# Inhibitors Targeting the Enzymatic Activity and Biological Function of Pin1

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**Abstract:** Pin1, a phospho-Ser/Thr-Pro specific PPIase, participates in many biological processes. Recently, through designing substrate mimetics, or library screening, several classes of Pin1 inhibitors have been discovered. Some polyaromatic compounds, including juglone, as well as peptide mimetics containing both proline and phosphate, have been demonstrated to inhibit biological functions of Pin1.

**Keywords:** Pin1, inhibitor, PPIase, substrate mimetics, combinatorial library, phosphorylation.

## THE CHEMICAL ASPECT OF PEPTIDE BOND ISOMERISATION AND PPIASES

Peptide bond resonance first discussed by Linus Pauling in 1948 has revealed the exclusive rigidity of peptide bond among the linkages that form a protein backbone [1]. The resulting planar peptide bond and its partial double bond character cause restrictions in the number of energy minima in amide bond torsion [2]. The *cis* ($\omega \approx 0^oC$) and *trans* ($\omega \approx 180^oC$) isomers (Fig. **1**) are separated by a barrier corresponding to the perpendicular high energy state of $\omega \approx 90^oC$. Meanwhile, the steric hindrance of the $\alpha$-C of neighbouring amino acids in the *cis* conformation could account for the low percentage of secondary *cis* peptide bonds. Nineteen of the twenty gene-coded amino acids form secondary amidic peptide bonds, whereas the proline forms an imidic peptide bond at the N-terminal side. The two lowest energy arrangements of the peptidyl prolyl bond, *cis* and *trans*, are of comparable thermodynamic stability. The dynamic properties of peptide bond isomerisation [3] (peptidyl prolyl bond in particular) cause a slowly interconverting conformational multiplicity, and thus have a great influence on protein structure and folding.

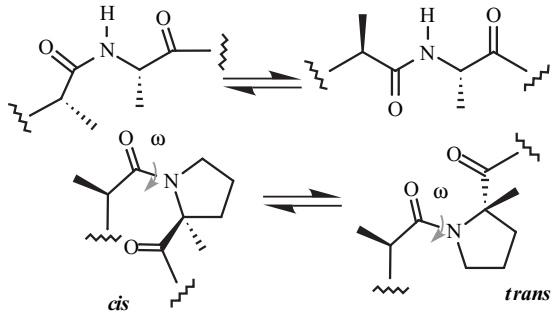


**Fig. (1).** Cis/trans isomerisation of peptide bond and peptidyl prolyl bond.

Protein folding was considered to be an autonomous process, not requiring enzymes or auxiliary substances. However, in recent years, several families of proteins have been discovered as folding helpers, including chaperones [4], protein disulfide isomerases [5], peptidyl prolyl *cis*/*trans* isomerases [6] (PPIases, EC 5.1.2.8), and amide peptide bond *cis*-*trans* isomerases (APIases) [7]. The *cis*/*trans* isomerisation of peptidyl prolyl bond is often a rate-limiting step in protein folding and PPIases catalyse the reaction in both directions. There are three known families of PPIases, cyclophilin (Cyp) [8], FK506 binding protein (FKBP) [9, 10], and parvulin (Par) [11-13]. Notably, Cyp18 and FKBP12 are immunophilins because they are the cellular receptors for immunosuppressive drugs cyclosporin A (CsA) [14, 15] and FK506 (Fig. **2**) [9, 10], respectively. However, the inhibition of Cyp18 and FKBP12 by the immunosuppressants is required but not sufficient for immunosuppression [16]. The Cyp18/CsA and FKBP12/FK506 share a common cellular target Ser/Thr protein phosphatase calcineurin [17, 18]. Inhibition of calcineurin by the immunophilin/drug complexes contributes to the immunosuppressive activities of both drugs. Cyclophilins and FKBPs are big families of PPIases and abundant in the cells. They have been found to participate in many important biological events, which have been covered by several excellent reviews [6, 19, 20].

## THE BIOLOGICAL EFFECTS OF HUMAN PARVULIN PIN1

Pin1 and Par14 are the two known human parvulins. Recently, Pin1 has been the subject of many interesting studies in the fields of cell cycle [21], cancer pathogenesis [22], and Alzheimer's disease [23, 24]. All these biological effects of Pin1 are related to its high substrate specificity and binding affinity toward pS/T-P (phosphorylated Ser/Thr-Pro motif) [25]. Pin1 is a two-domain protein [26]. While the substrate specificity is determined by its catalytic site within the PPIase domain exclusively [26], its binding specificity is mediated through both the WW domain [27], which exhibits high affinity to pS/T-P, and the PPIase domain that possesses relatively weak affinity to the same core motif. Because phosphorylation of S/T-P is an important signalling mechanism that controls a plenty of cellular processes, the preference of Pin1 toward pS/T-P places it as a general mediator in these events [21].

### Cell Cycle

A new phosphorylation regulatory mechanism of cell cycle has been proposed based on the interaction of Pin1 with a subset of mitotic phosphoproteins [21]. For instance,

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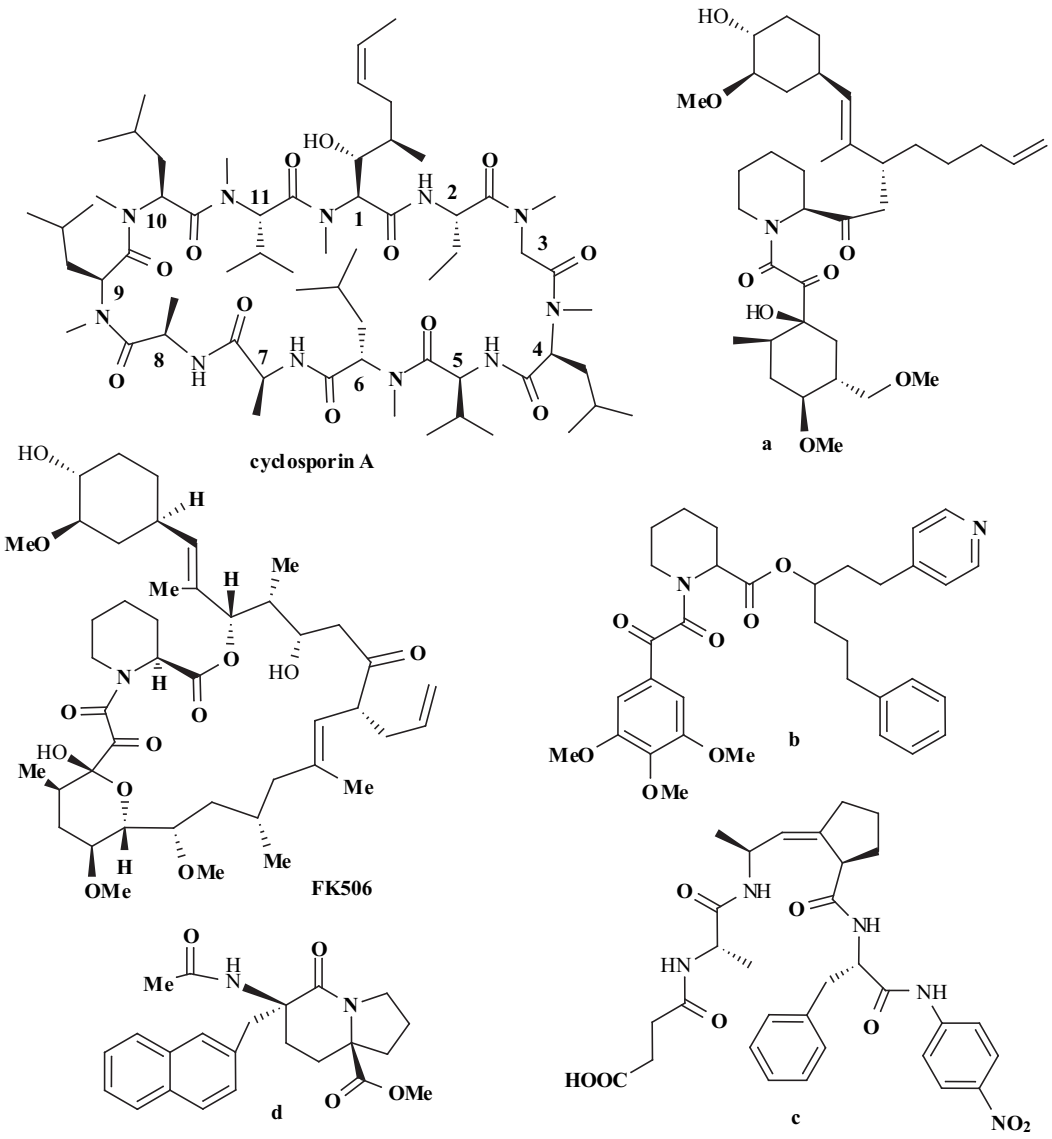


**Fig. (2).** Inhibitors for cyclophilins and FKBPs.

Pin1 may modulate cell cycle control through interaction with phosphorylated Cdc25 and its activator, Plx1 [28]. Depletion of Pin1 in budding yeast and tumour cell lines causes mitotic arrest and apoptosis [29]. Furthermore, Pin1 is required for the DNA replication checkpoint and G2/M transition in *Xenopus* extracts [30].

## Cancer Pathogenesis

The proline directed phosphorylation is also a central signalling mechanism controlling malignant transformation, as well as normal cell proliferation. Pin1 is overexpressed in several human cancer cells such as prostate, breast, cervical, brain, lung, and colon cancer [22, 31, 32]. Given that Pin1 could interact with a large number of proteins phosphorylated on the S/T-P motif, different mechanisms concerning Pin1's involvement in oncogenesis have been proposed. Results from human breast cancer and Pin1-knockout mice strongly suggest that Pin1 is essential for the regulation of cyclin D1, an oncoprotein that is common in breast cancer, through both transcriptional [32, 33] and post-transcriptional mechanisms [34]. Most recently, Zacchi *et al*. [35] and Zheng *et al*. [36] reported the stabilisation effect of Pin1 on p53, a tumour suppressor important in the cell decision either to arrest cell cycle progression or to induce apoptosis in response to a variety of stimuli, through interaction of Pin1 with p53 on several pS/T-P motifs.

## Alzheimer's Disease

Another intriguing function of Pin1 is its involvement in Alzheimer's disease, through interaction with hyperphosphorylated microtubule-associated protein tau, the main component of paired helical filaments that is an important neuropathological hallmark of Alzheimer's disease [24]. On one hand, Pin1 is able to restore the ability of hyperphosphorylated tau to bind to microtubules and promote microtubule assembly. On the other hand, depletion of soluble Pin1 by hyperphosphorylated tau induces mitotic arrest and apoptotic cell death. Most recently, Liou *et al*. reported that Pin1$^{-/-}$ mice exhibit progressive age-dependent neuropathy such as motor and behavioural deficits and tau

filament formation [23]. Notably, $Pin1^{-/-}$ mice represent the first Alzheimer's disease model generated through gene knockout.

## CHEMISTRY AND MOLECULE BASIS OF PIN1 BIOLOGICAL EFFECTS

One of the most interesting features of Pin1 is that both the catalytic and WW domains recognise pS/T-P specifically [37]. Due to the physiological and pathological importance of Pro-directed phosphorylation, Pin1 regulates a great number of cellular processes. However, the regulatory mechanism remains largely unknown, especially the function of Pin1 as PPIase.

As enzymes, PPIases accelerate the peptidyl prolyl *cis*/*trans* isomerisation in the direction of chemical equilibrium. No catalyst would have effect on a system that has already reached the equilibrium, e.g., a folded protein. Therefore, the catalytic effect of PPIase on a folded protein must be coupled with an energy event such as phosphorylation or dephosphorylation, which disturbs the *cis*/*trans* equilibrium of peptidyl prolyl bond. Probably the most exquisite example of the effect of PPIases on folded proteins have been shown by Zhou *et al*. [38]. One of the major pro-directed phosphatases PP2A is conformation-specific and effectively dephosphorylates only the *trans* pS/T-P isomer. Pin1 catalyses the peptidyl prolyl *cis* to *trans* isomerisation of pS/T-P motifs within peptides and proteins, therefore facilitates their dephosphorylation by PP2A [38]. Similarly, FKBP12 has been shown to accelerate the phosphorylation of oligopeptide substrate by MAP kinase ERK2 [39].

The influences of phosphorylation on peptidyl prolyl *cis*/*trans* isomerisation have been investigated in either peptides [40] or proteins [41]. Proteolysis assay [35, 42] has been successfully used as an indirect method to probe conformational change. Given that protein posttranslational modifications such as phosphorylation and dephosphorylation are highly complex and dynamic cellular processes, a direct demonstration of the resulted structural changes, from single peptide bond conformation to entire protein topology, represent daunting challenges for biochemical and biophysical researches. In particular, the involvement of PPIases catalytic activity in these events, as well as the structural basis for the high interconversion barrier of *cis*/*trans* isomerisation in the absence of PPIases, remains to be addressed in molecular details.

## INHIBITORS OF PPIASES

To answer the questions concerning Pin1 biological function, Pin1 inhibitor would be a powerful tool for either *in vitro* or *in vivo* studies. Moreover, Pin1 seems to be a good target for anticancer drug development since depletion of Pin1 induces mitotic arrest followed by apoptosis [29]. The studies of immunosuppressive drugs CsA and FK506 revealed an important signalling pathway in T cell activation [17], while CsA, FK506, and their derivatives have been widely used to probe the biological function of cyclophilins, FKBPs, and calcineurin. Design and synthesis of potent Pin1 inhibitors would help us to understand the role of Pin1 as a central regulator in many Pro-directed phosphorylation events, such as cell cycle, oncogenesis and Alzheimer's disease pathology.

Based on the structures of known natural products inhibiting cyclophilin and FKBP as well as the substrate preferences of the enzymes, various designed inhibitors of both families of PPIase have been reported. Non-macrocyclic compounds containing the minimal FKBP12-binding elements of FK506 were prepared and are capable of binding to FKBP12 with low nanomolar affinity [43-46] (Fig. **2a**). N-(glyoxy) pipecolyl esters [47, 48] (Fig. **2b**) and N-(glyoxy) prolyl esters were reported with low nanomolar up to picomolar affinities for FKBP. Replacement of the diketo portion of FK506 with other functionality, such as sulphonamide [47], urea [49] or *(E)*-alkene [50] resulted in compounds with inhibition constants in the low micromolar range. In general, peptidic inhibitors of FKBP are less potent than N-(glyoxy) pipecolyl derivatives [51, 52]. In the case of cyclophilin, several peptide bond surrogates were used to generate substrate derived inhibitors like fluoroolefins [53], *(Z)*-alkenes [54] (Fig. **2c**), ground-state analogues [55] (Fig. **2d**), and thioxo peptides [56]. However, the $K_i$ values were much higher than the respective value for CsA. A relatively general approach to convert substrate to inhibitor has been investigated through mapping the stereospecificity of PPIases [57]. It was demonstrated that a substrate derived diastereomer containing a D-amino acid on the position directly preceding proline represents a competitive inhibitor for several PPIases with substrate like affinities.

Immunosuppressive drugs cyclosporin A and FK506 are potent inhibitors for cyclophilins and FKBPs, respectively, and their structures have provided the basis for many designed inhibitors [58]. However, there is no natural product known as reversible Pin1 inhibitor. Moreover, although both the crystal [26, 59] and NMR [60] structures of Pin1 are available, two different catalytic site conformations within the PPIase domain have been suggested. Whereas Verdecia *et al*. [59] have resolved the crystal structure of Pin1 with a double phosphorylated peptide binding to the WW domain and observed an extended conformation of the $\alpha 1/\beta 1$ catalytic loop, Ranganathan *et al*. [26] reported the crystal structure of Pin1 with a dipeptide Ala-Pro binding to the active site and detected a closed conformation (Fig. **3**). The NMR structure [60] shows agreements with the structure proposed by Ranganathan *et al*. Without exact information concerning active site conformation and the binding preference to small molecule ligand, several approaches have been developed to discover Pin1 inhibitors, including combinatorial library screening and design of substrate mimetics.

## JUGLONE

In a first attempt to discover Pin1 inhibitor, the brown walnut dye juglone (5-hydroxy-1, 4-naphtochinone) (Fig. **4**) has been shown to inhibit several parvulins, including *E. coli* parvulin, yeast Ess1/Ptf1, as well as human Pin1, without inhibition of PPIases of other families [61]. Inactivation mechanism involves partial unfolding of the active site following covalent modification of active site cysteine.

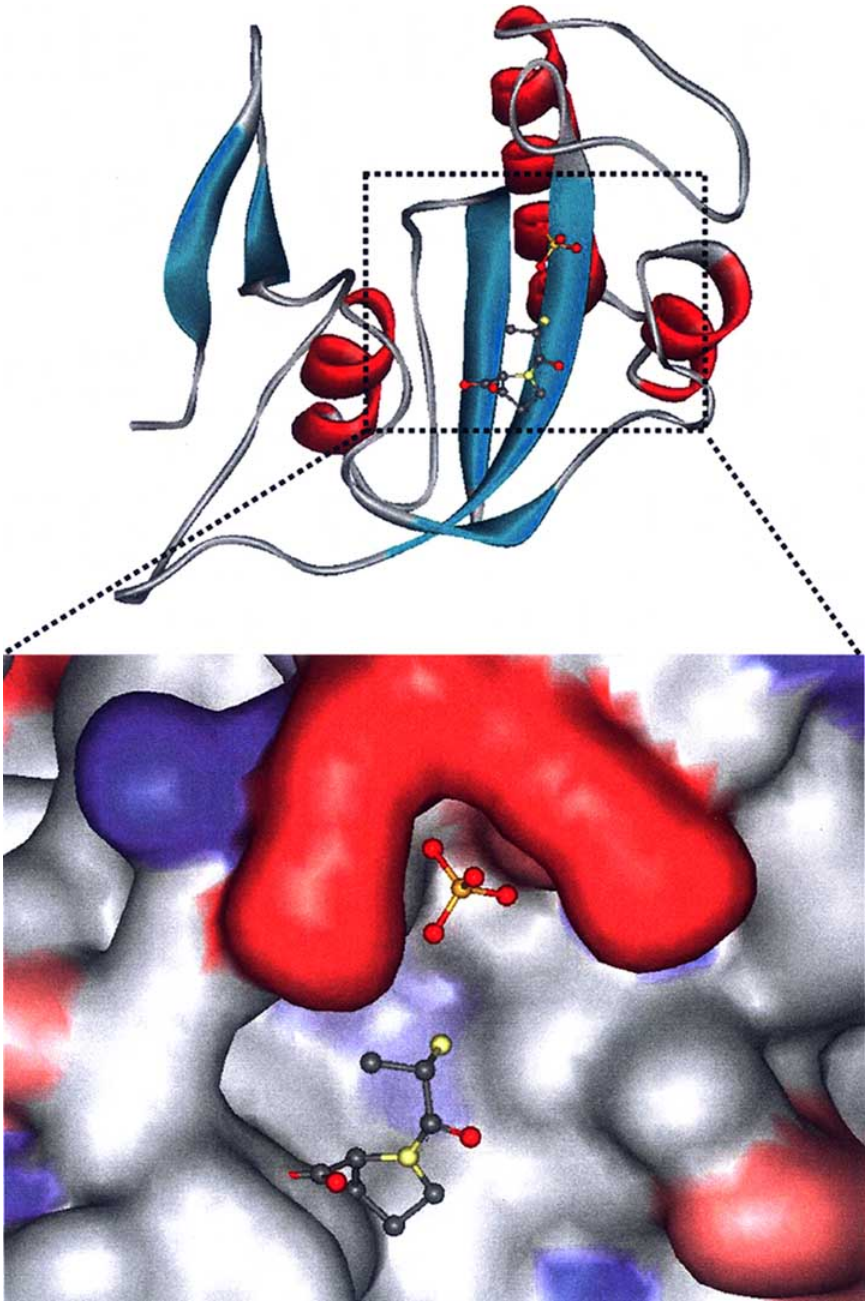

**Fig. (3).** The crystal structure of Pin1 in complex with Ala-Pro (up): The dipeptide Ala-Pro is shown as ball and stick. The substrate-binding pocket of Pin1 (down): A basic cluster (red) represents the potential interaction site for phosphate moiety, while a hydrophobic pocket binds to the proline ring.

Juglone has been tested in several studies of Pin1 biological functions. In neuroblastoma cells, whereas a strong correlation between Pin1 overexpression and an increase in cyclin D1 level has been found, inhibition of Pin1 by juglone resulted in a decrease of cyclin D1 [62]. In agreement with the expected important role of Pin1 in oncogenesis, juglone has been shown to block entry into mitosis of human lung carcinoma cells, as discovered in a high-throughput screening of small molecules that suppress cell-cycle at different phases [63]. Rippmann *et al*. have reported that juglone treatment induced apoptosis in HeLa cells [64]. Furthermore, the result was consistent with experiments that used other Pin1 depletion methods such as an overexpression of Pin1 antisense RNA or overexpression of dominant-negative Pin1.

Although juglone has proved to be a useful agent in probing Pin1 function, the high chemical reactivity of juglone toward other proteins (to the SH of cysteine in particular) prevents reliable conclusions being made exclusively from its inhibition data. For instance, Ess1, the yeast homologue of Pin1, exerts its effect on RNA processing through interaction with the phosphorylated CTD [65] (C-terminal domain of the large subunit of RNA polymerase II that contains from 26 to 52 copies of

YSPTSPS motif). However, in human Hela cells, juglone has been shown to block the initiation by RNA polymerase, but through a Pin1 independent mechanism [66]. Therefore, potent and reversible Pin1 inhibitors would be preferred for pinning down the cellular Pin1 specifically.

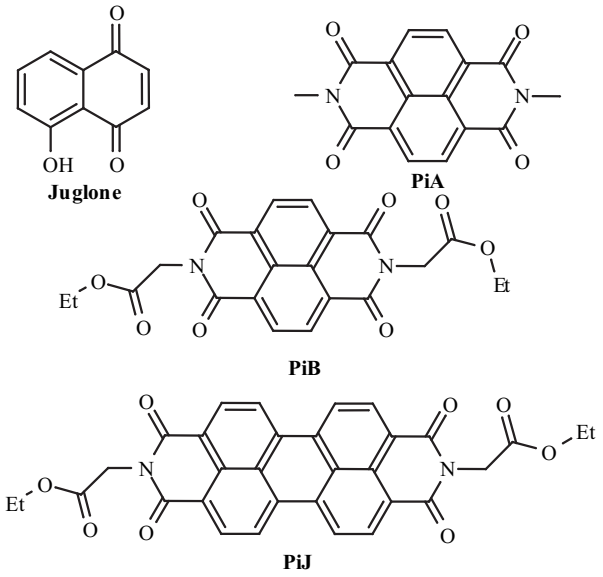


**Fig. (4).** Pin1 inhibitors: juglone and several cyclic compounds discovered in library screening.

## COMBINATORIAL LIBRARY SCREENING

The inhibition of Pin1 by juglone and its specificity toward parvulin among the PPIases have inspired to search for small organic molecules with polyaromatics structure. Meanwhile, many compounds have been found to possess potent anti-cancer cell activity from combinatorial libraries screening. However, the cellular targets of some compounds remain unknown. Therefore, to examine the effects of these compounds on Pin1 would illustrate whether the Pin1 inhibition could be accounted for their anticancer activity. Uchida *et al*. [67] have reported that a set of compounds inhibit Pin1 PPIase activity *in vitro* with low μM $IC_{50}$ values in a screening of a library containing 1000 chemically synthesised compounds. Among the inhibitors, PiB and PiA gave $IC_{50}$ values of 1.5 μM and 2 μM, respectively (Fig. **4**). Based on the core structure of PiA and PiB, several derivatives have been synthesised and PiJ was found to inhibit Pin1 as efficiently as PiB. Furthermore, both PiB and PiJ inhibit Par14 with low μM $IC_{50}$ as well, despite the peptide used in the assay, Suc-Ala-Glu-Pro-Phe-MCA, is a relatively poor substrate for Par14, as compared with its substrate property for Pin1. Molecule modelling experiments indicated that PiB and PiJ bind to the active site of Pin1 and Par14.

To verify the Pin1 inhibition of these compounds in cells, their effects in embryo fibroblasts of Mice (MEF) were examined. Mice lacking Pin1 developed normally [68]. Nevertheless, they displayed a range of cell-proliferative abnormalities and the breast epithelial compartment of $Pin1^{-/-}$ adult females failed to undergo the massive proliferative changes associated with pregnancy [34]. Although $Pin1^{-/-}$ MEF did not exhibit remarkable phenotype, wild type and $Pin1^{-/-}$ MEF showed different sensitivity to PiB and PiJ [67]. Furthermore, cells with a low Pin1 level were less sensitive to inhibitor treatment, as compared with the cells expressing a high level of Pin1. It seems that some other proteins could exert very similar functions as Pin1. Cyclophilin and Par14 are possible candidates. On one hand, Par14, another member of the human parvulins, is significantly up-regulated of $Pin1^{-/-}$ MEF. On the other hand, cyclophilin A and Ess1 (yeast homologue of Pin1) have been shown to function in parallel pathways in yeast [69]. Nevertheless, other targets could not be excluded since the inhibition of Par14 in the $Pin1^{-/-}$ MEF by PiB or PiJ exhibited relatively weak effect on cell proliferation, as compared with their inhibitory effect on wild type. Meanwhile, wild type and $Pin1^{-/-}$ MEF did not show different susceptibilities to cyclosporin A treatment. Furthermore, given the different substrate specificities of Pin1, Par14, and cyclophilin, the mechanism through which they perform a similar function as folding helpers or binding partners remains largely unknown.

## SUBSTRATE MIMETIC

Design of substrate mimetic represents another approach for discovering Pin1 inhibitors. Ac-Ala-Ala-Ser($PO_3H_2$)-Pro-Arg-NH-Np is known to be a very good substrate for Pin1 catalysis [40]. To investigate whether the peptide itself or a fragment of the sequence represents the core motif for Pin1 recognition, we developed a novel PPIase activity assay based on the different absorption coefficients between *cis* and *trans* isomers [70]. The method avoids the incorporation of any artificial chromogenic moiety and allows measuring the substrate feature of short peptides such as dipeptides and tripeptides. The dipeptide Ala-Pro, which binds to the Pin1 active site with a *cis* prolyl bond as observed in the crystal structure [26], as well as the Ser($PO_3H_2$)-Pro, are not substrates for Pin1, but competitive inhibitors. While a tripeptide seems to be the minimal requirement for Pin1 catalysis, the pentapeptide represents the optimal substrate length.

Incorporations of non-natural amino acids into peptides would be expected to impair the substrate property and to enhance the binding affinity and stability. For instance, replacing L-Ser with D-Ser in a substrate for casein kinase II reduced the catalysed phosphorylation significantly [71]. Incorporations of D-amino acid [56] and thioxo amide bond [57] have previously been shown to convert PPIase substrates to corresponding inhibitors. Moreover, the resulting thioxo peptides exhibited increased stability against cellular proteolytic activity. To substitute a phospho-D-Ser for a phospho-L-Ser impaired the PPIase substrate feature of Ac-Ala-Ala-Ser($PO_3H_2$)-Pro-Arg-NH-Np and no Pin1 catalysis was observed at high Pin1 concentration [70]. Based on this strategy, several non-natural peptides containing thioxo amide bond or phospho-D-Ser have been synthesised (Fig. **5a** and **b**). These compounds showed Pin1 inhibition with $IC_{50}$ values in the low μM range and exhibited high stability against cellular phosphatase activity. Furthermore, the phosphorylated peptidic mimetics display high specificity to Pin1, without effect on cyclophilin 18 and FKBP12.

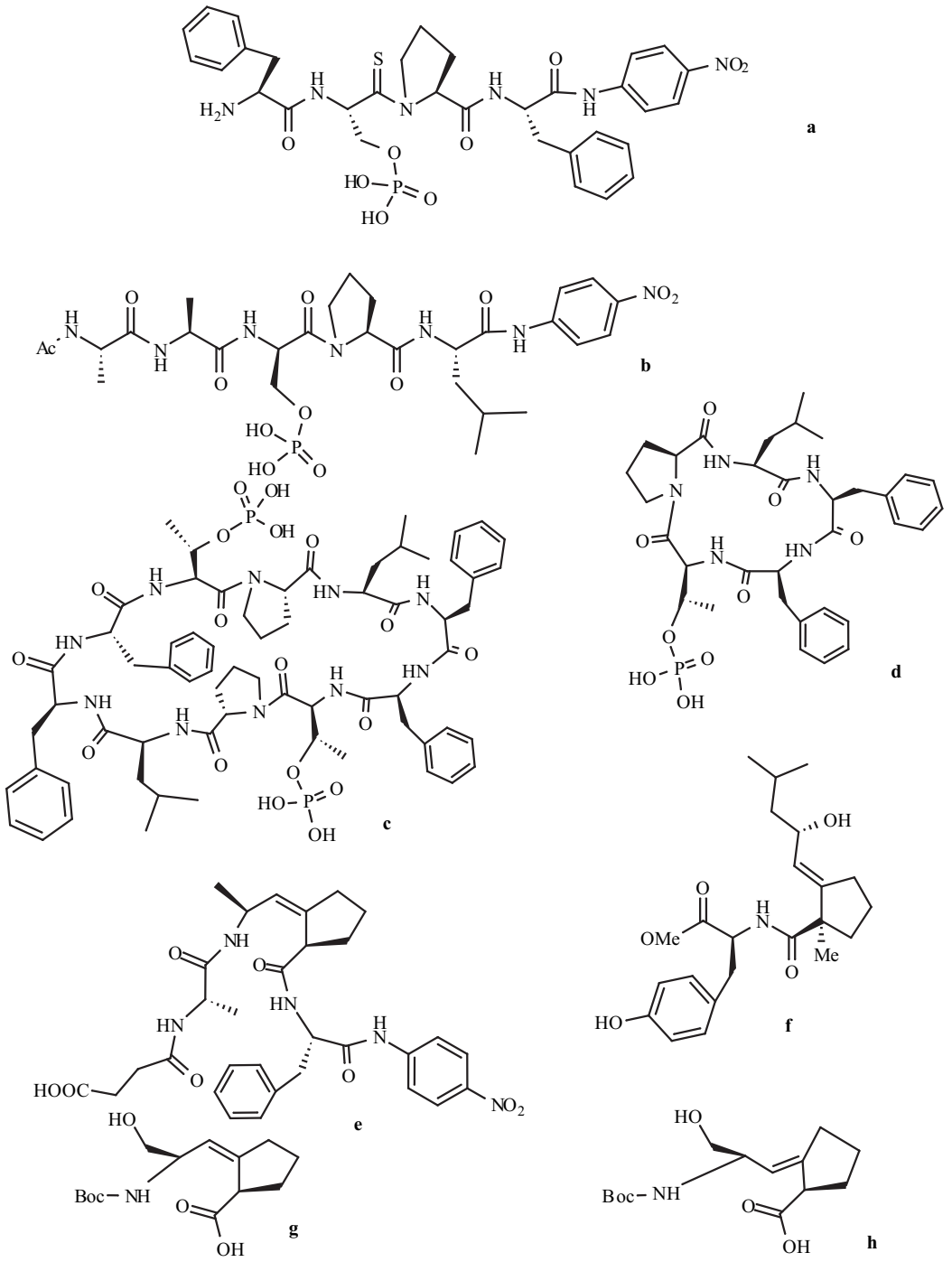

**Fig. (5).** Pin1 substrate mimetics as the inhibitors.

The Pin1 inhibitory efficiencies of mono- or poly-phosphorylated cyclopeptides have also been investigated (unpublished results). On the one hand, like thioxo peptides and D-peptides, cyclopeptides have attracted much interest in recent years because they are good candidates as inhibitors and drugs due to their proteolysis resistance. On the other hand, multiple phosphorylations on several S/T-P motifs within a peptide or protein could enhance its affinity to Pin1. In the case of CTD peptide YSPTSPS, the double phosphorylated form YpSPTpSPS displayed lower dissociation constant to Pin1, Pin1 WW domain, and Pin1 PPIase domain, as compared with its mono-phosphorylated forms YSPTpSPS and YpSPTSPS [59]. Given the fact that cyclisation would reduce the contacting interface between a peptide and a protein, we investigated the Pin1 inhibition by cyclic decapeptides and pentapeptides (Fig. **5c** and **d**). We synthesised a double phosphorylated cyclic decapeptide cyc [Phe-Phe-Thr($PO_3H_2$)-Pro-Leu-Phe-Phe-Thr($PO_3H_2$)-Pro-Leu], and found that it inhibits Pin1 with an $IC_{50}$ of 4.4 μM, whereas mono-phosphorylated cyclic pentapeptide cyc [Phe-Phe-Thr($PO_3H_2$)-Pro-Leu] inhibits Pin1 PPIase activity with an $IC_{50}$ of 18.2 μM (unpublished results). As expected, without phosphorylation, both cyclic peptides possess minor Pin1 inhibition.

## OTHER PEPTIDE BOND SURROGATES

The protease inhibitors could provide some interesting lessons for the design of PPIase inhibitor because the reactions catalysed by both enzymes occur on the peptide bond. Recently, some important progresses have been made in the design and synthesis of potent inhibitors for HIV protease [72]. One general strategy is to use transition state or peptidic bond mimetics, such as hydroxyethylamine dipeptide isosteres or (*E*)-alkene dipeptide isostere, to improve the binding affinity and bioavailability. For instance, Tamamura *et al*. [73] reported a purely nonpeptidic protease inhibitor based on the combination of hydroxyethylamine dipeptide isosteres and (*E*)-alkene dipeptide isostere replacing the peptide backbone in the parent structure. Etzkorn *et al*. have stereoselectively synthesised an Ala-*cis*-Pro (*Z*)-alkene isostere (Fig. **5e**) that inhibited the PPIase activity of cyclophilin 18 with an $IC_{50}$ of 6.5 μM [54]. An (*E*)-alkene *trans*-Pro mimic (Fig. **5f**) was shown to inhibit FKBP12 with an inhibition constant of 8.6 μM [50].

The successes in the discovery of protease and PPIase inhibitors through incorporating peptide bond surrogate have inspired the use of a similar strategy in the design of Pin1 inhibitor. Wang *et al*. [74] have recently reported a synthetic strategy of serine-*cis*-proline and serine-*trans*-proline isosteres. The resulting Boc protected (*Z*)-alkene and (*E*)-alkene (Fig. **5g** and **h**) would be ideal building blocks for constructing peptidic mimetics for Pin1 inhibition.

## CONCLUSION

The biological functions of Pin1 as a general regulator for cell cycle and proliferation, its involvement in Alzheimer's disease, as well as the role as a folding helper, have inspired many interesting studies recently. Whereas the potent inhibitors of cyclophilins and FKBPs have helped to illustrate the functions of both families of PPIases, no specific inhibitor was known for the parvulins. Screenings of small organic compound libraries have resulted in potent inhibitors such as juglone, PiA, and PiB. Furthermore, the biological effects of these compounds also indicate the involvement of Pin1 in cellular processes such as cell cycle. Nevertheless, to distinguish the Pin1 inhibition by these inhibitors from their effects on other cellular proteins would be necessary to verify their biological potencies. On the other hand, the substrate based approach has resulted in peptidic mimetics with high specificity to Pin1. To improve the inhibitory efficiency, bioavailability and cell membrane permeability of the phosphorylated compounds, construction and screening of libraries based on the known inhibitor's structures would be a reasonable approach in the future. Because Pin1 is a two-domain protein, a molecule consisting of two parts targeting each domain respectively would be expected to exhibit remarkably enhanced inhibition and specificity to Pin1 based on polyvalent interaction [75].

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