http://dx.doi.org/10.5012/bkcs.2013.34.12.3874

Inhibition of PTPN2 by PTP inhibitor V

Younghyun Kim and Sayeon Cho*

*College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea. *E-mail: sycho@cau.ac.kr*
Received August 30, 2013, Accepted September 16, 2013

Key Words : PTPN2 (TC-PTP), PTP inhibitor V (PHPS1)

Protein phosphorylation is closely linked to critical pathways in numerous human diseases, which is regulated by the coordinated activities of kinases and phosphatases.[1] Protein phosphorylation occurs predominantly on serine, threonine, and tyrosine residues of proteins and plays important roles in the regulation of physiological processes including gene expression, proliferation, differentiation, cell cycle arrest, and apoptosis.[2] About 30% of proteins are phosphorylated in the mammalian cell. In case of eukaryotic cells, tyrosine phosphorylation occupies only 0.01-0.05% of total protein phosphorylation, while most of protein phosphorylation occurs on serine or threonine residues. However, growth factor stimulation or tumorigenesis enhances the level of tyrosine phosphorylation up to 1-2% of total protein phosphorylation.[3] Protein phosphorylation is a reversible process that is controlled by protein kinases and protein phosphatases. In particular, protein tyrosine phosphatases (PTPs) are involved in most signaling pathways and thus play important roles in eukaryotes.[4] PTPs superfamily comprises 107 enzymes in human genome. Based on the amino acid sequences of their catalytic domains, the PTPs can be grouped into four main families.[1] Since PTPs play critical roles for cell homeostasis and thus diseases, chemical inhibitors that regulate PTPs have been extensively investigated to be used as therapeutic reagents.

PTP inhibitor V, also known as phenyl hydrazono pyrazolone sulfate 1 (PHPS1), is 4-{*N*′-[3-(4-nitrophenyl)-5-oxo-1-phenyl-1,5-dihydro-pyrazol-(4*Z*)-ylidene]-hydrazino}-benzenesulfonic acid (Fig. 1(a)). PTP inhibitor V was originally identified as a potent cell-permeable inhibitor that acts as active-site targeting, reversible, competitive inhibitor of SHP-2 with the IC_{50} value of 2.1 μM.[5] PTP inhibitor V inhibits ECPTP, PTP1B and SHP-1 (IC_{50} = 5.4, 19, 30 μM, respectively).[5] To investigate the effect of PTP inhibitor V on other PTPs, we performed *in vitro* phosphatase assays with purified recombinant PTPs (Table 1). We found that protein tyrosine phosphatase non-receptor type 2 (PTPN2) was inhibited by PTP inhibitor V while other PTPs were not.

PTPN2 that is also called T cell protein tyrosine phosphatase (TC-PTP) belongs to class I Cys-based PTPs as the intracellular non-receptor PTPs with a high degree of sequence and structural homology within the catalytic domain.[6,7] The mRNA levels of PTPN2 increase at G_1 phase and return to basal level after G_1 phase during cell cycle progression.[8] PTPN2 is positively involved in cell proliferation.[9] Abnormal expression of PTPN2 is correlated with several diseases such as diabetes, obesity, and cancer.[10] PTPN2 has been reported to modulate cytokine receptor signaling, including IFN-γ signaling.[11,12] In another case, PTPN2 negatively regulates and inactivates Src tyrosine kinases to suppress

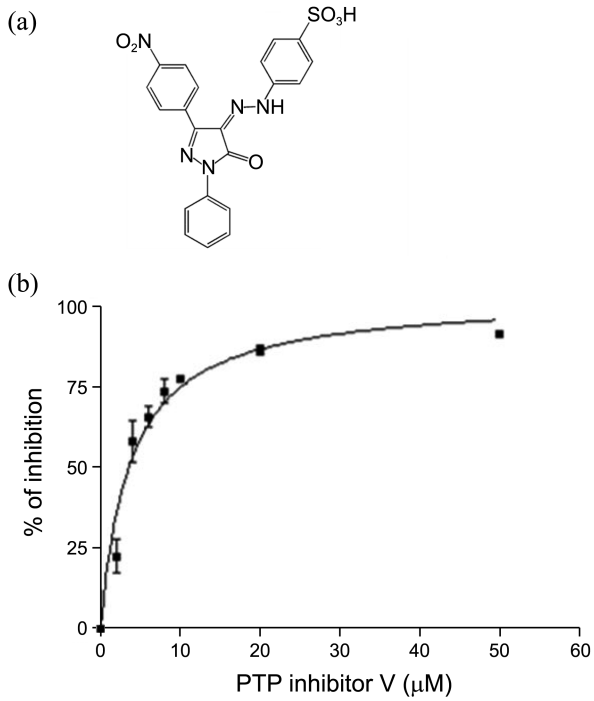

Figure 1. Chemical structure of PTP inhibitor V and its inhibitory effect on PTPN2. (a) Chemical structure of PTP inhibitor V. (b) Recombinant PTPN2 was incubated with various concentrations of PTP inhibitor V at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental section.

Table 1. Inhibition of PTPs by PTP inhibitor V. Inhibitory 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 experiment was performed in triplicate.

Protein tyrosine phosphatase	IC_{50} (μM)
DUSP18	> 10 (n = 3)
DUSP23	> 10 (n = 3)
DUSP26	> 10 (n = 3)
ACP1	>10 (n = 3)
PTPN2	3.79 ± 0.59 (n = 3)

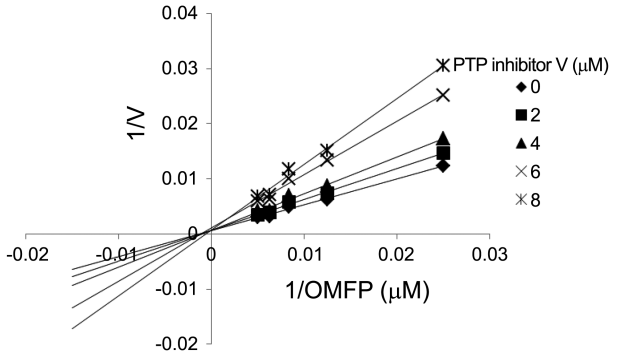

Figure 2. Kinetic analysis of PTPN2 inhibition by PTP inhibitor V. The inhibition of PTPN2 by PTP inhibitor V was carried out as described in Experimental section. Lineweaver-Burk plots of PTPN2 were generated from the reciprocal data.

downstream signaling through ERK and production of interleukin 6.[13]

To characterize the inhibitory pattern of PTPN2 by PTP inhibitor V, PTPN2 was treated with various concentrations of PTP inhibitor V. The half maximal inhibitory concentration (IC_{50}) of PTPN2 was determined as 3.79 ± 0.59 μM by the curve fitting program Prism 3.0 (GraphPad Software) (Fig. 1(b)). In subsequent investigations, kinetic analyses on the basis of the Michaelis–Menten equation were performed with PTP inhibitor V and PTPN2 to provide experimental evidence for the mechanism of PTPN2 catalysis and for binding of the inhibitor to the active site of the phosphatase. The Lineweaver–Burk plots show that the K_i was 2.85 μM (Fig. 2). The results also show that PTP inhibitor V functions as a competitive inhibitor of PTPN2, suggesting that PTP inhibitor V down-regulates the catalytic activity of PTPN2 by binding to the catalytic site. To test whether PTP inhibitor V inhibits PTPN2 expressed in mammalian cells, human embryonic kidney (HEK) 293 cells were transfected with

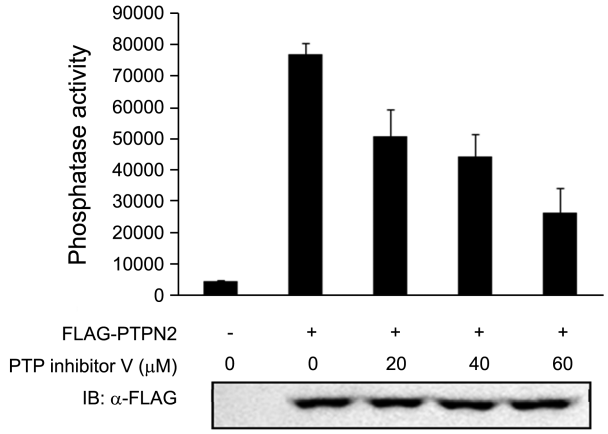

Figure 3. Inhibitory effect of PTP inhibitor V on PTPN2 expressed in mammalian cells. HEK 293 cells were transfected with FLAG-PTPN2 expression plasmid and incubated for 48 h. HEK 293 cell lysates were subjected to immunoprecipitation with anti-FLAG M2-agarose. Immunoprecipitated PTPN2 was incubated with various concentrations of PTP inhibitor V and OMFP at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental section.

FLAG-tagged PTPN2 expression plasmid. After transfection, cells were lysed with PTP lysis buffer. PTPN2 was immunoprecipitated from cell lysates using anti-FLAG M2-agarose. Phosphatase activities of immunoprecipitated PTPN2 were then determined using OMFP as a substrate in the presence of PTP inhibitor V (Fig. 3). The results showed that PTP inhibitor V effectively inhibited PTPN2 expressed in HEK 293 cells.

In previous report, PTPN2 phosphatase activity was effectively regulated by other chemical inhibitor such as ethyl-3,4-dephostatin.[14] In this study, we newly indentified that PTP inhibitor V inhibits PTPN2 phosphatase activity. It has been reported that a negative correlation between sensitivity to IFN-γ and PTPN2 activity was detected in the case of chronic myelogenous leukemia.[15,16] Therefore, PTP inhibitor V seems to play a potential regulator of tumorigenesis by inhibiting PTPN2 phosphatase activity. Furthermore, this study for the effect of PTP inhibitor V on PTPN2 will have an advantageous effect in PTPN2-related diseases such as chronic myelogenous leukemia.

Experimental Section

Antibodies and Reagents. Anti-FLAG M2 antibody was purchased from Sigma-Aldrich (St. Louis, MO). Linear-formed polyethylenimine (PEI) used for transfection into HEK 293 cell was purchased from Polysciences, Inc. (Warrington, PA). PTP inhibitor V was purchased from Merck KGaA (Darmstadt, Germany).

Cell Culture and Transfection. 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, Waltham, MA) and penicillin/streptomycin in the presence of 5% CO_2. For transfection, 1×10^6 cells were seeded in each 60 mm cell culture dish before the day of transfection and transfected with DNA using PEI.

Purification of Recombinant Protein. 6 x His-tagged PTPN2 was constructed in pET28a(+) plasmid (Novagen, Darmstadt, Germany) and transformed into BL21(DE3)-RIL *Escherichia coli*. Recombinant protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 22 °C for overnight. Cells were harvested and then lysed by sonication in 50 mM Tris-HCl (pH 6.8), 300 mM NaCl, 1% IGEPAL CA-630 (NP-40), 1 mM phenylmethanesulfonyl fluoride (PMSF). The lysates were clarified at 10,000 rpm for 20 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), 500 mM NaCl, 50 mM imidazole and eluted with 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 200 mM imidazole.

***In vitro* Phosphatase Assays and Kinetic Analysis.** Phosphatase activity was measured using the substrate OMFP (Sigma-Aldrich, St. Louis, MO) in a 96-well microtiter plate assay based on methods described previously.[17] PTP inhibitor V and OMFP were solubilized in dimethyl sulfoxide (DMSO). All reactions were performed at the final concentration of

1% DMSO. The final reaction mixture (100 μL) was optimized for enzyme activity and composed of 30 mM Tris-HCl (pH 7.0), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.4 mM dithiothreitol (DTT), 0.132% bovine serum albumin (BSA), and 100 nM of PTPs. Reactions were started by addition of OMFP and incubated for 30 min at 37 °C. Fluorescence emission from the product was measured with a multi-well plate reader (Biotek, excitation filter, 485 nm; emission filter, 535 nm). The reaction was linear over the time period of the experiment and was directly proportional to both enzyme and substrate concentrations. Half-maximal inhibition constant (IC_{50}) was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activities. 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, San Diego, CA). All experiments were performed in triplicate and were repeated at least three times.

Immunoblotting Analysis. Cell lysates were run in SDS-10% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked in 5% skim milk for 1 h and incubated with the appropriate primary antibodies, followed by incubation with the appropriate secondary antibodies conjugated horseradish peroxidase (HRP). The protein bands were visualized by the ECL detection system (Pierce, Rockford, IL).

Immunoprecipitation and *in vitro* Phosphatase Activity Assay. For immunoprecipitation, HEK 293 cells were transiently transfected with FLAG-PTPN2 expression plasmid for 48 h. After incubation, cells were washed several times with 1 x phosphate buffer saline (PBS, pH 7.4) to remove the inhibitor remained in the media. The cells were lysed with the lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.5% IGEPAL CA-630, 0.5% Triton X-100, 1% Glycerol, 1 mM EDTA, 1 mM PMSF, and 3 μM DTT for 15 min at 4 °C. Cell lysates were centrifuged at 13,000 rpm for 30 min at 4 °C and the soluble fractions were immunoprecipitated with anti-FLAG M2-agarose for 3 h at 4 °C. After binding, the beads were washed with lysis buffer and the bound proteins were incubated with PTP inhibitor V and OMFP. The reaction buffer was comprised of 30 mM Tris-HCl (pH 7.0), 75 mM NaCl, 1 mM EDTA, 0.4 mM DTT, and 0.132% BSA. The reaction mixtures were incubated at 37 °C for 30 min and fluorescence was measured using a microplate reader (excitation filter, 485 nm; emission filter, 535 nm).

Inhibition Study. The inhibition constant (K_i) to PTPN2 for the inhibitor was determine by measuring the initial rates at several OMFP concentration 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 slops obtained were replotted against the inhibitor concentrations. The K_i value was obtained from the slopes of these replots.[18]

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

Acknowledgments. This research was supported by the Chung-Ang University Excellent Student Scholarship and in 2012 and by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science, ICT & Future Planning (NRF-2011-0030029 and NRF-2012R1A1B3001937).

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. Ciesla, J.; Fraczyk, T.; Rode, W. *Acta Biochim. Pol.* **2011**, *58*, 137.
3. Hunter, T.; Sefton, B. M. *Proc. Natl. Acad. Sci. USA* **1980**, *77*, 1311.
4. Van Vactor, D.; O'Reilly, A. M.; Neel, B. G. *Curr. Opin. Genet. Dev.* **1998**, *8*, 112.
5. 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.
6. Jia, Z.; Barford, D.; Flint, A. J.; Tonks, N. K. *Science* **1995**, *268*, 1754.
7. Asante-Appiah, E.; Ball, K.; Bateman, K.; Skorey, K.; Friesen, R.; Desponts, C.; Payette, P.; Bayly, C.; Zamboni, R.; Scapin, G.; Ramachandran, C.; Kennedy, B. P. *J. Biol. Chem.* **2001**, *276*, 26036.
8. Tillmann, U.; Wagner, J.; Boerboom, D.; Westphal, H.; Tremblay, M. L. *Mol. Cell Biol.* **1994**, *14*, 3030.
9. Radha, V.; Nambirajan, S.; Swarup, G. *FEBS Lett* **1997**, *409*, 33.
10. Dube, N.; Tremblay, M. L. *Biochim. Biophys. Acta* **2005**, *1754*, 108.
11. Myers, M. P.; Andersen, J. N.; Cheng, A.; Tremblay, M. L.; Horvath, C. M.; Parisien, J. P.; Salmeen, A.; Barford, D.; Tonks, N. K. *J. Biol. Chem.* **2001**, *276*, 47771.
12. Simoncic, P. D.; Lee-Loy, A.; Barber, D. L.; Tremblay, M. L.; McGlade, C. J. *Curr. Biol.* **2002**, *12*, 446.
13. van Vliet, C.; Bukczynska, P. E.; Puryer, M. A.; Sadek, C. M.; Shields, B. J.; Tremblay, M. L.; Tiganis, T. *Nat. Immunol.* **2005**, *6*, 253.
14. Seo, H.; Cho, S. *Bull. Korean Chem. Soc.* **2011**, *32*, 2477.
15. Shimizu, T.; Miyakawa, Y.; Iwata, S.; Kuribara, A.; Tiganis, T.; Morimoto, C. ; Ikeda, Y.; Kizaki, M. *Exp. Hematol.* **2004**, *32*, 1057.
16. Shimizu, T.; Miyakawa, Y.; Oda, A.; Kizaki, M.; Ikeda, Y. *Exp. Hematol.* **2003**, *31*, 601.
17. Tierno, M. B.; Johnston, P. A.; Foster, C.; Skoko, J. J.; Shinde, S. N.; Shun, T. Y.; Lazo, J. S. *Nat. Protoc.* **2007**, *2*, 1134.
18. Shi, Z.; Tabassum, S.; Jiang, W.; Zhang, J.; Mathur, S.; Wu, J.; Shi, Y. *Chembiochem* **2007**, *8*, 2092.