Inhibition of Acid Phosphatase 1 (ACP1) Activity by NSC-87877

Mina Song, Sung Jin Park, and Sayeon Cho*

College of Pharmacy and Research Institute for Translational System Biomics, Chung-Ang University, Seoul 156-756, Korea
**E-mail: sycho@cau.ac.kr*
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Protein tyrosine phosphorylation plays a crucial role in the regulation of diverse processes such as cellular metabolism, proliferation, differentiation, growth, migration, and invasion of normal and malignant cells.[1,2] The protein phosphorylation level is regulated by the balance between activities of protein kinases and phosphatases. The protein tyrosine phosphatase (PTP) superfamily comprises almost 100 enzymes. Based on the amino acid sequences of their catalytic domains, the PTPs can be grouped into four main families: (i) classical PTPs and dual-specificity protein tyrosine phosphatases (DUSPs), (ii) tyrosine-specific low molecular weight phosphatases, (iii) the cdc25 family, and (iv) Eyes absent (EyA) protein that was recently discovered to be tyrosine-, or dual serine- and tyrosine-specific protein phosphatases.[3]

Growing evidence indicates that modulation of their enzymatic activity could have a role in regulating a large spectrum of cellular functions and disease susceptibility.[4] Therefore, chemical compounds that regulate the activity of PTPs can be of value as potent therapeutic reagents.

ACP1 is a polymorphic enzyme member of the cytosolic low molecular weight protein tyrosine phosphatases that are ubiquitously expressed in various tissues.[1,5] Its crystal structure has been reported.[6] It is a 18-kDa enzyme that is composed of two active isoforms (IF1 and IF2); these are derived by alternative splicing of a single transcript.[7,8] IF1 and IF2 differ in substrate specificity and in their sensitivity, *in vitro*, to some modulators. ACP1 interacts with several receptor tyrosine kinases and docking proteins, including platelet-derived growth factor receptor (PDFG-R),[9] ephrin A2 receptor (Eph2A),[10] and β-catenin.[11] ACP1 is involved in control of mitogenic and adhesive signals[9,12] and considered as a negative regulator of growth factor-induced cell proliferation (antioncogene), although in some instances it also functions as a positive signal (oncogene).[1,13,14]

8-Hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid (NSC-87877) was originally identified as a potent inhibitor of Src homology region 2 (SH2) domain-containing phosphatase 1 (SHP-1) and SHP-2 PTPs (Fig. 1).[15] They are characterized by containing two SH2 N-terminal domains and a C-terminal protein tyrosine phosphatase domain. In particular, SHP-2 is involved in the activation of ERK1/2 by epidermal growth factor (EGF).[16] SHP-2 is involved in the function of several growth factors and metabolic pathways, suggesting its role in disease pathways such as leukemia, diabetes, neurodegeneration, and cancer.[17,18] Since there might be more phosphatases that can be targets of NSC-87877, we screened 15 PTPs by *in vitro* phosphatase assays to identify PTPs that are inhibited by NSC-87877. An inhibition curve was plotted for each PTP and the inhibitory concentration 50 (IC_{50}) values were calculated. As shown in Table 1, ACP1 was inhibited with IC_{50} value of 9.3 μM. The IC_{50} values for other PTPs were higher than 100 μM, suggesting that NSC-87877 has low inhibitory effect on these phosphatases.

We compared the inhibitory effects of NSC-87877 on SHP-1 and ACP1. When both PTPs were treated with various concentrations of NSC-87877, phosphatase activities were decreased by the inhibitor in a dose-dependent manner (Fig. 2A and B). Molecular modeling and site-directed mutagenesis studies suggested that NSC-87877 binds to the catalytic cleft of SHP-2.[15] Kinetic studies with NSC-87877 and ACP1 revealed a competitive inhibition, suggesting that NSC-87877 binds to the catalytic cleft of ACP1 in the same manner as SHP-2 (Fig. 3). The K_m value of ACP1 for OMFP was 60 μM, and the K_i was 34.9 μM.

We next examined whether the inhibitory action of NSC-87877 on ACP1 influences the phosphorylation level of mitogen-activated protein kinases (MAPKs). We performed *in vitro* dephosphorylation assays with the active MAPKs that were phosphorylated at the activation loop. Active recombi-

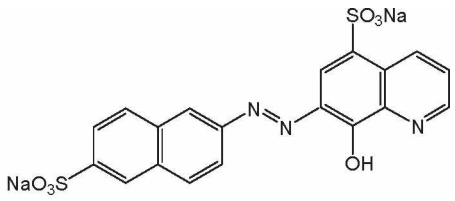

Figure 1. Chemical structure of NSC-87877

Table 1. Inhibition of PTPs by NSC-87877. Inhibitory IC_{50} of enzyme activity was determined for the various PTPs including PTPN2, SSH3, DUSP22, SHP-1, and ACP1. Data are presented as mean ± S.E.M. IC_{50} of full-length SHP-1 determined in this experiment is about 100 times higher than that of truncated SHP-1 (residues 205-597) previously reported,[15] which might be due to differences of substrates, forms of SHP-1, and buffer components used in the reactions.

Protein tyrosine phosphatase	IC_{50} (μM)
PTPN2	> 200 (n=3)
SSH3	> 200 (n=3)
DUSP22	> 100 (n=3)
SHP-1	40 ± 17.32 (n=3)
ACP1	9.3 ± 2.8 (n=3)

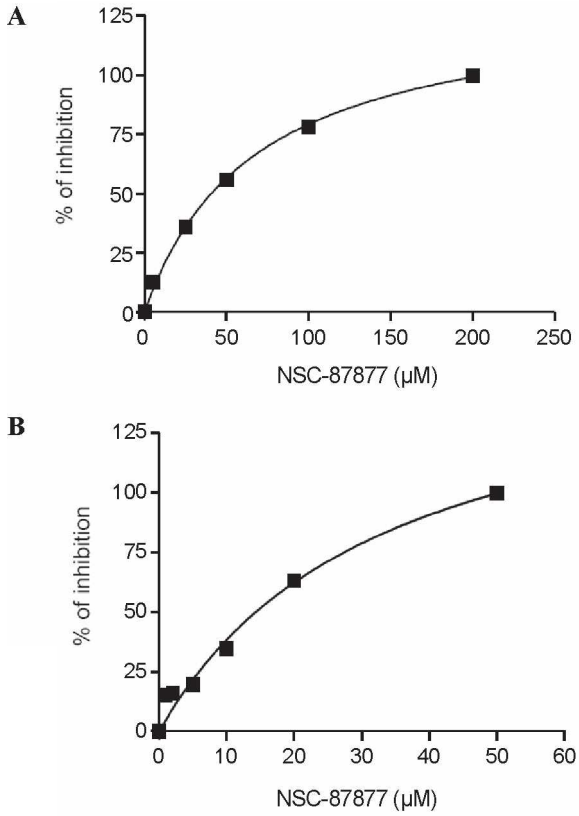

Figure 2. Inhibitory effect of NSC-87877 in ACP1 (A and B) SHP-1 (100 nM) and ACP1 (180 nM) were incubated with various concentrations of NSC-87877 at 37 °C for 30 min, respectively. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental section.

nant MAPKs were incubated *in vitro* with the recombinant ACP1 that was purified from bacteria. Then, the levels of phosphorylated MAPKs were determined with Western blotting analysis using anti-phospho-MAPK antibodies. As shown in Fig. 4, ACP1 failed to dephosphorylate recombinant p38, ERK, and JNK. These data indicate that MAPKs are not substrates of ACP1.

In the present study, we screened PTPs to search for targets of NSC-87877 by performing *in vitro* PTP activity assays. We

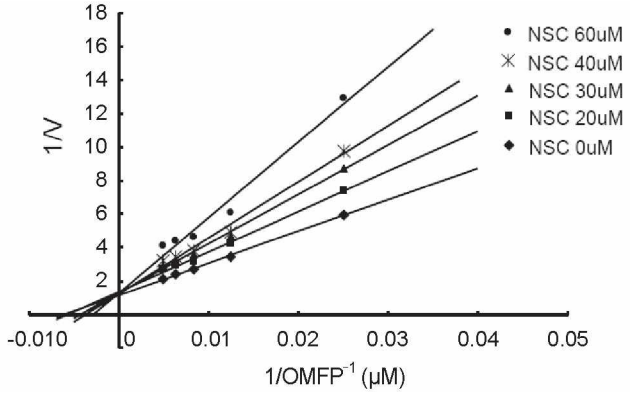

Figure 3. Kinetic analysis of ACP1 inhibition by NSC-87877. Lineweaver-Burk plots of ACP1 were generated from the reciprocal data.

found that NSC-87877 inhibits activity of ACP1 in a dose-dependent manner and is a potent competitive-inhibitor of ACP1. Among PTPs, a role for ACP1 in tumorigenesis has recently been suggested. *In vivo* studies demonstrated that ACP1 is a positive regulator of both tumor onset and development.[14] Kikawa *et al.* demonstrated that overexpression of ACP1 is sufficient to confer transformation upon nontransformed epithelial cells.[10] For these reasons, we suggest that ACP1 could be a therapeutic target.

Experimental Section

Reagents and antibodies. Anti-ERK, anti-phospho-JNK (specific for phospho-Thr183 and phospho-Tyr185), anti-phospho-p38 (phospho-Thr180 and phospho-Tyr182), and anti-phospho-ERK (phospho-Thr202 and phospho-Tyr204) antibodies were purchased from Cell Signaling Technology. Anti-JNK and anti-p38 antibodies were from Santa Cruz Biotechnology. Active JNK and active p38 proteins were from Upstate Biotechnology. Active ERK protein was from Millipore.

Purification of 6 x His-tagged protein. PTP expression plasmids were constructed in pET-28a (+) and transformed into BL21(DE3)-RIL *E.coli*. Expression of recombinant protein was induced with 1mM isopropyl-β-D-thiogalactopyranoside at 37 °C or 30 °C for 8 ~ 10 h. Cells were harvested and then

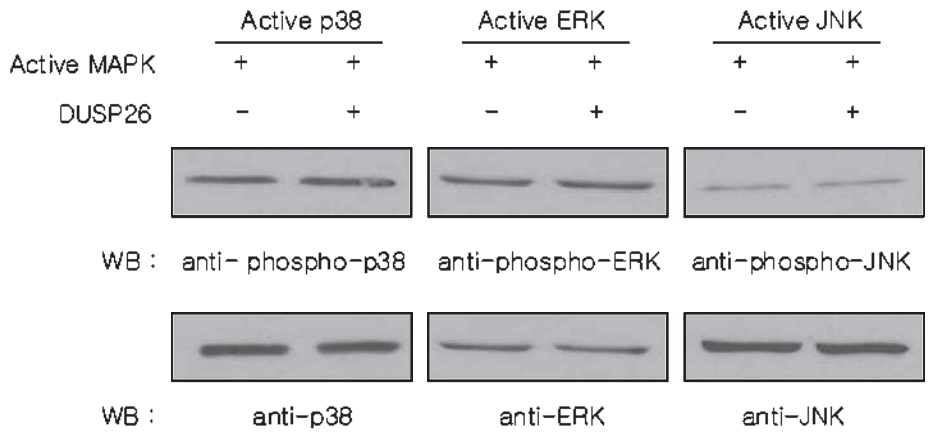

Figure 4. Effect of ACP1 on active MAPKs phosphorylation status. Recombinant active MAPKs (p38, ERK, or JNK) were incubated with or without ACP1 at 37 °C for 30 min. Dephosphorylation levels of MAPKs were determined by Western blotting analysis.

lysed by sonication in 50 mM Tris-HCl (pH 8), 300 mM NaCl, 1% Tergitol-type NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF). The lysate was clarified at 4000 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 with 20 mM Tris-HCl (pH 8), 500 mM NaCl, 50 mM imidazole and eluted with 20 mM Tris-HCl (pH 8), 500 mM NaCl, 200~300 mM imidazole. Recombinant proteins were dialyzed overnight against 20 mM Tris-HCl, 100 mM NaCl, 30% glycerol, 0.5 mM PMSF before storage at -80 °C.

***In vitro* PTP activity assay and kinetic analysis.** The activity of phosphatases was measured using the substrate 3-O-methylfluorescein phosphate (OMFP; Sigma) at concentrations varying with the K_m of each enzyme in a 96-well microtiter plate assay based on methods described previously.[19] The NSC-87877 (Calbiochem) and OMFP were solubilized in H_2O 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), 0.4 mM dithiothreitol (DTT), 0.33% bovine serum albumin (BSA) and 100 nM of PTPs. Reactions were initiated by the addition of OMFP and the incubation time was 30 min at 37 °C. Fluorescence emission from the reaction product was measured with a multiwell plate reader (GENios Pro; excitation filter, 485nm; emission filter, 535nm). 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 (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). All experiments were performed in triplicate and were repeated at least three times.

Dephosphorylation of recombinant active MAPKs by ACP1. 6 x His-tagged ACP1, at a final concentration of 1 μg, was combined with recombinant phosphorylated p38 (10 ng), ERK (10 ng), or JNK (40 ng) in 30 mM Tris-HCl (pH 7), 75 mM NaCl, 1 mM EDTA, 0.4 mM DTT , 0.33% BSA, and incubated for 30 min at 37 °C. The samples were subjected to Western blotting analysis to examine the phosphorylation state of MAPKs using phospho-MAPK antibodies.

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 as previously described.[20] The immunoreactive bands were visualized using an ECL system (Pierce).

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