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Discovery of novel selenium derivatives as Pin1 inhibitors by high-throughput screening

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ABSTRACT

Peptidyl prolyl cis/trans isomerization by Pin1 regulates various oncogenic signals during cancer progression, and its inhibition through multiple approaches has established Pin1 as a therapeutic target. However, lack of simplified screening systems has limited the discovery of potent Pin1 inhibitors. We utilized phosphorylation-dependent binding of Pin1 to its specific substrate to develop a screening system for Pin1 inhibitors. Using this system, we screened a chemical library, and identified a novel selenium derivative as Pin1 inhibitor. Based on structure-activity guided chemical synthesis, we developed more potent Pin1 inhibitors that inhibited cancer cell proliferation.

1. Introduction

Peptide bonds at the N-terminus of a proline take two conformations - *cis* or *trans*. Such conformational changes affect the structure of proteins, thereby, inducing important effects on their activity, subcellular localization, and stability. The isomerization of this bond occurs slowly and spontaneously, and can be catalyzed by peptidyl-prolyl cis-trans isomerase (PPIase) [1]. PPIases are categorized into three families: cyclophilins, FKBPs (FK506 binding proteins), and parvulins [2].

Pin1 (peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) is a unique PPIase belonging to the parvulin family, and it isomerizes peptide bond between phospho-(Ser/Thr) and Pro [3,4]. At the N-terminus, it has a WW domain that recognizes phosphopeptides, while at the C-terminus it has the catalytic PPIase domain [5]. Since Ser/Thr-Pro is a substrate motif for mitotic protein kinases, like CDKs and MAP kinases [6,7], the isomerization of this motif is known to be important for mitotic progression, and rapid cell growth [3,8]. Therefore, Pin1 is considered an attractive target for cancer therapy. In addition, Pin1 appears to be essential for maintaining the stemness of cancer cells [9,10], by preventing proteasomal degradation of Notch1 and Notch4 [9]. Inhibition of Pin1, either by small–molecule inhibitors or through RNAi, has led to inhibition of proliferation, and decrease in aggressiveness and size of tumor, in both humans and animals [11,12].

In the past decade, numerous Pin1 inhibitors, such as Juglone [13], PiB [14], D-peptide [15], EGCG [12], 974-B [16], ATRA [11] and bicyclic peptide **37** [17], have been discovered using various approaches. However, none of the Pin1-specific inhibitors have been developed for clinical usage, yet. The lack of a proper high-throughput screening (HTS) system, required to screen large libraries, has prevented the discovery of more potent Pin1 inhibitors.

To this end, we have designed a novel HTS system to assess the binding of fluorescence-labeled Pin1 protein to plate bound target phosphopeptides. Using this method, we screened over 1000 compounds, and found a selenium-containing compound that inhibited Pin1 activity at a submicromolar concentration. Furthermore, through structure-activity relationship studies, we identified its more potent derivatives, which inhibit cancer cell proliferation, and are expected to be lead compounds in cancer therapy.

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2. Materials and methods

2.1. Materials

Peptide substrates for Pin1 assay (Suc-Ala-Glu-Pro-Phe-pNA), and FKBP12 assay (Suc-Ala-Leu-Pro-Phe-pNA) were purchased from Bachem, Switzerland, and Peptide Institute Inc., Japan, respectively. Phosphopeptides for Pin1 binding assay were derived from human Wee1A sequence C-QVNINPFpTPDSLL, and a cysteine was added to the N-terminus for covalent binding to maleimide-coated 96-well plates. These phosphopeptides, and their non-phosphorylated derivatives, were chemically synthesized at RIKEN BSI, Research Resources Center, Japan. Recombinant FKBP12 was purchased from Abcam, USA, while FK506 was obtained from RIKEN NPDepo, Japan. Dehydrated *N*,*N*-dimethylformamide (DMF) was purchased from Wako Pure Chemical Industries, Ltd., Japan.

2.2. Pin1 binding assay

Primary screening for modifiers of Pin1 binding was performed, essentially, as described in our previous research [18]. Briefly, PIN1 ORF, fused with monomeric Azami Green (mAG) at the N-terminus, was inserted into a pRSET vector [19], and transformed into *E. coli* BL21 star (DE3) cells. Pin1 binding phosphopeptides were covalently bound to maleimide-activated 96-well plates (Pierce). The bacterial lysates expressing mAG-Pin1 fusion protein, or its mutants, were mixed with candidate compound (66.6 μg/mL), added to phosphopeptide bound wells, and incubated at 4 °C overnight. Bound Pin1 was quantified using spectrofluorometry, after washing with 0.05% NP-40 and PBS.

2.3. PPIase assay

PPIase assay was performed as described earlier by L. Hennig et al. [13]. Briefly, 15 nM of His-Pin1, or 33 nM of FKBP12, was dissolved in 35 mM HEPES buffer (pH 7.5), containing BSA (0.27 mg/mL) and chymotrypsin (33 U/mL), at 4 °C. After 5 min of incubating Pin1, or FKBP12, with or without inhibitor, peptide substrate (final concentration 120 μM) was added. After mixing the resulting solution, PPIase activity was measured by monitoring proteolytic release of *p*-nitroaniline at 390 nm, for 3 min, using Shimadzu UV-mini 1240 UV−Vis scanning spectrophotometer.

2.4. Protein purification

His-Pin1, inserted in pET-28a vector, was transformed into *E. coli* BL21 star (DE3) cells. The bacteria were grown on LB media, and induced for protein expression using 0.4 mM IPTG for 4 h, after which they were pelleted and lysed, using sonication. His-Pin1 fusion protein was purified using the Ni-NTA column, then quantified and used for the assay.

2.5. Chemical synthesis

The general scheme and techniques for compound synthesis are shown in the supplementary information. All solvents and reagents purchased were of the highest commercial quality, and were used as such. Reactions were analyzed through thin layer chromatography (TLC) using 0.25 mm Merck precoated silica gel plates, whereas for preparative TLC, 0.5 mm Merck precoated silica gel plates were used. ^{1}H NMR spectra were recorded on a JEOL JNM-ECA-500 (500 MHz) spectrometer, using $CDCl_3$ (with tetramethylsilane (0 ppm)) as the internal standard. Abbreviations were used to explain the multiplicities (singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (b)). ^{13}C NMR spectra were recorded on a JEOL JNM-ECA-500 (125 MHz) spectrometer with $CDCl_3$ as a solvent, and the internal standard (77.0 ppm). HRESIMS analysis was conducted on a Waters Synapt G2 System.

2.6. Cell culture

MDA-MB-231 cell line, from ATCC, and iCSCL-10A cells [20,21], were routinely cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% FBS and 1% penicillin-streptomycin, at 37 °C, and incubated in a 5% CO_2 incubator with humidified atmosphere. For proliferation assay of MDA-MB-231, cells were seeded at 4000 cells/well in a 96-well plate. After an overnight incubation, cells were treated with either DMSO or drugs, and then reincubated for 48 h. Cell viability was measured using Cell Count Reagent SF (Nacalai Tesque, Japan).

For proliferation assay of iCSCL-10A, cells were seeded at 4000 cells/well in a 96-well plate for monolayer formation, and treated with drugs the next day, for 72 h. For tumorsphere assay, iCSCL-10A cells were seeded at 16000 cells/well in an ultra-low attachment plate, using serum free media [21]. The cells were treated with drugs on the 4th day, and analyzed after 72 h. The viability in monolayer and tumorsphere of iCSCL-10A was measured using CellTiter-Glo 2.0 Assay (Promega, Madison, Wisconsin). For all assays, viability was measured using Wallac, ARVO SX plate reader, and represented as % of DMSO treated control.

3. Results and discussion

3.1. Establishment of a high-throughput screening system to identify Pin1 inhibitors

Earlier, we established a screening system to identify small molecule inhibitors of phosphorylation-dependent protein−protein interactions [22,23]. Using this system, we isolated inhibitors of polo box domain (PBD) of polo like kinase 1 (Plk1) and Skp2-p27 interaction [18,24]. Since Pin1 has a WW domain that specifically binds to phosphorylated peptides, and this interaction is required for the activity of Pin1 [5,25], we tried to establish a screening system to isolate small molecules that inhibit the binding of WW domain of Pin1 to the target sequence.

It is known that Pin1 specifically binds, and catalyzes, somatic Wee1 [26]. Thus, we chose synthetic phosphopeptides derived from human Wee1A sequence as targets for the binding assay. The phosphopeptides, and their corresponding non-phosphorylated control peptides, were covalently bound to the maleimide-activated 96-well plates, and bacterial lysate expressing mAG-Pin1 was incorporated into the wells. The binding of mAG-Pin1 to the phosphopeptides was detected through spectrofluorometry (Fig. 1a, Wt (WC) black bar). It was deduced that this binding was phosphorylation-dependent, since no significant binding to the non-phosphorylated peptide was observed (Wt white bar). In addition, the two tryptophan (W) residues in the WW domain are known to be essential for its binding to target [27]. When one of them, in Pin1 WW domain (W34), was mutated to alanine (AC mutant), the intensity of the signal was diminished, indicating that the binding is dependent on WW domain.

The cysteine residue (C113), in the C-terminus catalytic domain of Pin1, is essential for PPIase activity, as C113S mutant did not have any PPIase activity (not shown). When we examined the effect of this mutation (WS) in the binding assay, unexpectedly, an increase in the signal was detected. Significant increase in the binding was also observed in the WW domain only mutant, where codon at C57 of Pin1 was changed to stop codon (W*). During the enzymatic action of Pin1, the binding of Pin1 to its target phosphopeptide, through the WW domain, may be released after the completion of

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