http://dx.doi.org/10.5012/bkcs.2012.33.9.3142

Inhibition of Dual-specificity Phosphatase 22 (DUSP22) by PRL-3 inhibitor I

Anna Ju and Sayeon Cho*

*College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea. *E-mail: sycho@cau.ac.kr*
Received June 7, 2012, Accepted June 27, 2012

Key Words : DUSP22, PRL-3 inhibitor I, PTP inhibitor

Protein phosphorylation is an essential mechanism for human health and disease, which is regulated by the coordinated activities of kinases and phosphatases.[1] Protein phosphorylation occurs predominantly on serine, threonine, and tyrosine residues of eukaryotic proteins and plays critical roles in the regulation of physiological processes including gene expression, proliferation, differentiation, cell cycles arrest, and apoptosis.[2]

Mitogen-activated protein (MAP) kinases are critical mediators in signal transduction pathways regulating embryogenesis, cell differentiation, cell proliferation, and cell death in response to extracellular stimuli and stress.[3,4] Extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) are the major mammalian MAP kinases that are regulated by some of protein tyrosine phosphatase (PTP) family members. PTP superfamily comprises 107 enzymes. Based on the amino acid sequences of their catalytic domains, the PTPs can be grouped into four main families: (i) classical PTPs and dual-specificity phosphatases (DUSPs), (ii) tyrosine-specific low molecular weight phosphatases, (iii) the cdc25 family, and (iv) Eyes absent (EyA) protein that was recently discovered to be tyrosine-, or dual serine- and tyrosine-specific protein phosphatases.[1] DUSPs are a subfamily of mammalian class I cysteine-based PTP superfamily, which dephosphorylate both tyrosine and serine/threonine residues. Some DUSPs have been characterized for the role in MAPK signaling pathways.[5] Since PTPs play critical roles for cell homeostasis, chemical inhibitors that regulate DUSPs have been extensively investigated to be used as therapeutic reagents.

PRL-3 inhibitor I is a rhodanine derivative and inhibits phosphatase of regenerating liver-3 (PRL-3) *in vitro*.[6] PRL-3 is a nonclassical protein tyrosine phosphtase and overexpression of PRL-3 plays a role in tumorigenesis and metastasis in various types of cancer cells. PRL-3 is associated with promoting cell motility, invasion activity, and metastasis. However, the biological and mechanistic evidence for PRL-3 function has not been understood well.[7,8] The half maximal inhibitory concentration (IC_{50}) of PRL-3 inhibitor I is known to be 0.9 μM against PRL-3 (Fig. 1(a)). To investigate the role of PRL-3 inhibitor I on other PTPs, we performed *in vitro* phosphatase assays with purified recombinant PTPs (Table 1). We identified that dual-specificity phosphatase 22 (DUSP22) is inhibited by PRL-3 inhibitor I while other PTPs are not.

DUSP22, also called JNK-stimulatory phosphatase 1 (JSP1) and JNK pathway-associated phosphatase (JKAP), positively regulates MAPK signaling pathways in contrast to most phosphatases which negatively regulate MAPK signaling pathways.[9] Overexpression of DUSP22 in HEK 293T cells activated JNK but not p38 and ERK.[10] Activation of JNK by DUSP22 is associated with activation of MKK4 which is an immediate JNK upstream activating kinase. In addition, a kinase inactive form of MKK4 (MKK4KR) abolished DUSP22-induced activation of JNK in COS-1 cells. Furthermore, the inactive mutant of DUSP22 failed to induce JNK activation.[11] This suggests that DUSP22 does not directly activate JNK but supports an enzyme activity of

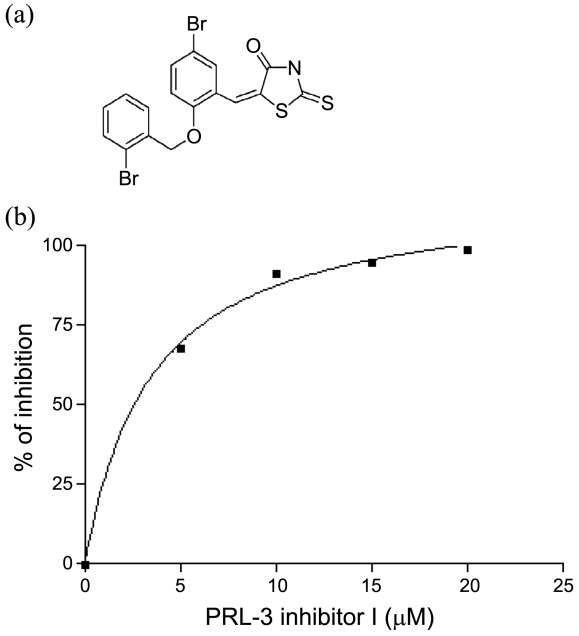


Figure 1. (a) Chemical structure of PRL-3 inhibitor I (5-[[5-Bromo-2-[(2-bromophenyl)methoxy]phenyl]methylene]-2-thioxo-4-thiazolidinone). (b) Inhibitory effect of PRL-3 inhibitor I on DUSP22. DUSP 22 was incubated with various concentrations of PRL-3 inhibitor I at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental Section. The half maximal inhibitory concentration (IC_{50}) of DUSP22 was about 3.46 ± 0.55 μM.

Abbreviations: ACP1 (Acid Phosphatase-1), CDC25B (cell division cycle 25 homolog B) EPM2A (epilepsy, progressive myoclonus type 2A, Lafora disease, laforin), PTPN2 (Protein tyrosine phosphatase, non-receptor type 2), PTPN6 (protein tyrosine phosphatase, non-receptor type 6).

Table 1. Inhibition of PTPs by PRL-3 inhibitor I

PTPs	IC_{50} (μM)
ACP1	No Inhibition
CDC25B	No Inhibition
DUSP13	No Inhibition
DUSP18	No Inhibition
DUSP23	No Inhibition
DUSP26	No Inhibition
DUSP3	No Inhibition
DUSP6	No Inhibition
EPM2A	No Inhibition
PTPN2	No Inhibition
PTPN6	No Inhibition
DUSP22	3.46 ± 0.55

Inhibitory IC_{50} of enzyme activity was determined for the various PTPs. PTPs was incubated with 0 or 10 μM of PRL-3 inhibitor I at 37 °C for 30 min. Fluorescence emission was measured with a multiwell plate reader. The experiment was performed in triplicate.

kinase upstream of MKK4.[11] Since DUSP22 is a specific regulator of JNK activation in response to stress, growth, and apoptosis, regulators of JNK activation like DUSP22 can be a reasonable therapeutic drug target.[10]

The phosphatase activity of DUSP22 was decreased by PRL-3 inhibitor I in a dose-dependent manner, whereas others were not (data not shown). The half maximal inhibitory concentration (IC_{50}) of DUSP22 was determined about 3.46 ± 0.55 μM by the curve fitting program Prism 3.0 (GraphPad Software) (Fig. 1(b)). Next, we confirmed that DUSP22 is involved in the regulation of JNK signaling and PRL-3 inhibitor I inhibits DUSP22 activity towards JNK. To examine whether DUSP22 plays a role as a JNK activator *in vivo*, HEK 293 cells were transfected with FLAG-tagged DUSP22 wild-type (WT) expression plasmid. After 48 h of transfection, cells were treated with various concentrations of PRL-3 inhibitor I for 3 h. Then the levels of endogenous phospho-JNK were determined with immunoblotting analysis. As shown in Figure 2, we found the level of phospho-JNK was effectively decreased by PRL-3 inhibitor I in a dose-dependent manner. The results indicate that PRL-3 inhibitor I reduces the level of phospho-JNK by inhibiting the enzymatic activity of DUSP22.

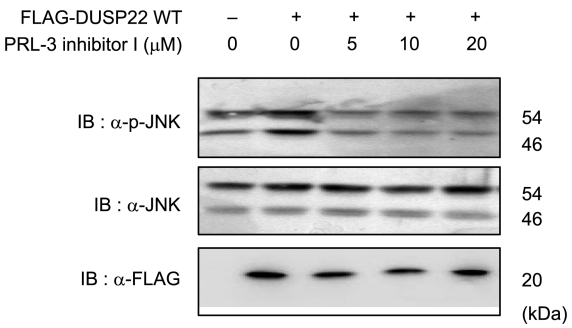


Figure 2. PRL-3 inhibitor I inhibits DUSP22-dependent JNK activation. HEK 293 cells were transfected with 1.5 μg of DUSP22 WT expression plasmid. After 48 h transfection the cells were treated with various concentrations of PRL-3 inhibitor I (0, 5, 10, 20 μM) for 3 h. The levels of phospho-JNK and JNK were detected by immunoblotting.

Taken together, we confirmed DUSP22 was effectively inhibited by PRL-3 inhibitor I *in vivo* and *in vitro* and DUSP22-mediated JNK signaling could be controlled by PRL-3 inhibitor I. Since JNK signaling pathway is implicated in critical physiological processes such as embryonic morphogenesis, cell survival, and apoptosis, the regulation of DUSP22-mediated JNK signaling by PRL-3 inhibitor I could be used as therapeutic drugs for JNK signaling linked human diseases such as tumorigenesis, cardiac hypertrophy, ischemia-reperfusion injury, diabetes, hyperglycemia-induced apoptosis, neurodegenerative diseases like Parkinson's disease.[8,11-13]

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 DUSP22-WT was constructed in pET28a plasmid (Novagen, Madison, WI) for protein expression in *Escherichia coli* and FLAG-tagged DUSP22-WT 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.[14]

***In vitro* PTP Activity Assays and Kinetic Analysis.** Protein phosphatase activity was measured using the substrate 3-Omethylfluorescein phosphate (OMFP; Sigma, St. Louis, MO) at the approximate K_m of the enzyme in a 96-well microtiter plate based on methods described previously.[15] PRL-3 inhibitor I and OMFP were solubilized in H_2O and DMSO, respectively, and all reactions were performed at a final concentration of 1% DMSO. The final reaction mixture (100 μL) was optimized for enzyme activity and comprised of 30 mM Tris-HCl (pH 7), 75 mM NaCl, 1 mM ethylenediaminetetra acetic acid (EDTA), 0.4 mM dithiothreitol (DTT), 0.132% bovine serum albumin (BSA) and 100 nM of PTPs. Reaction immediately begins by addition of OMFP and the incubation time was 30 min at 37 °C using a fluorescence plate reader set at an excitation of 485 nm and emission of 535 nm. Half maximal inhibition constant (IC_{50}) was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activity. Half-maximal inhibition

constants 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.

Immunoblotting Analysis. Transfected cells with or without DUSP22 WT expression plasmid 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 ^{o}C for 5 min. Samples 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).

Acknowledgments. This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korea government (MEST) (No. 2011-0030029) and by Basic Research Program through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology (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. Pearson, G.; Robinson, F.; Beers Gibson, T.; Xu, B. E.; Karandikar, M.; Berman, K.; Cobb, M. H. *Endocr. Rev.* **2001**, *22*, 153.
4. Raman, M.; Chen, W.; Cobb, M. H. *Oncogene* **2007**, *26*, 3100.
5. Pulido, R.; Hooft van Huijsduijnen, R. *FEBS J.* **2008**, *275*, 848.
6. 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.
7. Liang, F.; Liang, J.; Wang, W. Q.; Sun, J. P.; Udho, E.; Zhang, Z. Y. *J. Biol. Chem.* **2007**, *282*, 5413.
8. 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.
9. Schwertassek, U.; Buckley, D. A.; Xu, C. F.; Lindsay, A. J.; McCaffrey, M. W.; Neubert, T. A.; Tonks, N. K. *FEBS J.* **2010**, *277*, 2463.
10. Chen, A. J.; Zhou, G.; Juan, T.; Colicos, S. M.; Cannon, J. P.; Cabriera-Hansen, M.; Meyer, C. F.; Jurecic, R.; Copeland, N. G.; Gilbert, D. J.; Jenkins, N. A.; Fletcher, F.; Tan, T. H.; Belmont, J. W. *J. Biol. Chem.* **2002**, *277*, 36592.
11. Shen, Y.; Luche, R.; Wei, B.; Gordon, M. L.; Diltz, C. D.; Tonks, N. K. *Proc. Natl. Acad Sci. U S A* **2001**, *98*, 13613.
12. Qi, M.; Elion, E. A. *J. Cell Sci.* **2005**, *118*, 3569.
13. Uehara, T.; Honda, K.; Hatano, E.; Terao, R.; Iimuro, Y.; Yamamoto, N.; Yamamoto, M.; Kaneda, Y.; Yamaoka, Y. *J. Hepatol.* **1999**, *30*, 836.
14. Kim, H.; Seo, H.; Cho, S. *Bull. Korean Chem. Soc.* **2011**, *32*, 4103.
15. 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.