http://dx.doi.org/10.5012/bkcs.2012.33.10.3505

Inhibition of DUSP13B Activity by NSC 663284

In-Seon Lee, Anna Ju, and Sayeon Cho*

*College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea. *E-mail: sycho@cau.ac.kr*
Received July 6, 2012, Accepted July 23, 2012

Key Words : DUSP13B, NSC 663284, PTP inhibitor

Dual-specificity phosphatases (DUSPs) are group of enzymes that belong to the superfamily of protein-tyrosine phosphatases and catalyze dephosphorylation of proteins at both phosphorylated tyrosine and serine/threonine residues.[1,2] Several members of the DUSP family have distinct substrate specificity, subcellular localization, and induced expression pattern by extracellular stimuli.[3] These phosphatases play an important role in the regulation of intracellular signaling cascades governing cell growth, differentiation, and apoptosis.[2,4] Among several phosphatases, DUSP13 encodes two distinct DUSPs in alternative reading frames, called MDSP (muscle-restricted DUSP) and TMDP (testis- and skeletal muscle-specific DUSP). They are called DUSP13A and DUSP13B, respectively.[5] DUSP13 can dephosphorylate phosphotyrosine and phosphoserine/threonine residues, but cellular targets of DUSP13 proteins are yet to be characterized. Our recent report showed that DUSP13A interacts with and activates apoptosis signal-regulating kinase 1 (ASK1) regardless of its phosphatase activity.[6] DUSP13B is up-regulated at the meiotic stage and expressed stage-specifically during spermatogenesis.[7] According to the study of Katagiri *et al.*, contrary to DUSP13A, DUSP13B inactivates mitogen-activated protein kinase (MAPK) activation in the order of selectivity, JNK = p38 > ERK in cells, while DUSP13A did not show MAPK phosphatase activity.[1]

6-Chloro-7-[[2-(4-morpholinyl)ethyl]amino]-5,8-quinolinedione (NSC 663284) is the first of a series of quinolinediones that were known as a potent cell permeable Cdc25 phosphatase inhibitor II and exhibited mixed inhibition kinetics against Cdc25A, Cdc25B2, and Cdc25C (IC_{50} = 29, 95, and 89 nM for Cdc25A, Cdc25B2, and Cdc25C, respectively) (Fig. 1).[8,9] NSC 663284 inhibition kinetics reflect the potential for interactions at the two anionic binding sites.[10] NSC 663284 arrested cells at both the G1 and G2/M phase and inhibit dephosphorylation and activation of cdk1 and cdk2. By inhibiting Cdc25 isoforms, NSC 663284 blocks proliferation of several human tumor cell lines.[9,10]

We examined the inhibitory effect of NSC 663284 on DUSP13B using *in vitro* phosphatase activity assay. DUSP13B was treated with various concentrations of NSC 663284, its phosphatase activity was decreased dose-dependently by the inhibitor (Fig. 2(a)). Inhibition curve of IC_{50} was described from dose-dependent manner plots. This result indicates that NSC 663284 inhibits DUSP13B with IC_{50} of 3.84 ± 0.86 μM. In subsequent experiment, we performed kinetics study of NSC 663284 and DUSP13B. The kinetic analyses were based on the Michaelis-Menten equation and provide experimental evidence of a competitive inhibition. A Lineweaver-

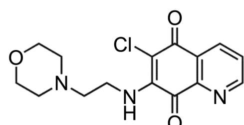

Figure 1. Chemical structure of NSC 663284.

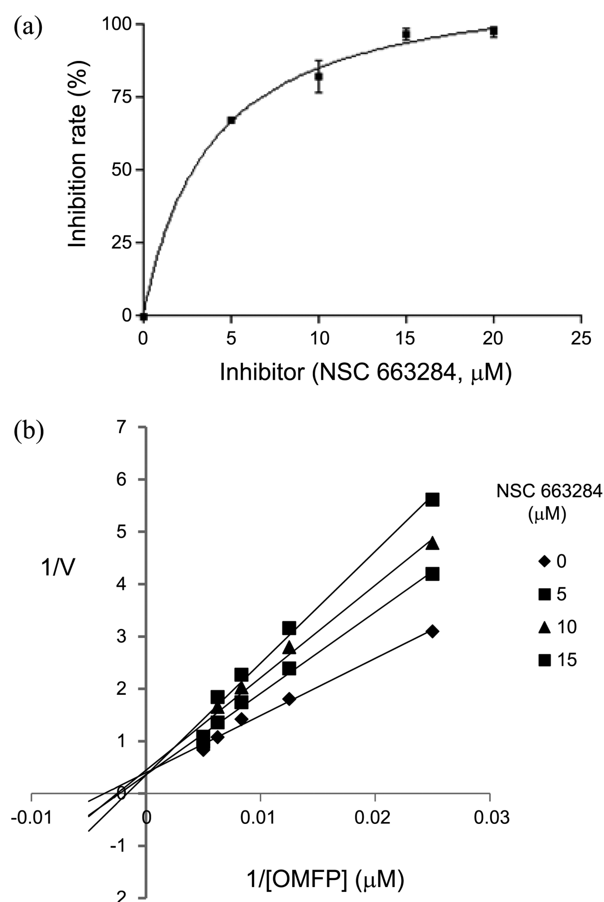

Figure 2. Inhibitory effect of NSC 663284 on DUSP13B. (a) The half maximal inhibitory constant of enzyme was determined to 3.84 ± 0.86 μM. DUSP13B (100 nM) was incubated with various concentrations of NSC 663284 at 37 °C for 30 min. Fluorescence emission from the product was measured with a multi-well plate reader as described in Experimental Section. (b) Lineweaver-Burk plots of DUSP13B were generated from the reciprocal data.

Burk analysis of NSC 663284 inhibition was carried out, and the pattern of inhibition corresponds to that expected for competitive inhibition (Fig. 2(b)). The plots show that the Ki value of NSC 663284 for DUSP13B was 7.8 μM. These results suggest that NSC 663284 regulates the catalytic activity of DUSP13B as a competitive inhibitor by binding to the catalytic site of the phosphatase.

Next, we investigated whether NSC 663284 regulates activity of DUSP13B on dephosphorylation of MAPK *in vitro*. We performed *in vitro* dephosphorylation assays with active ERK2, JNK, and p38 that were phosphorylated in the activation loops to identify a direct substrate of DUSP13B. Active MAPK proteins were incubated with recombinant DUSP13B purified from bacteria, then phosphorylated levels of MAPKs were determined with immunoblot analysis using anti-phospho-ERK, JNK, or p38 antibodies. DUSP13B failed to dephosphorylate MAPKs, which suggests that MAPKs are not substrates of DUSP13B (Fig. 3). These results suggest that recombinant DUSP13B does not dephosphorylate active ERK, JNK, and p38 *in vitro*. Our results of DUSP13B activity on MAPKs are contradictory to previous report that DUSP13B inactivates MAPK activation in cells. These results raise the possibility that DUSP13B regulates upstream factors of MAPK for signal transduction *in vivo*. Subsequently, to determine whether DUSP13B is inhibited by NSC 663284 in cells, human embryonic kidney (HEK) 293 cells were transfected with FLAG-DUSP13B expression plasmid and treated with various concentrations of NSC 663284. After 3 h of NSC 663284 treatment, cells were lysed with PTP lysis buffer. DUSP13B was immunoprecipitated from cell lysates with anti-FLAG M2 affinity gel. Then, phosphatase activities of immunoprecipitated DUSP13B were determined by *in vitro* phosphatase assays using OMFP as a substrate (Fig. 4). Phosphatase activities were inhibited in a dose-dependent manner. The results showed that NSC 663284 effectively permeated into the cells and inhibited DUSP13B activity. Taken together, these data suggest that NSC 663284 inhibits phosphatase activity of DUSP13B *in vivo*. The inhibitory activity of NSC 663284 is independent of MAPK signal pathway in our study.

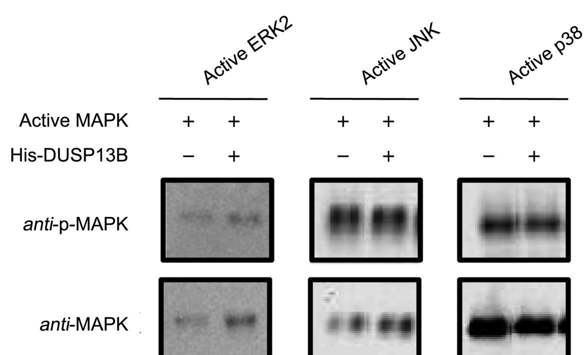

Figure 3. Phosphatase activity of DUSP13B on MAPK *in vitro*. DUSP13B was incubated with recombinant active MAPK for 30 min at 37 °C. Phosphorylation levels of ERK, JNK, and p38 were determined by immunoblot analysis as described in Experimental Section.

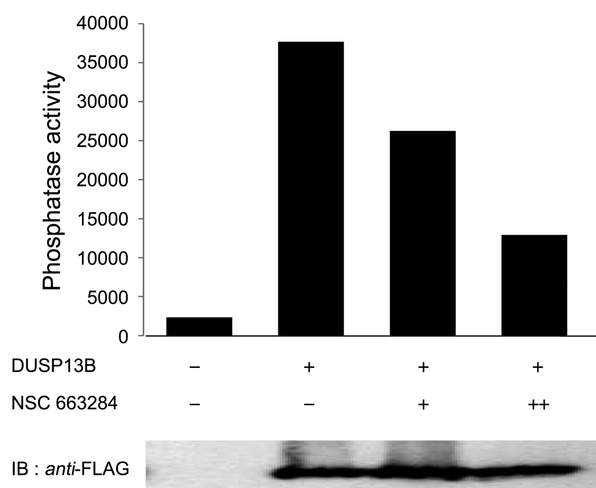

Figure 4. Inhibition of DUSP13B by NSC 663284 *in vivo*. Human embryonic kidney (HEK) 293 cells were transfected with FLAG-DUSP13B expression plasmid and incubated for 48 h, and then treated with various concentrations of NSC 663284. After 3 h of treatment, HEK 293 cell lysates were subjected to immunoprecipitation with anti-FLAG M2 affinity gel. Immunoprecipitated DUSP13B was incubated with OMFP at 37 °C for 30 min. Fluorescence emission from the product was measured with a multi-well plate reader as described in Experimental Section.

Protein phosphatase inhibitors are very important for their pharmacological potency. NSC 663284 is a well-known inhibitor of Cdc25 phosphatases that coordinate cell cycle progression and cellular signaling. NSC 663284 prevents proliferation of several human tumor cell lines.[8] In this study, we identified NSC 663284 as a potent competitive inhibitor of DUSP13B and demonstrated that it could inhibit DUSP13B activity in intact cell models. Thus, although the cellular target proteins of DUSP13B are yet to be identified, this study provides therapeutic potential that NSC 663284 could be a candidate of therapeutic reagent for spermatogenesis disorder.

Experimental Section

Reagents and Antibodies. Monoclonal anti-FLAG M2 mouse antibody and anti-FLAG M2 affinity gel were purchased from Sigma (St. Louis, MO). Anti-phospho-ERK and JNK antibodies were from Cell Signaling Technology (Danvers, MA). Anti-phospho-p38 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). NSC 663284 was from Sigma.

Plasmid and Recombinant Proteins. FLAG-tagged DUSP13B was constructed in pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA). His-DUSP13B was constructed in pET28a (+) plasmid and transformed into BL21 (DE3)-RIL *E. coli*. Recombinant protein purification for *in vitro* PTP activity assay was performed as previously described.[11]

***In vitro* Phosphatase Assays and Kinetic Analysis.** Phosphatase activity was measured using the substrate 3-O-methylfluorescein phosphate (OMFP; Sigma, St. Louis, MO). NSC 663284 and OMFP were solubilized in DMSO. All

reactions were performed at the final concentration of 1% DMSO. The final incubation mixture (150 μL) was optimized for enzyme activity and comprised of 30 mM Tris-HCl (pH 7.0), 75 mM NaCl, 1 mM EDTA, 0.4 mM DTT, 0.33% bovine serum albumin (BSA) and 100 nM of recombinant PTPs. Reactions were initiated 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). Kinetic analysis was performed as previously described.[12]

Dephosphorylation of Recombinant Active MAPKs by DUSP13B. Active ERK2 was kindly provided from Dr. Jeong (KRIBB, Korea). Active JNK and active p38 was from Merck Millipore (Billerica, MA). Dephosphorylation assays were carried out as previously described.[13] Subsequently, the reaction mixtures were analysed by immunoblot analysis using anti-phospho-ERK, JNK, and p38 antibodies, respectively.

Immunoblot Analysis. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane (Whatman, Springfield Mill, UK). Then the membranes were blocked with 5% skim milk for 1 h and incubated with an appropriate antibody, followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma). The protein bands were visualized by the ECL detection system (Pierce, Rockford, IL).

Immunoprecipitation and *in vitro* Phosphatase Activity Assay. For the immunoprecipitation, human embryonic kidney (HEK) 293 cells were transiently transfected with FLAG-DUSP13B expression plasmid and NSC 663284 was treated to the cells for 3 h. After incubation, cells were washed several times with 1 X 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% 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 soluble fractions were immunoprecipitated with anti-FLAG M2 affinity gel for 5 h at 4 °C. After binding, the beads were washed with lysis buffer and the bound proteins were incubated with OMFP, phosphatase substrate. The reaction buffer was comprised of 30 mM Tris-HCl (pH 7.0), 75 mM NaCl, 1 mM EDTA, 0.4 mM DTT, 0.33% BSA. The reaction mixtures were incubated at 37 °C for 30 min and fluorescence measured using microplate reader (excitation filter, 485 nm; emission filter, 535 nm).

Acknowledgments. We thank Dae Gwin Jeong for generously providing active ERK protein. This research was supported by Basic Research Program through the National Research Foundation (NRF) funded by the Ministry of Education, Science and Technology (2012R1A1B3001937).

References

1. Katagiri, C.; Masuda, K.; Nomura, M.; Tanoue, K.; Fujita, S.; Yamashita, Y.; Katakura, R.; Shiiba, K.; Nomura, E.; Sato, M.; Tanuma, N.; Shima, H. *Molecular and Cellular Biochemistry* **2011**, *352*, 155.
2. Patterson, K. I.; Brummer, T.; O'Brien, P. M.; Daly, R. J. *Biochem. J.* **2009**, *418*, 475.
3. Lang, R.; Hammer, M.; Mages, J. *J. Immunol.* **2006**, *177*, 7497.
4. Shang, X.; Vasudevan, S. A.; Yu, Y.; Ge, N.; Ludwig, A. D.; Wesson, C. L.; Wang, K.; Burlingame, S. M.; Zhao, Y. J.; Rao, P. H.; Lu, X.; Russell, H. V.; Okcu, M. F.; Hicks, M. J.; Shohet, J. M.; Donehower, L. A.; Nuchtern, J. G.; Yang, J. *Oncogene* **2010**, *29*, 4938.
5. Chen, H. H.; Luche, R.; Wei, B.; Tonks, N. K. *J. Biol. Chem.* **2004**, *279*, 41404.
6. Park, J. E.; Park, B. C.; Kim, H. A.; Song, M.; Park, S. G.; Lee, D. H.; Kim, H. J.; Choi, H. K.; Kim, J. T.; Cho, S. *Cellular. and Molecular Life Sciences* **2010**, *67*, 2619.
7. Nakamura, K.; Shima, H.; Watanabe, M.; Haneji, T.; Kikuchi, K. *Biochem. J.* **1999**, *344 Pt 3*, 819.
8. Guo, J.; Parise, R. A.; Joseph, E.; Lan, J.; Pan, S. S.; Joo, B.; Egorin, M. J.; Wipf, P.; Lazo, J. S.; Eiseman, J. L. *Anticancer. Res.* **2007**, *27*, 3067.
9. Pu, L.; Amoscato, A. A.; Bier, M. E.; Lazo, J. S. *J. Biol. Chem.* **2002**, *277*, 46877.
10. Lazo, J. S.; Aslan, D. C.; Southwick, E. C.; Cooley, K. A.; Ducruet, A. P.; Joo, B.; Vogt, A.; Wipf, P. *J. Med. Chem.* **2001**, *44*, 4042.
11. Lee, I.; Seo, H.; Cho, S. *Bull. Korean Chem. Soc.* **2010**, *31*, 2110.
12. Park, J. E.; Park, B. C.; Song, M.; Park, S. G.; Lee, D. H.; Park, S. Y.; Kim, J. H.; Cho, S. *Biochem. Biophys. Res. Commun.* **2009**, *387*, 795.
13. Song, M.; Park, S.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*, 236.