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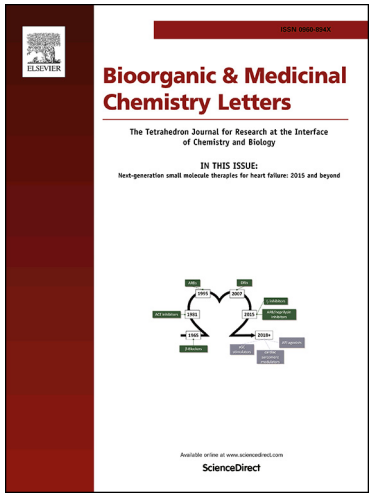

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An irreversible inhibitor of peptidyl-prolyl *cis*/*trans* isomerase Pin1 and evaluation of cytotoxicity

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ABSTRACT

Pin1 (protein interacting with never in mitosis A-1) is a member of the peptidyl prolyl isomerase (PPIase) family, and catalyzes *cis*-*trans* isomerization of pThr/Ser-Pro amide bonds. Because Pin1 is overexpressed in various cancer cell lines and promotes cell growth, it is considered a target for anticancer agents. Here, we designed and synthesized a covalently binding Pin1 inhibitor (*S*)-**2** to target Pin1's active site. This compound inhibited Pin1 in protease-coupled assay, and formed a covalent bond with Cys113 of Pin1, as determined by ESI-MS. The acetoxymethyl ester of (*S*)-**2**, i.e., **6**, suppressed cyclin D1 expression in human prostate cancer PC-3 cells, and exhibited cytotoxicity. Pin1-knockdown experiments indicated that a target for the cytotoxicity of **6** is Pin1.

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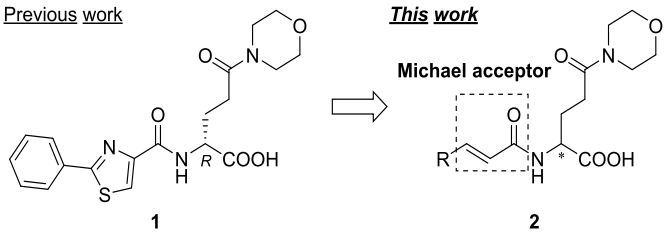


Fig. 1 Design of a covalent Pin1 inhibitor **2**

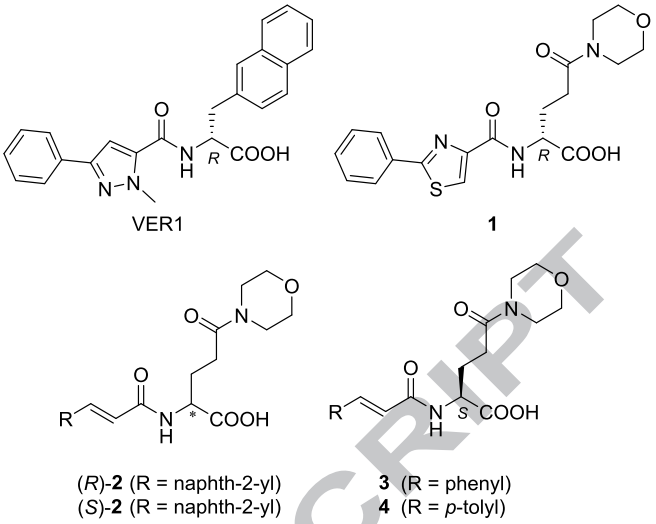


Fig. 2 Structures of VER1 and synthesized inhibitors

Pin1 (protein interacting with never in mitosis A-1) is a member of the peptidyl prolyl isomerase (PPIase) family, and specifically catalyzes *cis*–*trans* isomerization of pThr-Pro or pSer-Pro amide bonds in its substrate proteins. There are three subfamilies of PPIase, cyclophilins (Cyps), FK506-binding proteins (FKBPs), and purvulins. Pin1 is a member of the purvulin family and is the only enzyme that catalyzes isomerization of phosphorylated substrates in humans.[1, 2] Pin1 is involved in the regulation of kinase signaling processes by altering the ratio of *cis*-/*trans*-conformers of phosphorylated proteins[3] For example, signal transduction pathways involving cyclin-dependent kinases and MAP kinases, as well as cell-cycle controllers, are regulated by Pin1 activity.[4] Substrates of Pin1 include cancer-related signaling proteins such as cyclin D1, NF-kB, and p53.[5–8] Furthermore, Pin1 is overexpressed in various types of cancer cells, including prostate cancer, rectal cancer, hepatic cancer, and esophageal cancer.[2] It was also reported that the prognosis of prostate cancer is related to the expression level of Pin1 in the cancer cells.[9] Thus, Pin1 may be a new therapeutic target for these cancers. Pin1 is also involved in the pathogenesis of Alzheimer's disease by isomerizing phosphorylated tau proteins, resulting in a reduction of tau-dependent fibril formation. Thus, Pin1 catalyzes a unique reaction, and contributes to the temporal regulation of protein phosphorylation, acting like a 'molecular timer'.[10] The catalytic domain of Pin1, containing the cation-recognition site, consists of Lys63, Arg68, and Arg69, which serve to stabilize the phosphoryl moiety of the substrate peptide via electrostatic effect.

Several Pin1 inhibitors have been reported.[11–19] Among them, KPT-6566 is a covalent inhibitor targeting Cys113 in the catalytic site of Pin1; it has a quite potent inhibitory activity (K_i = 625.2 nM, k_{inact} = 0.466 min^{-1}).[20] Although irreversible enzyme inhibitors can be problematic from the viewpoint of toxicity, they are potent, and some are in clinical use. We previously described Pin1 inhibitors with a D-glutamic or D-aspartic acid structure bearing a cyclic aliphatic amine moiety (Fig. 1).[21] Here, based on the structure of amino acid type inhibitors, we designed and synthesized an irreversible inhibitor **2** which was expected to covalently bind to Cys113, one of the active site residues of Pin1, identified by ESI-MS analysis after trypsin digestion. A membrane-permeable derivative **6** suppressed expression of cyclin D1, which is stabilized by Pin1, in human prostate cancer PC-3 cells. It also inhibited cell growth of PC-3 cells, and this inhibition was suppressed in Pin1-KD PC-3 cells. These results indicated that the membrane-permeable derivative **6** successfully enters the cell, where it is hydrolyzed to **2**, which selectively inhibits Pin1, stabilizing cyclin D1 and suppressing the growth of PC-3 cells. Although the inhibitory activity and cell cytotoxicity were less than that of KPT-6566, in this paper, we showed how to rationally design an irreversible inhibitor based on a known inhibitor and chemical reaction.

It was reported that the role of Cys113 in Pin1's catalytic activity is to attack the carbonyl carbon of the amide bond or to serve as a hydrogen bond donor to the carbonyl oxygen of the amide bond.[22] We previously developed Pin1 inhibitors that have a D-glutamic or D-aspartic acid structure bearing a cyclic aliphatic amine. These compounds probably bind to Pin1's active site via three interactions: ionic interaction of carboxylate of the inhibitor with the cationic pocket of Pin1, hydrophobic interaction of the aromatic groups with the proline-binding pocket of Pin1, and interaction of another aryl group with the hydrophobic surface of the Pin1 catalytic site. Among these compounds, compound **1**, which was tethered to 2-phenylthiazole, showed potent Pin1-inhibitory potency (Fig. 2). The results of docking simulation between Pin1 and **1** suggest that Cys113 of Pin1 is located close to the 2-phenylthiazolyl group of **1**. Based on all the above considerations, we designed an irreversible inhibitor **2** having a *trans*-2-(2-naphthyl)ethenyl group as a Michael acceptor to covalently bind to Cys113 of Pin1, instead of a 2-phenylthiozolyl group. Further, to confirm the importance of the stereochemistry at the α-carbon, we synthesized and evaluated (*R*)-**2** and (*S*)-**2**. The structure and purity of the synthesized compounds were confirmed by means of ^{1}H NMR, ^{13}C NMR, HRMS, HPLC, and elemental analysis.

The Pin1-inhibitory activity of the synthesized compounds was evaluated by means of the proteinase-coupled assay method.[23] Briefly, the indicated concentrations of test compound (as a DMSO solution; the final DMSO concentration was 5% v/v) were preincubated with 0.2 mM DTT, 100 μg/mL BSA, and 22 nM Pin1 in 150 μL of 35 mM HEPES-KOH (pH 7.8) for 10 min at 10 ºC, followed by enzymatic reaction with 250 μM synthetic substrate peptide (suc-Ala-Glu-Pro-Phe-pNA). *C*-Terminal hydrolysis of the substrate peptide was then initiated by addition of an excess amount of α-chymotrypsin (150 μL of 0.8 mg/mL protease in 35 mM HEPES-KOH, pH 7.8). The absorbance of the released *p*-nitroaniline (pNA) at 390 nm was recorded for 10 min with a spectrophotometer. α-Chymotrypsin rapidly digested the substrate peptide initially present in *trans* form (rapid phase), and then slowly hydrolyzed the *trans* form as it was generated via conversion of the *cis*- to *trans*-form by Pin1 (isomerization phase). The observed reaction rate of the isomerization phase was thus taken as the Pin1 activity. The inhibitory activity was expressed as $((k_{(inh)} - k_0)/(k_{(noinh)} - k_0)) \times 100$ (%), where $k_{(inh)}$ is the observed pseudo-first-order rate constant in the presence of an inhibitor, $k_{(noinh)}$ is that without inhibitor, and k_0 is that in the absence of Pin1.

Table 1. IC_{50} values of synthesized inhibitors and VER1

inhibitors	IC_{50} (μM)
VER1	3.9[16]
1	5.4[21]
(*R*)-**2**	8.9
(*S*)-**2**	3.2
3	>100
4	>100

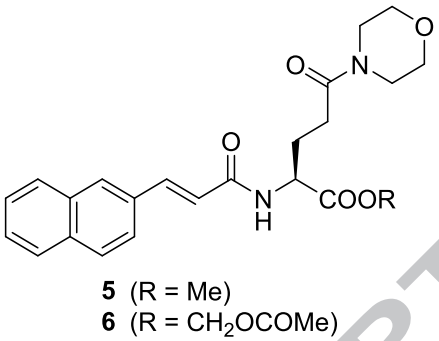
As shown in Table 1, the inhibitory activity of (*S*)-**2** was comparable with those of lead compound **1** and a potent Pin1 inhibitor VER1 reported by Vernalis (Fig. 2).[16] Interestingly, (*S*)-**2** showed 2.8 times stronger inhibitory activity than (*R*)-**2**. To examine why (*S*)-**2** was more potent than (*R*)-**2**, we conducted docking simulation with Glide software (Schrödinger, Fig. S1). The G-score, which is a docking score based on the free energy change, of (*S*)-**2** was calculated to be –8.12 while that of (*R*)-**2** was –5.98, indicating that (*S*)-**2** is a better fit to the Pin1 active site. Aiming to optimize the size of naphthyl group of (*S*)-**2**, we converted its naphthyl group, which was expected to interact with the hydrophobic surface of Pin1, to smaller aryl groups, such as phenyl (**3**) and *p*-tolyl (**4**). However, these compounds did not show potent inhibitory activity. This result indicated that the size of substituent which interacted with the hydrophobic surface was more important factor than stereochemistry of alpha carbon. From these results, we chose (*S*)-**2** as the optimized inhibitor, and used it in the following experiments.

First, to confirm that Cys113 forms a covalent bond with (*S*)-**2**, we analyzed the reacted solution by means of ESI-MS (Fig. 4). The incubated solution of Pin1 and (*S*)-**2** was digested with trypsin, and the products were labeled with excess 2-iodoacetamide (IAA), and subjected to ESI-MS analysis. As shown in Fig. 3, in the presence of (*S*)-**2**, the expected fragment (m/z 2520) was observed, while in the absence of (*S*)-**2** only the peak at m/z 2180 was seen. From these results, it was suggested that Pin1 inhibition by (*S*)-**2** is due to Michael addition of the inhibitor with Cys113 of Pin1.

Next, we measured the potency of (*S*)-**2** in terms of the k_{inact}/K_i ratio, where K_i is the affinity of the initial non-covalent interaction and k_{inact} is the rate of the subsequent bond-forming reaction.[24] We measured these kinetic parameters of (*S*)-**2** and (*R*)-**2**. As shown in Table 2, the k_{inact} values of the compounds were calculated to be 3.42×10^{-7} s^{-1} and 9.41×10^{-8} s^{-1}, respectively. The K_i values were estimated to be 1.37 μM and 5.47 μM, respectively. From these results, the values of the index of irreversible inhibition were calculated to be 0.249 and 0.017, meaning that (*S*)-**2** is a more potent irreversible inhibitor than (*R*)-**2**.

Table 2. Kinetic parameters of (*R*)- and (*S*)-**2** for Pin1 inhibition

	k_{inact} (s^{-1})	K_i (μM)	k_{inact}/K_i ($M^{-1}\cdot s^{-1}$)
(*S*)-**2**	3.42×10^{-7}	1.37	0.249
(*R*)-**2**	9.41×10^{-8}	5.47	0.017



5 (R = Me)
6 (R = CH_2OCOMe)

Fig. 4 Structures of membrane-permeable

Therefore, we next aimed to employ (*S*)-**2** for cell-based assays. Because (*S*)-**2** showed poor cell membrane permeability, we synthesized the methyl ester (**5**) and acetoxymethyl ester (**6**) of (*S*)-**2**, since these compounds are expected to be hydrolyzed by intracellular esterases to form (*S*)-**2** after uptake into cells (Fig. 4).

We confirmed that the synthesized inhibitors alter the expression level of cyclin D1, which is upregulated by Pin1 in PC-3 prostate cancer cells.[25] After incubation of PC-3 cells with each inhibitor for 24 hours, the lysate was subjected to Western blotting analysis. As shown in Fig. 5, compound **6** and VER1, a reference compound, suppressed the expression of cyclin D1, whereas compound **5** did not. This is likely because the acetoxymethyl ester is more easily hydrolyzed than the methyl ester under intracellular conditions.

Next, we conducted cell viability assay of compound **5**, **6,** and VER1 against not only PC3, but also human colon cancer cell, HCT116, and human normal diploid cells, TIG1 using a water-soluble tetrazolium (WST-8, Fig. 6) in order to evaluate the cytotoxicity. From the results of Western blotting for each cells, PC3 cells expressed the most amount of Pin1 in these three cells,

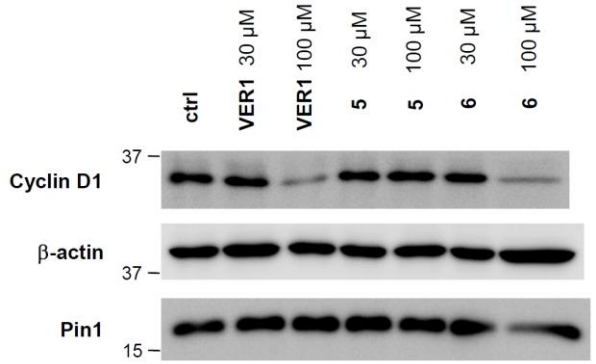
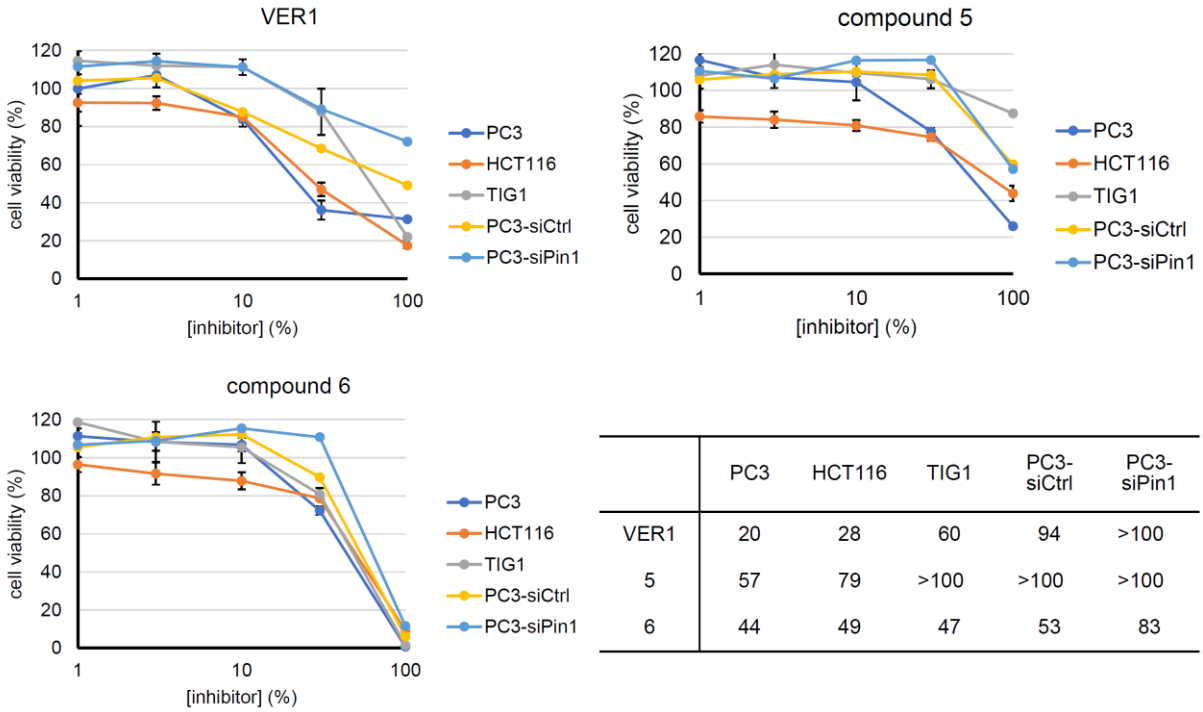


Fig. 5 Western blotting to quantify cyclin D1 in PC-3 cells treated with (*S*)-**2**. PC-3 cells were incubated with (*S*)-**2** for 24 hours, then the lysate was subjected to Western blotting experiments.

(a) Pin1 sequence
MADEEK/LPPGWEK/R/MSR/SSGR/VYYFNHITNASQWER/PSGNSS
SGGK/NGQGEPAR/VR/CSHLLVK/HSQSR/R/PSSWR/QEK/ITR/TK/E
EALELINGYIQK/IK/SGEEDFESLASQFSD**C**$_{113}$SSAK/AR/GDLGAFSR/
GQMQK/PFEDASFALR/TGEMSGPVFTDSGIHIILR/TE

(b) (c)



	PC3	HCT116	TIG1	PC3-siCtrl	PC3-siPin1
VER1	20	28	60	94	>100
5	57	79	>100	>100	>100
6	44	49	47	53	83

Fig. 6 Cell viability assay using WST-8. After incubation of each cell with the indicated compounds for 48 hours, the cell viability was measured using WST-8.

whereas ITG1 was the least. (Fig. S2). After incubation of each cell in the presence of each inhibitor for 48 hours, the cells were treated with WST-8 at 37 ºC for 2 hours. The cell viability was calculated from the absorption at 450 nm. As shown in Fig. 6, VER1, compound **5**, and compound **6** all showed slightly cytotoxic activity against cancer cell lines, PC3 and HCT116. A possible reason why **6** is less potent than VER1 may be that the hydrolysis of **6** was rate-limiting for the inactivation reaction. Compound **5** showed the weakest cytotoxicity because hydrolysis of methyl ester was probably slower than that of acetoxymethyl ester. Further, compound **6** showed slight toxicity to not only cancer cells, PC3 and HCT116, but also human normal cells, TIG1. Probably, compound **6** represented off-target effect after hydrolysis even in TIG1 cells. To confirm that these compounds inhibited intracellular Pin1, we conducted Pin1 knockdown in PC-3 cells (PC3-siPin1), and examined the effect on the IC_{50} value. After knockdown of Pin1 in PC-3 cells with siRNA, cell viability assay was conducted (Fig. S3, 6). The IC_{50} value of compound **6** for PC3-siPin1 cells was 83 µM, while that of PC-3 cells treated with a control siRNA (PC3-siCtrl) was 53 µM. This result indicated that compound **6** did not mainly affected Pin1 to show its moderate toxicity in cellular condition.

In conclusion, we designed and synthesized a new covalent (irreversible) Pin1 inhibitor, (*S*)-**2**. Its acetoxymethyl ester, **6,** suppressed cyclin D1 expression in PC-3 cells and exhibited moderate cytotoxicity. Probably, the cellular main target of compound **6** was not Pin1, and identification of the target is currently in progress.

Acknowledgments

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Supplementary Material

Supplementary material that may be helpful in the review process should be prepared and provided as a separate electronic file. That file can then be transformed into PDF format and submitted along with the manuscript and graphic files to the appropriate editorial office.