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Inhibitors

Small Molecule Pin1 Inhibitor Blocking NF-κB Signaling in Prostate Cancer Cells

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Abstract: Prolyl-isomerase 1 (Pin1) is a conserved enzyme that regulates cell processes such as cell cycle progression, transcriptional regulation, and apoptosis. However, overexpression of Pin1 is correlated with a higher probability of prostate tumor recurrence. We utilized a molecular docking technique to identify Pin1 inhibitors from a database of natural product and natural product-like compounds. The action of the hit compounds against Pin1 activity was studied using multiple methods, including a fluorometric enzymatic assay, co-immunoprecipitation, western blotting, cell thermal shiftm, and other techniques. We have identified compound **1** as a natural-product-like inhibitor of Pin1 activity via structure-based virtual screening and showed that compound **1** could target Pin1 and disrupt the interaction between Pin1 and the p65 subunit of NF-κB in cells. Furthermore, compound **1** reduced nuclear p65 (Thr254) phosphorylation and attenuated NF-κB activity in cells. Finally, compound **1** induced apoptosis in prostate cancer cells. Compound **1** represents a natural product-like Pin1 inhibitor that acts via targeting the Pin1–NF-κB interaction.

The reversible phosphorylation of proteins is a central regulatory mechanism in cells.[1] Prolyl-isomerase 1 (Pin1) subjects Ser/Thr-Pro phosphorylated proteins to post-phosphorylation prolyl isomerization, which in turn regulates cell processes such as cell cycle progression, transcriptional regulation, and apoptosis.[2] Pin1 is overexpressed in malignancies such as prostate, breast, and lung cancers.[3] Moreover, overexpression of Pin1 is correlated with a higher probability of prostate tumor recurrence.[4] Mechanistically, overexpression of Pin1 activates oncogenic signaling molecules including cyclin D, p65 and β-catenin, through altering protein activation and stabilization.[5] This has stimulated the development of Pin1 inhibitors for the potential treatment of cancer.[5] There are several Pin1 small molecule inhibitors were discovered, such as the naphthoquinone juglone from walnut trees,[6] EGCG from green tea[7] and dipentamethylene thiuram monosulfide.[8] Another class of Pin1 inhibitors was developed from cell-active analogs of pepticinnamin C, a farnesyl transferase inhibitor synthesized by actinomycetes.[9] Peptidic Pin1 inhibitors have been generated by modifying the isomerized peptidyl bond, with the carbonyl oxygen being replaced by a sulfur atom, which converts the substrate into an effective inhibitor.[10] **PiB**, a fused tetracyclic tetraone, inhibits Pin1 and suppresses the growth of cancer cells.[10,11] *Cis* and *trans* Peptide Mimetic Inhibitors utility of both *cis*- and *trans*-locked alkene isosteres as close geometric mimics of peptides bound to Pin1.[12] Another reduced-Amide Inhibitor of Pin1 binds in a conformation resembling a twisted-amide transition state.[13] PPIase domain of Pin1 catalyzes the *cis*/*trans* isomerization of prolyl bonds to regulate protein activation and stabilization through recognize the phosphoserine/phosphothreonine-proline motifs. A peptidic Pin1 inhibitor, (2S,4R)-4-fluoroproline, can induce C–H-π interaction to enhance the affinity of the peptide for the WW domain.[14] However, no Pin1 inhibitors have been applied for prostate cancer clinical therapy. To date, only one Pin1 inhibitor, all-*trans* retinoic acid (ATRA), has been applied for acute promyelocytic leukemia (APL) therapy.[15] Natural product and natural product analogues offer a rich source of bioactive scaffolds for the development of new drugs.[16] Meanwhile, structure-based virtual screening has been widely applied to early-stage drug discovery campaigns in recent years.[17] In silico technologies can potentially accelerate drug discovery[18] and reduce the cost of drug development.[19] In this work, we utilized molecular docking technique to identify Pin1 inhibitors from a database of natural product and natural product-like compounds and evaluated the Pin1 inhibitory activity of the hit compounds using biological assays. The high-resolution crystal structure of Pin1 in complex with (R,E)-2-(2- naphthamido)-5-phenylpent-4-enoic acid (PDB: 3JYJ) was utilized to generate a molecular model for our investigations.[20] Over 90,000 natural product and natural product-like compounds were screened against this model in silico. 9 compounds (**1**–**9**) ex-

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hibiting binding scores less than −30.0 (Figure 1) were selected for biological validation using an in vitro Pin1 activity assay. In this assay, the ability of the compounds to inhibit the conformational change of a Pin1 substrate by Pin1 is measured.

From the preliminary biological evaluation, compound **1** is a promising inhibitor of Pin1 activity (Figure 2a). In a dose-response experiment, compound **1** inhibited Pin1 activity with an EC_{50} value about 1.07 μM, whereas a reported Pin1 inhibitor **PiB** had an EC_{50} value of ca. 5 μM (Figure 2b). To our knowledge, no biological activities of compound **1** have been reported in the literature. An analogue of **1**, a shikimic acid derivative, was identified as an Aurora kinase inhibitor against Aurora A-associated tumors, however, its molecular mechanism of action was not clear.[21] To further investigate the mechanism of action of compound **1**, molecular modelling of compound **1** with Pin1 was performed. The docking results indicated that compound **1** was situated in the Pin1 active site (Figure 3). The hydroxyl group of the cyclohexene motif of compound **1** serves as a hydrogen bond acceptor in bonding with the side chain of the Gln131. Moreover, the carbonyl oxygen atom of the cyclohex-1-enecarboxamide motif of compound **1** was predicted to form a hydrogen bond with the side chain of Arg69 of Pin1, while the N-H group of another amide moiety of the compound was expected to form a hydrogen bond with the backbone of Asp112. Additionally, the terminal aromatic

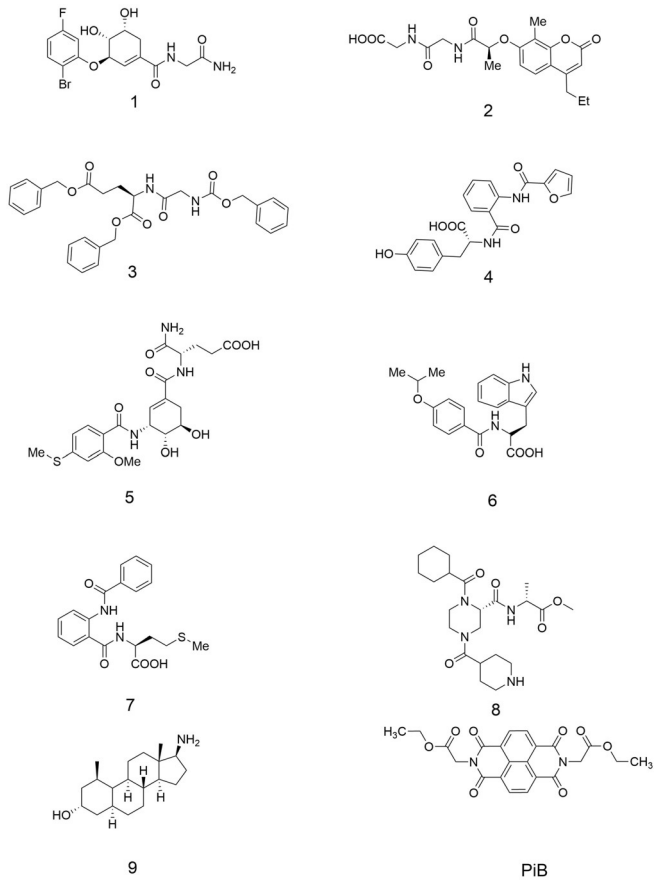

Figure 1. Chemical structures of natural product-like compounds **1**–**9** and **PiB**.

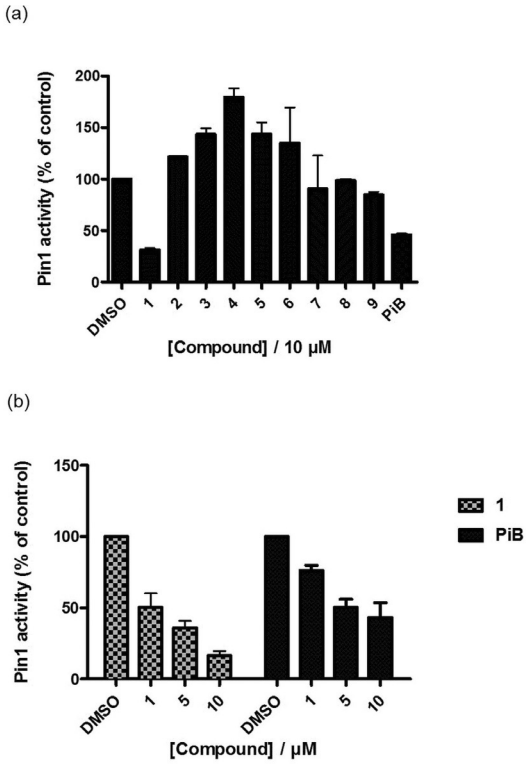

Figure 2. Effect of compounds **1**–**9** and **PiB** on Pin1 activity. (a) Inhibition of Pin1 activity by compounds **1**–**9** and **PiB**. (b) Inhibition of Pin1 activity by compound **1** and positive control **PiB** in a dose dependent manner. Pin1 activity was determined by measuring fluorescence intensity at Ex/Em = 490 nm/520 nm. Error bars represent the standard error of the mean of the results from three independent experiments.

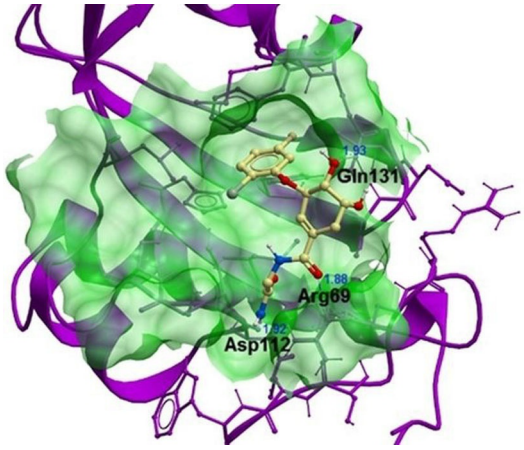

Figure 3. Low-energy binding conformation of compound **1** bound to Pin1 as generated by molecular docking.

system of compound **1** extends into a small pocket, forming extensive hydrophobic contacts with the residues lining the binding site. It indicated that compound **1** may target Pin1 directly. In another docking study, **PiB** was also predicted to hydrogen bond to Arg69 and Lys119 of Pin1.[11] The low-energy binding conformations of the other compounds in the docking hit list, compounds **2**–**8**, are show in Figure S1. However, unlike

compound **1**, none of the other compounds (**PiB** or compounds **2**–**8**) could form simultaneous hydrogen bond interactions with Gln131, Arg69 and Asp112 at the same time. Therefore, the higher activity of compound **1** compared to the other hit compounds might be attributed to the multiple hydrogen bond interactions formed between **1** and the active site of Pin1, which increases the affinity of the interaction of compound **1** for the enzyme. Moreover, only compound **1** contains a fluorine atom in its structure, which is predicted to be situated in a small pocket near Gln131. This group might be able to form unique non-polar interactions with the hydrophobic residues lining the binding pocket that are unavailable to the other molecules.

The cellular thermal shift assay (CETSA) experiment can monitor and quantify the extent of drug engagement to a protein target of interest within a cell or cell lysate. To further evaluate whether Pin1 is engaged by **1** in PC3 cell lysates, CETSA was performed. The amount of Pin1 protein in the soluble fraction was quantified by Western blotting (Figure 4a). Both DMSO and drug-treated Pin1 become denatured at higher temperatures and precipitate, decreasing the amount of protein remaining in the soluble fraction. However, drug-treated Pin1 was observed to denature at a higher temperature than DMSO-treated Pin1, suggesting that the compound binds to and stabilizes Pin1 (Figure 4b). This result indicates that compound **1** is able to engage Pin1 even in the complicated cell lysate environment.

Pin1 binds to the pThr254-Pro motif of the p65 subunit of NF-κB and isomerizes the phosphorylated p65 subunit, resulting in increased nuclear accumulation and enhanced NF-κB activity that is implicated in driving human malignancies.[22] An immunoprecipitation assay was conducted to investigate the effect of compound **1** on the Pin1–p65 interaction in cells.

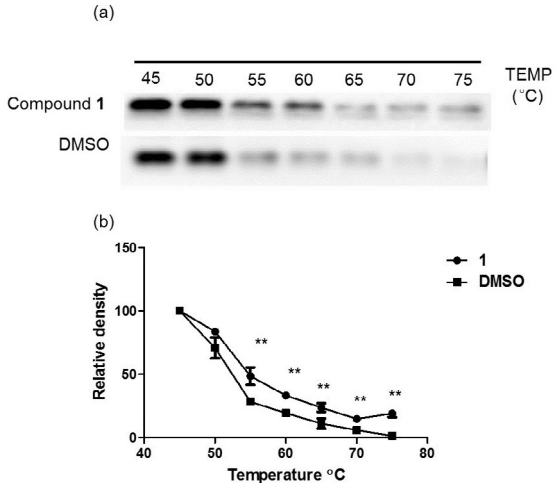

Figure 4. Compound **1** stabilizes Pin1 in PC3 cells. (a) Stabilization of Pin1 by **1** at 10 μM as revealed by Western blotting. (b) The band intensity of Pin1 in the soluble fraction at different temperatures. The data were normalized to the Pin1 level of the control group at 45 °C and are expressed as the means ± SD of three individual experiments. The data were analyzed using Image Lab. Significant differences versus control at the same temperature are indicated by $^{**}p < 0.01$.

Human prostate cancer PC3 cells were pre-treated with compound **1** and Pin1–p65 complexes were immunoprecipitated using protein A agarose beads coated with anti-p65 antibody. Western blotting analysis using anti-Pin1 and anti-p65 antibodies revealed that compound **1** could disrupt the binding of Pin1 to p65 in PC3 cells (Figure 5a,b). As the Pin1–p65 interaction enhances NF-κB signaling in human malignancies, including prostate cancer,[22] we further investigated the effect of compound **1** on NF-κB activity in cells. PC3 cells were transiently transfected with the NF-κB luciferase reporter gene and treated with compound **1** for 6 h. The results showed that the activation of NF-κB was attenuated NF-κB activity about 35% at 5 μM which is corresponding to Pin1 interaction reduce about 50% at 1 μM (Figure 5c). These results suggest that compound **1** could attenuate NF-κB activity presumably through disrupting the binding between Pin1 and p65.

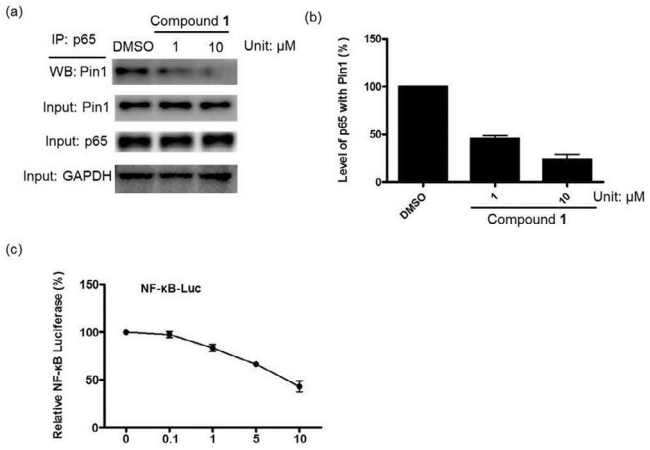

Figure 5. Inhibition of Pin1–p65 interaction in cells. (a) Compound **1** suppressed the Pin1–p65 interaction in PC3 cells as revealed by co-immunoprecipitation. The PC3 cells were treated with the indicated concentration of compound **1** for 6 h. Protein lysates were incubated with anti-p65 magnetic beads, and the precipitated proteins were revealed by western blot with anti-Pin1 antibodies. 20% protein lysates were labelled as input were revealed by western blot with anti-p65 and anti-GAPDH antibodies. (b) The band intensity of the binding between Pin1 and p65. The data were normalized to the DMSO control group and are expressed as the means ± SD of three individual experiments. The data were analysed using Image Lab. (c) Inhibition of NF-κB activity by compound **1**. After PC3 cells were transfected with NF-κB-luciferase plasmid for 24 h, the cells were treated with compound **1** for 6 h. The transcriptional activity was determined by measuring the activity of firefly luciferase. Error bars represent the standard error of the mean of the results from three independent experiments.

As Pin1 increases the nuclear localization of phosphorylated p65, we next investigated the effect of compound **1** on the nuclear localization of phosphorylated p65. After treatment of PC3 cells with **1** for 6 h, the levels of phospho-p65(Thr254) in the nucleus and total p65 in the cytoplasm were determined by Western blotting. The results showed that compound **1** significantly attenuated nuclear phospho-p65(Thr 254) levels, but had no effect on total p65 in the cytoplasm (Figure 6). This indicates that compound **1** could decrease nuclear p65 phosphorylation and decrease nuclear p65 accumulation, presumably as a result of its suppression of Pin1 activity. These results

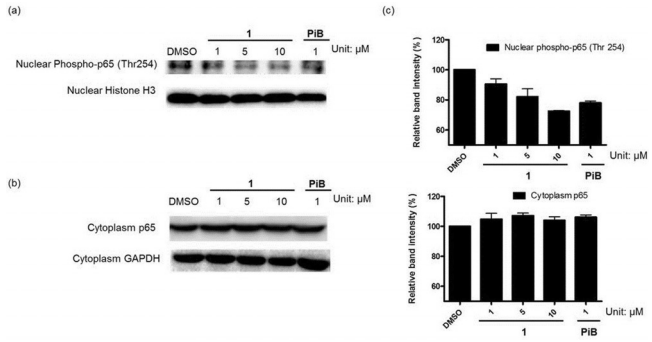

Figure 6. Effect of compound **1** on p65 phosphorylation in PC3 cells. (a) Compound **1** decreased nuclear phospho-p65 level in PC3 cells treated with PiB or compound **1**. Protein lysates were analyzed by Western blotting with the indicated antibodies. (b) Compound **1** has no effects on cytoplasm p65 level in PC3 cells treated with PiB or compound **1**. Protein lysates were analyzed by Western blotting with the indicated antibodies. (c) The band intensity of nuclear phospho-p65 and cytoplasm p65 level. The data were normalized to the DMSO control group and are expressed as the means ± SD of three individual experiments. The data were analyzed using Image Lab.

are consistent with those of the immunoprecipitation and luciferase assays above.

Site-directed mutagenesis was also performed to confirm whether interaction with the resides Arg69, Asp112 and Gln131 were necessary for the effects of compound **1** on Pin1. The results showed that compound **1** had less effect against the Pin1-promoted nuclear accumulation of phosphor-p65 (Thr254) in PC3 cells expressing mutant Pin1 (D112C, R69V or Q131V) compared to wild-type Pin1 (Figure S2) This suggests that compound **1** forms interactions with Asp112, Arg69 and Gln131 in Pin1, and is consistent with the molecular modeling results described above indicating that compound **1** interacts with those residues.

The activation of NF-κB signaling mediated by Pin1 in cancer cells correlates with increased expression of anti-apoptotic Bcl-2 family proteins and resistance apoptosis.[24] Additionally, Pin1 can increase the activity of Bcl-2, a critical suppressor of apoptosis, and decrease the activity of Bax, a pro-apoptotic regulator. Thus, the levels of Bax and Bcl-2 are important factors in determining whether cells will undergo apoptosis.[25] Interestingly, compound **1** could suppress Bcl-2 levels while inducing Bax levels in PC3 cells (Figure S3). This result suggested that compound **1** could promote apoptosis presumably through its effects on Pin1 activity. In addition, we also monitored the effect of compound **1** on caspase 3, caspase 7 and caspase 9 which are associated with apoptosis. The results showed that compound **1** decreased caspase 3, caspase 7 and caspase 9 levels, presumably through cleavage leading to caspase activation, indicating that compound **1** can induce apoptosis (Figure S3). Moreover, inhibition of Pin1 would led to a suppression of its oncogenic substrates, including cyclin D1 and PKM2.[15] As expected, after treatment of PC3 cells with compound **1**, suppression of cyclin D1 and PKM2 protein levels was observed (Figure S4). Moreover, the cytotoxicity of **1** was investigated in PC3 and normal liver LO2 cell lines. Cells were exposed to compound **1** (0.01 to 20 μM) for 72 h, and cellular proliferation was assessed by the MTT assay. The results showed that **1** inhibited cell proliferation in a dose-dependent manner in PC3 cells with an IC_{50} value of ca. 4.7 μM, while compound **1** had no discernible toxicity on LO2 cells at up to 10 μM (Figure S5).

In conclusion, we have identified compound **1** as a potential Pin1 natural product-like inhibitor by structure-based virtual screening. A co-immunoprecipitation assay revealed that compound **1** could disrupt the Pin1–p65 interaction in PC3 cells, while CETSA revealed that compound **1** engaged Pin1 directly in PC3 cell lysates. Furthermore, compound **1** also reduced activity of p65, leading to the inhibition of p65 phosphorylation and the induction of apoptosis in cells. We anticipate that compound **1** may serve as a useful scaffold for the further development of highly potent inhibitors of Pin1, as potential agents for the treatment of prostate tumor. To our knowledge, compound **1** is the first-in-class natural product-like Pin1 inhibitor that acts via targeting the Pin1–p65 interaction.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: enzymes · natural products · Pin1 · protein-protein interactions · virtual screening

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