

Regulation of Dual-Specificity Phosphatase 14 (DUSP14) by NSC-87877

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The cellular balance of protein tyrosine phosphorylation is controlled through the actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The human genome encodes around 100 PTPs.¹ PTPs are generally divided into two classes: protein serine/threonine phosphatase and tyrosine specific phosphatase. The dual-specificity protein phosphatases (DUSPs) are also a subclass of PTP families. They dephosphorylate both tyrosine and serine/threonine residues on the same protein. Members of DUSPs exhibit distinct substrate specificities, different tissue distribution and subcellular localization, and different modes of inducibility of their expressions by extracellular stimuli.^{2,3,4,5} Some of them, known as mitogen-activated protein kinase (MAPK) phosphatases (MKPs), have been found to dephosphorylate phospho-tyrosine and -threonine residues within the activation loop of MAPKs. Thus, MKPs function as negative regulators of MAPK-mediated signaling in diverse cellular processes.^{3,6} MAPKs are important signaling enzymes that regulate proliferation, cell survival and death, differentiation, development, immune function, gene expression, and other intracellular events. The four major subfamilies of MAPK in mammalian cells are p38, extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and extracellular signal regulated kinase-5 (ERK5).^{7,8}

Numerous studies indicate that PTPs are involved in most biological processes and modulation of their enzymatic activity is critical in regulating disease susceptibility.⁹

Therefore, chemical compounds that regulate the activities of PTPs may constitute a therapeutic approach for the treatment of diseases such as cancer, diabetes, and inflammation.

NSC-87877 was identified as a potent inhibitor of Src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1) and SHP-2 PTPs (Fig. 1).² Since there might be more phosphatases that can be targets of NSC-87877, we screened PTPs by performing *in vitro* phosphatase assays. Among several PTPs, DUSP14 was found to be inhibited by NSC-87877.

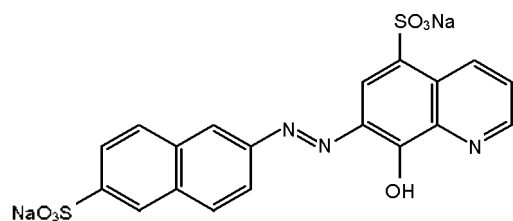


Figure 1. Chemical structure of NSC-87877

To confirm whether NSC-87877 was able to inhibit DUSP14, DUSP14 was treated with various concentrations of NSC-87877. DUSP14 activity was decreased by the inhibitor in a dose-dependent manner. The IC_{50} of DUSP14 was found to be about $4.5 \pm 2.01 \mu\text{M}$ (Fig. 2A). Molecular modeling and site-directed mutagenesis studies suggested that NSC-87877 binds to the catalytic cleft of SHP-2.² Kinetic studies were performed with NSC-87877 and DUSP14 to examine the mechanism of inhibition. Analysis of the mode of inhibition indicated a competitive inhibition with K_i of $4.14 \mu\text{M}$ (Fig. 2B).

DUSP14 dephosphorylates ERK, JNK, and p38, suggesting that DUSP14 behave as a general MAPK phosphatase.¹⁰ To examine that NSC-87877 could function as a DUSP14

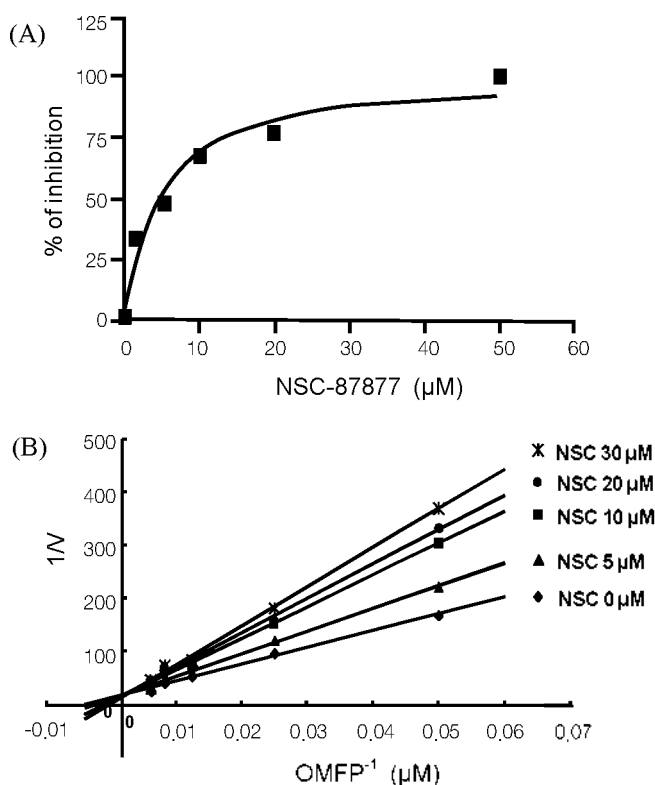


Figure 2. Inhibitory effect of NSC-87877 in DUSP14 and kinetic analysis of DUSP14 inhibition by NSC-87877. (A) DUSP14 was incubated with various concentrations of NSC-87877 at 37°C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental section. (B) Lineweaver-Burk plot of DUSP14 was generated from the reciprocal data.

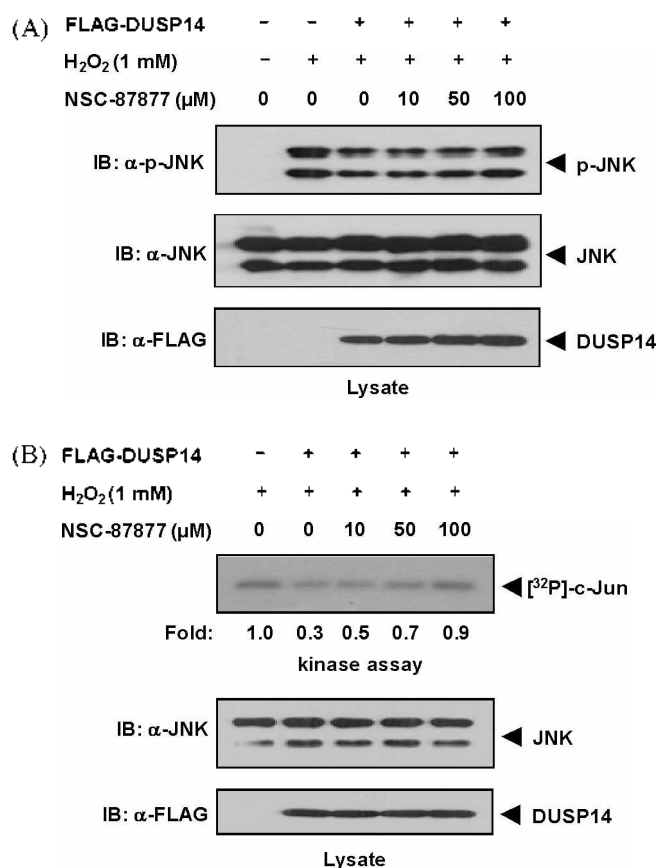


Figure 3. NSC-87877 inhibits the action of DUSP14 on JNK *in vivo*. (A) Transfected HEK 293 cells were pre-treated with various concentrations of NSC-87877 (0 - 100 μM) for 3 h and then stimulated with H₂O₂ (1 mM, 30 min). Cell lysates were analyzed by Western blotting with appropriate antibodies as described. (B) After transfection, HEK 293 cells were pretreated with various concentrations of NSC-87877 (0 - 100 μM) for 3 h and then stimulated with H₂O₂ (1 mM, 30 min). Immunoprecipitation and Western blotting were processed as described in Experimental section. The immune complex kinase assay was carried out as described under Experimental section. The relative JNK kinase activity is shown.

inhibitor and thus activates JNK in cells, we measured the *in vivo* phosphorylation level of JNK. HEK 293 cells were transfected with FLAG-DUSP14 expression plasmid, pre-incubated with or without NSC-87877, and then treated with H₂O₂ to phosphorylate JNK. The phosphorylation level of the endogenous JNK determined with Western blotting analysis. As shown in Fig. 3A, DUSP14 down-regulated phosphorylation level of the endogenous JNK but the treatment of NSC-87877 recovered JNK phosphorylation level. We next carried out immune complex kinase assays to further confirm the regulatory effect of NSC-87877 on JNK activity *in vivo*. HEK 293 cells were transfected with FLAG-DUSP14 and pre-treated with or without NSC-87877, and then stimulated with H₂O₂. NSC-87877 activates JNK kinase activity by inhibiting DUSP14 *in vivo* (Fig. 3B). These results suggest that NSC-87877 recovers DUSP14-suppressed JNK activity by inhibiting DUSP14.

In the present study, we found that NSC-87877 inhibits activity of DUSP14 in a dose-dependent manner and is a

potent competitive-inhibitor of DUSP14. The phosphorylation level of the endogenous JNK was decreased by DUSP14, and treatment with NSC-87877 inhibited DUSP14-mediated dephosphorylation of JNK. Immune complex kinase assays suggest that NSC-87877 activates JNK through inhibition of DUSP14. The inhibitory effect of NSC-87877 on DUSP14 is about ten times lower than that on SHP2, which might be due to structural difference in catalytic clefts of PTPs. However, the inhibitory activity of NSC-87877 on DUSP14 is effective enough to be considered for further study, when compared with other phosphatase inhibitors. Since DUSP14 functions as a negative regulator of CD28 signaling through regulation of MAPKs in T cells,¹⁰ this study provides novel insights into potential therapeutic application of NSC-87877 and development of new inhibitors derived from NSC-87877 for DUSP14-related T cell regulation.

Experimental Section

Antibodies. Anti phospho-JNK (specific for phospho-Thr183 and phospho-Tyr185) was purchased from Cell Signaling Technology (Danvers, MA). Anti-JNK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

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) 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).

Purification of 6 x His-tagged proteins. Purification of recombinant proteins was carried out as previously described.¹¹

***In vitro* Phosphatase assays and Kinetic analysis.** The activity of phosphatases was measured using the substrate 3-O-Methylfluorescein Phosphate (OMFP; Sigma, St. Louis, MO) at concentrations varying with the *K_m* of each enzyme in a 96-well microtiter plate assay based on methods described previously.¹² The NSC-87877 (Calbiochem, San Diego, CA) and OMFP were solubilized in H₂O and DMSO, respectively. All reactions were performed at a 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), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT), 0.33% bovine serum albumin (BSA) and 100 nM of PTPs. Reactions were initiated by addition of OMFP and the incubation time was 30 min at 37 °C. Fluorescence emission from product was measured with a multi-well plate reader (GENios Pro; excitation filter, 485 nm; emission filter, 535 nm). The reaction was linear over the time period of the experiment and was directly proportional to both enzyme and substrate concentration. Half-maximal inhibition constant (IC₅₀) was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activity. Half-maximal inhibition constants and best curve fit for Lineweaver-Burk plots were determined by using the curve fitting program Prism 3.0 (GraphPad Software, San Diego, CA). All experiments were performed in triplicate and repeated at least

three times.

Western blotting analysis. Samples were run in SDS-10% polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was blocked in 5% nonfat skim milk and incubated with an appropriate antibody, followed by incubation with a secondary antibody conjugated to horseradish peroxidase. The immunoreactive bands were visualized using an ECL system (Pierce, Rockford, IL).

Immune complex kinase assays. For immune complex kinase assays, HEK 293 cells were co-transfected with HA-JNK and FLAG-DUSP14 expression plasmids. After 48 h of transfection, cells were pretreated with NSC-87877 (0-100 μ M, 3 h) and then stimulated with H₂O₂ (1 mM, 30 min). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with anti-HA agarose beads. The beads were washed once with the PTP lysis buffer, twice with a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM DTT, and 1 mM PMSF, and then once with a solution containing 20 mM Tris-HCl (pH 7.5) and 20 mM MgCl₂. The beads were then resuspended in kinase reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, and 1 mM DTT) containing 20 μ M ATP and 0.3 μ Ci of [γ -³²P]ATP with 1 μ g of GST-c-Jun for 30 min at 30 °C. The products of kinase reactions were separated by SDS-PAGE. The gels were dried and exposed to film.

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