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Inhibition of Dual-specificity Phosphatase 14 (DUSP14) by PTP Inhibitor V

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Post-translational modification is one of the most important steps for cellular homeostasis in living organisms.[1] Particularly, protein phosphorylation is an essential process for regulating cell growth, proliferation, differentiation, cell cycle, and apoptosis.[2] Protein phosphorylation usually arises in serine, threonine, or tyrosine residues and is regulated by protein kinases.[3] Protein tyrosine phosphorylation is regulated through the actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).[4,5]

PTPs are responsible for dephosphorylation and comprise 107 genes in human genome.[6] The dual-specificity phosphatases (DUSPs) belong to a subfamily of PTPs and have ability to dephosphorylate phospho-Ser, phospho-Thr, and phospho-Tyr. They can be classified into six subgroups on the basis of sequence similarity such as slingshots, PRLs (phosphatases of regenerating liver), Cdc14 phosphatases (Cdc is cell division cycle), PTENs (phosphatase and tensin homologues deleted on chromosome 10), myotubularins, MKPs (mitogen-activated protein kinase phosphatases) and atypical DUSPs.[7] Among them, MKPs negatively regulate the mitogen-activated protein kinases (MAPKs) by protein dephosphorylation. In addition, atypical DUPSs share some characteristics with MKPs and have been shown to regulate MAPKs.[8-10] The MAPK pathway is one of the key signal-transduction signalings in mammalian cells.[11] The major subfamilies of MAPK are the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERK), and p38.[12] Since PTPs are involved in the cellular equilibrium, chemical compounds that regulate the enzymatic activity of PTPs have been searched to be used as therapeutic reagents for diseases such cancer, inflammation, and diabetes.

PTP inhibitor V, also known as PHPS1, is phenylhydrazonopyrazolone sulfonate that was reported as the specific inhibitor for SHP-2 over the intimately related SHP-1 and PTP1B.[13] To investigate whether PTP inhibitor V inhibits other phosphatases, we performed *in vitro* phosphatase assays with recombinant PTPs and PTP inhibitor V. Out of four PTPs we tested, DUSP14 was identified to be inhibited by PTP inhibitor V. DUSP14, also named MKP-L (MKP-1-like protein tyrosine phosphatase) and MKP-6, is a member of atypical DUSP family.[14]

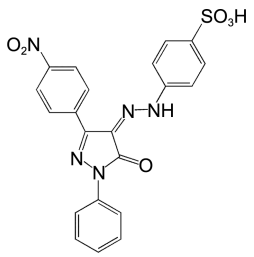

Figure 1. Chemical structure of PTP inhibitor V.

When DUSP14 was treated with various concentrations of PTP inhibitor V and then its activity was measured using OMFP as a substrate, DUSP14 activity was decreased by PTP inhibitor V in a dose-dependent manner. An inhibition curve was plotted for DUSP14 with various concentrations of PTP inhibitor V and the half maximal inhibitory concentration (IC_{50}) value was calculated using the curve fitting program PRISM 3.0 (Fig. 2(a)). The results suggest that PTP inhibitor V inhibits DUSP14 with the IC_{50} of 3.9 ± 1.0 μM. To examine the DUSP14 inhibitory mechanism by PTP inhibitor V, we performed kinetic studies based on the Michaelis-Menten equation. The results show that the PTP

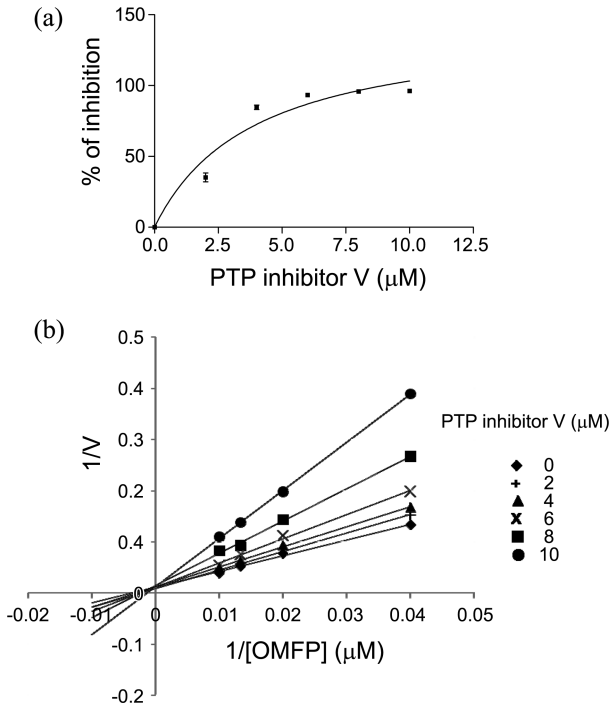

Figure 2. Inhibitory effect of PTP inhibitor V on DUSP14 and Kinetic analysis of DUSP14 inhibition by PTP inhibitor V. (a) DUSP14 was incubated with various concentrations of PTP inhibitor V at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental section. (b) Lineweaver-Burk plots of DUSP14 were generated from the reciprocal data.

inhibitor V is a competitive inhibitor with K_i of 3.42 μM (Fig. 2(b)), suggesting that PTP inhibitor V inhibits the enzymatic activity of DUSP14 through binding to the catalytic site.

To confirm the inhibitory effect of PTP inhibitor V on DUSP14, human embryonic kidney (HEK) 293 cells were transiently transfected with FLAG-tagged DUSP14 WT or kinetically inactive DUSP14 mutant expression plasmid. Overexpressed DUSP14 was immunoprecipitated from cell lysates with anti-FLAG M2 affinity gel. Immunoprecipitated DUSP14 was treated with various concentrations of PTP inhibitor V and then its activity was measured using OMFP as a substrate. The results showed that PTP inhibitor V inhibits phosphatase activity of DUSP14 expressed mammalian cells (Fig. 3(a)). We next confirmed whether the inhibitory regulation of PTP inhibitor V on DUSP14 targeted the dephosphorylation of MAPKs. DUSP14 has been known to dephosphorylate JNK, ERK, and p38, suggesting that DUSP14 acts as a general MAPK phosphatase.[14] We used JNK as a substrate for DUSP14 to further investigate whether PTP inhibitor V regulates JNK activation by inhibition of DUSP14 activity

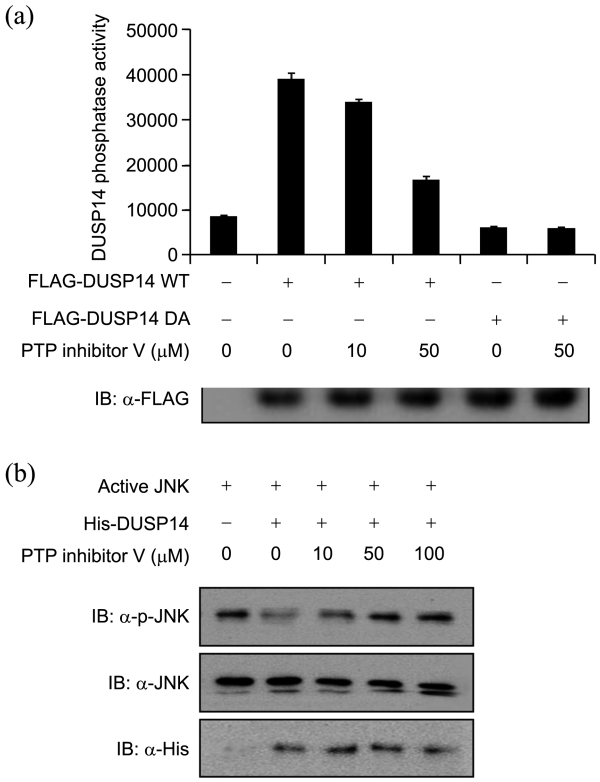

Figure 3. Inhibition of DUSP14 expressed in mammalian cells *in vitro* and DUSP14-mediated JNK dephosphorylation by PTP inhibitor V. (a) HEK 293 cells were transfected with 3 μg of FLAG-DUSP14 WT or DUSP14 D80A expression plasmid. After 48 h of transfection, cell lysates were subjected to immunoprecipitation with anti-FLAG M2 agarose. Immunoprecipitated DUSP14 was incubated with various concentrations of PTP inhibitor V at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental Section. (b) DUSP14 WT (1 μg) was pre-mixed with PTP inhibitor V (0, 10, 50, or 100 μM) and then incubated with active JNK. JNK phosphorylation level was determined by immunoblotting analysis.

in vitro. After treatment of active JNK with DUSP14 in the presence of various concentrations of PTP inhibitor V, samples were subject to immunoblotting with phospho-JNK and JNK antibodies. As shown in Figure 3(b), PTP inhibitor V effectively protects JNK phosphorylation by inhibiting DUSP14 phosphatase activity *in vitro*.

Based on our previous studies, DUSP14 enzymatic activity was negatively regulated by several chemical inhibitors such as NSC-87877, NSC-95397, and ethyl-3,4-dephostatin.[15-18] Here, we newly found that PTP inhibitor V inhibits DUSP14 phosphatase activity. DUSP14 was first characterized through a yeast two-hybrid system to verify novel proteins that interact with the T-cell co-stimulatory factor CD28. DUSP14 expression is increased strongly in T cells stimulated through CD28. CD28 signaling is known to be related to induction of interleukin-2 (IL-2) in T-cells. Remarkably, this CD28-mediated DUSP14 induction reduces IL-2 production.[14,19] In addition, IL-2 production is inhibited by negative regulation of CD28 signaling through the dephosphorylation of JNK in T cells, suggesting that DUSP14 acts as a negative-feedback regulator in T cell proliferation.[20] In the present study, we found that PTP inhibitor V effectively reduces DUSP14 phosphatase activity to activate JNK phosphorylation. Therefore, this study provides that PTP inhibitor V is a potential regulator in CD28 signaling through the inhibition of DUSP14 and may be used as a therapeutic reagent for immune diseases that are related to DUSP14-mediated T-cell inactivation.

Experimental Section

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, Waltham, MA). All of media were supplemented with 10% fetal bovine serum (FBS, Thermo Scientific) and penicillin/streptomycin in the presence of 5% CO_2. For transfection, 4×10^5 cells were seeded before the day of transfection and transfected with DNA using OmicsFect™ (Omics Biotechnology, Taiwan).

Antibodies and Plasmid Constructions. Anti-JNK was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-SAPK/JNK (specific for phospho-Thr183 and phosho-Tyr185) was from Cell Signaling Technology (Danvers, MA). Monoclonal Anti-FLAG was from Sigma-Aldrich. His-tagged DUSP14-WT was constructed in pET28a plasmid (Novagen, Madison, WI) for protein expression in *Escherichia coli* and FLAG-tagged DUSP14-WT and DUSP14 D80A mutant were constructed in pcDNA3.1 (Invitrogen, Carlsbad, California).

Purification of the six-His-tagged Proteins. PTP expression plasmids were constructed in pET-28a (+) and transformed into BL21 (DE3)-RIL *E. coli*. Recombinant proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 20 °C for 16 h. Cells were harvested and then lysed by sonication in 50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF). The lysates were clarified at 13,000 rpm for 30 min at 4 °C. The supernatant was applied by gravity

flow to a column of Ni–NTA resin (PEPTRON, Daejon, Korea). The resin was washed with 20 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 20 mM imidazole and then eluted with 20 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 250 mM imidazole. The eluted proteins were dialyzed overnight against 20 mM Tris–HCl, 150 mM NaCl, 20% glycerol, and 0.5 mM PMSF before storage at −80 °C.

***In vitro* Phosphatase Assays and Kinetic Analysis.** Phosphatase activities were measured using the substrate 3-*O*-methylfluorescein phosphate (OMFP; Sigma, St. Louis, MO) at concentrations that varied according to the K_m (Michaelis constant) of each enzyme in a 96-well microtiter plate assay based on methods described previously.[21] PTP inhibitor V and OMFP were solubilized in DMSO. All reactions were performed at a final concentration of 1% DMSO. The final incubation mixture (150 μL) was optimized for enzyme activity and was composed of 30 mM Tris–HCl (pH 7.0), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT), 0.33% bovine serum albumin (BSA) and 100 nM of each PTP. Reactions were initiated by addition of OMFP and incubated for 30 min at 37 °C. Fluorescence emission from the product was measured with a multiwall plate reader (Synergy H1; excitation filter, 485 nm; emission filter, 535 nm). The reaction was linear over the experimental time period and was directly proportional to both enzyme and substrate concentration. The half-maximal inhibition constant (IC_{50}) was defined as the concentration of inhibitor that caused a 50% decrease in the PTP activity. Half-maximal inhibition constants and best curve fit for Lineweaver-Burk plots were determined by using the curve fitting program PRISM 3.0 (GraphPad Software). All experiments were performed in triplicate and were repeated at least three times.

Inhibition Study. The inhibition constant (K_i) to DUSP14 phosphatase for the inhibitor was determined by measuring the initial rates at several OMFP concentrations for each fixed concentration of the inhibitor. The data were fitted to the following equation to obtain the inhibition constant of reversible competitive inhibitors. The slopes obtained were replotted against the inhibitor concentrations. The K_i value was obtained from the slopes of these replots.

$$1/V = K_m (1 + [I]/K_i) V_{max} [S] + 1/V_{max}$$

Effect of PTP Inhibitor V on DUSP14 Expressed in Mammalian Cells. HEK 293 cells were transfected with FLAG-DUSP14 WT or DUSP14 D80A expression plasmid. After 48 h of transfection, cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in PTP lysis buffer (0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% glycerol, 1 mM PMSF, and 1 μg/mL aprotinin) for 30 min at 4 °C. Cleared cell lysates from centrifugation were mixed with washed FLAG M2-agarose (Sigma-Aldrich, St. Louis, MO) and incubated for 16 h at 4 °C using rotation device. After incubation, FLAG M2-agarose was washed three times with PTP lysis buffer and measured their phosphatase activities.

Dephosphorylation Assays with Active Phosphorylated JNK. The six-His-tagged DUSP14 (1 μg) was combined with active phosphorylated JNK (10 ng) in PTP assay buffer (30 mM Tris–HCl (pH 7.0), 75 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 0.33% BSA) and incubated for 30 min at 37 °C in a 30 μL reaction volume. To determine whether PTP inhibitor V inhibits the DUSP14 effect on JNK *in vitro*, 100 nM of DUSP14 was mixed with 10 ng of active phosphorylated JNK and various concentrations of PTP inhibitor V (0, 10, 50, or 100 μM) in a 30 μL reaction volume and incubated for 30 min at 37 °C. The products of dephosphorylation reactions were subjected to SDS-PAGE and then immunoblotted with an anti-phospho-JNK antibody.

Immunoblotting Analysis. Transfected cells with or without FLAG-DUSP14 WT or DUSP14 mutant expression plasmids for 48 h were washed twice with phosphate buffered saline (PBS) buffer and lysed in 1x SDS sample buffer (12 mM Tris-HCl pH 6.8, 0.4% SDS, 5% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue) and boiled at 100 °C for 5 min. Sampled were run in SDS-10% polyacrylamide gels and transferred to nitrocellulose membrane. Then the membranes were blocked with 5% skim milk for 1 h and incubated with an appropriate antibody, followed by incubation with a HRP-conjugated secondary antibody. The protein bands were visualized by the ECL detection system (Pierce, Rockford, IL).

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