http://dx.doi.org/10.5012/bkcs.2012.33.12.4277

Inhibition of SHP-2 Activity by PRL-3 Inhibitor I

Anna Ju, Huiyun Seo, and Sayeon Cho*

*College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea. *E-mail: sycho@cau.ac.kr*
Received October 4, 2012, Accepted October 17, 2012

Key Words : SHP-2, PRL-3 inhibitor I, PTP inhibitor

The equilibrium of cellular protein tyrosine phosphorylation is regulated by the balanced action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).[1] Protein tyrosine phosphorylation controls fundamental cellular processes such as growth, differentiation, migration, and invasion of malignant cells.[2] Based on the amino acid sequences of the catalytic domains, over 100 protein tyrosine phosphatases can be classified into four separate families: (i) class I cysteine-based PTPs containing classical PTPs and dual-specificity protein phosphatases (DSPs), (ii) class II cysteine-based PTPs: tyrosine-specific low molecular weight phosphatases, (iii) class III cysteine-based PTPs containing CDC25 homology (CH2) domain, and (iv) Eyes absent (EyA) proteins as members of aspartic acid-based PTPs.[3]

Mitogen-activated protein (MAP) kinases are critical signal transduction enzymes involved in numerous cellular functions.[4] Mammalian MAP kinases are grouped into at least four: extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38α/β/γ/δ) and ERK5 which are regulated through upstream kinases, MAP kinase kinases (MAPKKs).[4] These MAP kinase activities are regulated by dual phosphorylation on a tyrosine and a threonine residue through signal cascades. Protein phosphatases normally dephosphorylate either phospho-tyrosine or phospho-serine and phospho-threonine of their substrates. Since MAP kinases comprise both phospho-threonine and phospho-tyrosine on the activation loop, protein phosphatases are potential regulators of MAP kinases.[5] In other words, protein phosphatases are critical in modulating the magnitude and duration of MAP kinase activities.[6] Therefore, regulating PTPs by chemical compounds could play a pivotal role in development of novel therapeutics.[7]

Src homology region 2 (SH2) domain-containing phosphatase 2 (SHP-2), encoded by the *PTPN11* gene, is a ubiquitously expressed nonreceptor protein tyrosine phosphatases as a member of class I containing two cytosolic *N*-terminal SH2 domains (N-SH2, C-SH2), a catalytic (PTP) domain, and a *C*-terminal tail with tyrosyl phosphorylation sites and a proline-rich motif.[8,9] In the basal state, *N*-terminal SH2 domain is wedged into the phosphatase domain and blocks its enzymatic active site.[9,10] Normally, auto-inhibitory effect of tandem SH2 domain leads to decrease of catalytic efficiency.[11] Sustained SHP-2 activity becomes increased by upstream of stimulation of EGF and other growth factors, cytokines, resulting from trapped SH2 domains by phosphorylated docking protein.[12] SHP-2 has been known to involved in the ERK signaling pathway in response to some growth factors such as epidermal growth factor and insulin-like growth factor.[13] SHP-2 gain-of-function mutations are unquestionably associated with various types of cancers such as Noonan syndrome and leukemia.[14,15] Therefore, understanding of SHP-2-mediated signaling pathways and discovering SHP-2 inhibitors might be important therapeutic application for the treatment of diseases evoked by SHP-2 mutation.

In this study, we performed *in vitro* phosphatases assays to identify PTPs that are inhibited by novel phosphatase of regenerating liver-3 (PRL-3) inhibitor I (Figure 1). PRL-3 inhibitor I is a rhodanine derivative and inhibits phosphatase of regenerating liver-3 (PRL-3) that is highly expressed in some types of cancer cells and associated with promoting cell motility, invasion activity, and metastatic ability.[16-18]

To investigate whether PRL-3 inhibitor I regulates other PTPs, we used 6 x His-tagged recombinant PTPs overexpressed in and purified from bacteria. We treated recombinant PTPs with PRL-3 inhibitor I and carried out *in vitro* phosphatase assays using 3-*O*-methylfluorescein phosphate (OMFP) as a substrate. In recent study, we found that enzymatic activity of DUSP22 was selectively inhibited by PRL-3 inhibitor I.[19] Through the additional study, we observed that SHP-2 is inhibited by PRL-3 inhibitor I. Then, the inhibition curve of SHP-2 was plotted and a half maximal inhibitory concentration (IC_{50}) value was calculated. When SHP-2 was treated with various concentrations of PRL-3 inhibitor I, it resulted in approximately 90% reduction of the phosphatase activity with IC_{50} of 5.35 ± 2.20 μM by the curve fitting program Prism 3.0 (GraphPad Software) (Figure 2(a)).

In the next step, we calculated the inhibition constant (K_i) value using the equations from the Lineweaver-Burk plots. It showed that the K_m value of SHP-2 for OMFP was 102 μM and the K_i was 5.06 ± 1.56 μM (Figure 2(b)). The result suggests that PRL-3 inhibitor I binds to SHP-2 and inhibits

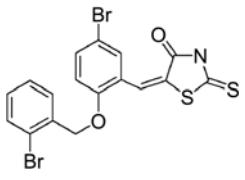

Figure 1. Chemical structure of PRL-3 inhibitor I. 5-[[5-Bromo-2-[(2-bromophenyl)methoxy]phenyl]methylene]-2-thioxo-4-thiazolidinone.

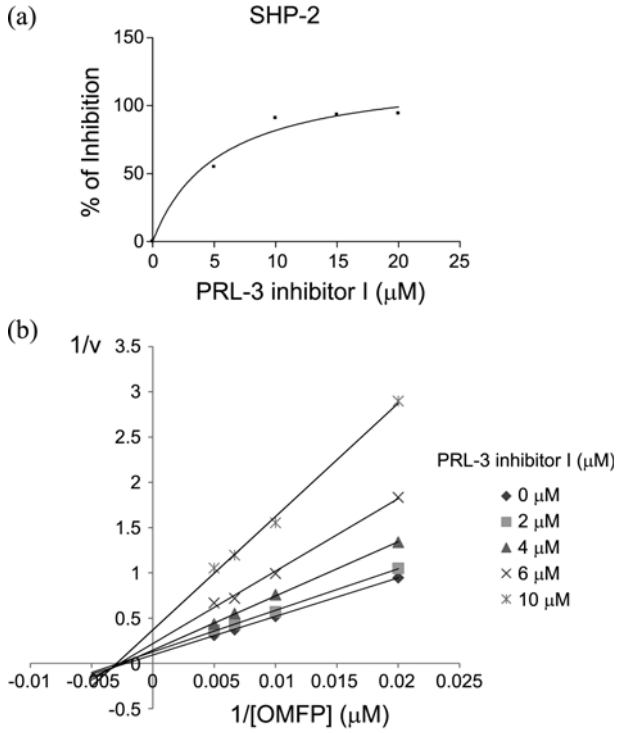

Figure 2. Inhibitory effect of PRL-3 inhibitor I on SHP-2. (a) SHP-2 was incubated with various concentrations of PRL-3 inhibitor I at 37 °C for 30 min and fluorescence emission was measured (an excitation of 485 nm and emission of 535 nm) as described in Experimental section. (b) Kinetics analysis of SHP-2 inhibition by PRL-3 inhibitor I was performed based on the theory of Lineweaver-Burk plots and the K_i value was calculated.

the enzymatic activity of SHP-2 in a noncompetitive way, which implies that PRL-3 inhibitor I binds to SHP-2 for inhibition without blocking the binding of the substrate to the catalytic site of SHP-2.

We also examined whether inhibitory effect of PRL-3 inhibitor I on SHP-2 is valid in mammalian cells and involved in ERK signaling pathway. To test if phosphorylation of ERK is regulated with the treatment of PRL-3 inhibitor I, human embryonic kidney (HEK) 293 cells were transiently transfected with or without plasmid carrying FLAG-SHP-2 (residues 205-593) which has only catalytically active domain without SH2 domains that negatively regulate SHP-2 activity. After 48 h of transfection, HEK 293 cells were treated with various concentrations of PRL-3 inhibitor I (0, 1, 5, 20 μM) for 3 h, immunoblotting analysis was performed to determine phospho-ERK levels *in vivo*. We observed gradual reduction of phospho-ERK levels in HEK 293 cells by inhibiting SHP-2 activity. These results demonstrate that PRL-3 inhibitor I effectively inhibits SHP-2-mediated phosphorylation of ERK *in vivo*.

It is noteworthy that discovery of novel inhibitors of protein tyrosine phosphatases is critical for therapeutic purpose since numerous diseases such as cancer, neurological disorders, and diabetes, result from failing to function of PTPs.[20] Since SHP-2 plays significant roles in biological processes and involved in several diseases such as Noonan syndrome

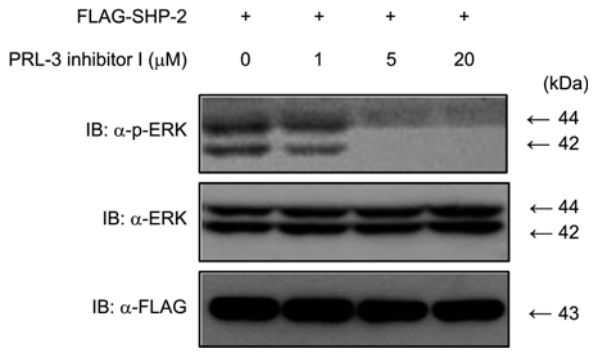

Figure 3. Effect of SHP-2 on ERK phosphorylation. HEK 293 cells were transfected with 1.5 μg of FLAG-SHP-2 (residues 205-593) and, after 48 h of transfection, HEK 293 cells were treated with varying concentrations of PRL-3 inhibitor I (0, 1, 5, 20 μM) for 3 h. Cells lysates were analyzed by immunoblotting with appropriate antibodies.

(NS), acute leukemia, and solid tumors,[21,22] further study for the role of SHP-2 and its regulation at the molecular level is important.[23] One of the most known inhibitors of SHP-2 is NSC-87877 (8-hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid). NSC-87877 inhibits the ligand-stimulated SHP-2 activation, resulting in blocking the ERK signaling pathway.[8,24] NSC-87877, however, has inhibitory activity on other PTPs, suggesting that the inhibitor may have multiple effects on other signaling pathways.[25-29] In this study, we confirmed that PRL-3 inhibitor I which regulates SHP-2 function could be a promising target for drug development.

Experimental Section

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells were maintained at 37 °C 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 transient transfection, 4×10^5 cells were seeded in 60 mm cell culture dish, grown overnight, and transfected with DNA using polyethylenimine (Polysciences, Inc., Warrington, PA).

Antibodies and Expression Plasmids. Anti-ERK and anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) were from Cell Signaling Technology (Danvers, MA). Monoclonal anti-FLAG was from Sigma-Aldrich. His-tagged SHP-2 (residues 205-593) was constructed in pET28a plasmid (Novagen, Madison, WI) for protein expression in *Escherichia coli* and FLAG-tagged SHP-2 (residues 205-593) was constructed in pcDNA3.1 (Invitrogen, Carlsbad, California).

Purification of 6x 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.[30]

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

St. Louis, MO) in a 96-well microtiter plate based on methods described previously.[31] PRL-3 inhibitor I and OMFP were solubilized in DMSO and all reactions were performed at a 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 mM and the incubation time was 30 min at 37 °C, fluorescence levels of released product 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 Prism 3.0 (GraphPad Software). The inhibition constant (K_i) to SHP-2 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 equations 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 SHP-2 (residues 205-593) for 48 h, cells were washed twice with phosphate buffered saline (PBS) and lysed on ice in following buffer: 150 mM NaCl, 20 mM Tris-HCl (pH 8.0) 0.5% NP-40, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF. Samples were boiled at 100 °C for 5 min and run in SDS-10% polyacrylamide gels and transferred onto nitrocellulose membrane. Then the membranes were blocked with 5% nonfat dry milk for 1 h and incubated with an appropriate antibody, followed by incubation with a HRP-conjugated secondary antibody with 5% skim milk. The immunoreactive bands were visualized by the ECL detection system (Pierce, Rock-ford, IL).

Acknowledgments. This study was supported by a grant of the National Project for Personalized Genomic Medicine, Ministry for Health & Welfare, Republic of Korea (A111218-CP03) and by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2012R1A2A2A01047338).

References

1. Tiganis, T.; Bennett, A. M. *Biochem. J.* **2007**, *402*, 1.
2. Malentacchi, F.; Marzocchini, R.; Gelmini, S.; Orlando, C.; Serio, M.; Ramponi, G.; Raugei, G. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 875.
3. Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. *Cell* **2004**, *117*, 699.
4. Chang, L.; Karin, M. *Nature* **2001**, *410*, 37.
5. English, J.; Pearson, G.; Wilsbacher, J.; Swantek, J.; Karandikar, M.; Xu, S. C.; Cobb, M. H. *Exp. Cell Res.* **1999**, *253*, 255.
6. Kondoh, K.; Nishida, E. *Biochim. Biophys. Acta* **2007**, *1773*, 1227.
7. Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2010**, *31*, 2721.
8. Chen, L.; Sung, S. S.; Yip, M. L.; Lawrence, H. R.; Ren, Y.; Guida, W. C.; Sebti, S. M.; Lawrence, N. J.; Wu, J. *Mol. Pharmacol.* **2006**, *70*, 562.
9. Mohi, M. G.; Neel, B. G. *Curr. Opin. Genet. Dev.* **2007**, *17*, 23.
10. Hof, P.; Pluskey, S.; Dhe-Paganon, S.; Eck, M. J.; Shoelson, S. E. *Cell* **1998**, *92*, 441.
11. Eck, M. J.; Pluskey, S.; Trub, T.; Harrison, S. C.; Shoelson, S. E. *Nature* **1996**, *379*, 277.
12. Cunnick, J. M.; Mei, L.; Doupnik, C. A.; Wu, J. *J. Biol. Chem.* **2001**, *276*, 24380.
13. Araki, T.; Nawa, H.; Neel, B. G. *J. Biol. Chem.* **2003**, *278*, 41677.
14. Tartaglia, M.; Mehler, E. L.; Goldberg, R.; Zampino, G.; Brunner, H. G.; Kremer, H.; van der Burgt, I.; Crosby, A. H.; Ion, A.; Jeffery, S.; Kalidas, K.; Patton, M. A.; Kucherlapati, R. S.; Gelb, B. D. *Nat. Genet.* **2001**, *29*, 465.
15. Mohi, M. G.; Williams, I. R.; Dearolf, C. R.; Chan, G.; Kutok, J. L.; Cohen, S.; Morgan, K.; Boulton, C.; Shigematsu, H.; Keilhack, H.; Akashi, K.; Gilliland, D. G.; Neel, B. G. *Cancer Cell* **2005**, *7*, 179.
16. Zeng, Q.; Dong, J. M.; Guo, K.; Li, J.; Tan, H. X.; Koh, V.; Pallen, C. J.; Manser, E.; Hong, W. *Cancer Res.* **2003**, *63*, 2716.
17. Saha, S.; Bardelli, A.; Buckhaults, P.; Velculescu, V. E.; Rago, C.; St Croix, B.; Romans, K. E.; Choti, M. A.; Lengauer, C.; Kinzler, K. W.; Vogelstein, B. *Science* **2001**, *294*, 1343.
18. Ahn, J. H.; Kim, S. J.; Park, W. S.; Cho, S. Y.; Ha, J. D.; Kim, S. S.; Kang, S. K.; Jeong, D. G.; Jung, S. K.; Lee, S. H.; Kim, H. M.; Park, S. K.; Lee, K. H.; Lee, C. W.; Ryu, S. E.; Choi, J. K. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2996.
19. Ju, A.; Cho, S. *Bull. Korean Chem. Soc.* **2012**, *33*, 3142.
20. Bialy, L.; Waldmann, H. *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 3814.
21. Chan, R. J.; Feng, G. S. *Blood* **2007**, *109*, 862.
22. Xu, R. Z. *Cell Research* **2007**, *17*, 295.
23. Ren, Y.; Chen, Z.; Chen, L.; Fang, B.; Win-Piazza, H.; Haura, E.; Koomen, J. M.; Wu, J. *Genes Cancer* **2010**, *1*, 994.
24. Deb, T. B.; Wong, L.; Salomon, D. S.; Zhou, G.; Dixon, J. E.; Gutkind, J. S.; Thompson, S. A.; Johnson, G. R. *J. Biol. Chem.* **1998**, *273*, 16643.
25. Park, S. J.; Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*, 3098.
26. Song, M.; Ho, S. *Bull. Korean Chem. Soc.* **2009**, *30*, 1858.
27. Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*, 1190.
28. Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*, 924.
29. Song, M.; Park, J. E.; Park, S. G.; Lee do, H.; Choi, H. K.; Park, B. C.; Ryu, S. E.; Kim, J. H.; Cho, S. *Biochem. Biophys. Res. Commun.* **2009**, *381*, 491.
30. Kim, H.; Seo, H.; Cho, S. *Bull. Korean Chem. Soc.* **2011**, *32*, 4103.
31. 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.