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Specific Inhibition of DUSP14 by NSC-95397 *in vitro*

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The regulation of cellular balance by protein phosphorylation plays a critical role in a variety of biological processes, including gene expression, proliferation, differentiation, cell cycle arrest, and apoptosis.[1] Among them, phosphorylation and dephosphorylation are strong tools for manipulating the stabilization and activity of proteins. Protein phosphorylation is regulated by protein kinases and protein phosphatases through phosphorylation or dephosphorylation of serine, threonine, or tyrosine residues of a protein.[2] The addition of phosphate group to an enzyme can activate or halt the enzymatic activity. Also, phosphorylated protein can gain the functional capability to interact with other proteins.[3] The human genome contains 107 protein tyrosine phosphatase (PTP) genes.[4] Dual-specificity phosphatases (DUSPs) as a heterogeneous group of protein phosphatases belong to a subclass of PTP families and dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues.[1] In particular, mitogen-activated protein kinase phosphatases (MKPs) have function as negative regulators of mitogen-activated protein kinase (MAPK)-mediated signaling in many cellular processes.[5,6]

Based on previous studies, the modulation of PTP enzymatic activity is involved in regulating diverse cellular biological functions and disease susceptibility.[7] Thus, chemical compounds that regulate the activity of PTPs may have ability of being therapeutic reagents for the treatment of diseases such cancer, inflammation, cancer, and diabetes.

NSC-95397 (2,3-bis-[2-hydroxyethylsulfanyl]-[1,4]naphthoquinone) is reported to inhibit Cdc25 family of DUSPs, including Cdc25A, -B, and -C and VH1-related dual-specificity phosphatase or protein tyrosine phosphatase 1B (Fig. 1).[8] Since there might be more phosphatases that are regulated by NSC-95397, we performed *in vitro* phosphatase assays with 17 PTPs to identify NSC-95397 targets. Of them, DUSP14 was the only phosphatase that is inhibited by NSC-95397 (Table 1). The inhibitory concentration 50 (IC_{50}) values for other PTPs were much higher than for DUSP14, suggesting that NSC-95397 selectively inhibits DUSP14 over other PTPs.

When DUSP14 was treated with various concentrations of NSC-95397, the inhibitory activity of NSC-95397 on DUSP14 was dose-dependent. An inhibition curve was plotted for DUSP14 with various concentrations of NSC-95397 and IC_{50} value was calculated using the curve fitting program PRISM 3.0 (Fig. 2). The results suggest that NSC-95397 inhibits DUSP14 with the IC_{50} of 7.13 ± 0.12 μM.

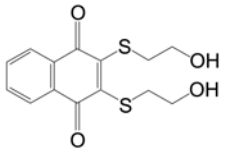

Figure 1. Chemical structure of NSC-95397.

Table 1. Selective inhibition of PTPs by NSC-95397 *in vitro*

DUSP3	> 100
DUSP6	> 200
DUSP13A	> 200
DUSP13B	> 200
DUSP18	> 200
DUSP22	> 200
DUSP23	> 200
DUSP26	> 200
PTPN2	> 200
PTPN6	> 200
EPM2A	> 200
SSH3	> 200
PTP4A	> 200
PTPRO	> 200
PTPN7	> 200
ACP1	> 50
DUSP14	7.13 ± 0.12

Each experiment was performed in triplicate. *In vitro* PTP activity assay was processed as in Experimental Section. Data are presented as means ± SEM.

We next examined the kinetics of inhibition against DUSP14. In subsequent experiments, kinetic analyses based on the Michaelis-Menten equation were performed with NSC-95397 and DUSP14 to provide experimental evidence for the mechanism of DUSP14 catalysis and for binding of the inhibitor to the active site of the phosphatase. It has been reported that NSC-95397 acts as a partial mixed inhibitor of Cdc25 isoforms.[8] We tested if NSC-95397 inhibits DUSP14 in the same way that the inhibitor acts on Cdc25 proteins. Lineweaver-Burk plots show that the K_m value of DUSP14 for small molecule substrate *O*-methyl fluorescein phosphate (OMFP) was 31.25 ± 2.64 μM and the K_i was 200 nM (Fig. 3). The *in vitro* K_i for DUSP14 is slightly higher than the values for Cdc25 proteins.[8] Unlike Cdc25 proteins, however, DUSP14 showed best fit with a competitive inhibition kinetic model against NSC 95397, suggesting that NSC-

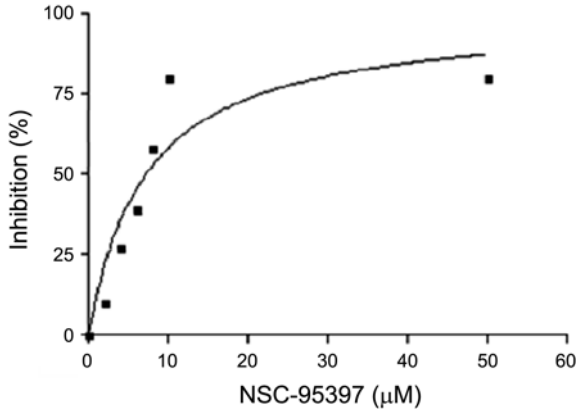

Figure 2. Inhibitory effect of NSC-95397 on DUSP14. DUSP14 was incubated with various concentrations of NSC-95397 for 30 min at 37 °C. Fluorescence emission from the product was measured with a multi-well plate reader as described in Experimental section.

95397 down-regulates the catalytic activity of DUSP14 by binding to the catalytic site. This result suggests that NSC-95397 binds to the catalytic cleft of DUSP14 in a different manner from Cdc25 isoforms (Fig. 3).

In conclusion, we found that NSC-95397 inhibits activity of DUSP14 *in vitro* in a dose-dependent manner and is a potent competitive-inhibitor of DUSP14. In addition, NSC-95397 inhibits DUSP14 with high specificity since other PTPs tested in this study were not inhibited by NSC-95397. DUSP14 was first identified through a yeast two-hybrid system to identify novel proteins that interact with the T-cell costimulatory factor CD28. In T cells, CD28 signaling is known to be related to induction of interleukin-2 (IL-2), and moreover CD28-mediated DUSP14 induction protects IL-2 production. In addition, DUSP14 has been suggested to act as a negative regulator of CD28 signaling through inactivation of MAPKs in T cells.[9] Taken together, our results suggest that NSC-95397 might regulate important cell signaling pathways through regulation of DUSP14 substrates such as MAPKs. Therefore, our results obtained in this work reveal that NSC-95397 might be a potential drug target for some diseases which are caused by DUSP14-mediated T-cell inactivation.

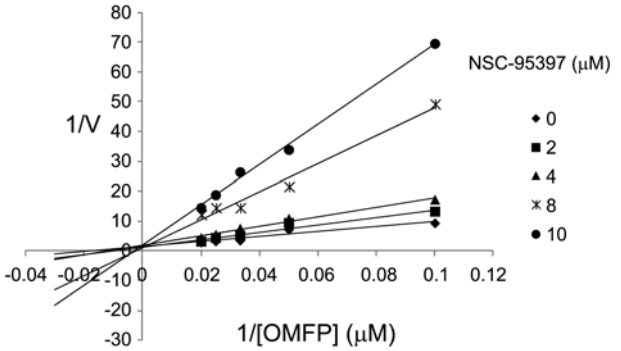

Figure 3. Kinetic analysis of DUSP14 inhibition by NSC-95397. Lineweaver-Burk plots of DUSP14 were generated from the reciprocal data.

Experimental Section

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 phenylmethylsulphonyl 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, Daejon, 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.

***In vitro* Phosphatase Assays and Kinetic Analysis.** Phosphatase activities were measured using the substrate 3-*O*-methylfluorescein phosphate (OMFP; Sigma, St. Louis, MO) at concentrations that varied according to the K_m of each enzyme in a 96-well microtiter plate assay based on methods described previously.[10] NSC-95397 and OMFP were solubilized in DMSO. All reactions were performed at a final concentration of 1% DMSO. The final incubation mixture (150 μL) was optimized for enzyme activity and was composed of 30 mM Tris–HCl (pH 7.0), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT), 0.33% bovine serum albumin (BSA) and 100 nM of each PTP. Reactions were initiated by addition of OMFP and incubated for 30 min at 37 °C. Fluorescence emission from the product was measured with a multiwall plate reader (Synergy H1; excitation filter, 485 nm; emission filter, 535 nm). The reaction was linear over the experimental time period and was directly proportional to both enzyme and substrate concentration. The half-maximal inhibition constant (IC_{50}) was defined as the concentration of 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). All experiments were performed in triplicate and were repeated at least three times.

Inhibition Study. The inhibition constant (K_i) to DUSP14 phosphatase for the inhibitor was determined by measuring the initial rates at several OMFP concentrations for each fixed concentration of the inhibitor. The data were fitted to the following equation to obtain the inhibition constant of reversible competitive inhibitors. The slopes obtained were replotted against the inhibitor concentrations. The K_i value was obtained from the slopes of these replots.[11]

$$1/V = K_m (1 + [I]/K_i) V_{max} [S] + 1/V_{max}$$

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