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Dipentamethylene thiuram monosulfide is a novel inhibitor of Pin1

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ABSTRACT

Pin1 is involved in eukaryotic cell proliferation by changing the structure and function of phosphorylated proteins. PiB, the Pin1 specific inhibitor, blocks cancer cell proliferation. However, low solubility of PiB in DMSO has limited studies of its effectiveness. We screened for additional Pin1 inhibitors and identified the DMSO-soluble compound dipentamethylene thiuram monosulfide (DTM) that inhibits Pin1 activity with an EC50 value of 4.1 μM. Molecular modeling and enzyme kinetic analysis indicated that DTM competitively inhibits Pin1 activity, with a K_i value of 0.05 μM. The K_D value of DTM with Pin1 was determined to be 0.06 μM by SPR technology. Moreover, DTM specifically inhibited peptidyl–prolyl *cis*/*trans* isomerase activity in HeLa cells. FACS analysis showed that DTM induced G0 arrest of the HCT116 cells. Our results suggest that DTM has the potential to guide the development of novel antifungal and/or anticancer drugs.

Introduction

Peptidyl–prolyl *cis*/*trans* isomerases (PPIases) catalyze the *cis*/*trans* isomerization of peptidyl–prolyl bonds in proteins both in vivo and in vitro and are required for a variety of functions [1–3]. PPIases are classified into three families, cyclophilins, FK506 binding proteins (FK506), and parvulins. Pin1 belongs to the parvulin family. It is the only PPIase known to have a high specificity to substrates with phosphorylated serine and threonine side chains preceding proline (pSer/pThr-Pro) [4,5]. In addition to the PPIase domain, Pin1 contains the WW domain that interacts with pSer/pThr-Pro motifs in proteins [5,6].

It has been reported that Pin1 functions in many cellular processes through its ability to bind to and promote phosphorylation-dependent isomerization of pSer/Thr-Pro peptide. Pin1 mainly interacts with many of the MPM-2 antigens in mitotic lysates, but it also interacts with other cell cycle regulatory molecules: CDC25C [7], Wee1 [8], Myt1 [9], CK2 [10], Plk1 [11], tis21 [12], cyclin D1 [13], and Raf-1 [14]. Pin1 interacts with transcription factors: NFAT [15], c-Jun [16], c-Fos [17], p54nrb [18], the hSpt5 subunit of the DRB sensitivity-inducing factor [19], and steroid receptor SRC-3 [20]. In addition to its effects on the function of specific transcription factors, Pin1 also has more general roles in transcription. It binds the phosphorylated C-terminal domain of the largest subunit of RNA polymerase II [21] and is required for the activation of checkpoint molecules, p53 [17], p73 [22], and Sil [23].

The depletion of Pin1 from *Saccharomyces cerevisiae* and *Candida albicans* causes cell death [24,25], but the depletion of Pin1 from *Drosophila melanogaster* [26], mice [27], and a strain of *S. cerevisiae C110* does not cause death [28]. Pin1−/− mice have a higher risk of developing Alzheimer's disease [29,30] and testicular and retinal atrophies; in these animals, the breast epithelial compartment failed to undergo the normal changes associated with pregnancy [13,27]. Deletion of Pin1 and p53 in mice did not cause cell death, but it induces hyperplasia in the thymus [31]. Pin1−/− mice are viable because other PPIases substitute for Pin1, thereby helping the organism to develop. Cyclophilin A and Par14, the second parvulin-type of PPIases, are slightly overexpressed in Ess1, yeast ortholog of Pin1-depleted yeast, and in the Pin1−/− mouse embryonic fibroblasts (MEF), respectively, [28,32]. Ess1/Pin1-depleted budding and fission yeast cells are more sensitive than wild-type yeast cells to cyclosporin A (CsA), which inhibits cyclophilin PPIase activity [28]. Taking the characteristics of Pin1 into consideration, we hypothesize that treatment of fungal diseases with CsA and the Pin1 PPIase inhibitors will be toxic for the fungi but not for the patients. We therefore decided to screen for Pin1 inhibitors.

We identified a series of Pin1 and Par14 inhibitors with reduced solubility in DMSO [32]. Therefore, we continued the effort to find

Abbreviations: Ac, acetyl; CsA, cyclosporin A; CypA, cyclophilin A; DTM, dipentamethylene thiuram monosulfide; FACS, fluorescence-activated cell sorter; MEF, mouse embryonic fibroblast; pNA, para-nitroanilide; PPIase, peptidyl–prolyl *cis*/*trans* isomerase; SPR, surface plasmon resonance; Suc, succinyl; TFE, trifluoroethanol.

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a DMSO-soluble compound that specifically inhibits Pin1 PPIase activity.

Material and methods

PPIase inhibitors. We purchased dipentamethylene thiuram monosulfide (DTM, Fig. 1A) from ICN Biomedicals Inc. (Aurora, OH, USA), CsA from Wako Pure Chemicals, Ltd. (Japan), substrate peptides from Bachem (Weil am Rhein, Germany), and chymotrypsin and trypsin from Sigma–Aldrich (Tokyo, Japan). FK506 was a gift from Astellas Pharma Inc. (Tokyo, Japan).

Cells. HeLa and HCT116 cells were kindly provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. We cultured the cells in DMEM containing 10% FCS. MEF of wild-type mice were obtained as described previously [32].

Preparation of protein samples for PPIase assay. The GST–Pin1 was produced by *Escherichia coli* as described previously [33]. We cultured MEFs in DMEM containing 10% FCS for 3 days in a culture dish (diameter: 150 mm) and harvested them in 500 μL NP40 buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1% (v/v) NP40; 5 mM EDTA; 1 mM PMSF; 50 mM NaF; 1 mM Na3VO4) supplemented with protease inhibitor cocktail (Nacalai Inc. Kyoto, Japan). The cells were homogenized by Ultra-Turrax T8 (IKA, Staufen, Germany). We determined the protein concentration of the samples using the BCA protein assay kit (Pierce, Illinois, USA). Total protein concentration of all samples was between 2–7 mg/mL.

PPIase assay. We performed the PPIase assay using the protease coupled assay as described previously [33]. Total sample volume was 1500 μL; sample buffer was 35 mM Hepes (pH 7.5). We prepared stock solutions of all substrates (30 mg/mL) in 0.47 M LiCl/TFE (anhydrous). Stock solutions of proteases (100 mg/mL for chymotrypsin and 50 mg/mL for trypsin) were prepared in 35 mM Hepes (pH 7.8). For routine assay of Pin1 PPIase, we used the chromogenic substrate Suc-AEPF-pNA. We measured the Pin1 activity with the substrate (61 μM) in presence of chymotrypsin (0.2 mg/mL) and Pin1 (10 nM) at 5 °C and recorded the reaction using Agilent 8453 UV–vis spectrophotometer (CA, USA). We used the signal difference between 390 and 510 nm to calculate the first order rate constants.

To measure the total cyclophilin related PPIase activity in MEF lysate, we used the substrate Suc-AAPF-pNA. Additionally, we added a final concentration of 33 μM FK506 to the substrate (0.03 mg/mL) and the protease (chymotrypsin, 0.2 mg/mL) during the measurement to inhibit FKBP related activities. The FKBP activity was measured with the substrate Suc-ALPF-pNA (0.03 mg/mL) in the presence of chymotrypsin (0.2 mg/mL) and 33 μM CsA. To determine the PPIase activity originating from Pin1, the phosphorylated substrate Ac-AA(pS)PR-pNA (0.02 mg/mL) in combination with trypsin (0.1 mg/mL), 33 μM CsA, and 33 μM FK506 were used.

SPR analysis. We performed binding affinity experiments using a Biacore T100 detector. Immobilization of anti-GST antibodies to CM5 Sensor Chips was performed as described previously [34]. We performed GST–Pin1 capture by injecting GST–Pin1 into anti-GST antibodies at concentrations of 20 μg/mL in HBS-EP buffer (10 mM Hepes, pH 7.4; 150 mM NaCl; 3 mM EDTA; 0.005% Tween 20) for 10 min at a flow rate of 10 μL/min. DTM (10 mM) in DMSO was diluted to yield 2.5–250 nM compounds in HBS-EP buffer containing 0.1% DMSO. A typical analysis cycle consisted of sample injection for 90 s, buffer flow for 120 s (dissociation phase), and buffer injection for 30 s to check for the sample carryover. We set the flow rate at 30 μL/min, and the flow cell temperature at 25 °C. We determined the binding affinity (K_D) of DTM to Pin1 by the steady-state affinity model using the Biacore T100 evaluation software (version 1.1.1).

Computational methods. In silico work was performed on an Intel® Pentium® Windows XP personal computer with the Molecular Operating Environment (MOE; version 2007.0902) software developed by the Chemical Computing Group Inc. (Montreal, Canada). Using MOE, we attempted to dock Pin1 (PDB code: 1PIN). At first,

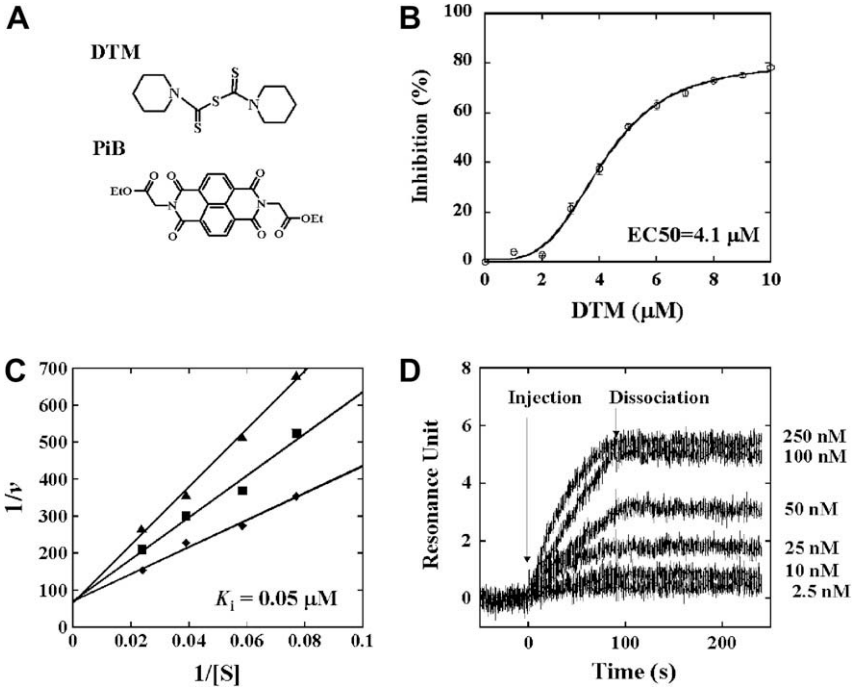

Fig. 1. Inhibition of Pin1 PPIase activity by DTM. (A) Structures of Pin1 inhibitors; DTM and PiB. (B) Inhibition of Pin1 PPIase with DTM. Pin1 was incubated with different concentrations of DTM. The Pin1 PPIase activity was measured by the protease-coupled PPIase assay. (C) Lineweaver–Burk plot for analysis of K_i of DTM. The Pin1 PPIase was assayed with DTM [0 μM (♦), 0.25 μM (■), and 0.5 μM (▲)]. (D) Sensorgrams of the binding of DTM to GST–Pin1 measured by the Biacore assay. Representative sensorgrams obtained from the injection of DTM at concentrations of 2.5, 10, 25, 50, 100, and 250 nM into GST-Pin1 immobilized on the CM5 chip.