

In vivo Effect of NSC-95397 as a DUSP14 Inhibitor

Huiyun Seo and Sayeon Cho*

College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea. *E-mail: sycho@cau.ac.kr
Received October 11, 2011, Accepted October 21, 2011

Key Words : NSC-95397, Dual-specificity phosphatase 14, PTP inhibitor

Dual-specificity phosphatases (DUSPs) as a heterogeneous group of protein phosphatases belong to a subclass of PTP (protein tyrosine phosphatase) family and dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues.¹ DUSPs play as major modulators of critical signal transduction pathways that are deregulated in many diseases. Particularly, mitogen-activated protein kinase phosphatases (MKPs) have function as negative regulators of mitogen-activated protein kinase (MAPK)-mediated signaling in many cellular processes.^{2,3} DUSP14 also known as MKP6 was first identified through a yeast two-hybrid system to identify novel proteins that interact with the T-cell costimulatory factor CD28. DUSP14 is expressed ubiquitously, but elevated expression has been observed in certain types of cells and tissues.⁴ DUSP14 is one of 19 atypical DUSPs that contain the consensus DUSP C-terminal catalytic domain but lack the N-terminal CH2 (Cdc25 homology 2) domain.

The MAPK subfamily, including the c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERK), and p38, has a diverse crucial role that regulates proliferation, cell survival and death, differentiation, development, immune function, gene expression, and other intracellular events.^{5,6} Several PTPs are able to function as oncoproteins or tumor suppressors by dephosphorylating specific MAPKs and modifying the pathway.⁷ DUSP14 dephosphorylates and inactivates MAPKs.⁸

NSC-95397 (2,3-bis-[2-hydroxyethylsulfanyl]-[1,4]naphthoquinone) was originally reported to inhibit Cdc25 family of DUSPs, including Cdc25A, B, and C and VH1-related dual-specificity phosphatase or PTP1B.⁹ In recent experiments, we showed that NSC-95397 effectively and specifically down-regulates DUSP14 phosphatase activity *in vitro*.¹⁰ In this study, we show that NSC-95397 regulates MAPK signaling *in vivo* through inhibition of DUSP14.

We first examined whether the inhibitory function of NSC-95397 on DUSP14 regulates the phosphorylation of MAPKs. It has been reported that DUSP14 can dephosphorylate phospho-ERK, JNK, and p38 *in vitro*.⁸ However, in T-cells expressing the catalytically inactive mutant DUSP14 (C111S), only the phosphorylation of ERK and JNK increased whereas that of p38 remains unchanged, suggesting that ERK and JNK are the valid targets of DUSP14 *in vivo*.⁸ In this study, we used JNK as a substrate for DUSP14 since DUSP14 dephosphorylates active phospho-JNK both *in vitro* and *in vivo*. After incubation of recombinant phospho-

JNK with DUSP14 in the presence of various concentrations of NSC-95397, samples were examined by immunoblotting with phospho-JNK and JNK antibodies (Fig. 1). NSC-95397 prevents *in vitro* JNK dephosphorylation by DUSP14.

To investigate whether NSC-95397 negatively regulates DUSP14 phosphatase activity *in vivo*, HEK 293 cells were transiently transfected with FLAG-tagged DUSP14 WT or catalytically inactive mutant (D80A) expression plasmid. Transfected HEK 293 cells were pretreated with 0-100 μ M NSC-95397 for 3 h before cells were lysed with PTP lysis buffer. DUSP14 was immunoprecipitated from cell lysates using *anti*-FLAG M2-agarose. Phosphatase activity of immunoprecipitated DUSP14 was then determined using 3-*O*-methylfluorescein phosphate (OMFP) as a substrate as described previously.¹¹ As shown in Figure 2, NSC-95397 inhibits DUSP14 phosphatase activity *in vivo*. The results indicate that NSC-95397 effectively penetrates the cells and inhibits DUSP14 activity.

We further examined whether NSC-95397 inhibited DUSP14 activity against endogenous JNK in cells. HEK 293 cells were transfected with FLAG-tagged DUSP14 WT or catalytically inactive mutant (D80A) expression plasmid, pretreated with NSC-95397 for 3 h, and then stimulated with H₂O₂ to phosphorylate JNK. The levels of phospho-JNK were determined using immunoblotting analysis. As shown Figure 3(a), DUSP14 WT-mediated dephosphorylation of JNK was significantly inhibited by NSC-95397, whereas DUSP14 (D80A) mutant showed no effect on phosphorylated JNK levels. The results suggest that NSC-95397 effectively inhibits DUSP14 to protect endogenous phospho-JNK.

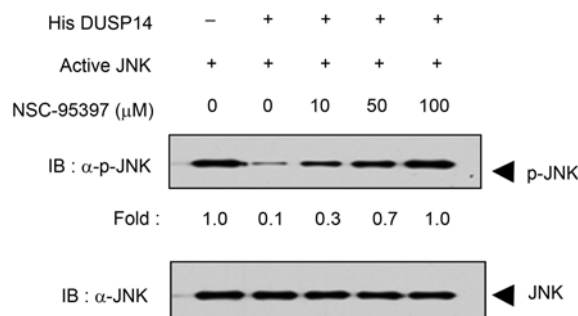


Figure 1. NSC-95397 down-regulates JNK inhibition mediated by DUSP14 *in vitro*. DUSP14 (1 μ g) was pre-mixed with NSC-95397 (0, 10, 50, or 100 μ M) and then incubated with active JNK. JNK phosphorylation level was determined by immunoblotting analysis.

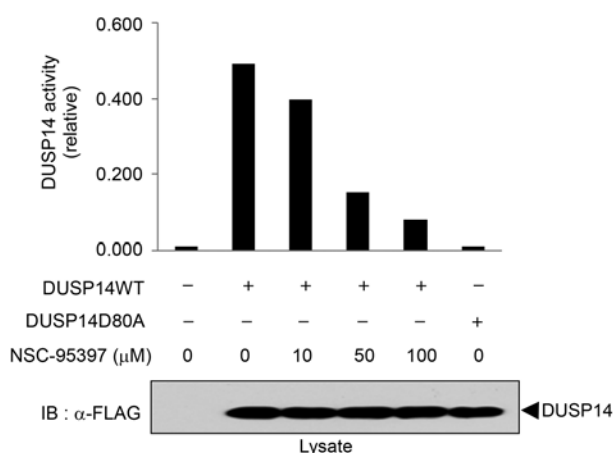


Figure 2. NSC-95397 inhibits the *in vivo* activity of DUSP14 in a dose-dependent manner. Transfected HEK 293 cells were pretreated with NSC-95397 (0, 10, 50, or 100 μM) and DUSP14 activity was determined by the immune complex DUSP14 assay as described in Experimental Section. The relative DUSP14 activity is shown.

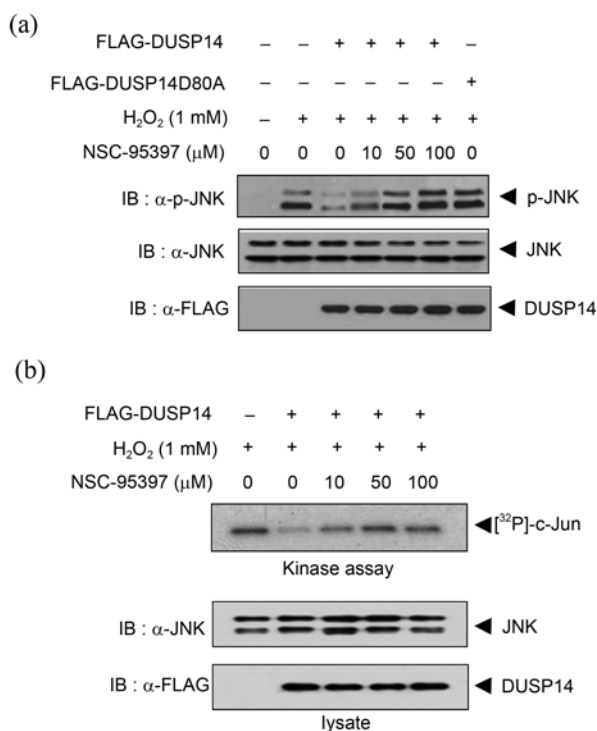


Figure 3. NSC-95397 inhibits the action of DUSP14 on JNK *in vivo*. (a) Transfected HEK 293 cells were pretreated with various concentrations of NSC-95397 (0, 10, 50, or 100 μM) for 3 h and then stimulated with H₂O₂ (1 mM, 1 h). Cell lysates were analyzed by immunoblotting with appropriate antibodies. (b) After transfection, HEK 293 cells were pretreated with various concentrations of NSC-95397 (0, 10, 50, or 100 μM) for 3 h and then stimulated with H₂O₂ (1 mM, 1 h). Immunoprecipitation and *in vitro* kinase assay were processed as in Experimental section.

We next investigated whether NSC-95397 potentiates the kinase activity of JNK by inhibiting DUSP14 *in vivo*. HEK 293 cells were transfected with FLAG-DUSP14 and then pretreated with NSC-95397 prior to stimulation with H₂O₂.

Cell lysates were immunoprecipitated with *anti*-JNK polyclonal antibody and then JNK kinase activities were determined by *in vitro* kinase assays using GST-c-Jun as a substrate (Fig. 3(b)). These results indicate that NSC-95397 protects JNK kinase activity by inhibiting DUSP14 *in vivo*.

In conclusion, this study suggests that NSC-95397 specifically inhibits dephosphorylation of JNK catalyzed by DUSP14. In CD28 signaling for induction of interleukin-2 (IL-2), DUSP14 expression is increased apparently in T cells stimulated by CD28.⁸ This enhanced DUSP14 decreases IL-2 production. In addition, IL-2 production is decreased by negative regulation of CD28 signaling through inactivation of JNK in T cells, suggesting that DUSP14 acts as a negative-feedback regulator in T cell proliferation. Thus, the results obtained in this study provide pharmacological evidence that NSC-95397 might be applied to the development of novel therapeutics for immune diseases related to DUSP14-mediated T-cell inactivation.

Experimental Section

Reagents and Antibodies. *Anti*-phospho-JNK (specific for phospho-Thr183 and phospho-Tyr185) and His antibodies were purchased from Cell Signaling Technology (Danvers, MA). *Anti*-JNK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Active JNK protein was from Upstate Biotechnology (Lake Placid, NY). NSC-95397 was from Sigma-Aldrich (St. Louis, MO). *Anti*-FLAG M2 antibody and *anti*-FLAG M2-agarose beads were from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and penicillin/streptomycin in the presence of 5% CO₂. For transient transfection, 1.4 × 10⁶ cells were plated in each 60-mm cell culture plate, grown overnight, and transfected with DNA using LipofectAMINE (Invitrogen).

Plasmid Constructions. FLAG-tagged DUSP14 wild-type and DUSP14 (D80A) mutant were constructed in pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA). His-tagged DUSP14 WT was constructed in pET-28a (+) plasmid (Novagen, Darmstadt, Germany) for protein expression in *E. coli*. GST-c-Jun (1-79) was constructed in pGEX 6p-1 plasmid (Amersham Biosciences, Little Chalfont, UK) for *in vitro* kinase assays.

Purification of the six-His-tagged Proteins. PTP expression plasmids were constructed in pET-28a (+) and transformed into BL21 (DE3)-RIL *E. coli*. Recombinant proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 20 °C for 16 h. Cells were harvested and then lysed by sonication in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were clarified at 13,000 rpm for 30 min at 4 °C. The supernatant was applied by gravity flow to a column of Ni-NTA resin

(PEPTRON, Daejeon, Korea). The resin was washed with 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 20 mM imidazole and then eluted with 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 250 mM imidazole. The eluted proteins were dialyzed overnight against 20 mM Tris-HCl, 150 mM NaCl, 20% glycerol, and 0.5 mM PMSF before storage at -80°C .

Dephosphorylation Assays with Active Phosphorylated JNK. The six-His-tagged DUSP14 (1 μg) was combined with active phosphorylated JNK (10 ng) in PTP assay buffer (30 mM Tris-HCl (pH 7.0), 75 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 0.33% BSA) and incubated for 30 min at 37°C in a 30 μL reaction volume. To determine whether NSC-95397 inhibits the DUSP14 effect on JNK *in vitro*, 100 nM of DUSP14 was mixed with 10 ng of active phosphorylated JNK and various concentrations of NSC-95397 (0, 10, 50, or 100 μM) in a 30 μL reaction volume and incubated for 30 min at 37°C . The products of dephosphorylation reactions were subjected to SDS-PAGE and then immunoblotted with an *anti*-phospho-JNK antibody.

***In vitro* Kinase Assays.** The six-His-tagged DUSP14 (1 μg) was pre-mixed with various concentrations of NSC-95397 (0, 10, 50, or 100 μM) in PTP assay buffer for 15 min at 37°C and then further incubated in the presence of active phosphorylated JNK (10 ng) for 15 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/0.3 μCi [γ - ^{32}P]ATP and 1 μg of GST-c-Jun as a substrate. After 30 min at room temperature, reactions were terminated by the addition of SDS-PAGE sample buffer and the products of the kinase reactions were separated by SDS-PAGE. The gels were dried and exposed to X-ray film.

Immune Complex Kinase Assays. For the immune complex kinase assays, HEK 293 cells were pretreated with

NSC-95397 (0-100 μM , 3 h) and then stimulated with H_2O_2 (1 mM, 1 h). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with an *anti*-JNK antibody. The immune complexes were then resuspended in kinase reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgCl_2 , 1 mM DTT) containing 20 μM ATP and 0.3 μCi of [γ - ^{32}P]ATP with 1 μg of GST-c-Jun for 30 min at room temperature. The products of the kinase reactions were separated by SDS-PAGE. The gels were dried and exposed to X-ray film.

Acknowledgments. The authors thank Jae Eun Park for technical assistance. 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).

References

1. Camps, M.; Nichols, A.; Arkinstall, S. *FASEB J.* **2000**, *14*, 6-16.
2. Dickinson, R. J.; Keyse, S. M. *J. Cell Sci.* **2006**, *119*, 4607-4615.
3. Keyse, S. M. *Curr. Opin. Cell Biol.* **2000**, *12*, 186-192.
4. Boutros, T.; Chevet, E.; Metrakos, P. *Pharmacol. Rev.* **2008**, *60*, 261-310.
5. Gille, H.; Sharrocks, A. D.; Shaw, P. E. *Nature* **1992**, *358*, 414-417.
6. Lewis, T. S.; Shapiro, P. S.; Ahn, N. G. *Adv. Cancer. Res.* **1998**, *74*, 49-139.
7. Tonks, N. K. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 833-846.
8. Marti, F.; Krause, A.; Post, N. H.; Lyddane, C.; Dupont, B.; Sadelain, M.; King, P. D. *J. Immunol.* **2001**, *166*, 197-206.
9. Lazo, J. S.; Nemoto, K.; Pestell, K. E.; Cooley, K.; Southwick, E. C.; Mitchell, D. A.; Furey, W.; Gussio, R.; Zaharevitz, D. W.; Joo, B.; Wipf, P. *Mol. Pharmacol.* **2002**, *61*, 720-728.
10. Seo, H.; Cho, S. *Bull. Korean Chem. Soc.* **2011**, *32*, 4435-4437.
11. Park, J. E.; Park, B. C.; Song, M.; Park, S. G.; Lee do, H.; Park, S. Y.; Kim, J. H.; Cho, S. *Biochem. Biophys. Res. Commun.* **2009**, *387*, 795-799.