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Virtual screening identifies a PIN1 inhibitor with possible anti-ovarian cancer effects

Running title: PIN1 inhibitor with anti-cancer effects

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ABSTRACT

PIN1 is a peptidyl-prolyl isomerase that binds phospho-Ser/Thr-Pro motifs in proteins and catalyzes the *cis–trans* isomerization of proline peptide bonds. PIN1 is overexpressed in several cancers including high-grade serous ovarian cancer. Since few therapies are effective against this cancer, PIN1 could be a therapeutic target but effective PIN1 inhibitors are lacking.

To identify molecules with *in vivo* inhibitory effects on PIN1, we used consensus docking to model existing PIN1-ligand X-ray structures and to screen a chemical database for candidate inhibitors. Ten molecules were selected and tested in cellular assays, leading to the identification of VS10 that bound and inhibited PIN1. VS10 treatment reduced viability of ovarian cancer cell lines by inducing proteasomal PIN1 degradation, without effects on *PIN1* transcription, and also reduced levels of downstream targets β-catenin, cyclin D1 and pSer473-Akt.

VS10 is a selective PIN1 inhibitor that may offer new opportunities for treating PIN1-overexpressing tumors.

1 INTRODUCTION

In cancer, molecularly targeted therapy is attractive since it may not have the adverse effects commonly associated with chemotherapy. On the other hand, blocking only one molecular pathway may be ineffective since cancer cells have many alternative routes for staying alive and multiplying, allowing the neoplasm to progress lethally. The inhibition of proteins that control multiple oncogenic pathways could be the solution (Ciarcia et al., 2013).

One protein that regulates multiple cellular pathways is PIN1 (peptidylprolyl cis/trans isomerase, NIMA-interacting 1). This enzyme catalyzes the *cis–trans* conformational switch of the proline peptide bond in Ser/Thr-Pro motifs, which are target sequences of kinases and phosphatases (Lu et al., 1996). Phosphorylation and dephosphorylation of Ser/Thr-Pro motifs is a common signaling mechanism in cell growth and transformation, and the activities of different kinases and phosphatases depend on the conformational state of the proline peptide bond in their target motifs (Lu et al., 2007). Hence, isomerization of Ser/Thr-Pro motifs affects phosphorylation status of many proteins, with profound effects on their functions and stability (Lucchetti et al., 2013; La Montagna et al., 2012, 2013, Rizzolio et al., 2012, 2013; Russo Spena et al., 2018).

Isomerization and phosphorylation of Ser/Thr-Pro motif-containing proteins is implicated in the activation of oncogenes and inactivation of tumor suppressor genes (Lu et al., 2007). Indeed, in multiple tumors, PIN1 expression was found to be upregulated and to correlate with poor prognosis (Bao et al., 2004). In mice, Pin1 overexpression induced chromosome instability and tumorigenesis (Suizu et al., 2006), while *Pin1* knockout was not lethal but caused various cell-proliferative alterations, such as testicular and retinal atrophy and lower body weight (Liou et al., 2002). Moreover, in Pin1 knockout female pregnant mice the mammary gland has a severe deficiency in the development and proliferation of mammary epithelial cells (Liou et al., 2002). We recently discovered that PIN1 is overexpressed in human serous ovarian cancer, and showed that its inhibition induces tumor cell death and tumor shrinkage in a

immunocompetent mouse model of metastatic ovarian cancer (Russo Spena et al., 2018). These observations suggest that PIN1 is a strong candidate for targeted therapy.

Over the past 10 years, several PIN1 inhibitors have been developed and shown to have high potency in biochemical assays (Moore and Potter, 2013). However, many of these molecules have limited activity in cells due to the presence of the doubly negative-charged phosphate group, which mimics natural phospho-substrates but limits their cell permeability (Guo et al., 2009). Non-phosphate inhibitors with low- to sub-micromolar activity in cells have also been described (Campaner et al., 2017; Subedi et al., 2016; Zhao et al., 2016). Among these, *all-trans* retinoic acid (ATRA) has been extensively studied. ATRA is approved for the treatment of acute promyelocytic leukemia (APL), which is almost caused by aberrant promyelocytic leukemia-retinoic acid receptor α (PML-RARα) (Johnson and Redner, 2015). As reported by Wei et al. (Wei et al., 2015), PIN1 binds PML-RARα and stabilizes it, but ATRA inhibits PIN1 and leads to its degradation, thereby destabilizing PML-RARα and inhibiting the growth of APL cells. These same researchers tested ATRA on triple negative breast cancer cells and found that it inhibited their growth via PIN1 inhibition. Yet ATRA has a short half-life (Regazzi et al., 1997) and is not specific for PIN1 (Notario et al., 2003; Ochoa et al., 2003; Schenk et al., 2014), limiting its use.

One approach for discovering new PIN1 inhibitors is virtual screening using a consensus docking protocol. Docking is an *in silico* modeling technique that predicts the most energetically favored position of a ligand bound to a protein, while consensus docking combines the results of different docking methods to obtain better results from both a qualitative and a quantitative point of view (Poli et al., 2016; Tuccinardi et al., 2014b). We found that the consensus approach was better than single-docking methods in predicting ligand binding poses, and that as the consensus level (i.e. the number of docking methods yielding the same pose) of a docking pose increased, so did its reliability. The reliability of this approach was shown in virtual screening campaigns that identified new non-covalent inhibitors for three different enzymes (Chiarelli et al., 2018; Poli et al., 2015; Tuccinardi et al., 2014a).

Encouraged by the results obtained with both the *in silico* and experimental tests, we applied consensus docking to the identification of new PIN1 inhibitors. We found a new compound, VS10, with micromolar efficacy and possible anticancer effects. Treatment of ovarian cancer cells with VS10 led to PIN1 degradation by the proteasome and reduced the cellular levels of the PIN1 downstream targets β-catenin, cyclin D1 and pSer473-Akt. These data suggest that VS10 is a potential new therapeutic agent in PIN1-overexpressing tumors.

2 Materials and Methods

2.1 Molecular modeling

The 12 available human PIN1–ligand X-ray complexes were retrieved from the Protein Data Bank (Berman et al., 2000). For all complexes, the ligand was extracted from its X-ray structure and subjected to a conformational search. To test the reliability of consensus docking in predicting the position of the ligand binding site, each ligand was docked in all the PIN1 3D structures using ten docking procedures, namely AutoDock 4.2.3, DOCK 6.7, FRED 3.0, Glide 5.0 (SP and XP), GOLD 5.1 (ASP, ChemScore, GoldScore and PLP), and AutoDock Vina 1.1, as previously described (Poli et al., 2018; Tuccinardi et al., 2015). The reliability of these docking procedures was evaluated in cross-docking analyses. For each procedure, we calculated the average root-mean-square deviation (RMSD) between the position of the ligand predicted by the docking and the known, experimental position, for all the ligands docked into all of the binding sites. The procedure with the lowest average RMSD was considered the most reliable. Details about the docking procedures and the cross-docking analyses are given in Supplementary Materials and Methods.

To study the effects of consensus docking on the docking evaluations, for each ligand docked into each PIN1 binding site, we clustered the results of the ten docking procedures, to search for common binding modes. For this purpose, consensus level was defined as the number of docking poses that clustered together. At each consensus level, we calculated average RMSD and the percentage of compounds retained ("survived").

To screen for new PIN1 inhibitors using consensus, a hierarchical workflow was used to apply the ten docking procedures to a subset of the Enamine database (HTS Collection) comprising the approximately 32,500 compounds with at least one negative charge. Compounds with a consensus level of ten were selected. To verify the stability of their binding mode as predicted by docking calculations, we did 10 ns molecular dynamic simulations with explicit water (Supplementary Materials and Methods). We calculated the average RMSD of the position of each ligand during the simulation compared to their

initial docking pose, and analyzed the stability of the interactions predicted by docking. Compounds with an average RMSD <2.0 Å were selected and purchased from Enamine (Monmouth Junction, NJ, USA) for study in cellular assays.

2.2 Cell lines and reagents

Human OVCAR3 and SKOV3 cell lines were purchased from ATCC (Manassas, VA, USA). Human OVCAR5 and NIH3T3 cell lines were provided by Gustavo Baldassarre (Aviano, Italy, EU) while the A2780 human ovarian cancer cell line was provided by Donatella Aldinucci (Aviano, Italy, EU). Cell lines were grown in RPMI-1640 medium with 10% fetal bovine serum. All the cell lines tested negative for mycoplasma contamination by PCR analysis and gel electrophoresis.

Antibodies used in western blotting included: mouse anti-HSP70 (1:1000; cat. no. sc 24) and rabbit anti-PIN1 (1:250; cat. no. sc-15340) from Santa Cruz Biotechnology (Santa Cruz, CA USA); mouse anti-human cyclin D1 (1:1000; cat. no. 556470) from BD Pharmingen (Franklin Lakes, NJ, USA); rabbit anti-β-catenin (1:1000; cat. no. 8480S), rabbit anti-pSer473-Akt 1:1000; cat. no. 4060s) and rabbit anti-β-actin (1:1000; cat. no. 4967S) from Cell Signaling Technology (Danvers, CO, USA). Secondary antibodies were mouse anti-rabbit IgG (1:5000; cat. no. 31464) and goat anti-mouse IgG (1:5000; cat. no. 31432) from Thermo Fisher Scientific (Waltham, MA, USA).

2.3 Half-maximal inhibitory concentration (IC_{50})

Compounds identified by virtual screening were tested for PIN1 inhibitory activity using the *in vitro* fluorescent SensoLyte Green Pin1 Assay Kit (AS-72240; AnaSpec, Fremont, CA, USA). Compounds were serially diluted 1:10 starting from 1 mM. ATRA was used as positive control.

IC_{50} was also calculated from cell viability. Briefly, cells were plated in 96-well plates at 5 x 10^2 cells/well. The next day, cells were treated with VS10 in 1:2 serial dilutions from 300 μM to 2.3 μM.

After 96 h, cell viability was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, WI, USA) with the Infinite M1000 PRO microplate reader (Tecan, Mannedorf, Switzerland). IC_{50} was calculated using Prism software (GraphPad, La Jolla, CA, USA).

2.4 Cell treatments

To test effects on PIN1 protein stability, NIH3T3 cells were plated in 100 X 20 mm tissue culture dishes (1.5 X 10^5 cells per dish). One day later, cells were treated with 0, 35 and 70 μM compound VS10 for 48 h, then with 10 μM MG132 or vehicle (DMSO) for 6 h (Roberti et al., 2011). In other experiments, cells were treated with 70 μM VS10 or vehicle (DMSO) for 24 h, followed by 10 μg/mL cycloheximide for 0, 3, 6, 12 and 24 h. After treatments, cells were collected for western blotting and RT-PCR.

To test effects on PIN1 targets, OVCAR3 cells were seeded in 100 X 20 mm tissue culture dishes (5 X 10^5 cells per dish). One day later, cells were treated with 70 μM VS10 or 10 μM ATRA. After treatment, cells were collected for western blotting.

2.5 Western blotting

A total cell extract was obtained by lysing cells with RIPA buffer plus protease and phosphatase inhibitors (Complete-EDTA-free Protease Inhibitor Cocktail; Roche, Basel, Switzerland), incubating on ice for 20 min, sonicating for 5 s, and centrifuging at 12,000 rpm for 20 min at 4 °C. Equal amounts of protein (30 μg) were separated on TruPAGE Precat Gels 4-12% (Sigma-Aldrich, St. Louis, MI, USA). Proteins were transferred onto nitrocellulose membranes (Amersham Protran 0.45 μm; GE Healthcare Life Sciences, Chicago, IL, USA). Free protein-binding sites were blocked for 30 min with 5% non-fat dried milk in TBS containing 0.1% Tween-20 (TBS-T). The membranes were incubated with primary antibodies against PIN1 and β-actin at 4 °C overnight, washed three times with TBS-T, and incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. Bound antibodies were detected

using LiteAblot PLUS Enhanced Chemiluminescent Substrate (EuroClone Life Sciences, Pero, Italy, EU). The results were analyzed with the ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA).

2.5 RNA extraction and PCR

Total RNA was prepared from murine NIH3T3 cells using the Smarter Nucleic Acid Sample Preparation kit (Stratec Molecular; Berlin, Germany, EU). Total RNA (400 ng) was reverse transcribed in a 10 µl reaction using GoScript Reverse Transcription System kit (Promega, Fitchburg, WI, USA). cDNA (0.1 volume) was amplified using GoTaq G2 Polymerase and Master Mix (Promega, Fitchburg, WI, USA). Murine *Hprt* was used as a control. Amplification reactions were carried out in a final volume of 20 µl as follows: 5 min at 95 °C; 20 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C x 30 cycles. The products were analyzed via 3% agarose gel electrophoresis.

3 RESULTS

3.1 Virtual screening prioritizes ten new PIN1 inhibitors

As a first step to screening for new PIN1 inhibitors, we tested the reliability of consensus docking in predicting the position of the ligand binding site for existing PIN1–ligand X-ray complexes. Ligands were extracted from their X-ray complexes and then docked in all the structures using 10 docking procedures. A total of 12 ligand-protein structures were analyzed, with 1440 docking calculations. Reliability was assessed from the average root-mean-square deviation (RMSD) between the position of the ligand predicted by docking and their known position. As shown in Figure 1, the docking procedures had an average RMSD in the range of 3.7-4.9 Å, with AutoDock4 having the best result (smallest deviation).

Then, the results of each docking procedure (data for each ligand docked into each PIN1 binding site) were clustered to search for common binding modes. As the consensus level increased from 2 (i.e., taking into account all the ligand−protein combinations that showed at least two out of ten docking poses clustered together) to the maximum value of 10, the average RMSD decreased from 3.7 Å to 0.7 Å (Figure 2). The best reliability achieved with consensus docking (0.7 Å at consensus 10) is about 5-times better than that obtained by using the best docking procedure in the cross-docking analysis (3.7 Å with AutoDock). However, as the consensus level increased, the percentage of all ligand−protein combinations retained ("survived") decreased, from 99% at a consensus level of 2 to 5% at a consensus of 10. These results mean that the quality of docking predictions increases with the consensus level, and that consensus docking improves the prediction of the ligand docking pose.

Consensus docking was then used in virtual screening for new PIN1 inhibitors. The 10 docking procedures were applied to a filtered Enamine database, and 32 compounds (out of about 32,500 compounds screened) reached a consensus of 10. These 32 compounds were subjected to molecular dynamic simulations, to examine the stability of their binding. A total of 10 compounds had an average RMSD (between the position of the 32 ligands during the simulation and their initial docking poses) <2.0

Å. These compounds were obtained for testing in biological assays to evaluate their PIN1 inhibitory activity.

3.2 Compound VS10 is a potent PIN1 inhibitor

Ten compounds selected by virtual screening were tested for inhibitory action against PIN1 isomerization in a fluorescent assay with a logarithmic dilution from 1 mM to 1 nM. The half-maximal inhibitory concentration (IC_{50}) was >100 μM for nine compounds (Table 1). One compound (called VS10) had a mean IC_{50} = 13.4 μM (SD = 1.24 μM). The positive control, ATRA, showed an IC_{50} of 33.2 μM (in the range of published results (Liao et al., 2017)), which is about 2-fold less than that of VS10.

Figure 3 shows the binding of VS10 in the PIN1 binding site. The carboxylic group of the ligand has ionic interactions with R69 and K63, the thiophene ring interacts with C113, and the phenyl ring is inserted into a lipophilic cleft mainly delimited by L122, M130, F134 and H157. The 3-methylbenzofuran-2-carboxamide fragment makes an H-bond with the hydroxyl oxygen of S154 and is partially exposed to water.

3.3 Compound VS10 reduces cancer cell viability

The activity of VS10 was tested in human OVCAR3 and OVCAR5 cell lines, as models of high-grade serous ovarian cancer, and in human SKOV3 and A2780 ovarian cancer cell lines. Cells were exposed to serial dilutions of the drug for 96 h, and IC_{50} values were calculated from cell viability. VS10 showed IC_{50} values ranging from 53.9 to 76.4 μM (Table 2).

3.4 Compound VS10 induces PIN1 protein degradation

Knowing that high affinity or covalent PIN1 inhibitors induce the protein's degradation, we examined the effects of VS10 on PIN1 levels in NIH3T3 fibroblasts. Treatment of NIH3T3 cells with two

concentrations of VS10 seemed to reduce the steady-state amount of this protein, and this effect was blocked by MG132 proteasomal inhibitor (Figure 4a). When cells were first treated with VS10 and then with cycloheximide (to inhibit protein synthesis), the level of PIN1 protein decreased over time (Figure 4b, right panel); this effect was not seen when cells were treated with DMSO (vehicle) and cycloheximide (Figure 4b, left panel). VS10 had no effect on the level of *PIN1* mRNA (Figure 4c).

Finally, the effects of PIN1 inhibition on three PIN1 targets were assessed in the OVCAR3 cell line. Western blotting showed that VS10 treatment decreased the levels of β-catenin, cyclin D1 and pSer473-Akt proteins (Figure 5).

4 Conclusions

In this study, we used consensus docking following by molecular dynamic simulations to identify possible PIN1 inhibitors. By applying this procedure to a dataset of ligands, we selected 10 potential PIN1 inhibitors for analysis. An *in vitro* assay for inhibitory activity against PIN1 isomerization revealed that VS10 had an IC_{50} in the low micromolar range (13.4 ± 1.2 μM). This molecule was selected for further study and was found to have inhibitory effects on four ovarian cancer cell lines, with IC_{50} values ranging from 53.9 to 76.4 μM.

Structurally, VS10 includes a 3-methylbenzofuran-2-carboxamide fragment, a thiophene and a phenyl ring. Molecular modeling showed that each VS10 moiety interacts with distinct residues in the PIN1 catalytic site: the VS10 carboxylic group forms ionic bonds with PIN1 R69 and K63; the thiophenic ring interacts with C113; the phenyl ring is inserted into a lipophilic cleft mainly delimited by L122, M130, F134 and H157; and the 3-methylbenzofuran-2-carboxamide fragment forms an H-bond with the hydroxyl oxygen of S154.

VS10 treatment of NIH3T3 cells induced PIN1 degradation via the proteasome, as shown by the fact that treatment with MG132 (a proteasome inhibitor) restored the normal level of PIN1 in VS10-treated cells. These results are similar to those obtained with another PIN1 inhibitor, ATRA (Wei et al., 2015). The inhibitory effects of VS10 on PIN1 seen here did not involve a change in *PIN1* transcription. As a consequence of decreased PIN1 expression, the levels of the PIN1 downstream targets β-catenin, cyclin D1 and pSer473-Akt also decreased.

Several studies reported an intriguing correlation between the expression levels of PIN1 and its downstream targets in maintaining the survival and proliferation of cancer cells, with PIN1 regulating cyclin D1 expression both directly and indirectly as a consequence of its interactions with β-catenin and pSer473-Akt (Liao et al., 2009; Liou et al., 2002; Ryo et al., 2001). In particular, PIN1 prevents β-catenin degradation by adenomatous polyposis coli (APC) (Ryo et al., 2001). On the other hand, β-catenin stabilization and activation may result from PIN1-mediated activation of Akt (Liao et al., 2009). In

accordance, we found that β-catenin, cyclin D1 and pSer473-Akt were deregulated in VS10-treated cells. The simultaneous alteration of different pathways regulated by PIN1 and involved in cancer progression suggests that VS10 is a candidate drug.

In conclusion, VS10 has the potential to be a more efficient PIN1 inhibitor than existing molecules, with possible clinical application in PIN1-overexpressing cancers such as high-grade serous ovarian cancer. Further studies are required to test the efficacy and safety of this molecule.

Competing interests

The authors declare no competing interests.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website.

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Zhao H, Cui G, Jin J, Chen X, Xu B. 2016. Synthesis and Pin1 inhibitory activity of thiazole derivatives. Bioorg Med Chem 24(22):5911–5920.

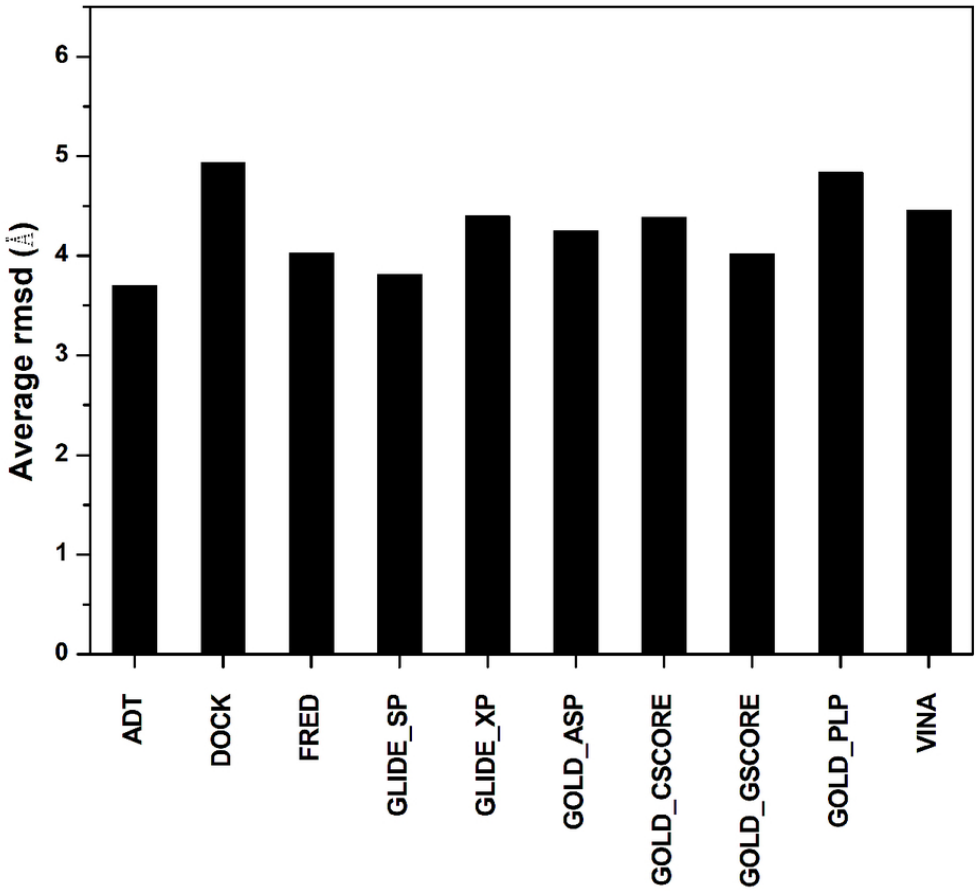


Figure. 1. Average root-mean-square deviations (RMSDs) for ten cross-docking procedures.

80x72mm (300 x 300 DPI)

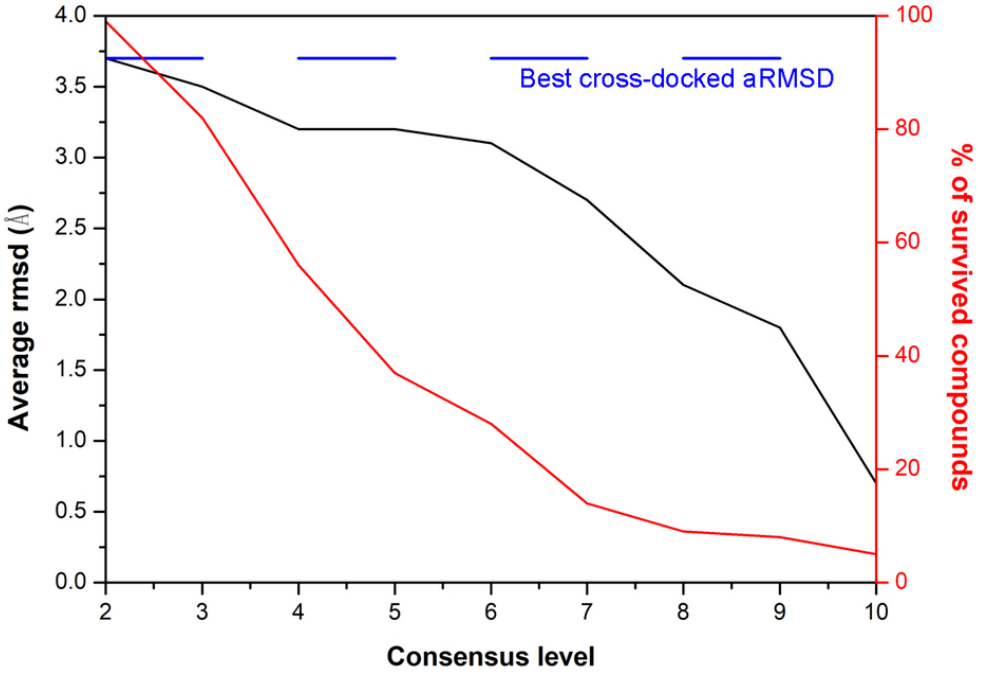


Figure 2. Results of consensus docking. Black line, average RSMD of the consensus docking; red line, percentage of survived compounds; blue interrupted line, best average RMSD obtained with the single docking program AutoDock.

80x55mm (300 x 300 DPI)

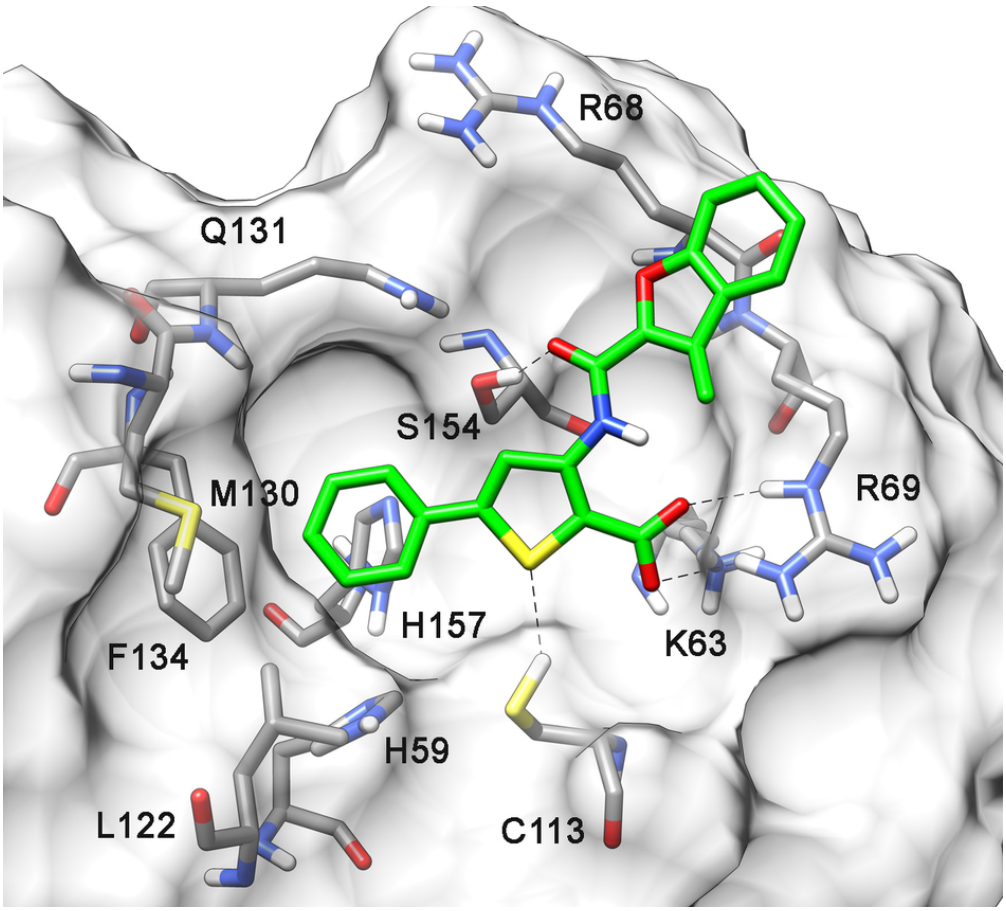


Figure 3. Putative binding pose of VS10 (green) in the binding site of PIN1. The most relevant ligand-protein interactions are marked.

80x72mm (300 x 300 DPI)

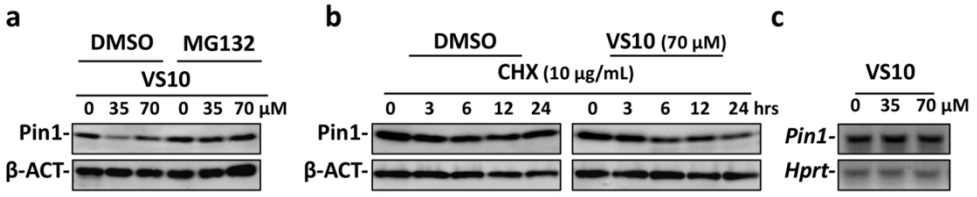


Fig. 4. Compound VS10 targets PIN1 to the proteasome. (a) NIH3T3 fibroblasts were treated with 35 μM and 70 μM VS10 for 48 h, followed by 10 μM proteasomal inhibitor MG132 or vehicle for 6 h. (b) Fibroblasts were treated with 70 μM VS10 or vehicle for 24 h followed by 10 μg/mL cycloheximide (CHX) for the indicated times. (c) Cells were treated as in (a), and PIN1 mRNA was amplified by PCR.

139x31mm (300 x 300 DPI)

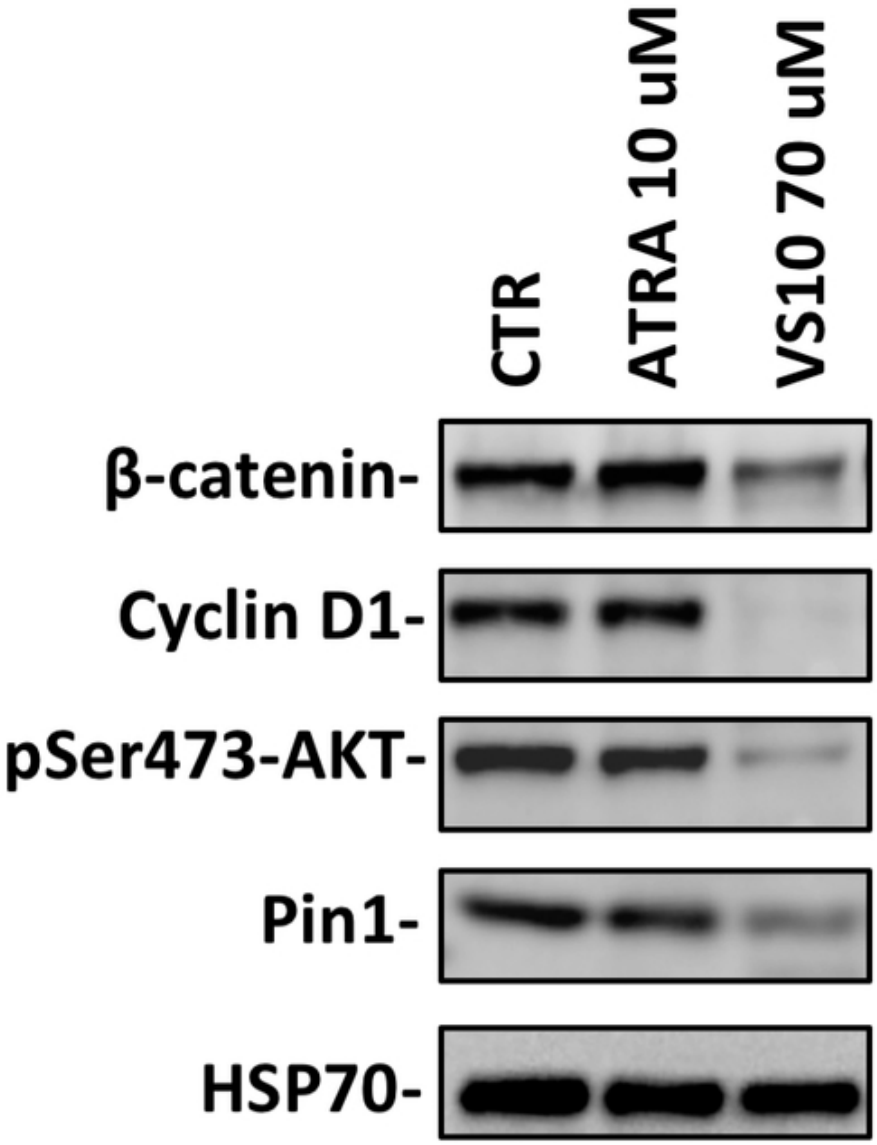
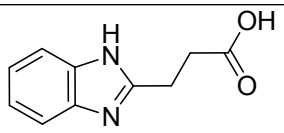
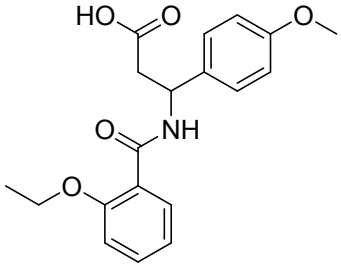
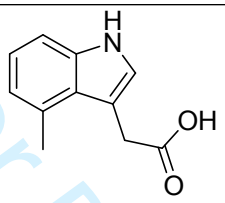
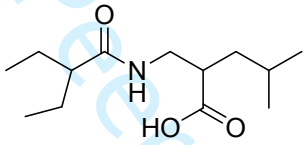
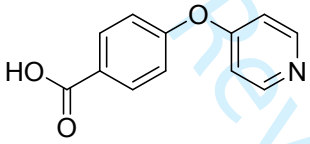
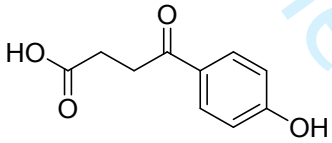
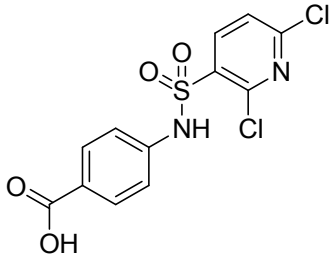


Figure 5. Compound VS10 has inhibitory effects on PIN1 target proteins. OVCAR3 cells were treated with 10 μM ATRA (control) or 70 μM VS10, then lysed and subjected to western blotting. HSP70 was used as a control for sample loading.

43x56mm (300 x 300 DPI)

Name	Structure	IC_{50} (μM)
VS1		>100
VS2		>100
VS3		>100
VS4		>100
VS5		>100
VS6		>100
VS7		>100

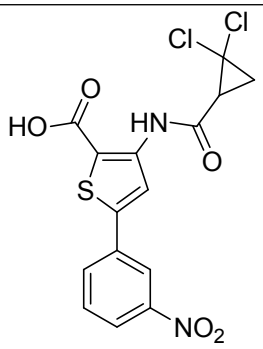
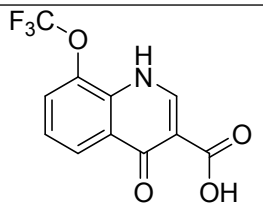
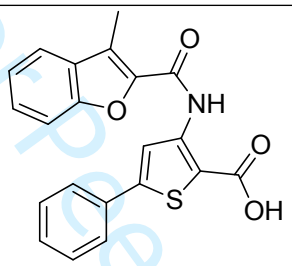
VS8		>100
VS9		>100
VS10		13.4 (1.2)
ATRA		33.2 (1.8)

Table 1. Structures and half-maximal inhibitory concentrations (IC_{50}) on human PIN1 isomerization (SensoLyte Green assay), for ten compounds selected by virtual screening and for ATRA (positive control). Values are mean (SD).

Cell line	IC_{50} (µM)
OVCAR3	53.9 (26.0)
OVCAR5	75.0 (25.7)
SKOV3	76.4 (14.5)
A2780	53.9 (21.5)

Table 2. IC_{50} of VS10 in ovarian cancer cell lines. Values are mean (SD).