NSC-87877 Inhibits Dual-specificity Phosphatase 23 (DUSP23) that Regulates ERK

Mina Song and Sayeon Cho*

*College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea. *E-mail: sycho@cau.ac.kr*
Received June 8, 2009, Accepted June 26, 2009

Key Words: DUSP23, NSC-87877, PTP inhibitor

Phosphorylation and dephosphorylation of proteins comprise important regulatory pathways that are involved in the control of cell growth, differentiation adhesion and death.[1,2,3] This process requires the combined action of protein kinases and phosphatases. Protein phosphatases are generally divided into two classes: protein serine/threonine phosphatase and tyrosine specific phosphatase. The sequencing of the human genome has revealed 107 protein tyrosine phosphatases (PTPs).[4] The dual-specificity phosphatases (DUSPs) are subclass of the PTP superfamily and can dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within the same protein. DUSPs can be grouped into six subgroups on the basis of sequence similarity that include slingshots, phosphatases of regenerating liver (PRLs), Cdc14 phosphatases, phosphatase and tensin homologues deleted on chromosome 10 (PTENs), myotubularins, mitogen-activated protein kinase phosphatases (MKPs) and atypical DUSPs.[5] MKPs exhibit distinct substrate specificities for various mitogen-activated protein kinases (MAPKs), different tissue distribution, subcellular localization, and different modes of inducibility of their expressions by extracellular stimuli.[6,7,8,9] The three major groups of MAPK that are expressed in mammals are p38, extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK). MAPKs are key signaling enzymes that regulate proliferation, cell survival and death, differentiation, development, immune function, gene expression, and other signaling cascades.

Growing data presented that modulation of PTP enzymatic activities could have a role in regulating disease susceptibility.[10] Therefore, chemical compounds that regulate the activities of PTP may constitute a therapeutic approach for the treatment of diseases such as cancer, diabetes, and inflammation.

NSC-87877 was originally identified as a potent inhibitor of Src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1) and SHP-2 PTPs.[6] Fig. 1 shows the structure of NSC-87877. We used NSC-87877 to identify more phosphatases that are targets of NSC-87877. The phosphatase inhibition screening led to the discovery of several phosphatases that were effectively inhibited by NSC-87877.[11,12,13,14] In this report, we show that DSUP23 is another target of NSC-87877. NSC-87877 inhibited DUSP23 with the inhibitory concentration 50 (IC_{50}) about 11.25 ± 0.12 μM. When DUSP23 was treated with various concentrations of NSC-87877, DUSP23 inhibitory activity of NSC-87877 was dose-dependent (Fig. 2A). Analysis of the mode of inhibition indicated a competitive inhibition with K_i of 11.21 μM (Fig. 2B), suggesting that NSC-87877 binds to the catalytic cleft of DUSP23.

Next we asked whether the inhibitory action of NSC-87877 on DUSP23 influences the phosphorylation level of DUSP23 substrates. The MAPK substrates for DUSP23 remains to be clarified as two independent reports presented conflicting data.[15,16] To make clear substrate specificity of DUSP23, we

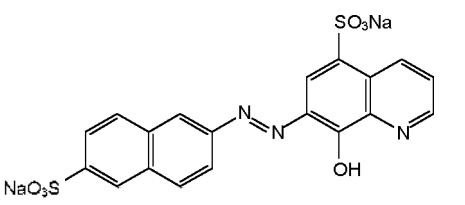

Figure 1. Chemical structure of NSC-87877.

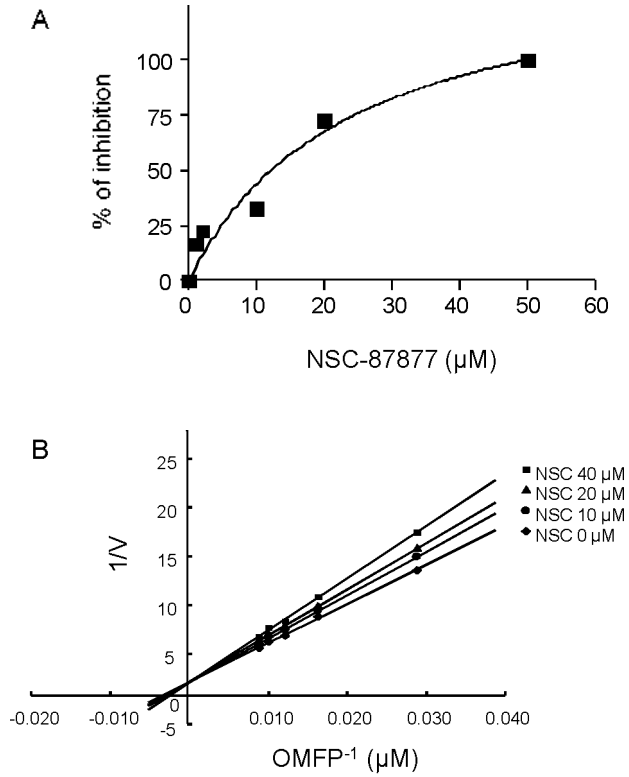

Figure 2. Inhibitory effect of NSC-87877 in DUSP23 and kinetic analysis of DUSP23 inhibition by NSC-87877 (A) DUSP23 was incubated with various concentrations of NSC-87877 at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental section. (B) Lineweaver-Burk plot of DUSP23 was generated from the reciprocal data.

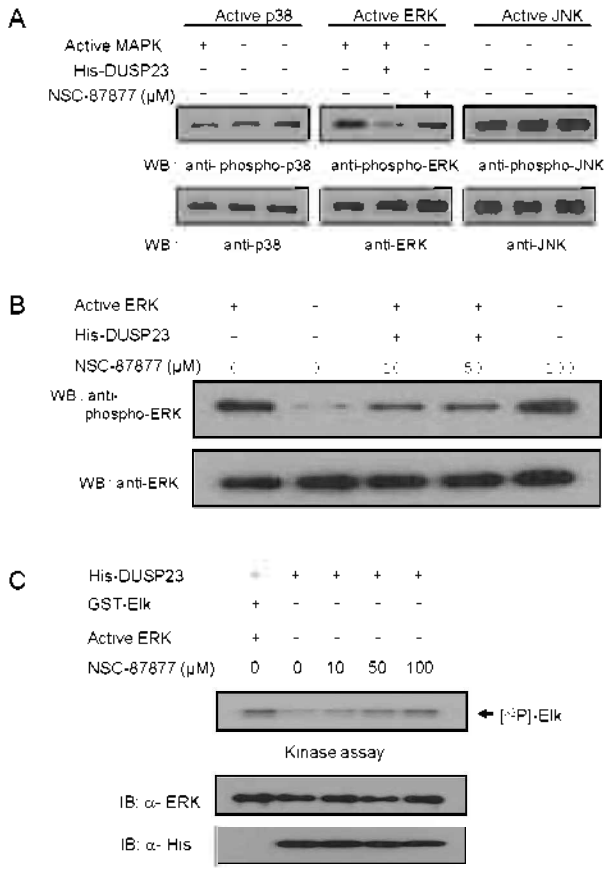

Figure 3. DUSP23 specifically dephosphorylates and inhibits ERK, and NSC-87877 inhibits the action of DUSP23 on ERK (A) Active recombinant MAPKs (p38, ERK, or JNK) were incubated *in vitro* with or without recombinant DUSP23 that was purified from bacteria at 37 °C for 30 min. Dephosphorylation levels of MAPKs were determined by Western blotting analysis using anti-phospho-MAPK antibodies. (B) DUSP23 and phospho-ERK were incubated with NSC-87877 (0, 50, or 100 μM) and the Western blotting analysis was performed as described in Experimental section. (C) DUSP23 (1 μg) was pre-mixed with various NSC-87877 concentrations as indicated and then incubated with active ERK. ERK activities were determined by kinase assays using GST-Elk as a substrate. Samples were resolved by SDS-PAGE and subjected to autoradiography.

performed *in vitro* dephosphorylation assays with the active recombinant MAPKs that were phosphorylated in the activation loops. As shown in Fig. 3A, DUSP23 effectively dephosphorylated the active phosphorylated ERK and the dephosphorylation of ERK by DUSP23 was blocked in the presence of NSC- 87877. In contrast, dephosphorylation of p38 and JNK by DSUP23 was not observed (Fig. 3A). These data suggest that both p38 and JNK are not direct targets of DUSP23.

The dose dependence of NSC-87877 on DUSP23-mediated ERK dephosphorylation was also examined. After treatment of phospho-ERK with DUSP23 in the presence of various concentrations of NSC-87877, the change in ERK phosphorylation level was determined with Western blotting analysis. As shown in Fig. 3B, DUSP23-mediated dephosphorylation of ERK was inhibited by NSC-87877 in a dose-dependent manner. Taken together, these data suggest that DUSP23 targets ERK and NSC-87877 treatment reduces DUSP23 phosphatase activity.

We additionally examined the effects of DUSP23 on ERK activity using an *in vitro* kinase assays. Kinase activity of ERK towards GST-Elk as a substrate was decreased in the present of DUSP23. And NSC-87877 blocked this inhibitory effect (Fig. 3C). These results imply that DUSP23 specifically dephosphorylates and inhibits ERK and NSC-87877 inhibits the action of DUSP23 on ERK.

In the present study, the results of our study suggest that NSC-87877 is a potent competitive inhibitor of DUSP23. DUSP23 dephosphorylates and inactivates ERK but has no activity on JNK and p38. Since NSC-87877 inhibited dephosphorylation of ERK by DUSP23, our results will provide the basis for developing a DUSP23-specific inhibitor that activates ERK signaling pathway.

Experimental Section

Antibodies. Anti-ERK, anti-phospho-JNK (specific for phospho-Thr183 and phospho-Tyr185), anti-phospho-p38 (phospho-Thr180 and phospho-Tyr182), and anti-phospho-ERK (phospho-Thr202 and phospho-Tyr204) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-JNK and anti-p38 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Active JNK and active p38 proteins were from Upstate Biotechnology (Lake Placid, NY). Active ERK protein was from Millipore (Bedford, MA).

Purification of 6 x His-tagged proteins. Purification of recombinant proteins were carried out as previously described.[13]

***In vitro* phosphatase assays and kinetic analysis.** *In vitro* Phosphatase assays and Kinetic analysis were carried out as previously described.[14]

Western blotting analysis. Western blotting was carried out as previously described.[15]

Dephosphorylation assays with active phosphorylated MAPKs. 6 x His-tagged DUSP23 (1 μg) was combined with active phosphorylated p38 (10 ng), ERK (10 ng), or JNK (20 ng) in PTP assay buffer (30 mM Tris-HCl (pH 7), 75 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 0.33% BSA) in a total reaction volume of 30 μL, and incubated for 30 min at 37 °C. To determine whether NSC-87877 down-regulates DUSP23 effect on ERK *in vitro*, 1 μg of DUSP23 was mixed with 10 ng active phosphorylated ERK and various NSC-87877 concentrations (0, 50, or 100 μM) in a 30 μL reaction volume and incubated for 30 min at 37 °C. The samples were subjected to Western blotting analysis to examine the phosphorylation state of MAPKs using the phospho-MAPK antibodies.

***In vitro* kinase assay.** 6 x His-tagged DUSP23 (1 μg) was pre-mixed with various NSC-87877 concentrations (0, 50, or 100 μM) in PTP assay buffer for 30 min at 37 °C and then further incubated in the presence of active phosphorylated ERK (10 ng) for 30 min at 37 °C. Kinase reactions were initiated by mixing the pre-incubated samples with kinase reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM DTT) supplemented with 20 μM ATP/10 μCi [γ-^{32}P]ATP and 1 μg of Elk as a substrate. After 30 min at 30 °C, reactions were terminated by addition of SDS-PAGE sample buffer and the products of kinase reactions

were separated by SDS-PAGE for autoradiography. The gels were dried and exposed to X-ray film.

Acknowledgments. This work was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A01-0385-A70604-07M7-00040B).

References

1. Yarden, Y.; Ullrich, A. *Annu. Rev. Biochem.* **1988**, *57*, 443-478.
2. Hunter, T. *Curr. Opin. Cell. Biol.* **1989**, *1*(6), 1168-1181.
3. Ullrich, A.; Schlessinger, J. *Cell* **1990**, *61*(2), 203-212.
4. Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. *Cell* **2004**, *117*(6), 699-711.
5. Patterson, K. I.; Brummer, T.; O'Brien, P. M.; Daly, R. J. *Biochem. J.* **2009**, *418*(3), 475-489.
6. 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*(2), 562-570.
7. Dickinson, R. J.; Keyse, S. M. *J. Cell Sci.* **2006**, *119*(Pt 22), 4607-4615.
8. Dixon, D.; Moyana, T.; King, M. J. *Exp. Cell Res.* **1998**, *240*(2), 236-243.
9. Srikanth, S.; Franklin, C. C.; Duke, R. C.; Kraft, R. S. *Mol. Cell Biochem.* **1999**, *199*(1-2), 169-178.
10. Fischer, E. H.; Charbonneau, H.; Tonks, N. K. *Science* **1991**, *253*(5018), 401-406.
11. Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*(5), 1190-1192.
12. Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*(4), 924-926.
13. Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*(1), 236-238.
14. 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*(4), 491-495.
15. Wu, Q.; Li, Y.; Gu, S.; Li, N.; Zheng, D.; Li, D.; Zheng, Z.; Ji, C.; Xie, Y.; Mao, Y. *Int. J. Biochem. Cell Biol.* **2004**, *36*(8), 1542-1553.
16. Takagaki, K.; Satoh, T.; Tanuma, N.; Masuda, K.; Takekawa, M.; Shima, H.; Kikuchi, K. *Biochem. J.* **2004**, *383*(Pt. 3), 447-455.
17. Cho, S. *Bull. Korean Chem. Soc.* **2005**, *26*(11), 1823-1825.