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NSC 663284 Inhibits SHP-2

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Diverse cellular mechanisms by extracellular stimuli in mammalian cells are mainly regulated by protein phosphorylation and dephosphorylation.[1] These cellular events are coordinated by protein phosphatases and protein kinases and thus mutations in those enzymes bring about various kinds of disorders resulting from abnormal phosphorylation.[2] Therefore, the finding and developing of the specific inhibitors that target protein phosphatase or kinases are essential parts for the treatment of cancer and chronic inflammatory disease. There are over 100 protein tyrosine phosphatase (PTP) superfamily genes in human genome, compared to 90 protein tyrosine kinase (PTK) genes.[3]

Mutations of the Src homology 2 (SH2) domain-containing phosphatase 2 (SHP-2) that is encoded by the *PTPN11* gene induce variety of human diseases such as Noonan syndrome (NS), Leopard syndrome, childhood hematologic malignancies and leukemias.[4-6] However, the biological mechanism of SHP-2 is not fully understood. SHP-2 has 2 N-terminal Src homolog 2 (SH2) domains, a catalytic protein tyrosine phosphatase region, and C-terminal tails.[7] The N-terminal SH2 domain regulates the enzyme activity of SHP-2. Under basal conditions, SHP-2 activity is self-inhibited resulting from the N-terminal SH2 domain interaction with the PTP domain.[8] On the other hand, SHP-2 binding partners including receptors and scaffolding adapters rescue SHP-2 from self-inhibition by binding to the N-terminal SH2 domain and releasing it from the PTP domain.[9] SHP-2 is the first identified proto-oncogene in the PTP subfamily and many gain of function mutations in SHP-2 involved in leukemia and solid tumors have been studied.[10] Therefore, it is worthwhile to discover SHP-2 targeting inhibitors and to understand its biological mechanisms for the treatment of related diseases.

NSC 663284 (6-chloro-7-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione) is known as a potent cell permeable Cdc25 phosphatase inhibitor II (Fig. 1).[11] Cdc25 dual specificity phosphatase plays a significant role in cell proliferation by regulating $G_1$and G_2/M phase. NSC 663284 arrests cell cycle-progression and acts as an anti-proliferation reagent by inhibiting Cdc25.[12] NSC 663284 inhibition kinetics with Cdc25 phosphatases reflect partial mixed competitive inhibition model, suggesting that binding of NSC 663284 affects the enzyme's binding affinity for the substrate.[11]

We examined whether other protein tyrosine phosphatases (PTPs) could be inhibited by NSC 663284 since there might

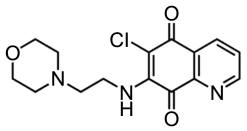

Figure 1. Chemical structure of NSC 663284. 6-chloro-7-(2-morpholin-4-ylethylamino)-quinoline-5,8-dione.

be more phosphatases that can be targets of NSC 663284. Our previous report showed that DUSP13B activity was inhibited by NSC 663284 *in vitro* and also down-regulated effectively in the presence of NSC 663284 in human embryonic kidney (HEK) 293 cells.[13] Several PTPs were tested by *in vitro* phosphatase assays. To measure inhibitory effect of NSC 663284 on PTPs, His-tagged recombinant PTPs purified from bacterial cells were incubated with 3-*O*-methylfluorescein phosphate (OMFP) as a substrate with or without 10 μM of NSC 66328 (Table 1). Of several PTPs, SHP-2 was conspicuously inhibited by NSC 663284. After SHP-2 activities were measured with various concentrations of NSC 663284, an inhibition curve was plotted and the half inhibitor concentration (IC_{50}) value was calculated. As shown in Figure 2(a), SHP-2 was inhibited with IC_{50} value of 0.32 ± 0.06 μM.

Next, kinetic studies with SHP-2 and NSC 663284 were

Table 1. Inhibition of PTPs by NSC 663284

PTPs	IC_{50} (μM)
ACP1	No Inhibition
DUSP3	No Inhibition
DUSP6	No Inhibition
DUSP18	No Inhibition
DUSP22	No Inhibition
DUSP26	No Inhibition
SSH3	No Inhibition
PTPN6	No Inhibition
DUSP13B	3.84 ± 0.86
SHP-2	0.32 ± 0.06

Inhibition of recombinant PTPs including ACP1, DUSP3, DUSP6, DUSP18, DUSP22, DUSP26, SSH3, PTPN6, DUSP13B, SHP-2 were measured by using *in vitro* phosphatase assay as described in Experimental Section. PTPs were incubated with 0 or 10 μM of NSC 663284 at 37 °C for 30 min. Then fluorescence emission from product was measured with a multi-well plate reader (GENios Pro; excitation filter, 485 nm; emission filter, 535 nm). DUSP13B was inhibited with IC_{50} value of 3.84 ± 0.86 μM.[13] In comparison, inhibitory effects of NSC 663284 on SHP-2 activity appeared to be about 10 times higher than DUSP13B.

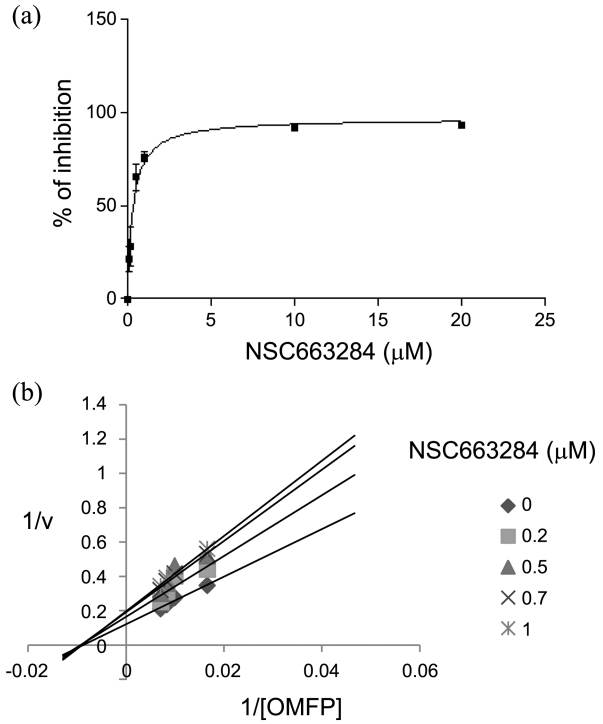

Figure 2. Inhibitory effect of NSC 663284 on SHP-2. (a) SHP-2 (100 nM) was incubated with various concentration of NSC 663284 and OMFP (100 μM) as substrates at 37 °C for 30 min. Fluorescence emission was measured (an excitation of 485 nm and emission of 535 nm) as described in Experimental section. (b) Kinetics analysis was performed based on the theory of Lineweaver-Burk plots and the K_i value was calculated. Lineweaver-Burk plots of SHP-2 were generated from the reciprocal data.

carried out based on the Michaelis-Menten equation. The K_m value of SHP-2 for OMFP was 102 μM. Lineweaver-Burk analysis of NSC 663284 showed that the K_i value is 1.05 ± 0.44 μM (Figure 2(b)). The results demonstrate that NSC 663284 acts as a non-competitive inhibitor of SHP-2, suggesting that NSC 663284 blocks SHP-2 enzymatic activity through binding to an allosteric site of enzyme and undergoing a conformation change. It is worth of noting that the kinetic action of NSC 663284 on SHP-2 is different from that on Cdc25 proteins.

In the following study, we investigated whether NSC 663284 regulates SHP-2 protein expressed in HEK 293 cells. HEK 293 cells were transfected with FLAG-tagged SHP-2 (residues 205-593) expression plasmids containing catalytic domain (residues 220-525) with deletion of the N-terminal SH2 domain. Cells were lysed and overexpressed FLAG-tagged SHP-2 protein was pulled down using anti FLAG M2-agarose. Then, the immunoprecipitated SHP-2 was incubated with various concentration of NSC 663284 inhibitor (0, 1, 5, 10 μM) and with OMFP as substrates at 37 °C for 30 min. As shown in Figure 3(a), enzymatic activity of pulled down SHP-2 was decreased drastically in a dose-dependent manner. Even, at the concentration of 5 μM inhibitor treatment, SHP-2 activity is reduced over 90% by NSC 663284.

Since SHP-2 is required for the activation of ERK signaling pathway, phosphorylation levels of ERK were measured to determine whether NSC 663284 blocks SHP-2-mediated ERK activation. After HEK 293 cells were transfected with FLAG tagged SHP-2 (residues 205-593) expression plasmids, cells were treated with NSC 663284 in a dose dependent manner (0, 10, 50 μM) for 3 h. The ERK phosphorylation

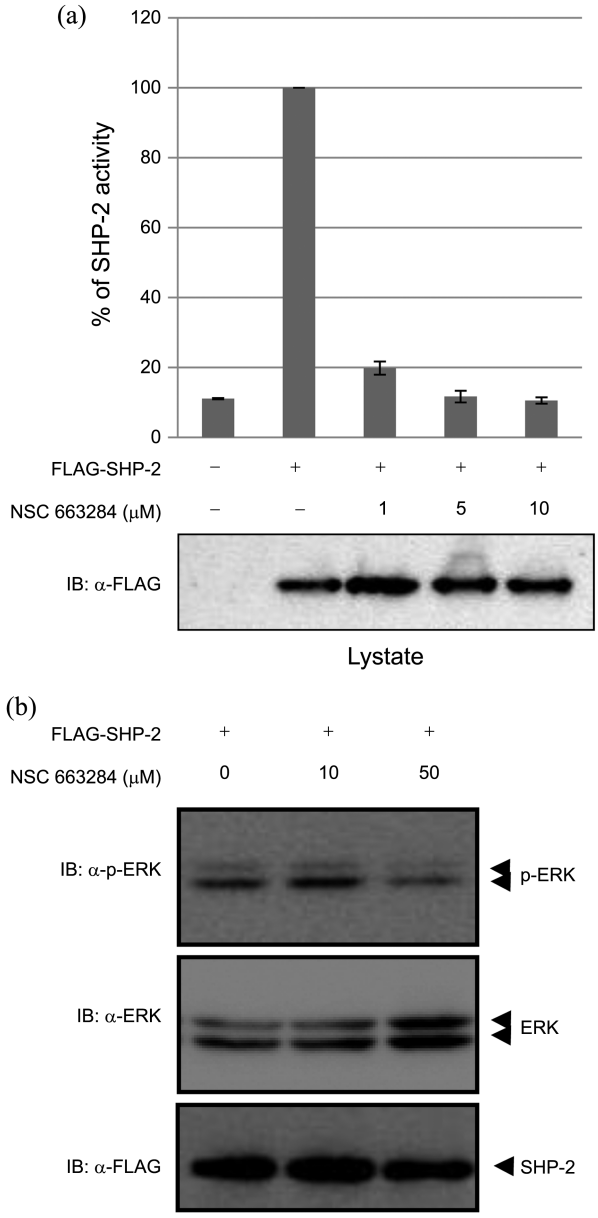

Figure 3. Effects of NSC 663284 on SHP-2 purified from HEK 293 cells and on SHP-2-regulated Phospho-ERK. (a) After transfection of SHP-2 expression plasmid for 48 h in HEK 293 cells, cell lysates were harvested and subjected to immunoprecipitation with anti-FLAG M2 agarose. Immunoprecipitated SHP-2 were incubated with various concentration of NSC 663284 (0, 1, 5, 10 μM) at 37 °C for 30 min. Fluorescence emission from product was measured with a multi-well plate reader. (b) After transfection of SHP-2 expression plasmid for 48 h in HEK 293 cells, cells were treated with various concentration of NSC 663284 (0, 10, 50 μM) for 3 h. Cell lysates were subjected to SDS-PAGE. Then the phosphorylation levels of ERK were monitored by immunoblotting with appropriate antibodies.

levels were detected by immunoblotting analysis. As shown in Figure 3(b), the phosphorylation levels of ERK were reduced with the increase of NSC 663284 concentration. The results suggest that NSC 663284 inhibits SHP-2 activity and thus SHP-2-mediated ERK activation in HEK 293.

In this study, we observed that NSC 663284 acts as an effective SHP-2 inhibitor. As PTPs have emerged as drug targets for many human diseases, discovering and developing of those kinds of PTP inhibitors are significantly important tasks. Especially, SHP-2 selective inhibitors could be promising anti-disease agents to treat diseases such as Noonan syndrome (NS) and sporadic leukemias.[14]

Experimental Section

Cell Culture. Human embryonic kidney (HEK) 293 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific) and penicillin/streptomycin (Life Technologies Corporation, Carlsbad, CA) in the presence of 5% CO_2.

Transfection and Plasmid Constructs. HEK 293 cells were transfected by polyethylenimine (Polysciences, Inc., Warrington, PA) with 1.5 μg of FLAG-tagged SHP-2 or empty vector (Mock). FLAG-tagged SHP-2 (residues 205-593) was constructed in pcDNA3.1 (Invitrogen, Carlsbad, CA). Bacterial expression His-tagged SHP-2 was constructed in pET28a (Novagen, Darmstadt, Germany).

Recombinant PTP Proteins. PTP expression plasmids constructed in pET-28a were transformed into BL21 (DE3)-RIL *E. coli*. Expression of recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 3 h or 30 °C for 6 h or 22 °C for overnight. Cells were harvested and then lysed by sonication in *E. coli* lysis buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1% Tergitol-type NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF)). After centrifuging at 10,000 rpm for 30 min at 4 °C, the supernatant was applied by gravity flow to a column of Ni-NTA resin (PEPTRON). The resin was washed twice with washing buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 50 mM imidazole) and eluted with elution buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 200-300 mM imidazole). The recombinant proteins were dialyzed with dialysis buffer overnight (20 mM Tris-HCl (pH 8.0), 75 mM NaCl, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 50% glycerol) before storage at −80 °C.

***In vitro* Phosphatase Assays and Kinetic Analysis.** The activity of phosphatases was measured using the substrate 3-Omethylfluorescein phosphate (OMFP; Sigma-Aldrich) in a 96-well microtiter plate assay based on methods described periviously.[15] NSC 663284 and OMFP were solubilized in DMSO and H_2O. All reactions were performed at a final concentration of 1% DMSO. The final incubation mixture (100 μL) were optimized for enzyme activity and composed of 30 mM Tris-HCl (pH 7), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.33% bovine serum albumin (BSA), 100 nM of PTP. Reactions were initiated by addition of OMFP and incubated for 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). Half-maximal inhibition constant (IC_{50}) 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). The inhibition constant (K_i) to SHP-2 for NSC 663284 was calculated using the equations from the Lineweaver-Burk plots by measuring the initial rates at several OMFP concentrations for each fixed concentration of the inhibitor. The slopes showed the noncompetitive inhibition pattern and the K_i value was obtained from the below equations of noncompetitive inhibition. All experiments were performed in triplicate and were repeated at least three times.

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

Effects of NSC 663284 on SHP-2 Purified from HEK 293 Cells. HEK 293 cells were transfected with 1.5 μg of FLAG-SHP-2 phosphatase expression plasmid. After 48 h of transfection, cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in PTP lysis buffer containing 0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% glycerol, 1 mM PMSF for 30 min at 4 °C. Cleared cell lysates were mixed with FLAG M2-agarose (Sigma-Aldrich, St.Louis, MO) and incubated for 1h at 4 °C using rotation device. FLAG M2-agarose was washed three times with PTP lysis buffer. Then, the phosphatase activities of the immunoprecipitated SHP-2 with various concentrations of NSC 663284 were measured as described above.

***In vivo* Effect of NSC 663284 on SHP-2 Phosphatase-regulated Phospho-ERK.** After HEK 293 cells were transiently transfected with or without FLAG tagged SHP-2 (residues 205-593) for 48 h, cells were treated with NSC 663284 (0, 10, 50 μM) for 3 h. Cells were washed twice with phosphate buffered saline (PBS) buffer and lysed on ice in lysis buffer containing 150 mM NaCl, 20 mM Tris (pH 8.0) 0.5% NP-40, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF. Samples were boiled at 100 °C for 5 min and run in SDS-10% polyacrylamide gels and transferred onto nitrocellulose membrane. Then the membranes were blocked with 5% nonfat skim milk for 1 h and incubated with an appropriate antibody with 5% BSA, followed by incubation with a HRP-conjugated secondary antibody with 5% skim milk. The immunoreactive bands were visualized by the ECL detection system (Pierce, Rockford, IL).

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