figure 3. Activity of peptide E against human cancer cells: (a) structure of peptide E; (b) inhibition of BT-474 breast cancer cell growth by peptide E (1 μM). The control experiment contained no peptide E. (c) Western blots showing the protein levels of PML and SMRT in HeLa cells in the presence of 0, 0.1, and 1 μM peptide E. Blotting with anti-β-actin antibodies showed equal protein loading across all lanes.

levels of both SMRT and PML (Figure 3c). In an earlier study, Wildemann et al. showed that inhibition of Pin1 by a linear phosphopeptide inhibitor dramatically decreased the survival rate at late stages of development in *Xenopus laevis*.[44] Taken together, these observations indicate that peptide E was internalized and inhibited Pin1 activity in vivo.

Design of Second-Generation Cell Permeable Pin1 Inhibitors. Encouraged by the in vivo activity of octaarginine-derivatized cyclic peptide E, we next explored the possibility of directly replacing the residues in peptide E that are noncritical for Pin1 binding with L-and/or D-arginine to improve its membrane permeability. Our screening results revealed that Pin1 has little selectivity at the X^1 position and prefers a positively charged L-arginine at the X^5 position (Table 1). Thus, we synthesized cyclic peptide F, which is derived from peptide A but contained D- and L-arginine at the X^1 and X^5 positions, respectively. In addition, the D-Ala at the pThr-2 position was replaced with a D-arginine. We also synthesized peptide G, which is identical to peptide F except that three additional D-arginine residues were added at the pThr + 4 to pThr + 6 positions to further increase the number of positive charges. Finally, peptide H was designed to contain the sequence motif RRQRRR, which is a part of the cell penetrating peptide RKKRRQRRR derived from HIV Tat protein.[45] Three unphosphorylated peptides (peptides I−K) were synthesized as negative controls. The peptides were purified by HPLC and tested for binding to Pin1 by ITC and inhibition of Pin1 activity. Peptides F, G, and H remained relatively potent inhibitors of Pin1, with K_D values in the range 0.033−0.98 μM, whereas peptides I−K showed no detectable inhibition against Pin1 (Table 2). To determine whether peptides F−K are membrane permeable, we labeled the peptides with fluorescein isothiocyanate (FITC) at the side chain of the invariant glutamate residue via a lysine linker (see Figure S5 in Supporting Information for structures). HeLa cells were incubated with 1 μM labeled peptides, washed, fixed, and visualized by fluorescence microscopy. We found that all of the peptides were able to enter the cells and were distributed in both the nucleus and
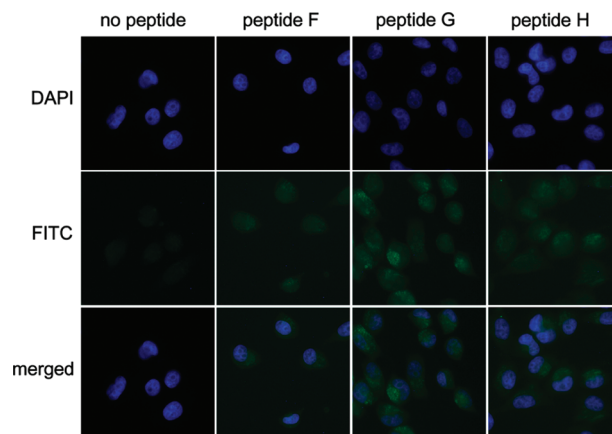
Figure 4. Fluorescence microscopy showing the uptake and intracellular distribution of FITC-labeled phosphopeptides F, G, and H in Hela cells. Hela cells were treated with 0 or 1.0 μM peptide for 48 h, fixed with 3.7% paraformaldehyde, and washed with PBS prior to fluorescence visualization. Cells were also stained with DAPI to visualize the nuclear DNA and merged with the FITC images to determine the intracellular distribution of fluorescent peptides.

cytoplasm (Figure 4 and Figure S6 in Supporting Information). Judging from the fluorescence intensities inside the cells, it appears that the efficiency of internalization correlates with the number of arginine residues in the peptide sequences. Peptides G and J, each of which has six arginine residues, were most efficiently taken up by the cells, whereas peptides F and I (each of which contains three arginine residues) were the least efficient. Next, peptides F and H were tested for their ability to inhibit proliferation of HeLa and BT-474 cells. Peptide H reduced the rate of HeLa cell growth by 31% after 6 days of treatment (at 2.5 μM), whereas the unphosphorylated control peptide (peptide K) had minimal effect (Figure 5). Peptides F (0.5 μM) and H (2.5 μM) also inhibited BT-474 cell growth by 37% and 32%, respectively, after 6 days (data not shown). Western blot analysis showed
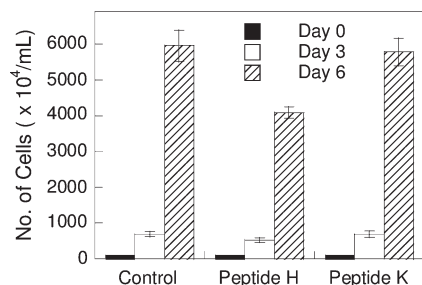
Figure 5. Proliferation of HeLa cells in the absence (control) or presence of 2.5 μM peptide H or K.

that treatment with peptide H (2.5 and 5.0 μM) increased the protein levels of SMRT and PML in BT-474 cells, while the control peptide K (5 μM) had minimal effect (data not shown).

Conclusion

By designing and screening a focused cyclic peptide library against human Pin1 catalytic domain, we have discovered potent cyclic peptidyl Pin1 inhibitors. These cyclic peptides were rendered cell permeable either by attachment of an octaarginine sequence to the side chain or by direct incorporation of arginine residues into the cyclic peptide backbone. To our knowledge, these peptides represent the first examples of macrocyclic inhibitors against Pin1. These compounds should provide useful tools for investigating the cellular functions of Pin1 and may be further developed into therapeutic agents. In addition, our study provided important structure−activity relationship information for future inhibitor design against Pin1.

Experimental Section

Materials and General Methods. Reagents for peptide synthesis were purchased from Advanced ChemTech (Louisville, KY), Peptides International (Louisville, KY), NovaBiochem (La Jolla, CA), or Anaspec (San Jose, CA) except for 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), which was from GenScript (Piscataway, NJ). TentaGel S NH_2 resin (90 μm, 0.29 mmol/g, and $\sim$100 pmol/bead) was purchased from Peptides International. Rink resin LS (100−200 mesh, 0.2 mequiv/g) was purchased from Advanced ChemTech. N-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSU) was from Advanced ChemTech. FITC and phenyl isothiocyanate (PITC, as 1 mL sealed ampules) were purchased from either Sigma-Aldrich (St. Louis, MO) or Pierce (Rockford, IL), and a freshly opened ampule was used in each experiment. N-Hydroxysuccinimidyl (NHS) ester of Texas Red-X was from Invitrogen (Carlsbad, CA), while (+)-biotin N-hydroxysuccinimide ester was from Sigma. Pin1 substrate Suc-Ala-Glu-Pro-Phe-pNA was from Peptides International (Louisville, KY), and α-chymotrypsin was from Aldrich. All solvents and other chemical reagents were purchased from Aldrich and were of analytical grade. Synthesis of N^α-Fmoc-Glu(δ-N-hydroxysuccinimidyl)-O-CH$_2$CH=CH$_2$ has previously been reported.[46] Purity of Pin1 inhibitors was determined to be $\geq$95% by analytical HPLC on a C-18 column eluted with a linear gradient of 10−50% acetonitrile in water containing 0.05% TFA.

Synthesis of Pin1 Inhibitor Library. The OBOC peptide library was synthesized on 1 g of TentaGel S NH_2 resin (90 μm, 0.26 mmol/g, $\sim$100 pmol/bead) as previously described.[46,47] Briefly, after the linker sequence (BBNBRM) was synthesized by standard Fmoc/HBTU chemistry, the beads were soaked overnight in water, drained, and rapidly dispersed into 15 mL of 1:1 (v/v) dichloromethane (DCM)/diethyl ether containing 0.5 equiv of N^α-Fmoc-Glu(δ-N-hydroxysuccinimidyl)-O-CH$_2$CH=CH$_2$.

After incubation for 30 min, the resin was drained, washed with DMF, and reacted with excess Fmoc-Glu(^{t}Bu)-OH and HBTU. To generate different ring sizes, the resin was equally split into four aliquots and zero to three Fmoc-D-Ala residues were coupled to the four aliquots. The resin from the four reactions was combined, and the random region was synthesized by using the split-and-pool method with 5 equiv of Fmoc-amino acids and HATU. The coupling reactions were repeated once to ensure complete reaction at each step. To differentiate isobaric amino acids during MS sequencing, 5% (mol/mol) of CD_3CO_2D was added to the coupling reactions of D-Ala, Lys, D-Leu, and Orn at the X^1, X^4, and X^5 positions, D-Glu at the X^2 position, and D-Pro at the X^3 position, whereas 5% $CH_3CD_2CO_2D$ was added to the coupling reactions of Nle.[39] After the addition of the N-terminal D-Ala residue, the allyl group on the C-terminal Glu residue was removed by treatment with tetrakis(triphenylphosphine)palladium, triphenylphosphine, formic acid, and diethylamine (1, 3, 10, 10 equiv, respectively) in anhydrous THF overnight at room temperature. Next, the N-terminal Fmoc group was removed by treatment with 20% piperidine in DMF. Finally, the surface peptides were cyclized by treating the resin with PyBOP/HOBt/NMM (5, 5, 10 respectively) in DMF for 3 h. Side chain deprotection was achieved by treatment with reagent K (TFA/thioanisole/water/phenol/1,2-ethanedithiol, 82.5:5:5:5:2.5 v/v) for 3 h. The resulting library was washed with DCM, DMF, 5% N,N-diisopropylethylamine in DMF, 1:1 (v:v) DCM/diethyl ether, DMF, and DCM extensively and stored at −20 °C.

Expression, Purification, and Labeling of Pin1 Protein. The full-length human S16A/Y23A mutant Pin1 was cloned into prokaryotic expression vector pMAL-c2X (New England Biolabs, Ipswich, MA) between EcoRI/XbaI restriction sites. This cloning procedure resulted in the fusion of Pin1 with an N-terminal maltose-binding protein (MBP). The MBP−Pin1 fusion protein was expressed in E. coli BL21(DE3) cells and purified by affinity chromatography on an amylose column. Labeling of the Pin1 mutant with biotin or Texas-Red was achieved by treating the protein with the NHS ester of biotin or Texas-Red. Briefly, 1 mg of MBP−Pin1 protein was concentrated to $\sim$3 mg/mL and incubated with 2 mol equiv of NHS-biotin dissolved in DMSO (10 mg/mL) in a reaction buffer containing 100 mM $NaHCO_3$ (pH 8.5) for 1 h at 4 °C. Any excess NHS-biotin was quenched by treatment with 10 μL of 1 M Tris buffer (pH 8.5). The reaction mixture was then passed through a Sephadex G-25 column (GE Healthcare, Piscataway, NJ) to remove any free biotin from the labeled protein. Texas-Red labeling of Pin1 was carried out in the same manner with NHS-Texas-Red in the dark. The labeling efficiency of Texas-Red was measured by comparing the absorbance at 280 and 595 nm (absorption maximum for Texas-Red), and a $\sim$1:1 protein/dye molar ratio was achieved under the above conditions. Both biotin- and Texas-Red-labeled Pin1 proteins had the same catalytic activity as the unlabeled protein.

Library Screening. Library screening against Pin1 was carried out by two different methods. The library resin (typically 50 mg) was swollen in DCM, washed extensively with DMF, doubly distilled H_2O, and HBST buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% Tween-20), and blocked overnight at 4 °C with 1 mL of blocking buffer (HBST buffer plus 0.1% gelatin). For fluorescence based screening (method A), Texas-Red labeled MBP−Pin1 protein was added to the library to a final concentration of 200 nM and the mixture (total volume of 3 mL) was incubated in a Petri dish for 6 h at 4 °C with gentle shaking. The library was viewed under an Olympus SZX12 fluorescence microscope (Olympus America, Center Valley, PA), and positive beads (red colored) were manually removed from the library with a micropipet. For biotin/SAAP based screening, the library was incubated with 200 nM biotinylated MBP−Pin1 for 6 h at 4 °C in micro-BioSpin column (0.8 mL, BioRad) with rotary

shaking. The protein solution was then drained, and the library was incubated with SAAP (1 μg/mL final concentration) in 1 mL of the blocking buffer for 10 min at 4 °C. The library was washed three times each with the blocking buffer and SAAP staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM MgCl$_2$, 20 μM ZnCl$_2$, and 0.05% Tween-20) and transferred to a Petri dish. Finally, 3 mL of the staining buffer and 300 μL of a BCIP stock solution (5 mg/mL) were added to the beads and the library was incubated at room temperature (with shaking). Turquoise color developed on positive beads in ∼20 min. The positive beads were manually collected under a dissecting microscope and individually sequenced by the PED-MS method.[39]

Synthesis of Individual Peptides. Each peptide was synthesized manually on 200 mg of Rink resin LS (0.2 mmol/g) in a manner similar to that employed for the library synthesis. Peptide cyclization was monitored by ninhydrin tests of any remaining amines. The peptides were cleaved off the resin and deprotected by the reagent K and triturated three times with cold Et$_2$O. The resulting crude peptides were purified by reversed-phase HPLC on a C$_{18}$ column, and the identity of the peptides was confirmed by MALDI-TOF mass spectrometric analyses. To prepare the octaarginine-conjugated peptide E, the relevant cyclic peptide containing a C-terminal lysine and the octaarginine peptide (Ac-CRRRRRRRR-NH$_2$) were synthesized separately by standard Fmoc/HATU chemistry. The cyclic peptide (1 mg), after deprotection and cleavage from resin, was reacted with 1 equiv of *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce, catalog no. 21857) in 200 μL of 1:1 (v/v) DMSO/NaHCO$_3$ buffer (50 mM, pH 8.5) mixture for 30 min at room temperature. The octaarginine peptide (1.5 mg) was reduced by treatment with 2 equiv of resin-bound TCEP (Pierce, catalog no. 77712) for 20 min. The reduced octaarginine peptide was collected by filtration through a micro-BioSpin column (0.8 mL, BioRad) and immediately added to the SPDP-derivatized cyclic peptide solution, and the reaction mixture was incubated at room temperature for 4−6 h. The resulting disulfide cross-linked cyclic peptide−octaarginine conjugate was purified by reversed-phase HPLC on a C$_{18}$ column. The authenticity of all peptides was confirmed by MALDI-TOF mass spectrometry.

Determination of Binding Affinity by ITC. Isothermal titration calorimetry experiments were performed using a MicroCal VP-ITC at 25 °C. Buffer exchange for protein samples was done by passing through a Sephadex G-25 column with 10 mM Hepes (pH 7.8). The same buffer was used to prepare solutions of peptides, to dilute the protein, and to evaluate the baseline heats of dilution. All solutions were degassed under vacuum before filling the sample cell and syringe. For titration experiments, 8 μM S16A/Y23A MBP-Pin1 protein was loaded into the sample cell and 100−200 μM peptide solution was filled into the syringe. The titration experiments were performed by 24 10-μL injections or 16 15-μL injections. Heat of dilution was measured in the same manner by titrating peptides into the buffer. After subtraction of the heat of dilution, the net enthalpy data were fitted to a single-site binding model using the MicroCal origin software package.

Pin1 Inhibition Assay. Pin1 substrate Suc-Ala-Glu-Pro-Phe-pNA (Peptide International, SAP-3947-PI) was dissolved in anhydrous trifluoroethanol (TFE) containing 0.5 M LiCl to make a 2 mM stock. Prior to use, LiCl was dried by heating in an oil bath at 140 °C for 24 h under vacuum. TFE was dried with MgSO$_4$ and stored in a sealed glass bottle containing 4 Å molecular sieves. α-Chymotrypsin (Sigma-Aldrich, C4129-1G) was dissolved in 1.2 mM HCl to make a stock solution of 60 mg/mL. A Pin1 stock solution (800 nM) was prepared in the reaction buffer (35 mM HEPES, pH 7.8). A typical assay reaction (total volume of 200 μL) contained Pin1 (80 nM), chymotrypsin (6 mg/mL), peptide substrate (50 μM), and varying concentrations of Pin1 inhibitor (0−100 μM). Pin1 inhibitor and Pin1 protein were preincubated for 10 min at 4 °C prior to addition to the reaction mixture. The reaction was initiated by the addition of chymotrypsin followed by substrate. The reaction progress was monitored at 390 nm on a UV/vis spectrophotometer (Perkin-Elmer Lambda 20) at 4 °C for 30 s. The IC$_{50}$ value was obtained by fitting the data into the following equation: $V = V_{max}/(1 + [I]/IC_{50})$.

Cell Culture and Western Blotting. HeLa cells were grown in standard DME (Sigma-Aldrich, D7777) supplemented with 10% FBS and 1% penicillin/streptomycin sulfate at 37 °C in 5% CO$_2$. BT-474 cells were grown in DME (ATCC Hybri-Care Medium 46-X) supplemented with 10% FBS. Cells were treated with 1 μM Pin1 inhibitor E, 1 μM peptide F or I, 2.5 μM peptide H or K, or DMSO for 48 h. Cells were washed twice with cold 1× PBS and lysed in RAPI buffer containing protease inhibitors. Protein concentrations were measured using Quick Start Bradford dye reagent, 1× (Bio-Rad, 500-0205), and equal amounts of protein were subjected to SDS−PAGE on 8% polyacrylamide gel. The proteins were then electrophoretically transferred to nitrocellulose membranes and processed for immunoblotting. Membranes were first blocked with 10% nonfat dry milk in PBST buffer for 1 h followed by incubating with specific primary antibodies for 1 h at room temperature or overnight at 4 °C. Antibodies used were anti-SMRT,[42] anti-PML (Santa Cruz sc-5621), anti-β-actin (Sigma A5441), and anti-Pin1.[42] The blots were washed three times with PBST washing buffer and incubated with HRP secondary IgG antibody for 30−60 min. The signals were detected by SuperSignal West Pico chemiluminescent substrate (Fisher 34080).

Cell Proliferation Assay. Cell proliferation assays were performed as previously described.[43] BT-474 cells were counted, and 5×10^3 cells/well were seeded into 96-well plates. The cell number at time zero was taken 8−10 h after plating. Cells were treated with 0−2.5 μM Pin1 inhibitor peptide and incubated in 5% CO$_2$ incubator for 2, 4, and 6 days with fresh medium provided every day. At each time point, cell proliferation was measured using the CyQUANT NF cell proliferation assay kit (Invitrogen C35006). The medium was removed by gentle aspiration using multichannel pipet, and 100 μL of 1× dye binding solution was dispensed into each well. After 45 min of incubation at 37 °C, the fluorescence intensity was measured at 495 nm with SPECTRA max M2 (Molecular Devices). Each assay was performed in replicate ($n = 6$).

Fluorescence Microscopy. HeLa cells were grown in 12-well plates with a cover glass as described above. The cells were treated with or without 1.0 μM FITC-labeled peptides F−K for 48 h followed by fluorescence microscopy as previously described.[43] Briefly, the cells were washed three times with 1× PBS, fixed with 3.7% paraformaldehyde at room temperature for 20 min, and washed three times with 1× PBS. The cells were permeabilized with 10% goat serum and 1% Triton X-100 in 1× PBS at room temperature for 10 min and washed three times with 1× PBS. To visualize DNA, the cells were treated with the mounting medium containing DAPI on the slides prior to fluorescence microscopy.

Supporting Information Available: Additional experimental details, MS spectra, ITC data, and HPLC tracings. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) 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.

(2) Lu, P. J.; Zhou, X. Z.; Shen, M. H.; Lu, K. P. Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science* **1999**, *283*, 1325–1328.

(3) Yaffe, M. B.; Schutkowski, M.; Shen, M. H.; Zhou, X. Z.; Stukenberg, P. T.; Rahfeld, J. U.; Xu, J.; Kuang, J.; Kirschner, M. W.; Fischer, G.; Cantley, L. C.; Lu, K. P. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science* **1997**, *278*, 1957–1960.

(4) Lu, K. P.; Zhou, X. Z. The prolyl isomerase Pin1: a pivotal new twist in phosphorylation signaling and disease. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 904–916.

(5) Crenshaw, D. G.; Yang, J.; Means, A. R.; Kornbluth, S. The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1. *EMBO J.* **1998**, *17*, 1315–1327.

(6) Stukenberg, P. T.; Kirschner, M. W. Pin1 acts catalytically to promote a conformational change in Cdc25. *Mol. Cell* **2001**, *7*, 1071–1083.

(7) Zhou, X. Z.; Kops, O.; Werner, A.; Lu, P. J.; Shen, M. H.; Stoller, G.; Kullertz, G.; Stark, M.; Fischer, G.; Lu, K. P. Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. *Mol. Cell* **2000**, *6*, 873–883.

(8) Okamoto, K.; Sagata, N. Mechanism for inactivation of the mitotic inhibitory kinase Wee1 at M phase. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3753–3758.

(9) Albert, A. L.; Lavoie, S.; Vincent, M. A hyperphosphorylated form of RNA polymerase II is the major interphase antigen of the phospho-protein antibody MPM-2 and interacts with the peptidyl-prolyl isomerase Pin1. *J. Cell Sci.* **1999**, *112*, 2493–2500.

(10) Xu, Y. X.; Manley, J. L. The prolyl isomerase Pin1 functions in mitotic chromosome condensation. *Mol. Cell* **2007**, *26*, 287–300.

(11) Wulf, G. M.; Ryo, A.; Wulf, G. G.; Lee, S. W.; Niu, T. H.; Petkova, V.; Lu, K. P. 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.

(12) Liou, Y. C.; Ryo, A.; Huang, H. K.; Lu, P. J.; Bronson, R.; Fujimori, F.; Uchida, T.; Hunter, T.; Lu, K. P. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1335–1340.

(13) Yeh, E. S.; Lew, B. O.; Means, A. R. The loss of PIN1 deregulates cyclin E and sensitizes mouse embryo fibroblasts to genomic instability. *J. Biol. Chem.* **2006**, *281*, 241–251.

(14) Yeh, E.; Cunningham, M.; Arnold, H.; Chasse, D.; Monteith, T.; Ivaldi, G.; Hahn, W. C.; Stukenberg, P. T.; Shenolikar, S.; Uchida, T.; Counter, C. M.; Nevins, J. R.; Means, A. R.; Sears, R. A signaling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat. Cell Biol.* **2004**, *6*, 308–318.

(15) Zacchi, P.; Gostissa, M.; Uchida, T.; Salvagno, C.; Avolio, F.; Volinia, S.; Ronai, Z.; Blandino, G.; Schneider, C.; Del Sal, G. The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. *Nature* **2002**, *419*, 853–857.

(16) Zheng, H.; You, H.; Zhou, X. Z.; Murray, S. A.; Uchida, T.; Wulf, G.; Gu, L.; Tang, X. R.; Lu, K. P.; Xiao, Z. X. J. The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. *Nature* **2002**, *419*, 849–853.

(17) Berger, M.; Stahl, N.; Del Sal, G.; Haupt, Y. Mutations in proline 82 of p53 impair its activation by Pin1 and Chk2 in response to DNA damage. *Mol. Cell. Biol.* **2005**, *25*, 5380–5388.

(18) Mantovani, F.; Piazza, S.; Gostissa, M.; Strano, S.; Zacchi, P.; Mantovani, R.; Blandino, G.; Del Sal, G. Pin1 links the activities of c-Abl and p300 in regulating p73 function. *Mol. Cell* **2004**, *14*, 625–636.

(19) Monje, P.; Hernandez-Losa, J.; Lyons, R. J.; Castellone, M. D.; Gutkind, J. S. 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.

(20) Ryo, A.; Suizu, F.; Yoshida, Y.; Perrem, K; Liou, Y. C.; Wulf, G.; Rottapel, R.; Yamaoka, S.; Lu, K. P. Regulation of NF-κB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol. Cell* **2003**, *12*, 1413–1426.

(21) Ryo, A.; Nakamura, N.; Wulf, G.; Liou, Y. C.; Lu, K. P. Pin1 regulates turnover and subcellular localization of β-catenin by inhibiting its interaction with APC. *Nat. Cell Biol.* **2001**, *3*, 793–801.

(22) Chen, S. Y.; Wulf, G.; Zhou, X. Z.; Rubin, M. A.; Lu, K. P.; Balk, S. P. Activation of β-catenin signaling in prostate cancer by peptidyl-prolyl isomerase Pin1-mediated abrogation of the androgen receptor−β-catenin interaction. *Mol. Cell. Biol.* **2006**, *26*, 929–939.

(23) Lu, K. P.; Hanes, S. D.; Hunter, T. A human peptidyl-prolyl isomerase essential for regulation of mitosis. *Nature* **1996**, *380*, 544–547.

(24) Winkler, K. E.; Swenson, K. I.; Kornbluth, S.; Means, A. R. Requirement of the prolyl isomerase Pin1 for the replication checkpoint. *Science* **2000**, *287*, 1644–1647.

(25) Rippmann, J. F.; Hobbie, S.; Daiber, C.; Guilliard, B.; Bauer, M.; Birk, J.; Nar, H.; Garin-Chesa, P.; Rettig, W. J.; Schnapp, A. Phosphorylation-dependent proline isomerization catalyzed by Pin1 is essential for tumor survival and entry into mitosis. *Cell Growth Differ.* **2000**, *11*, 409–416.

(26) 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.

(27) Hennig, L.; Christner, C.; Kipping, M.; Schelbert, B.; Rucknagel, K. P.; Grabley, S.; Kullertz, G.; Fischer, G. Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone. *Biochemistry* **1998**, *37*, 5953–5960.

(28) Daum, S.; Erdmann, F.; Fischer, G.; Feaux de Lacroix, B.; Hessamian-Alinejad, A.; Houben, S.; Frank, W.; Braun, M. Aryl indanyl ketones: efficient inhibitors of the human peptidyl prolyl cis/trans isomerase Pin1. *Angew. Chem., Int. Ed.* **2006**, *45*, 7454–7458.

(29) Braun, M.; Hessamian-Alinejad, A.; Feaux de Lacroix, B.; Alvarez, B. H.; Fischer, G. Novel spiroannulated 3-benzofuranones. Synthesis and inhibition of the human peptidyl prolyl isomerase Pin1. *Molecules* **2008**, *13*, 995–1003.

(30) Tatara, Y.; Lin, Y.-C.; Bamba, Y.; Mori, T.; Uchida, T. Dipenta-methylene thiuram monosulfide is a novel inhibitor of Pin1. *Biochem. Biophys. Res. Commun.* **2009**, *384*, 394–398.

(31) Guo, C.; Hou, X.; Dong, L.; Dagostino, E.; Greasley, S.; Ferre, R.; Marakovits, J.; Johnson, M. C.; Matthews, D.; Mroczkowski, B.; Parge, H.; Vanarsdale, T.; Popoff, I.; Piraino, J.; Margosiak, S.; Thomson, J.; Los, G.; Murray, B. W. Structure-based design of novel human Pin1 inhibitors (I). *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5613–5616.

(32) Zhang, Y.; Fussel, S.; Reimer, U.; Schutkowski, M.; Fischer, G. Substrate-based design of reversible Pin1 inhibitors. *Biochemistry* **2002**, *41*, 11868–11877.

(33) Wang, X. J.; Xu, B. L.; Mullins, A. B.; Neiler, F. K.; Etzkorn, F. A. Conformationally locked isostere of phosphoSer-cis-Pro inhibits Pin1 23-fold better than phosphoSer-trans-Pro isostere. *J. Am. Chem. Soc.* **2004**, *126*, 15533–15542.

(34) 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.

(35) Wildemann, D.; Erdmann, F.; Alvarez, B. H.; Stoller, G.; Zhou, X. Z.; Fanghanel, J.; Schutkowski, M.; Lu, K. P.; Fischer, G. Nanomolar inhibitors of the peptidyl prolyl cis/trans isomerase Pin1 from combi-natorial peptide libraries. *J. Med. Chem.* **2006**, *49*, 2147–2150.

(36) Zhang, Y.; Daum, S.; Wildemann, D.; Zhou, X. Z.; Verdecia, M. A.; Bowman, M. E.; Lucke, C.; Hunter, T.; Lu, K. P.; Fischer, G.; Noel, J. P. Structural basis for high-affinity peptide inhibition of human Pin1. *ACS Chem. Biol.* **2007**, *2*, 320–328.

(37) Zhang, Y.; Zhou, S. G.; Wavreille, A. S.; DeWille, J.; Pei, D. Cyclic peptidyl inhibitors of Grb2 and tensin SH2 domains identified from combinatorial libraries. *J. Comb. Chem.* **2008**, *10*, 247–255.

(38) Shen, M.; Stukenberg, P. T.; Kirschner, M. W.; Lu, K. P. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. *Genes Dev.* **1998**, *12*, 706–720.

(39) Thakkar, A.; Wavreille, A.-S.; Pei, D. Traceless capping agent for peptide sequencing by partial Edman degradation and mass spectrometry. *Anal. Chem.* **2006**, *78*, 5935–5939.

(40) Kofron, J. L.; Kuzmic, P.; Kishore, V.; Colonbonilla, E.; Rich, D. H. Determination of kinetic constants for peptidyl prolyl cis-trans isomerases by an improved spectrophotometric assay. *Biochemistry* **1991**, *30*, 6127–6134.

(41) Wavreille, A. S.; Pei, D. A chemical approach to the identification of tensin-binding proteins. *ACS Chem. Biol.* **2007**, *2*, 109–118.

(42) Reineke, E. L.; Lam, M.; Liu, O.; Liu, Y.; Stanya, K. J.; Chang, K. S.; Means, A. R.; Kao, H. Y. 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.

(43) Stanya, K. J.; Liu, Y.; Means, A. R.; Kao, H. Y. Cdk2 and Pin1 negatively regulate the transcriptional corepressor SMRT. *J. Cell Biol.* **2008**, *183*, 49–61.

(44) Wildemann, D.; Alvarez, B. H.; Stoller, G.; Zhou, X. Z.; Lu, K.-P.; Erdmann, F.; Ferrari, D.; Fischer, G. An essential role for Pin1 in *Xenopus laevis* embryonic development revealed by specific inhibi-tors. *Biol. Chem.* **2007**, *388*, 1103–1111.

(45) Vives, E.; Brodin, P.; Lebleu, B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* **1997**, *272*, 16010–16017.

(46) Joo, S. H.; Xiao, Q.; Ling, Y.; Gopishetty, B.; Pei, D. High-throughput sequence determination of cyclic peptide library mem-bers by partial Edman degradation/mass spectrometry. *J. Am. Chem. Soc.* **2006**, *128*, 13000–13009.

(47) Liu, T.; Joo, S. H.; Voorhees, J. L.; Brooks, C. L.; Pei, D. Synthesis and screening of a cyclic peptide library: discovery of small-molecule ligands against human prolactin receptor. *Bioorg. Med. Chem.* **2009**, *17*, 1026–1033.