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Inhibition of DUSP13A Activity by PTP Inhibitor V

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Protein phosphorylation is regulated by two groups of enzymes, protein kinases and protein phosphatases. Regulation of phosphorylation has been a target for development of therapeutic reagents for many human diseases. Most protein phosphatases are members of protein tyrosine phosphatases (PTPs). The human genome encodes 107 PTPs, which are classified into four families: (i) class I cysteine-based PTPs, contains 2 classical PTPs and VH1-like PTPs, (ii) class II cysteine-based PTPs; tyrosine-specific low molecular weight phosphatases (LMPTP), (iii) class III cysteine-based PTPs containing cell division cycle25 (CDC25) homology (CH2) domain, (iv) transcription factors Eyes absent (EYA) proteins as members of aspartic acid based PTPs.[1]

DUSPs (dual-specificity phosphatases) are members of VH-1 like PTPs and dephosphorylate both phosphotyrosine and phosphoserine/threonine residues. Sixty one members of DUSPs are known in the human genome.[2] Many DUSPs have been found to play roles in the regulation of mitogenic signal transduction for extracellular stimulation and to collaborate with protein kinases to regulate cell proliferation, differentiation, and apoptosis.[3] Among DUSPs, *DUSP13* was known to be located in human chromosome 10q22.2 and classified as small atypical DUSP.[4, 5] *DUSP13* encodes DUSP13A/MDSP (muscle-restricted DUSP) and DUSP13B/TMDP (testis- and skeletal muscle-specific DUSP) due to alternative splicing of the ORF.[3,6] DUSP13B is encoded by the downstream ORF. These two proteins share 42% identity in amino acid sequences. DUSP13A had no significant inhibitory effect on AP-1-dependent gene expression.[7] Our recent report showed that DUSP13A is involved in the activation of apoptosis signal-regulating kinase 1 (ASK1), but the phosphatase activity of DUSP13A was not necessary to activate ASK1.[8]

PTP Inhibitor V is 4-(*N*′-(3-(4-Nitrophenyl)-5-oxo-1-phenyl-1,5-dihydro-pyrazol-(4*Z*)-ylidene)-hydrazino)-benzenesulfonic acid (Fig. 1).[9] PTP Inhibitor V is known to inhibit SHP-2-dependent cellular functions and selectively penetrates into the substrate-binding site of SHP-2. The half maximal inhibitory concentration (IC_{50}) of PTP Inhibitor V is 2.1 μM against SHP-2.[10] PTP Inhibitor V also inhibits ECPTP, PTP1B, SHP-1, mycobacterium MptpA (IC_{50} = 5.4, 19, 30, and 39 μM, respectively).[10]

To study the action of PTP inhibitor V on other PTPs, we performed *in vitro* phosphatase assays with purified recombinant PTPs (Table 1). We found that DUSP13A was inhibited by PTP inhibitor V while other PTPs were not. We

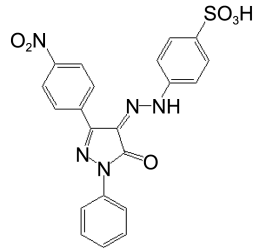

Figure 1. Chemical structure of PTP Inhibitor V. 4-(*N*′-(3-(4-Nitrophenyl)-5-oxo-1-phenyl-1,5-dihydro-pyrazol-(4*Z*)-ylidene)-hydrazino)-benzenesulfonic acid.

Table 1. Inhibition of PTPs by PTP inhibitor V

PTPs	IC_{50} (μM)
DUSP3	> 200
DUSP16	> 200
MTMR3	> 200
PTPN14	> 200
PTPN21	> 200
PTPN22	> 200
PTPRK	> 200
PTRRT	> 200
PTPRM	> 100
DUSP13A	3.64 ± 0.57

IC_{50} values of enzyme activity were determined for the various PTPs. PTPs were incubated with 0 or 10 μM of PTP inhibitor V at 37 °C for 30 min. Fluorescence emission was measured with a multiwell plate reader. The experiments were performed in triplicate.

then examined inhibition profile of PTP inhibitor V on DUSP13A. When DUSP13A were treated with various concentrations of PTP inhibitor V, DUSP13A phosphatase activities were decreased by the inhibitor in a dose-dependent manner. The IC_{50} of DUSP13A was 3.64 ± 0.57 μM as determined by the Prism 3.0 program (GraphPad Software) (Fig. 2(a)). In the next step, kinetic analyses based on the Michaelis-Menten equation were carried out. The Lineweaver-Burk showed that the K_m value of DUSP13A for OMFP was 104.1 μM and K_i value was 5.53 μM (Fig. 2(b)). The inhibition pattern suggests that PTP inhibitor V inhibits the enzymatic activity of DUSP13A in a noncompetitive manner, which suggests that PTP inhibitor V binds to a site away from the catalytic site of DUSP13A for inhibition. To confirm that PTP inhibitor V regulates DUSP13A expressed in human cells, human embryonic kidney (HEK) 293 cells

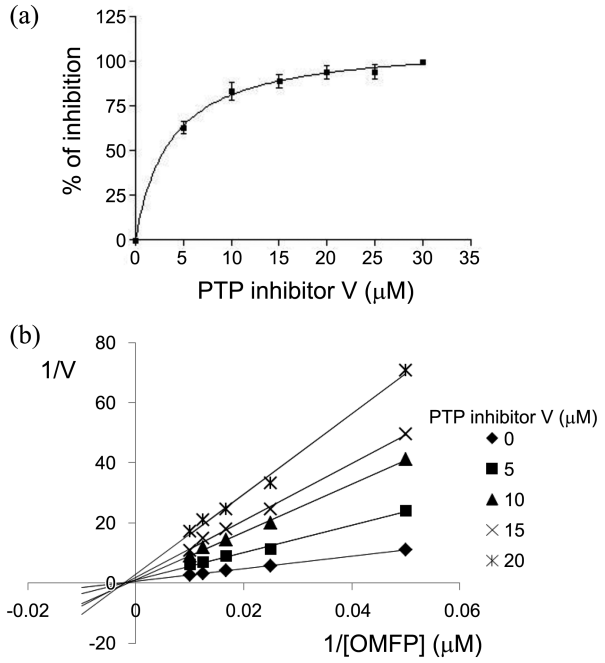

Figure 2. Inhibitory effect of PTP inhibitor V on DUSP13A. (a) DUSP13A was incubated with various concentrations of PTP inhibitor V at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental Section. (b) Kinetics analysis of DUSP13A inhibition by PTP inhibitor V was performed based on the theory of Lineweaver-Burk plots and the K_i value was determined.

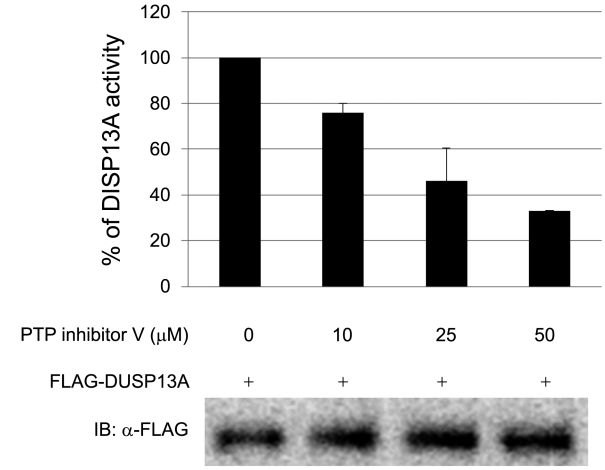

Figure 3. Inhibition of DUSP13A expressed in HEK 293 cells by PTP inhibitor V *in vitro.* HEK 293 cells were transfected with 1 μg of DUSP13A expression plasmid. After 48 h of transfection, HEK 293 cell lysates were subjected to immunoprecipitation with anti-FLAG M2 agarose. Immunoprecipitated DUSP13A 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. The values shown are relative activities compared to untreated samples after deducting the activities of cell lysates obtained from cells transfected with empty vector.

were transiently transfected with FLAG-tagged DUSP13A expression plasmid. After transfection, cells were lysed and immunoprecipitated with anti-FLAG M2 agarose bead. Immunoprecipitated DUSP13A was used for *in vitro* phosphatase assays in the presence of various concentrations of PTP inhibitor V and its phosphatase activities were determined (Fig. 3). The results showed that PTP inhibitor V effectively inhibited DUSP13A expressed in mammalian cells.

In this study, we identified that PTP inhibitor V functions as a selective and noncompetitive inhibitor of DUSP13A. PTP inhibitor V was originally shown to be a competitive inhibitor to SHP-2.[10] However, in this study, PTP inhibitor V acts as a noncompetitive inhibitor on DUSP13A, which means that the inhibitor binds to DUSP13A independent of the substrate binding. The precise mechanism how PTP inhibitor V is a competitive inhibitor of SHP-2 but a noncompetitive inhibitor of DUSP13A is not clear. In general, this type of noncompetitive inhibition implies that the inhibitor binds to an allosteric site different from the catalytic site. We speculate that PTP inhibitor V binds to the active site of DUSP13A but does not prevent the substrate from binding to the active site. The bound PTP inhibitor V inhibits the enzymatic reaction by blocking the product formation.

DUSP13 is known to be involved in the meiosis and spermatogenesis. DUSP13A is especially abundant in skeletal muscles. In addition, the expression of DUSP13 mRNA was increased in MCF-7 (Michigan Cancer Foundation-7) breast cancer cells treated with phorbol myristate acetate (PMA), suggesting that DUSP13 might be involved in the carcinogenesis of the breast cancer.[11] However, cellular targets and functional roles of DUSP13A are yet to be identified.[8] For these reasons, PTP inhibitor V might be useful for study of biological process of DUSP13A.

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) 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 polyethylenimine, linear MW ~25,000 (PEI, Polysciences, Warrington, PA).

Antibodies and Plasmid Constructions. Monoclonal anti-FLAG antibody was from Sigma-Aldrich (St. Louis, MO). 6 x His-tagged DUSP13A was constructed in pET28a plasmid (Novagen, Madison, WI) for protein expression in *Escherichia coli* (*E. coli*) and FLAG-tagged DUSP13A was constructed in pcDNA3.1(+) (Invitrogen, Carlsbad, California).

Purification of 6 x His Tagged Proteins. PTP expression plasmids were constructed in pET28a(+) and transformed into BL21(DE3)-RIL *E. coli*. Recombinant proteins were purified as previously described.[12]

***In vitro* PTP Activity Assays and Kinetic Analysis.** The activity of protein phosphatases was measured using the substrate 3-*O*-methylfluorescein phosphate (OMFP; Sigma-

Aldrich) in a 96-well microtiter plate based on methods described previously.[13] PTP inhibitor V and OMFP were solubilized in DMSO and all reactions were performed at the final concentration of 1% DMSO. The final incubation mixture (100 μL) was optimized for enzyme activity in assay buffer containing 30 mM Tris-HCl (pH 7), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.4 mM dithiothreitol (DTT), 0.033% bovine serum albumin (BSA) and 100 nM of PTPs. Reaction was initiated by the addition of OMFP to the final concentration of 100 μM and the incubation time was 30 min at 37 °C, fluorescence levels of released products were determined using a fluorescence plate reader (an excitation of 485 nm and emission of 535 nm). The half maximal inhibition constant (IC_{50}) was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activity by using the curve fitting program Prism3.0 (GraphPad Software). The inhibition constant (K_i) to DUSP13A phosphatase was calculated using the equations from the Lineweaver-Burk plots. The initial rates were measured at various OMFP concentrations for each fixed concentration of the inhibitor and the slopes showed the noncompetitive inhibition pattern. The K_i value was obtained from the below equation of noncompetitive inhibition. All experiments were performed in triplicate and repeated at least three times.

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

Immunoblotting Analysis. After HEK 293 cells were transiently transfected with or without FLAG-tagged DUSP13A for 48 h, cells were washed twice with phosphate buffered saline (PBS). The cells were lysed in PTP lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.5 % NP-40, 0.5% Triton X -100, 1 mM EDTA, 1% glycerol, 1 mM PMSF and 1 μg/mL aprotinin. Cell lysates were centrifuged at 13,000 rpm for 30 min at 4 °C and the supernatants were transferred to 1.5 mL Eppendorf tube. Samples were boiled at 100 °C for 5 min. Samples were run in SDS–10% polyacrylamide gels and transferred to nitrocellulose membrane (Whatman, Springfield Mill, UK). The membrane was blocked in 5% nonfat skim milk and incubated with an anti-FLAG antibody, followed by incubation with a secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were visualized using an ECL system (Pierce, Rockford, IL).

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References

1. Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. *Cell* **2004**, *117*, 699.
2. Patterson, K. I.; Brummer, T.; O'Brien, P. M.; Daly, R. J. *Biochem. J.* **2009**, *418*, 475.
3. Nakamura, K.; Shima, H.; Watanabe, M.; Haneji, T.; Kikuchi, K. *Biochem. J.* **1999**, *344 Pt 3*, 819.
4. Tonks, N. K. *FEBS J.* **2013**, *280*, 346.
5. Nunes-Xavier, C.; Roma-Mateo, C.; Rios, P.; Tarrega, C.; Cejudo-Marin, R.; Tabernero, L.; Pulido, R. *Anticancer Agents Med. Chem.* **2011**, *11*, 109.
6. Chen, H. H.; Luche, R.; Wei, B.; Tonks, N. K. *J. Biol. Chem.* **2004**, *279*, 41404.
7. Katagiri, C.; Masuda, K.; Nomura, M.; Tanoue, K.; Fujita, S.; Yamashita, Y.; Katakura, R.; Shiiba, K.; Nomura, E.; Sato, M.; Tanuma, N.; Shima, H. *Mol. Cell Biochem.* **2011**, *352*, 155.
8. Park, J. E.; Park, B. C.; Kim, H. A.; Song, M.; Park, S. G.; Lee do, H.; Kim, H. J.; Choi, H. K.; Kim, J. T.; Cho, S. *Cell Mol. Life Sci.* **2010**, *67*, 2619.
9. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug. Deliv. Rev.* **2001**, *46*, 3.
10. Hellmuth, K.; Grosskopf, S.; Lum, C. T.; Wurtele, M.; Roder, N.; von Kries, J. P.; Rosario, M.; Rademann, J.; Birchmeier, W. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7275.
11. Nunes-Xavier, C. E.; Tarrega, C.; Cejudo-Marin, R.; Frijhoff, J.; Sandin, A.; Ostman, A.; Pulido, R. *J. Biol. Chem.* **2010**, *285*, 26417.
12. Kim, H.; Seo, H.; Cho, S. *Bull. Korean Chem. Soc.* **2011**, *32*, 4103.
13. Tierno, M. B.; Johnston, P. A.; Foster, C.; Skoko, J. J.; Shinde, S. N.; Shun, T. Y.; Lazo, J. S. *Nature Protocols* **2007**, *2*, 1134.