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Pin1 and Par14 Peptidyl Prolyl Isomerase Inhibitors Block Cell Proliferation

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Summary

Disruption of the parvulin family peptidyl prolyl isomerase (PPIase) Pin1 gene delays reentry into the cell cycle when quiescent primary mouse embryo fibroblasts are stimulated with serum. Since Pin1 regulates cell cycle progression, a Pin1 inhibitor would be expected to block cell proliferation. To identify such inhibitors, we screened a chemical compound library for molecules that inhibited human Pin1 PPIase activity in vitro. We found a set of compounds that inhibited Pin1 PPIase activity in vitro with low μM IC50s and inhibited the growth of several cancer lines. Among the inhibitors, PiB, diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[lmn][3,8] phenanthroline-2,7-diacetate ethyl 1,3,6,8-tetrahydro-1,3,6,8-tetraoxo-benzo[lmn][3,8] phenanthroline-(2H,7H)-diacetate, had the least nonspecific toxicity. These results suggest that Pin1 inhibitors could be used as a novel type of anticancer drug that acts by blocking cell cycle progression.

Introduction

Peptidyl prolyl *cis-trans* isomerases (PPIase) catalyze the *cis-trans* isomerization of prolyl peptide bonds, and PPIase activity is required for a variety of functions including the assembly, folding, and transport of cellular proteins. PPIases belong to one of three families, the cyclophilins, FK506 binding proteins (FKBPs), and parvulins. Two kinds of bacterial parvulin homolog have been identified in human and mouse cells, Pin1 and Pin4/Par14 [1, 2]. The prokaryotic parvulins and mammalian Par14 consist of only a PPIase domain. Pin1 also belongs to the parvulin family but in addition to the C-terminal PPIase domain, it has a WW domain at its N terminus. PPIases with this type of WW-PPIase double-domain structure are conserved among almost all eukaryotes, including *Saccharomyces cerevisiae* (Ess1) [3–5], *Schizosaccharomyces pombe* (Pin1) [6], *Candida albicans* (Ess1) [7], *Drosophila melanogaster* (Dodo) [8], *Neurospora crassa* (Ssp1) [9], and mouse (Pin1) [10] and human (Pin1) [1], but true Pin1 homologs with a WW domain appear to be absent from plants (plants have Pin1-related proteins with the basic pocket in the catalytic domain, but they lack the WW domain). The WW domain is a small 40-residue protein-protein interaction domain [11]. There are four types of WW domain. Three of them recognize short proline-rich motifs, and the other recognizes phosphoserine (pSer) or phosphothreonine (pThr) proline motifs [12]. The Pin1 WW domain is the prototypic member of the latter group and interacts with pSer/pThr-Pro motifs in several proteins [13, 14]. Pin1 binds specifically to pSer/pThr-Pro sequences via its WW domain and isomerizes pSer/Thr-Pro bonds. Pin1 PPIase activity can alter protein conformation in a phosphorylation-dependent manner and/or promote protein dephosphorylation [15–19].

The human protein Pin1 was identified through a two-hybrid screen as a protein that interacts with *A. nidulans* NIMA. Although originally believed to be a cell cycle regulator, it is now known that Pin1 functions in many cellular processes through its ability to bind to and promote phosphorylation-dependent isomerization of pSer/pThr-Pro motifs. Pin1 interacts with many of the MPM-2 antigens in mitotic lysates, including Cdc25C [15, 16], Wee1 [16], and Myt1 [17]. Pin1 can also catalyze a conformational change in phosphorylated Cdc25 [18] and stimulate dephosphorylation of Cdc25 by PP2A [19]. Pin1 can either inhibit or stimulate the activity of phosphorylated Cdc25C, depending on which sites are phosphorylated [16, 18]. Pin1 might act in an analogous fashion as a phosphorylation-specific chaperone for other pSer/pThr-Pro-containing mitotic phosphoproteins, either promoting dephosphorylation or a conformational change, and thereby play a general role in mitotic progression.

In addition to functions in cell cycle control and proliferation, Pin1 is involved in other biological processes. Pin1 interacts with phosphorylated tau protein in the brain and can restore its activity to promote microtubule assembly in vitro [20]. The *Drosophila* homolog of Pin1, *dodo*, is required for proper dorsoventral patterning of the follicular epithelium during oogenesis through its ability to bind to and promote ubiquitin-mediated degradation of the CF2 transcription factor following its phosphorylation by ERK MAP kinase [21]. Pin1 physically interacts with phosphorylated NFAT and prevents dephosphorylation of the NFAT transcription factor, which is required for T cell activation [22]. Pin1 also regulates turnover and subcellular localization of β-catenin by inhibiting its interaction with APC [23]. Recently, Pin1 has been found to be overexpressed in breast cancer and to increase the expression of cyclin D1 as a result of its ability to bind to c-Jun phosphorylated at the Ser63/73-Pro motifs in its transactivation domain and thereby

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stimulate cyclin D1 promoter activity [24]. Pin1 can also increase the stability and nuclear localization of cyclin D1 through direct binding to pThr286-Pro [25]. We examined the expression level of Pin1 in 65 clinical samples with oral squamous cell carcinoma by immunohistochemical staining and showed that Pin1 level correlates with cyclin D1 level [26], which suggests that Pin1 is related to oncogenesis of oral squamous cell carcinoma as well as breast cancer.

In addition to its effects on the function of specific transcription factors, Pin1 also has more general roles in transcription. Pin1 binds the phosphorylated CTD of the largest subunit of RNA polymerase II [14, 27] and activates basal reporter gene transcription [28]. Ess1 also interacts with the phosphorylated CTD [29], and the *ptf1* temperature-sensitive *ESS1* allele has a defect in pre-mRNA 3′ end processing [30], suggesting a general role in transcription. Multicopy suppressor analysis of temperature-sensitive *ess1* mutants has revealed that Ess1 plays a role in general transcription/chromatin remodeling [31] and in gene silencing via the Sin3p-Rpd3p histone deacetylase [32]. Pin1 also plays a role in inhibition of endocytic membrane transport during mitosis [33]. These observations suggest that Pin1/Ess1 might play an indirect role in cell cycle control either through effects on transcription or on the activity of proteins required in a general housekeeping sense for growth and cell cycle progression. Thus, the diverse functions of Pin1/Ess1 in cell proliferation appear to be a result of its interaction with multiple phosphoproteins.

Although Ess1 has been reported to be essential for mitotic progression in *S. cerevisiae*, *C. albicans* [7], and in certain human tumor cells [1, 34], deletion of the *Drosophila* Pin1/Ess1 homolog, *dodo* [8], fission yeast [6], and the mouse homolog, *Pin1* [10], results in viable organisms, albeit with detectable phenotypes. This suggests that these organisms either have additional *Pin1* family genes, or else that they have PPIases that have overlapping functions with Pin1/Ess1. This idea would be consistent with the finding that *pin1*Δ budding and fission yeast cells are more sensitive than wild-type cells to cyclosporin A (CsA), which inhibits cyclophilin PPIase activity [5, 6]. We identified a second mammalian parvulin family PPIase, Par14. Although Par14 lacks the basic pocket needed for selection of phospho-Ser/Thr-Pro substrates, Par14 may substitute for Pin1 in a manner similar to cyclophilin PPIases in yeast.

In this manuscript, we identified a series of Pin1 and Par14 PPIase inhibitors and used them to show that Pin1 is important for cell cycle progression. Moreover, these inhibitors inhibited the growth of cancer cells expressing Pin1 and could potentially be developed into novel antifungal agents and anticancer drugs effective against some kinds of tumors.

Results

Expression analysis of Pin1$^{-/-}$ MEF

The Pin1$^{-/-}$ mouse embryo fibroblasts (MEF) grow a little slower than the wild-type MEF, but the difference is not that marked [10]. We speculated that other PPIases, such as the parvulin family PPIase Par14 or cyclophilins, may be upregulated and rescue cell growth. In order to examine whether the lack of Pin1 led to significant changes in gene expression, we investigated whether there were genes differentially expressed in wild-type and Pin1$^{-/-}$ MEF using a DNA microarray containing 9448 mouse cDNA clones. The threshold for a significant difference in the microarray hits was set at 1.7. None of the 9448 genes showed a dramatic change in expression in the Pin1$^{-/-}$ MEF compared to wild-type (Figure 1A). There were 33 up- and 66 downregulated genes in Pin1$^{-/-}$ MEF compared to Pin1$^{+/+}$ MEF. Figure 1A lists some of the genes that showed the greatest differences in expression. The p21 and p15 Cdk inhibitors and cyclin G were the most upregulated genes (2- to 3-fold) (Figure 1A). The results of quantitative RT-PCR analysis of these mRNAs coincided with the microarray analysis data (Figure 1A). Expression of insulin-like growth factor (IGF)-2 and cyclin D1 was downregulated in Pin1$^{-/-}$ MEF; the ratios were 3.0 and 1.7, respectively. A correlation between Pin1 levels and cyclin D1 expression was previously reported by Wulf et al. [24]. However, considering that Pin1 has been reported to be involved in transcriptional regulation, the differences in gene expression between Pin1$^{-/-}$ and wild-type MEF were not as significant as we expected. Moreover, Pin1$^{-/-}$ MEF had an identical cell cycle profile compared to Pin1$^{+/+}$ MEF (Figure 1B). In Pin1$^{-/-}$ MEF, another parvulin family PPIase, hPar14, was upregulated about 3-fold (Figure 1A). The cyclophilin C expression was not increased in Pin1$^{-/-}$ MEF. We have not examined the expression levels of various types of cyclophilins, but Pin1$^{+/+}$ MEF and Pin1$^{-/-}$ MEF did not show different susceptibilities against cyclosporin A treatment (data not shown). When the level of Par14 was examined by Western blotting, the Par14 level in Pin1$^{-/-}$ cells was about 2-fold higher than in Pin1$^{+/+}$ MEF (Figure 1C). The results suggested that Par14 may in part replace Pin1 function, and consequently we decided to develop inhibitors for both Pin1 and Par14.

Screening for Pin1 Inhibitors

The most suitable peptide for Pin1 screening would be a peptide containing a pSer/pThr-Pro motif, for example WFYpSPR (Kcat/Km: 20,160/mM s), but the peptide Ala-Glu-Pro-Phe (Kcat/Km: 3410/mM s) is good enough to be used as a substrate for Pin1 PPIase assays [35]. Moreover, this peptide is also a relatively good substrate for Par14 compared to other peptides we examined (Kcat/Km: 620/mM s) [2], meaning that it could also be used to screen for Par14 inhibitors. We assayed Pin1 PPIase using AEPF as the substrate. Although the reaction was very fast and the background was high, it was possible to assay Pin1 PPIase in this manner (Figure 2A). The method proved suitable for measurement of kinetic parameters, such as the peptide Km (Figure 2B). Using this method, we screened Pin1 PPIase inhibitors among 1000 chemically synthesized compounds. This library mainly consisted of the chemicals synthesized randomly, but it contained chemicals with double-ring structures, like juglone, which is a known Pin1 inhibitor, and chemicals developed as potential anticancer and antifungal drugs. Compounds with anticancer cell activity did not exhibit strong inhibitory activities against can-

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Ratio (-/- / +/+)		Up- Regulated Genes in Pin1 -/- MEF
Microarray	Real Time PCR	
3.3	nd	Calcium-Binding Protein A4
2.4	nd	Thrombospondin 1
2.2	3.0	Cyclin G
nd	3.0	Par14 (Parvulin- Like PPIase)
2.1	nd	CD24a Antigen
2.1	nd	Calcium Binding Protein A6
2.1	2.3	CDK2 Inhibitor (P21)
1.7	nd	CDK4 Inhibitor (P15)

nd; not determined

Figure 1. Expression Analysis of Pin1$^{-/-}$ MEF

(A) List of relevant genes upregulated in Pin1$^{-/-}$ MEF as determined by microarray and real-time PCR analysis. Poly (A)$^{+}$ RNA samples prepared from the cultured MEF were analyzed by DNA array (GEM Microarray, Incyte Genomics). The wild-type and Pin1$^{-/-}$ RNAs were labeled with CY3 and CY5, respectively, and hybridized to 9448 unique mouse cDNA clones. Quantitative PCR of p21, cyclin G, and Par14 was performed using the iCycler iQ Detection System (Bio-Rad) and interaction dye SYBR Green.

(B) FACS analysis of Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF. MEFs were suspended in a solution containing 50 μg propidium iodide/ml in 0.1% sodium citrate and 0.1% Triton X-100 and analyzed by FACS using the CellQuest program (Becton Dickinson).

(C) Western blot analysis of Pin1 and Par14 in Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF. The proteins (30 μg/lane) prepared from cancer cell lines and MEF were analyzed by SDS-PAGE and electroblotting onto a nitrocellulose membrane. The membrane was incubated with the 1/500-fold diluted primary antibody (rabbit anti-Pin1 and anti-Par14) and then with the 1/1000 secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG). The membrane was soaked in the solution of ECL Western blotting detection reagents and exposed to X-ray film.

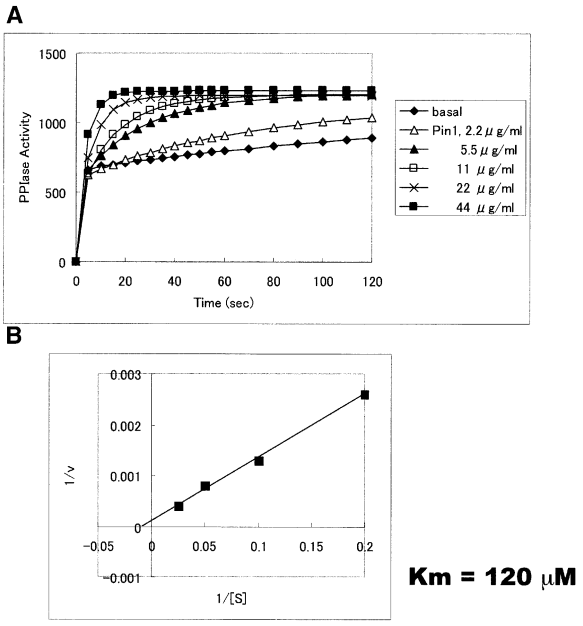


Figure 2. Pin1 PPIase Assay

(A) Pin1 PPIase assay. The assay was carried out measuring MCA fluorescence. Sixty-five microliters of assay buffer (50 mM HEPES, 100 mM NaCl, pH 7.0), 0–4 μl of Pin1 solution (11 mg/ml), and 10 μl chymotrypsin (60 mg/ml in 0.001N HCl) were mixed immediately, then 5 μl of the substrate solution, Suc-Ala-Glu-Pro-Phe-MCA, 100 μg/ml in trifluoroethanol containing 480 mM LiCl, was added. The reaction was performed for 0–120 s and stopped with 100 μl of acetic acid/methanol (1:1) followed by measurement of the MCA fluorescence (Ex/Em, 365 nm/460 nm) using a fluorescence microtiter plate reader.

(B) The Km of Pin1 was determined, and the screening conditions were established based on this Km value (120 μM).

didate target proteins, such as topoisomerases I and II, and we speculated that some of them might exert anticancer activity through Pin1 inhibition. We screened the compounds showing inhibition of Pin1 PPIase activity and roughly estimated the IC50 of them. Among these, we found a few relatively potent Pin1 inhibitors: PiA (2,7-dimethylbenzo[lmn][3,8]phenanthroline-1,3,6,8 (2H,7H)-tetrone) (IC50, 2.0 μM) and PiB (diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzo[lmn][3,8] phenanthroline-2,7-diacetate) (IC50, 1.5 μM) (Figure 3). We synthesized a series of compounds based on these structures (Figure 3). Among them, PiJ (diethyl-1,3,8,10-tetrahydro-1,3,8,10-tetraoxoanthra[2,1,9-def:6,5,10-d′e′f′]diisoquinoline-2,9-diacetate) inhibited Pin1 PPIase activity almost as strongly as PiB, with IC50s of about 1.5 μM. PiB and PiJ inhibited Par14 as potently as Pin1, with IC50s of 1.0 μM. Because Pin1 recognizes pSer or pThr in its targets, a phosphate mimic group might enhance inhibitor potency, although charged chemicals do not pass through cell membranes readily. However, derivatives of PiB with alkyl chains terminated with free COOH or $COOCF_3$ groups that have negative charge, such as PiC, PiD, and PiE, were no more potent as Pin1 inhibitors in the in vitro assay. The COOEt groups on PiB may be deesterified in the cell, generating a negative charge. Several of the inhibitors have symmetrical structures (e.g., PiB), which in principle would allow them to bind in either orientation and might increase potency.

The binding of the inhibitors to the PPIase domains of Pin1 [36] and Par14 [37, 38] was examined with the molecular modeling. Docking simulations made using DOCK 4.0 [39] suggested that PiJ and PiB can bind to the active centers of Pin1 (Figures 4A–4C) and Par14 (Figures 4D–4F), potentially interacting with or masking active site residues. Figure 4 shows models of PiB and PiJ bound to Pin1 and Par14 predicted by GOLD version 1.2, an automated ligand-docking program [40, 41].

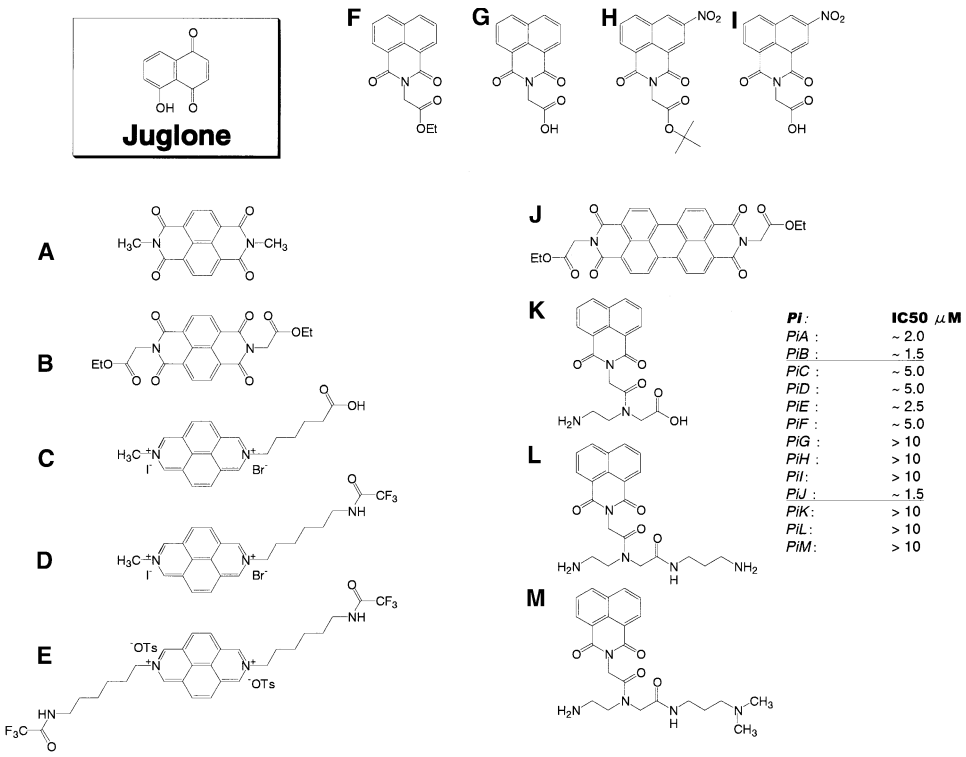


Figure 3. Structures of Pin1 Inhibitors

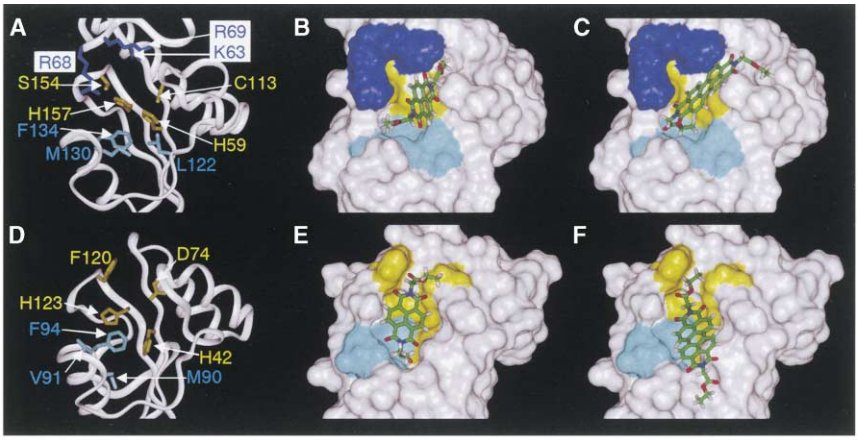
A known inhibitor, juglone, and the derivatives of the inhibitors we identified. PiA and B (IC50s, about 2 and 1.5 uM, respectively) and the derivatives were originally synthesized as inhibitors of topoisomerase I, but they did not exhibit such activities. All inhibitors consisted of a double-ring structure like juglone.

Judging from the docking scores (Figure 4G), these compounds can bind to both of Pin1 and Par14 with comparable affinities. They are docked into the substrate binding pocket and make close contacts with catalytic and substrate-recognition residues of the proteins (Figure 4G). These suggest that PiB and PiJ may inhibit the PPIase activities of Pin1 and Par14 in a competitive manner by masking their substrate binding sites, which is consistent with the observations made in the surface plasmon resonance analysis. Since the major components of the docking scores are contributed by van der Waals interactions, the aromatic rings of the compounds are probably important for binding, making hydrophobic interactions with the proteins. In addition, H^{ϵ} of Arg69 and H^{ϵ} of His157 of Pin1 and the backbone amide hydrogen (H^{N}) of Lys75 form hydrogen bonds with oxygen atoms of PiB, and H^{ϵ} and H^{N} of Gln131 and H^{γ} of Ser154 of Pin1 form hydrogen bonds with oxygen atoms of PiJ. Among these, H^{γ} of Ser154 and H^{ϵ} of His157 of Pin1 are suggested to be directly involved in the catalytic reaction [36]. Therefore, oxygen atoms of the compounds may be also important for specific binding to the proteins.

Effects of Pin1 Inhibitors on Cell Proliferation

To determine whether the Pin1 inhibitors reduced cell growth, their effects on the proliferation of a series of human cancer cell lines was tested. These inhibitors had antiproliferative activity against a variety of cancer cell lines. The IC50s for several cancer lines are summarized in Figure 5. Pin1 expression levels differed between the various cancer cell lines, whereas Par14 expression levels were similar in all the lines. Cells with a low level Pin1, such as HLE and HepG2 cells, were less sensitive to inhibitor treatment than cells expressing high levels of Pin1, such as HSC2, HCT116, and SKOV3 cells. The effects of PiB on the growth of wild-type and $Pin1^{-/-}$ MEFs were also examined. PiB inhibited proliferation of wild-type MEF (IC50, $\sim$1 μM), whereas the same concentration did not inhibit the growth of $Pin1^{-/-}$ MEFs (Figure 6). The proliferation of $Pin1^{-/-}$ MEF (B6 genetic background) reexpressing Pin1 following infection with an adenovirus vector was inhibited by PiB at a similar concentration to that required to inhibit growth of $Pin1^{+/+}$ MEF (Figure 6). The results suggest that one of the targets of PiB is Pin1 and that inhibition of Pin1 is apparently important for the antiproliferative activity of PiB. However, this does not rule out the possibility that PiB may have other targets in addition to Pin1. For example, Par14 is potentially another target of PiB (Figure 4). This idea is supported by the finding that siRNA depletion of Par14 inhibits the growth of $Pin1^{-/-}$ MEF but not $Pin1^{-/-}$ MEF reexpressing Pin1 (data not shown). The results suggest that other PPIases, including Par14, can substitute for Pin1, and the expression level of such proteins may influence the susceptibility of different cells to PiB inhibition.

To determine whether PiB caused an arrest at a specific cell cycle phase, $Pin1^{-/-}$ MEF cells infected with adenovirus vector or vector containing Pin1 cDNA were synchronized in G1 by serum starvation and then stimu-



G

*h*Pin1

Compound	Interacting atoms (ligand)	Interacting atoms (protein)	Distance Å	Type of interaction
PiJ	O (>C=O) O (>C=O) O (>C=O)	Arg69:NH2 Arg68:NH2 Arg68:NH1	3.84 4.00 3.48	hydrogen bond hydrogen bond hydrogen bond
PiB	O (>C=O)	Arg69:NH2	3.79	hydrogen bond

*h*Par14

Compound	Interacting atoms (ligand)	Interacting atoms (protein)	Distance Å	Type of interaction
PiJ	O (>C=O)	Lys119:NZ	3.05	hydrogen bond
PiB	O (>C=O)	Lys119:NZ	2.98	hydrogen bond

Figure 4. Molecular Modeling of Pin1 Inhibitors and Par14 Inhibitors

Crystal structure of Pin1 (A) and docking models of PiB (C) and PiJ (D) to Pin1. NMR structure of Par14 (D) and docking models of PiB (E) and PiJ (F) to Par14. In (A) and (D), the backbone structures are shown by ribbon representations. The side chains of catalytic and basic and hydrophobic substrate-recognition residues are shown by stick models and are colored yellow, blue, and cyan, respectively. Note that Par14 does not have the basic substrate-recognition residues [37], and there are thus no blue residues in (D). In (B), (C), (E), and (F), the surfaces of Pin1 and Par14 are shown and are colored with the residue colors used in (A) and (D). The compounds are represented by stick models where hydrogen, carbon, nitrogen, and oxygen atoms are colored white, green, blue, and red, respectively. The docking models were obtained as follows. The coordinates of Pin1 [36] and Par14 [37] were obtained from the Protein Data Bank (http://www.rcsb.org/pdb) (the identification codes are 1PIN and 1FJD, respectively). As for Pin1, only the PPIase domain (residues 45–163) was used in the calculation, and hydrogen atoms were attached using the Biopolymer module of the InsightII software (Accelrys Inc.). The protonation states of histidine residues in the active sites were modified based on the model of catalytic mechanism [36], where His59 (His42) and His157 (His123) of Pin1 (Par14) are protonated at N^{δ} and both of N^{δ} and N^{ϵ}, respectively. The models of PiB and PiJ were prepared using the Sybyl software (Tripos Inc.). The position of C^{α} of the substrate proline bound to Pin1 was used to define the search area. The protein atoms within 20 Å from this point were used in the docking calculation. As for Par14, corresponding point was calculated from superposition of C^{α} atoms of Pin1 onto those of Par14. The docking calculations were performed using GOLD version 1.2 with the standard default settings [40, 41]. Ten independent runs were carried out for each pair of ligand and protein, and the structure with the best score was adopted as the "docking model".

lated with serum to enter the cell cycle in the presence of 1 μM PiB. FACS analysis showed that the treated MEF did not arrest at a specific cell cycle phase (data not shown). Likewise, HCT116 cells did not arrest at specific cell cycle phase. The fraction of cells in S phase, however, was increased a little (Figure 7A). HCT116 cells treated with 1 μM PiB for 48 hr showed decreased expression of cyclins D1 and B1 (Figure 7B). In contrast, the levels of cyclins E and A were relatively unaffected (Figure 7B).

Discussion

Depletion of Pin1 from HeLa cells by antisense RNA expression and the depletion of Ess1 (yeast Pin1) from budding yeast by gene disruption both cause mitotic arrest and in HeLa cells. This leads to apoptosis [1, 34]. Given the ability of Pin1 to bind to and isomerize pSer/Thr-Pro motifs, this led to the suggestion that Pin1 regulates the G2/M transition in yeast and vertebrate cells by binding mitotic phase phosphoproteins, such as Cdc25, Wee1, Nek2, Cdc27, and topoisomerase IIa, and isomerizing the Ser/Thr-Pro peptide bond at sites phosphorylated by mitotic proline-directed kinases such as cyclin B/Cdc2 [16, 35]. We created mice lacking Pin1 by gene disruption and showed that $Pin1^{-/-}$ mice are viable, indicating that Pin1 is not essential for cell proliferation [10, 25]. However, $Pin1^{-/-}$ embryonic fibroblasts derived from these mice grow slower than normal MEF, and upon serum starvation they enter an irreversible G0 state [10]. These results suggest that there is a redundant function that can partially compensate for Pin1 in growing cells but cannot rescue $Pin1^{-/-}$ MEF from G0 arrest.

With regard to what other PPIases might compensate

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Pin1 Inhibitors	HSC2	HSC4	HCT116	Huh7	OVK2	SKOV3	HepG2	HLE
PiA	5.1	3.3	2.1	7.8	4.2	3.8	4.5	>10
PiB	0.7	2.8	1.2	2.5	1.0	1.1	4.8	>10
PiC	6.4	4.1	4.4	5.1	5.0	8.0	>10	>10
PiD	5.3	5.6	>10	5.1	5.7	5.5	>10	>10
PiE	7.2	5.8	>10	6.1	6.5	1.7	>10	>10
PiF	6.5	4.9	>10	6.3	5.2	>10	>10	>10
PiG	4.9	5.2	>10	4.5	5.5	>10	>10	>10
PiH	4.8	2.4	4.5	5.1	5.6	>10	>10	>10
Pi I	6.6	4.6	>10	5.1	5.1	4.1	7.1	>10
PiJ	0.8	1.5	1.2	1.1	1.3	1.5	5.5	>10
PiK	6.8	2.7	>10	>10	8.6	>10	>10	>10
PiL	7.2	5.2	>10	>10	7.7	>10	>10	>10
PiM	6.5	3.4	>10	>10	9.1	>10	>10	>10

(IC50; μM)

B

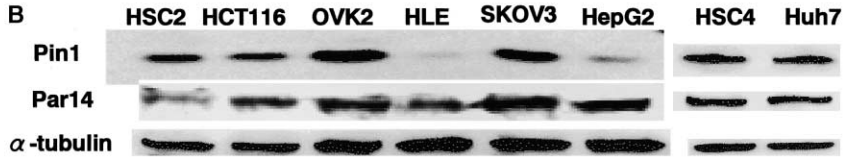
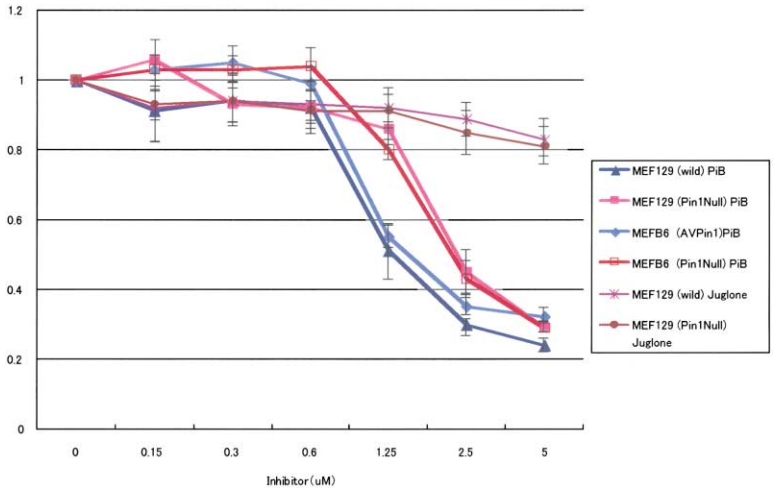


Figure 5. Effects of Pin1 Inhibitors on Cancer Cell Proliferation

(A) Results of anticell proliferation (MTT) assay. Cancer lines HSC2, HSC4, HCT116, Huh7, OVK2, SKOV3, HepG2, and HLE were treated with PiA derivatives (PiA-PiM). The IC50 for growth inhibition by these compounds for each cell line is listed.

(B) Expression levels of Pin1 and Par14 in these cancer lines used for the antiproliferation assay were detected by Western blot analysis (30 μg/lane).

for the lack of Pin1, the most obvious possibility is Par14. Under normal circumstances, their distinct peptide substrates preferences [2, 35], three-dimensional structures [36–38], and subcellular localizations [1, 42] suggest that Pin1 and Par14 PPIases have different biological roles in cells. However, the Par14 substrate profile is broad [2], and although the active site lacks the basic pocket present in Pin1 [37] that is needed for recognition of phosphorylated substrates [36] and dictates Pin1's more restricted substrate specificity, one might anticipate that Par14 will act on Pin1 targets, albeit less efficiently, particularly because Par14 lacks the WW domain



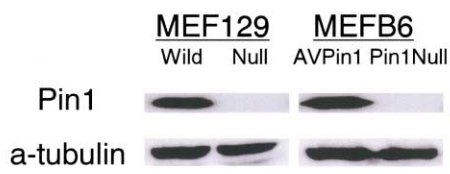
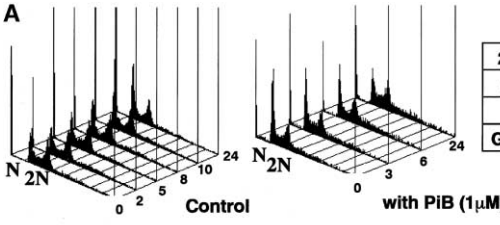


Figure 6. Effects of Pin1 Inhibitors on Pin1$^{-/-}$ MEFs

The effects of Pin1 inhibitors on cell proliferation were examined with MTT assay. Three thousand cells/well of the Pin1$^{-/-}$ MEF prepared from the original Pin1$^{-/-}$ mice and modified MEF prepared from Pin1$^{-/-}$ mice backcrossed onto the B6 genetic background and infected with adenovirus expression vector containing no insert or full-length Pin1 cDNA were plated in a 96-well plate and treated with PiB or juglone (0.15–5 μM).



24H	Control (%)	1 μM PiB (%)
G1	48.41	36.09
S	24.53	35.02
G2/M	27.75	29.67

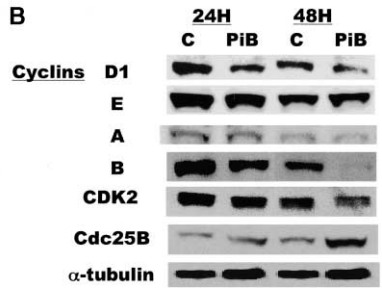


Figure 7. Effects of Pin1 Inhibitor on the Cell Cycle

(A) HCT116 cells synchronized at G0 by serum starvation were treated with 1 μM PiB for 48 hr, stained with propidium iodide, and analyzed by FACS. The percentages of the subpopulations of the cells are summarized in the table.

(B) The levels of the indicated cell cycle proteins in HCT116 cells treated with 1 μM PiB for 24 and 48 hr were analyzed by Western blotting. Forty micrograms of each cell lysate was loaded per lane followed by electrophoresis. The blot membranes were reacted with mouse monoclonal antibodies against cyclin D1, cyclin E, cyclin A, cyclin B, CDK2, CDC25B, and α-tubulin and then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody. ECL Western blotting detection reagents (Amersham, Arlington Heights, IL) were used according to the manufacturer's recommendations.

that allows Pin1 to bind to pSer/Thr-Pro-containing motifs. Cyclophilins also have a relatively broad substrate specificity [35], and cyclophilin PPIase activity compensates for the depletion of Ess1 in budding and fission yeasts [5, 6] even though Ess1 and cyclophilin belong to different PPIase subfamilies. It seems unlikely that FKBP family PPIase will substitute for Pin1 because FKBPs require a hydrophobic residue preceding the target proline.

To learn more about the phenotypes of Pin1$^{-/-}$ MEF, we looked for genes that are differentially expressed between Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF. Among the ~10,000 genes examined, no genes were found to be strongly upregulated in the Pin1$^{-/-}$ MEF. RNAs for several cell-cycle-related proteins, including p21 and cyclin G, which are p53-regulated genes [43, 44], were slightly upregulated. The most differentially expressed gene in the Pin1$^{-/-}$ MEF was Par14; Par14 RNA was increased about 3-fold (Figure 1A), and Par14 protein level was also increased in Pin1$^{-/-}$ MEF (Figure 1C). This provides further support for the idea that elevated Par14 might compensate for the lack of Pin1. On this basis, we reasoned that a small molecule able to inhibit both Pin1 and Par14 might block cell proliferation by causing cell cycle arrest.

We developed a screening method to isolate Pin1/Par14 PPIase inhibitors. The *cis* to *trans* isomerization of the Ser-Pro peptide bond proceeds fast, so we performed the assay at 4°C. The peptide substrate we chose was AEPF, because it is a peptide substrate that can be utilized by both Pin1 [11] and Par14 [2]. The reliability of this assay method was confirmed by enzyme kinetic analysis (Figure 2). Our chemical library screen identified PiA and PiB, originally synthesized as a derivative of PiA, as the most potent inhibitors with IC50s of ~2 μM and 1.5 μM, respectively. For comparison, juglone, the only previously reported Pin1 inhibitor, which is irreversible and rather nonspecific [45, 46], has an IC50 of 5 μM. PiA and PiB have double-ring structures like juglone, but otherwise their overall structures are completely different from the juglone quinone (Figure 3). PiA and PiB did not bind DNA and so did not exhibit inhibitory activities toward candidate target proteins such as topoisomerase I (data not shown). We synthesized several PiA/B derivatives and examined their IC50s. Among the derivatives, PiB and PiJ were the most potent inhibitors of Pin1 PPIase activity (Figure 3). PiB and PiJ interfered with the binding of the tetrapeptide AEPF to Pin1 (Figure 4A). PiB and PiJ were competitive inhibitors of Pin1 activity, whereas juglone was not. A model of the mechanism of enzymatic inhibition by PiB and PiJ is shown in Figure 4B. The molecular modeling results show that the compounds can potentially bind both Pin1 and Par14 at the active site (Figure 4), consistent with their ability to inhibit both PPIases.

PiB and PiJ were the most potent inhibitors of proliferation of several cancer lines (Figure 5A). Cancer lines expressing a low amount of Pin1 tended to be more sensitive to treatment with these inhibitors (Figure 5B). Par14 was expressed in all of the lines we examined, but there was no correlation between the Par14 level and the sensitivity to the inhibitors. These results support the idea that Pin1 is the critical target for these inhibitors, even though there are other potential target molecules such as Par14. The fact that the inhibitors did not block Pin1$^{-/-}$ MEF proliferation at a dose that was sufficient to inhibit growth of wild-type MEF (Figure 6) also suggests that Pin1 is a major target for PiB and PiJ. Cyclophilin C PPIase activity was only weakly inhibited by PiB and PiJ (IC50s >10 μM), making it unlikely that PiB and PiJ act on cyclophilins. FKBP PPIase activity was not inhibited at this dose (data not shown), consistent with the ability of cyclophilin 18 but not FKBP12 to act on AEPF [35].

To investigate how PiB and PiJ inhibit cell proliferation, we analyzed cell cycle events in the sensitive colon carcinoma line HCT116. Treatment with PiB increased the population of cells in S phase at 48 hr after cells were stimulated to enter the cell cycle following serum starvation, but there was not a specific cell cycle arrest (Figure 7A). The level of cyclins D1 and B1 was reduced in PiB-treated HCT116 cells, whereas the levels of

cyclins E and A, CDK2, and Cdc25B were largely unaffected (Figure 7B). The very low level of cyclin B1 will preclude entry into mitosis, but progression through other phases of the cycle may also be slowed and may account for the increase in S-phase cells.

Our results show that Pin1 is an important regulator of cell growth and cell cycle progression. Even though cells lacking Pin1 can cycle normally, presumably because other PPIases, such as Par14 or cyclophilins, can carry out Pin1 functions, Pin1-expressing cells rely on Pin1 to progress through the cell cycle. This finding is in keeping with recent evidence that the proliferation of cancer cells often remains dependent on functions that initiated their transformation, even though other growth-stimulatory pathways are in principle available to them. In this sense, Pin1-expressing cells are "addicted" to Pin1, whereas Pin1-deficient cells have "adapted" to doing without Pin1 function [47]. In this regard, it is interesting to note that Pin1 is overexpressed in a subset of tumor types and cancer cell lines [24, 26], and these cancers may be susceptible to treatment with Pin1 inhibitors.

In summary, we have identified inhibitors for Pin1/Par14 PPIases that inhibit cell proliferation. Pin1 inhibitors of this type could be useful as novel antitumor or antifungal drugs.

Significance

The phosphorylation of proteins is a vital biological signal in cells, critical for processes such as signal transduction, cell cycle progression, and apoptosis. It is well documented that the addition of a phosphate group can cause a protein to assume a different biological role from its unphosphorylated form. However, what is not understood is the structural basis for the observed functional changes as a result of phosphorylation. Peptidyl prolyl *cis-trans* isomerase (PPIase) catalyzes the *cis-trans* isomerization of prolyl peptide bonds, and PPIase activity is required for the assembly, folding, and transport of cellular proteins. Pin1 is a PPIase from the parvulin family that may be particularly important for cell signaling because, in addition to its PPIase domain, it has a WW domain at its N terminus that recognizes and interacts with phosphoserine (pSer) or phosphothreonine (pThr)-proline motifs in several proteins. Specifically, Pin1 binds to pSer/pThr-Pro sequences and isomerizes pSer/Thr-Pro bonds, altering the protein conformation in a phosphorylation-dependent manner and/or promoting protein dephosphorylation. This strongly supports a role for Pin1 as an integral component of the change in function of phosphorylated proteins. In mice, a knockout of the Pin1 gene resulted in viable offspring, albeit with detectable phenotypes, suggesting that mice have additional Pin1 genes or PPIases with overlapping functions. A second mammalian parvulin family PPIase, Par14, was identified. Although it lacks the basic pocket needed for substrate selection, Par14 may still substitute for Pin1. To investigate the roles of these proteins, we established a screen for Pin1 and Par14 inhibitors. A series of inhibitors were identified and were used to show that Pin1 is important for cell cycle progression. Moreover, these inhibitors slowed the growth of cancer cells expressing Pin1 and thus are promising drug leads that could be developed into novel anticancer drugs or, alternatively, potent antifungal agents.

Experimental Procedures

MEF Culture

Fetuses (13.5 days old) were obtained from wild-type and Pin1$^{-/-}$ mice. They were dissected, cut into small pieces, and digested with trypsin-EDTA. The mouse embryonic fibroblasts (MEF) obtained in this manner were seeded onto a 150 mm culture dish and grown in DMEM containing 10% fetal bovine serum for two passages. Then, cells at 60% confluence were cultured in DMEM containing 10% FCS for 3 days. The MEF were replated and harvested for experimental analysis. The cell cycle distributions of the Pin1$^{+/+}$ and Pin1$^{-/-}$ MEF were checked by FACS.

Microarray and Quantitative RT-PCR Analyses

Total RNA was prepared from the cultured MEF by extraction with acid phenol-guanidine-thiocyanate-chloroform, and poly (A)$^+$ mRNA was purified from the total RNA using oligo-(dT) cellulose chromatography. The poly (A)$^+$ mRNAs (OD260/OD280 = 1.9) were analyzed by DNA array (GEM Microarray, Incyte Genomics, Palo Alto, CA). The wild-type and Pin1$^{-/-}$ RNAs were labeled with CY3 and CY5, respectively, and hybridized to 9448 unique mouse cDNA clones from Incyte Genomics. Quantitative PCR was performed using the iCycler iQ Detection System (Bio-Rad, Richmond, CA) and interaction dye SYBR Green. PCR was performed with the following primers: p21, 5′-CACAGGCGGTTATGAAATTCACCC-3′, 5′-CAGAGCCC AGCTGGAGAAGAAGGG-3′; cyclin G, 5′-GTCTAGATGTCAGCCAAA GGT-3′, 5′-ATCAAATCAGTGCCGCCAGT-3′; and Par14, 5′-GAGTG ACAGTGCTGACAAGAAGGC-3′, 5′-ACCGGTGGGTCTGTAAACACA GGC-3′. The PCR conditions were 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. The amounts of the products at each step were monitored in real time.

Screening for Pin1 PPIase Inhibitors

Assay was carried out measuring the MCA fluorescence using Suc-Ala-Glu-Pro-Phe-MCA as substrate. The following reaction was performed in the cold room in 96-well microtiter dishes. One microliter compounds in DMSO (our own chemical library), 65 μl of assay buffer (50 mM HEPES, 100 mM NaCl, pH 7.0), 10 μl of Pin1 solution (11 μg/ml), and 10 μl chymotrypsin (60 mg/ml in 0.001N HCl) were mixed immediately, then 5 μl of the substrate solution, Suc-Ala-Glu-Pro-Phe-MCA (Japan Peptide Institute Co., Osaka; 100 μg/ml in trifluoroethanol containing 480 mM LiCl), was added. The reaction was performed for 20 s and stopped with 100 μl of acetic acid (100%)/methanol (1:1) followed by measurement of the MCA fluorescence (Ex/Em, 365 nm/460 nm) using a fluorescence microtiter plate reader (1420 Multilabel Counter, Wallac Co.).

MTT Assay

Three thousand cells per well of the Pin1$^{-/-}$ MEF prepared from the original Pin1$^{-/-}$ mice [5] and modified MEF prepared from Pin1$^{-/-}$ mice backcrossed onto the B6 genetic background and infected with adenovirus expression vector containing no insert or full-length Pin1 cDNA (kindly provided by Han-Kuei Huang) or several different cancer lines were plated in a 96-well plate (Becton Dickinson, Lincoln Park, NJ), and a variety of inhibitors were added to the cells. After the cells were incubated for 48 hr, 50 μl of a 3 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) was added. The cells were incubated with MTT for 6 hr at 37°C, after which 50 μl of a 25% (w/v) SDS solution with a pH of 2 was added. The plates were incubated overnight to dissolve the formazan crystals, and then the absorption at 540 nm was measured with a microplate reader (Bio-Rad, model 3550).

Western Blotting

The cancer cell lines and MEF were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, and 1% sodium deoxycholate) in the presence of protease inhibitors. Sample buffer containing 0.2% SDS and 0.6% 2-mercaptoethanol was added to 30 μg of each lysate; the samples were boiled for 10 min, then analyzed by SDS-PAGE and electroblotting onto a nitrocellulose membrane. The membrane was soaked in blocking solution (1× TBS, 0.05% Tween 20, 3% nonfat dried milk) for 1 hr and incubated with the primary antibody (rabbit anti-full-length Pin1 protein and anti-full-length Par14 protein antibodies [our preparation]) or mouse monoclonal antibodies against cyclin A, cyclin B, cyclin E, and CDK2 (BD Transduction Labs., Lexington, KY), cyclin D1 (Oncogene Research Products, Boston, MA), and α-tubulin (Sigma, St. Louis, MO) for 15 hr at 4°C. Then the membrane was washed three times with TBST (1× TBS, 0.05% Tween 20) and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG [Molecular Probes Inc., Eugene, OR]) for 1 hr. All the antibodies were diluted to 1:500. ECL Western blotting detection reagents (Amersham, Arlington Heights, IL) were used according to the manufacturer's recommendations.

FACS Analysis

HCT116 cells and MEF cells infected with adenovirus vector or the vector expressing Pin1 cDNA were cultured in DMEM containing 0.1% fetal bovine serum for 4 days, then in medium containing 5% fetal bovine serum with control (0.1% DMSO), 2, 5, and 10 μM PiB (0.1% DMSO) for 0, 6, 12, 24, and 48 hr. After observing the cells under a microscope, the cells were suspended in a solution containing 50 μg propidium iodide/ml in 0.1% sodium citrate and 0.1% Triton X-100 and analyzed by FACS using the CellQuest program (Becton Dickinson). To analyze the effect of inhibitors on G1 progression, serum-starved HCT116 cells were cultured in the presence of 1 μM PiB for 24 hr prior to serum stimulation.

Supplemental Data

A supplemental figure that shows how the IC50s of PiB and PiJ were determined is available. Please write to chembiol@cell.com for a PDF.

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