

Discovery of a Novel Shp2 Protein Tyrosine Phosphatase Inhibitor

Liwei Chen, Shen-Shu Sung, M. L. Richard Yip, Harshani R. Lawrence, Yuan Ren, Wayne C. Guida, Said M. Sebti, Nicholas J. Lawrence, and Jie Wu

Molecular Oncology and Drug Discovery Programs, H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida; and the Department of Interdisciplinary Oncology, University of South Florida College of Medicine, Tampa, Florida

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ABSTRACT

Shp2 is a nonreceptor protein tyrosine phosphatase (PTP) encoded by the *PTPN11* gene. It is involved in growth factor-induced activation of mitogen-activated protein (MAP) kinases Erk1 and Erk2 (Erk1/2) and has been implicated in the pathogenicity of the oncogenic bacterium *Helicobacter pylori*. Moreover, gain-of-function Shp2 mutations have been found in childhood leukemias and Noonan syndrome. Thus, small molecule Shp2 PTP inhibitors are much needed reagents for evaluation of Shp2 as a therapeutic target and for chemical biology studies of Shp2 function. By screening the National Cancer Institute (NCI) Diversity Set chemical library, we identified 8-hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid (NSC-87877) as a potent Shp2 PTP inhibitor. Molecular modeling and site-directed mutagenesis studies suggested

that NSC-87877 binds to the catalytic cleft of Shp2 PTP. NSC-87877 cross-inhibited Shp1 *in vitro*, but it was selective for Shp2 over other PTPs (PTP1B, HePTP, DEP1, CD45, and LAR). It is noteworthy that NSC-87877 inhibited epidermal growth factor (EGF)-induced activation of Shp2 PTP, Ras, and Erk1/2 in cell cultures but did not block EGF-induced Gab1 tyrosine phosphorylation or Gab1-Shp2 association. Furthermore, NSC-87877 inhibited Erk1/2 activation by a Gab1-Shp2 chimera but did not affect the Shp2-independent Erk1/2 activation by phorbol 12-myristate 13-acetate. These results identified NSC-87877 as the first PTP inhibitor capable of inhibiting Shp2 PTP in cell cultures without a detectable off-target effect. Our study also provides the first pharmacological evidence that Shp2 mediates EGF-induced Erk1/2 MAP kinase activation.

Shp2, encoded by the *PTPN11* gene, is a nonreceptor PTP with two SH2 domains (N-SH2, C-SH2) (Neel et al., 2003; Alonso et al., 2004). Molecular biology and genetic studies have shown that Shp2 mediates cell signaling by growth factors and cytokines, such as EGF, hepatocyte growth factor, and interleukin-6. In particular, Shp2 is involved in activation of Erk1/2 MAP kinase by EGF (Deb et al., 1998).

Shp2 is basally inactive because of auto-inhibition by its N-SH2 domain (Hof et al., 1998). In growth factor- and cytokine-stimulated cells, Shp2 binds to tyrosine-phosphorylated docking proteins through its SH2 domains, resulting in its

activation (Cunnick et al., 2001). Shp2 has been shown to bind to Gab1 (or Gab2) in cells stimulated with EGF, hepatocyte growth factor, or interleukin-6 (Maroun et al., 2000; Cunnick et al., 2001; Gu and Neel, 2003; Nishida and Hirano, 2003). Gab1-Shp2 interaction and Shp2 PTP activity are necessary for Erk1/2 activation by these growth factors (Cunnick et al., 2002; Neel et al., 2003). Although the mechanism by which growth factors activate Shp2 has been elucidated, those by which Shp2 produces downstream signals to activate Ras-Erk1/2 MAP kinase pathway and possibly other pathways are less clear and may be dependent on growth factor and cell context (Mohi et al., 2005).

Besides its role in growth factor and cytokine signaling, Shp2 has been implicated in pathogenicity of *Helicobacter pylori*. Cytotoxin-associated antigen A (CagA)-positive strains of *H. pylori* are strongly associated with gastritis and

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ABBREVIATIONS: PTP, protein tyrosine phosphatase; EGF, epidermal growth factor; MAP, mitogen-activated protein; Erk, extracellular signal-regulated kinase; CagA, cytotoxin-associated antigen A; JMML, juvenile myelomonocytic leukemia; NSC-87877, 8-hydroxy-7-(6-sulfonaphthalen-2-yl)diazenyl-quinoline-5-sulfonic acid; GST, glutathione S-transferase; PCR, polymerase chain reaction; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; NCI, National Cancer Institute; 3D, three-dimensional; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; NSC-668394, 7-[2-(3,5-dibromo-4-hydroxy-phenyl)ethylamino]quinoline-5,8-dione; NSC-5069, cacotheleine; PMA, phorbol 12-myristate 13-acetate.

gastric cancer. After being injected into host cells, CagA is retained on the plasma membrane and recruits Shp2 to induce transformation of gastric epithelial cells (Hatakeyama, 2004).

It is remarkable that *PTPN11* mutations have been found in Noonan syndrome, juvenile myelomonocytic leukemia (JMML), and several types of human malignancies (Bentires-Alj et al., 2004; Tartaglia and Gelb, 2005). Noonan syndrome is a developmental disorder characterized by facial anomalies, short stature, heart disease, skeletal defects, and hematological disorders (Tartaglia and Gelb, 2005). Germline *PTPN11* mutations are responsible for causing 50% of cases of Noonan syndrome. Some children with Noonan syndrome also develop JMML (Tartaglia et al., 2003). JMML is a progressive myelodysplastic/myeloproliferative disorder characterized by overproduction of tissue-infiltrating myeloid cells. Approximately 50% of cases of JMML have activating Ras mutations or homozygotic inactivation of the *NF1* gene, which encodes a Ras-GTPase activating protein, neurofibromin. Somatic mutations in *PTPN11* account for approximately 35% of JMML patients who do not have Ras or neurofibromin mutations (Kratz et al., 2005). It was reported recently that JMML-associated Shp2 mutants could transform murine bone marrow and fetal liver cells (Chan et al., 2005; Mohi et al., 2005; Schubbert et al., 2005) and caused fatal JMML-like disorder in BALB/c mice (Mohi et al., 2005). Although molecular etiologies of Noonan syndrome and JMML are becoming clear, several mechanistic issues regarding how Shp2 mutants cause Noonan syndrome and JMML remain unanswered. All Shp2 mutants found in Noonan syndrome and JMML are gain-of-function mutations, mostly resulting from weaker autoinhibition of the N-SH2 domain (Fragale et al., 2004; Keilhack et al., 2005).

In short, accumulated molecular biology and genetic evidence suggests that Shp2 is an important signaling component of growth factors, cytokines, and oncogenic bacteria. Gain-of-function Shp2 mutations are linked to childhood developmental disorder and juvenile leukemias. Therefore, Shp2 PTP is an important target for controlling growth factor receptor signaling and a potential target for development of novel therapies for Noonan syndrome, JMML, and possibly other Shp2-associated cancers.

PTP inhibitor development is an emerging area in the field of drug development (Bialy and Waldmann, 2005). Most efforts of PTP inhibitor discovery and design have so far been focused on PTP1B and Cdc25 inhibitors (Lazo et al., 2002; Zhang, 2002). No systematic effort to identify Shp2-selective PTP inhibitors has been reported. Although PTP1B inhibitors that cross-inhibit Shp2 have been found (Shen et al., 2001; Huang et al., 2003), none of them has demonstrated *in vivo* activity in cell cultures. By a combination of experimental and virtual screenings of the NCI Diversity Set chemical library, we have identified NSC-87877 as a potent Shp2 PTP inhibitor. It is significant that NSC-87877 is active in cell-based assays and has no detectable off-target effects in the EGF-stimulated Erk1/2 activation pathway.

Materials and Methods

Chemical Library. The NCI Diversity Set chemical library of 1981 compounds was provided by the NCI Developmental Therapeutics Program (<http://www.dtp.nci.nih.gov/branches/dscb/>

diversity_explanation.html). After the initial identification of NSC-87877 from the NCI Diversity Set, the authentic, 98% pure NSC-87877 was obtained from Acros Organics (Fairlawn, NJ) for subsequent experiments.

Recombinant PTP Proteins. Plasmids for expression of glutathione *S*-transferase (GST)-PTP fusion proteins of human Shp2 (residues 205–593), Shp1 (residues 205–597), and PTP1B (residues 1–435) were constructed in pGEX-2T by PCR subcloning techniques. A plasmid for GST fusion protein of human HePTP (residues 1–399) was constructed in pGEX-KG. GST-Shp2 PTP containing Lys-280-to-Val and Lys-280/Asn-281-to-Arg/Asp mutants were generated by PCR-based mutagenesis. Likewise, expression vectors for GST-full-length Shp2 (FL-Shp2) and GST-full-length-Shp2 containing Glu-76 to Lys (FL-E76K) mutation were prepared in pGEX-KG by PCR technique. All constructs were verified by DNA sequencing.

GST-PTP fusion proteins were expressed in *Escherichia coli* DH5 α and affinity purified with glutathione Sepharose. After elution from glutathione affinity columns, GST-fusion proteins were dialyzed with dialysis buffer (12.5 mM Tris-Cl, pH 7.5, 25 mM NaCl, 1 mM dithiothreitol, and 0.1% β -mercaptoethanol) at 4°C for 40 h and then stored in dialysis buffer plus 20% glycerol at –80°C. Recombinant CD45 (residues 584–1281) and LAR D1 domain were obtained from Calbiochem (San Diego, CA). Recombinant DEP1 was from Abcam Inc. (Cambridge, MA). GST fusion proteins of murine Shp2 (GST-Shp2 Δ N) and Shp1 have been reported previously (Cunnick et al., 1998, 2001) and were used in the chemical library screening and initial *in vitro* characterization.

PTP Activity Assay. PTP activity was measured using the fluorogenic 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP; Invitrogen, Carlsbad, CA) as the substrate. Unless otherwise specified, each reaction contained 25 mM MOPS, pH 7.0, 50 mM NaCl, 0.05% Tween 20, 1 mM dithiothreitol, 20 μ M DiFMUP, 10 nM Microcystin LR, 20 nM GST-PTP, and 5 μ l of test compound or dimethyl sulfoxide (solvent) in a total reaction volume of 100 μ l in black 96-well plates. Reaction was initiated by addition of DiFMUP, and the incubation time was 30 min at room temperature. DiFMUP fluorescence signal was measured at an excitation of 355 nm and an emission of 460 nm with a plate reader (Victor² 1420; PerkinElmer Wallac, Gaithersburg, MD). IC₅₀ was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activity. For IC₅₀ determination, eight concentrations of NSC-87877 at one-third dilution (~0.5 log) were tested. The ranges of NSC-87877 concentrations used in each PTP assay were determined from preliminary trials. Each experiment was performed either in triplicate or duplicate, and IC₅₀ data were derived from at least three independent experiments. The curve-fitting program Prism 4 (GraphPad Software, San Diego, CA) was used to calculate IC₅₀ values.

Computer Docking. Computer docking was performed using the X-ray crystal structure of human Shp2 (PDB identification code 2SHP) (Hof et al., 1998) using the GLIDE program (Grid-Based Ligand Docking from Energetics, as part of the FirstDiscovery Suite from Schrödinger, Inc., Portland, OR) (Friesner et al., 2004; Halgren et al., 2004). The N-SH2 domain of Shp2, which blocks the catalytic site, was removed from the 3D structure before the computer docking analysis. The GLIDE program relies on the Jorgensen OPLS-2001 force field. The optimal binding geometry for each model was obtained by using Monte Carlo sampling techniques coupled with energy minimization.

Preparation of HEK293 Cell Line for Doxycycline-Inducible Expression of a Gab1-Shp2 Chimera. Plasmid pcDNA5/FRT/TO-Gab1PH-Shp2 Δ N was constructed by subcloning the coding sequence for Flag-Gab1PH-Shp2 Δ N (Cunnick et al., 2002) from pcDNA3.1 into pcDNA5/FRT/TO (Invitrogen) through HindIII and ApaI sites. pcDNA5/FRT/TO-Gab1PH-Shp2 Δ N and pOG44 was then cotransfected into the Flp-In-T-Rex-293 cells (Invitrogen). Transfected cells were selected in Dulbecco's modified Eagle's medium (DMEM)/10% tetracycline-free fetal bovine serum medium containing 100 μ g/ml hygromycin. Individual hygromycin-resistant cell

lines were screened for doxycycline-inducible expression of Flag-tagged Gab1PH-Shp2ΔN by immunoblotting analysis of cell lysates with an anti-Flag antibody (M2; Sigma, St. Louis, MO). Among 24 hygromycin-resistant cell lines that we have screened, 21 showed doxycycline-inducible expression of Gab1PH-Shp2ΔN. One of these 21 cell lines was randomly selected for use in the subsequent experiments.

Cell Culture, Immunoprecipitation, and Immunoblotting. Cells were cultured in DMEM/10% fetal bovine serum. Subconfluent cells were serum-starved in DMEM/0.1% bovine serum albumin for 18 h before treatment with NSC-87877 and stimulation with EGF or PMA. Cells were lysed on ice with Lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 20 mM *p*-nitrophenyl phosphate, and 1% Triton X-100). Immunoprecipitation and immunoblotting analyses of Gab1, Shp2, paxillin, and Erk1/2 were performed essentially as described previously (Cunnick et al., 2001; Ren et al., 2004). Erk1/2 kinase assay was performed as reported except that endogenous Erk1/2 kinase activity was measured (Cunnick et al., 2001).

Immune Complex PTP Assay. Serum-starved cells were pre-treated with NSC-87877 (50 μM, 3 h) or dimethyl sulfoxide (solvent) and then stimulated with EGF (100 ng/ml, 5 min) or left untreated. Cells were lysed in ice-cold PTP lysis buffer [25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1:50 diluted protease inhibitor cocktail (Roche, Indianapolis, IN)]. Shp2 or Shp1 in cell lysate supernatants (0.5 mg/each) was immunoprecipitated with an antibody to Shp2 or an antibody to Shp1 (Santa Cruz Biotechnology, Santa Cruz, CA) plus protein A-Sepharose for 2 h at 4°C.

Immunoprecipitates were washed twice with the PTP lysis buffer and twice with reaction buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol) (Tartaglia et al., 2003). Each Shp2 or Shp1 immune complex was resuspended in 100 μl of reaction buffer containing 50 μM DiFMUP and then incubated at room temperature for 20 min. After a brief centrifugation, supernatants were transferred into 96-well plates and the DiFMU fluorescence signal was measured. The remaining immune complexes were used for immunoblotting analysis of Shp2 or Shp1.

Ras Activation Assay. Active Ras in MDA-MB-468 cells was detected by means of Ras-GTP bound to a GST fusion protein of the Ras-GTP binding domain of Raf fragment (GST-RBD) (Cunnick et al., 2002) followed by immunoblotting with an anti-Ras antibody (Santa Cruz Biotechnology).

Cytotoxicity and Cell Viability Assays. Cytotoxicity assay was performed using the CytoTox 96 nonradioactive cytotoxicity assay reagents (Promega, Madison, WI) following the supplier's instructions. Cells were plated in 96-well plates and allowed for attachment in DMEM (phenol red-free)/10% FBS for 24 h. Then, cells were incubated either in phenol red-free DMEM, DMEM/1% FBS, or DMEM/5% FBS in the presence or absence of 50 or 100 μM NSC-87877 for 24 h. Maximum lactate dehydrogenase release was achieved with the lysis solution provided in the kit. Cytotoxicity experiments were performed twice in triplicate ($n = 6$).

For cell viability assay, cells (4000 cells/well) were plated in 96-well plates in DMEM with or without 10% fetal calf serum (day 0). Inhibitors (NSC-87877, U0126, LY294002) were added on day 1 and again on day 3 with additional medium, such that the final concentration of inhibitors remained unchanged. Control wells contained medium or medium plus inhibitors. Relative viable cell number was determined on day 4 using the fluorometric CellTiter-Blue cell viability assay reagent (Promega).

Statistical Analysis. Statistical analyses for comparison of IC₅₀ values were performed using unpaired *t* test with Welch's correction using GraphPad Prism 4 software, whereas paired *t* test was used in other data analyses.

Results

Identification of NSC-87877 as a Shp2 PTP Inhibitor.

We screened the NCI Diversity Set chemical library, which contains 1981 compounds, for Shp2 PTP inhibitors using a GST-fusion protein of rodent Shp2 (GST-Shp2ΔN). Confirmed hits (>50% inhibition at 10 μM) that were 1) organometallic agents, 2) arsenic compounds, 3) previously identified PTP inhibitors (NSC-668394, NSC-5069) (Lazo et al., 2002), 4) potential carcinogens, 5) with lower IC₅₀ for Shp1 than that for Shp2 in the initial analyses, 6) a nonspecific protein binding agent, or 7) polymers were excluded from further analysis. Authentic compounds of the remaining seven hits were either obtained from independent sources or synthesized in house and then compared with the samples from the NCI Diversity Set library for inhibition of GST-Shp2. Only one of these compounds, NSC-87877 (Fig. 1), showed a similar or greater potency than the sample of the same chemical identification obtained from NCI.

The PTP inhibitory activity of NSC-87877 was then assessed against several human PTPs in vitro. As shown in Table 1, NSC-87877 potently inhibited Shp2 with an IC₅₀ of 0.318 ± 0.049 μM. NSC-87877 seemed to have no selectivity between human Shp2 and Shp1 in vitro. NSC-87877 showed approximately 5-, 24-, 206-, 266-, and 475-fold selectivity for Shp2 over PTP1B, HePTP, DEP1, CD45, and LAR, respectively (Table 1).

NSC-87877 Binds to the Shp2 Catalytic Cleft. NSC-87877 ranked among top 10% (175th) of the compounds with the best GLIDE scores for the docking to the human Shp2 PTP domain in our virtual screening of 2368 3D structures derived from the NCI Diversity Set. Computer docking of NSC-87877 (Fig. 2) suggested that the B-ring sulfonic acid group forms a hydrogen bond with the backbone NH group of Arg-465. Arg-465 is a conserved residue in the PTP signature motif (motif 9) VHCSXGXGR[T/S]G located at the base of the PTP catalytic cleft (Andersen et al., 2001). The A-ring sulfonic acid forms hydrogen bonds with the side-chain NH₃ group of Lys-280 and the side-chain NH₂ group of Asn-281.

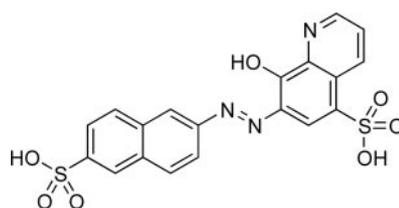


Fig. 1. Chemical structure of NSC-87877.

TABLE 1

Inhibition of PTPs by NSC-87877 in vitro

Each experiment was performed in triplicate or in duplicate. Data are presented as mean ± S.E.M.

PTP	IC ₅₀	IC ₅₀ Selectivity
	μM	
Shp2	0.318 ± 0.049 ($n = 11$)	1
Shp1	0.355 ± 0.073 ($n = 5$)	1
PTP1B	1.691 ± 0.407 ($n = 4$)	5
HePTP	7.745 ± 1.561 ($n = 4$)	24
DEP1	65.617 ± 4.120 ($n = 3$)	206
CD45	84.473 ± 16.185 ($n = 3$)	266
LAR	150.930 ± 9.077 ($n = 4$)	475
Shp2V280	1.110 ± 0.136 ($n = 6$)	3
Shp2RD	1.087 ± 0.162 ($n = 6$)	3

Lys-280/Asn-281 are nonconserved PTP residues located adjacent to the phosphotyrosine recognition loop (motif 1) (Andersen et al., 2001). The interaction between aromatic rings of the compound and the protein contributes to the binding through hydrophobic stabilization.

To evaluate this molecular model, we made two Shp2 PTP mutants containing changes in the Lys-280 and Asn-281 residues predicted to interact with NSC-87877. One (Shp2V280) mutant contained a K280V mutation and the other (Shp2RD) mutant contained a dual K280R/N281D mutation. *In silico* prediction gave both Shp2V280 and Shp2RD mutants a 0.6 kcal/mol increase (destabilization) in the GLIDE docking score. Sensitivity of mutated and wild-type Shp2 to NSC-87877 inhibition was then compared experimentally by the PTP assay. As shown in Table 1, Shp2V280 and Shp2RD were approximately 3-fold less sensitive to NSC-87877 inhibition ($p = 0.0015$ for comparison of IC_{50} values between Shp2 and Shp2V280; $p = 0.0062$ for comparison of IC_{50s} between Shp2 and Shp2RD). These data suggest that Lys-280 and/or Asn-281 are involved in NSC-87877 binding to Shp2.

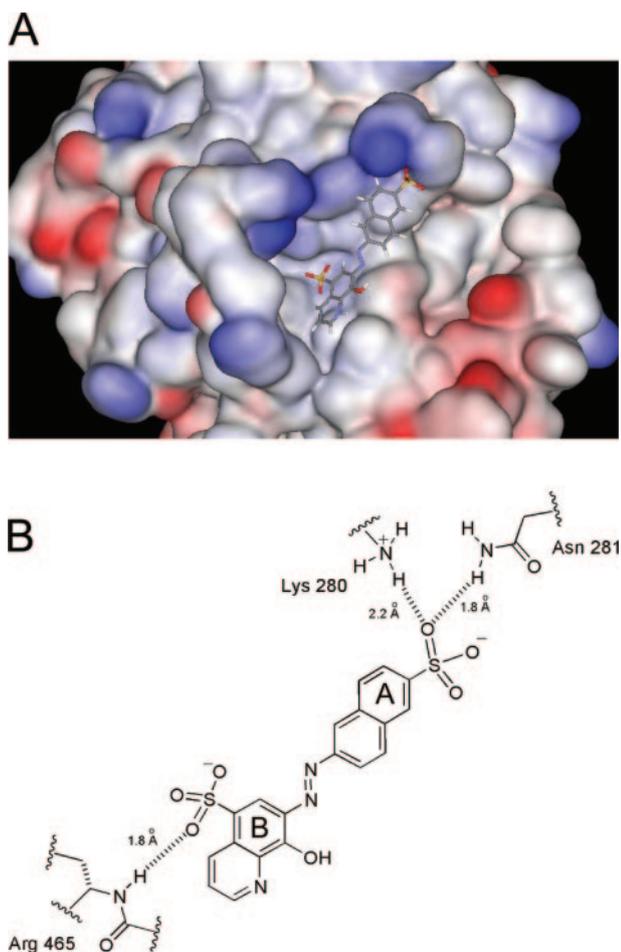


Fig. 2. Molecular model of NSC-87877 binding to the Shp2 PTP domain. A, the protein surface of Shp2 PTP domain is colored according to electrostatic potential. Positively charged areas are colored in blue, and negatively charged areas are colored in red. For NSC-87877, carbon atoms are colored in gray, oxygen in red, nitrogen in blue, hydrogen in white, and sulfur in yellow. B, the hydrogen bonds formed between the NSC-87877 and the protein, via Arg-465, Lys-280, and Asn-281 are shown schematically but not to scale. The hydrogen bonds are defined with a minimum donor angle of 90° and minimum acceptor angle of 60° and maximum length of 2.5 Å.

NSC-87877 Inhibits EGF-Stimulated Shp2 Activation. To determine whether NSC-87877 was able to inhibit Shp2 in the cells, serum-starved HEK293 cells were preincubated with or without NSC-87877 and then stimulated with EGF or mock-treated. Shp2 was immunoprecipitated from cell lysates and Shp2 PTP activity was then determined in the immune complexes using DiFMUP as the substrate. Shp2 PTP activity increased 2.6-fold in response to EGF stimulation in the absence of NSC-87877 pretreatment (Fig. 3). Incubation of NSC-87877 alone reduced the basal Shp2 PTP activity by 45%. The EGF-stimulated Shp2 activation was inhibited by 97% when cells were pretreated with $50 \mu\text{M}$ NSC-87877 (Fig. 3).

NSC-87877 Inhibits EGF-Induced Erk1/2 Activation. Molecular biology studies using overexpression of a PTP-inactive Shp2 mutant have suggested that Shp2 is involved in EGF-induced Erk1/2 activation (Deb et al., 1998). To evaluate whether NSC-87877 could inhibit Shp2-dependent cell signaling in the cells, we examined the effect of NSC-87877 on EGF-stimulated Erk1/2 activation. Serum-starved HEK293 cells were pretreated with 0 to $50 \mu\text{M}$ NSC-87877 for 3 h and then stimulated with EGF for 5 min. Erk1/2 activation was determined by immunoblotting analysis of cell lysates with an anti-phospho-Erk1/2 antibody. As shown in Fig. 4A, NSC-87877 inhibited EGF-stimulated Erk1/2 activation in a concentration-dependent manner. A 50% inhibition of EGF-stimulated Erk1/2 activation was observed at an average of $6 \mu\text{M}$ NSC-87877 in two independent experiments.

NSC-87877 Suppresses Erk1/2 Activation by a Gab1-Shp2 Chimera but Does Not Affect PMA-Induced Erk1/2 Activation. We found previously that expression of a chimeric protein (Gab1PH-Shp2 ΔN) consisting of the Gab1 PH domain and a constitutively active Shp2 N-SH2 domain deletion mutant resulted in Erk1/2 activation and that the Shp2 PTP activity is necessary for Erk1/2 activation by Gab1PH-Shp2 ΔN (Cunnick et al., 2002). To examine whether NSC-87877 could inhibit Shp2-dependent Erk1/2 activation that bypasses the ligand-receptor interaction, we established HEK293-derived cell lines (Dox-G1S2) containing doxycy-

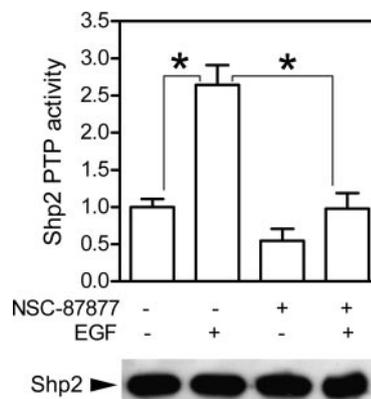


Fig. 3. Inhibition of EGF-stimulated Shp2 activation by NSC-87877. Serum-starved HEK293 cells were pretreated with or without NSC-87877 ($50 \mu\text{M}$), stimulated with EGF or mock-treated, and Shp2 PTP activity was determined by the immune complex Shp2 PTP assay as described under *Materials and Methods*. The relative Shp2 PTP activity is shown. Data were from two independent experiments performed in duplicate ($n = 4$). After determination of Shp2 PTP activity, Shp2 immunoprecipitates were analyzed by immunoblotting with an antibody to Shp2 (bottom). *, $p < 0.05$.

cline-inducible Gab1PH-Shp2ΔN using the Flp-In-T-Rex-293 cells. Figure 4B, top, shows a representative Dox-G1S2 cell line that demonstrated the property of doxycycline-inducible expression of Flag-tagged Gab1PH-Shp2ΔN. In the absence of doxycycline, there was little residual level of Gab1PH-Shp2ΔN in the cells and the level of active Erk1/2 was minimal. Induction of cells with doxycycline induced Gab1PH-Shp2ΔN expression and Erk1/2 activation (Fig. 4B). However, in NSC-87877-treated cells, Erk1/2 activation by Gab1PH-Shp2ΔN was inhibited. Coupled with data from Figs. 3 and 4A, this result suggests that the mechanism by which NSC-87877 inhibits EGF-induced Erk1/2 activation is not mediated by events upstream of Shp2, such as activation of EGF receptor. Furthermore, because no extracellular stimulation was involved in the Erk1/2 activation by Gab1PH-Shp2ΔN, the observation that NSC-87877 could inhibit Gab1PH-Shp2ΔN-induced Erk1/2 activation indicates that the target of NSC-87877 is intracellular.

Previous studies have shown that PMA-induced Erk1/2 activation is not affected by overexpression of a PTP-inactive Shp2 mutant (Yamauchi et al., 1995), suggesting that the

protein kinase C activator-mediated Erk1/2 activation is Shp2-independent. We therefore compared the effect of NSC-87877 on EGF- and PMA-stimulated Erk1/2 activation. Consistent with the data presented in Fig. 4A, EGF-stimulated Erk1/2 activation was inhibited by NSC-87877 (Fig. 4C). In contrast, NSC-87877 did not affect PMA-induced Erk1/2 activation (Fig. 4C). Protein kinase C is known to activate the Erk1/2 MAP kinase cascade by directly phosphorylating Raf-1 (Kolch et al., 1993; Carroll and May, 1994). Thus, our data indicating that NSC-87877 inhibited EGF-induced, but not PMA-induced, Erk1/2 activation suggest that the target of NSC-87877 is upstream of Raf-1, consistent with the notion that NSC-87877 is acting on Shp2 to inhibit Erk1/2 activation.

NSC-87877 Does Not Inhibit EGF-Induced Gab1 Tyrosine Phosphorylation and Gab1-Shp2 Association. Shp2 is activated in EGF-stimulated cells by binding to tyrosine-phosphorylated Gab1 (and Gab2 if it is also expressed) (Cunnick et al., 2001). To further assess whether NSC-87877 has an off-target effect upstream of Shp2 activation in the EGF-stimulated cells, we analyzed EGF-induced Gab1 tyrosine phosphorylation and Gab1-Shp2 association in HEK293 cells treated with NSC-87877 and/or EGF. HEK293 cells express Gab1 but not Gab2. Figure 5 shows that Gab1 was not tyrosine-phosphorylated and Shp2 was not detected in Gab1 immunoprecipitates in the absence of EGF stimulation. Gab1 became tyrosine-phosphorylated and Shp2 was coimmunoprecipitated with Gab1 upon stimulation of cells with EGF. Figure 5 also shows that NSC-87877 did not inhibit EGF-stimulated Gab1 tyrosine phosphorylation and subsequent binding of Shp2 to Gab1. Immunoblotting analysis of cell lysate supernatants used for immunoprecipitation of Gab1 indicated that an equal amount of Shp2 was present in each sample (Fig. 5, bottom). These results indicate that NSC-87877 does not affect EGF-activated signaling steps before Shp2 activation.

NSC-87877 Inhibits EGF-Stimulated Shp2 PTP, Ras, and Erk1/2 Activation in MDA-MB-468 Cells that Coexpress Shp2 and Shp1. HEK293 cells express Shp2 but not Shp1. Because NSC-87877 inhibits Shp2 and Shp1 with the similar IC₅₀ in vitro, we next tested the inhibitory effect of

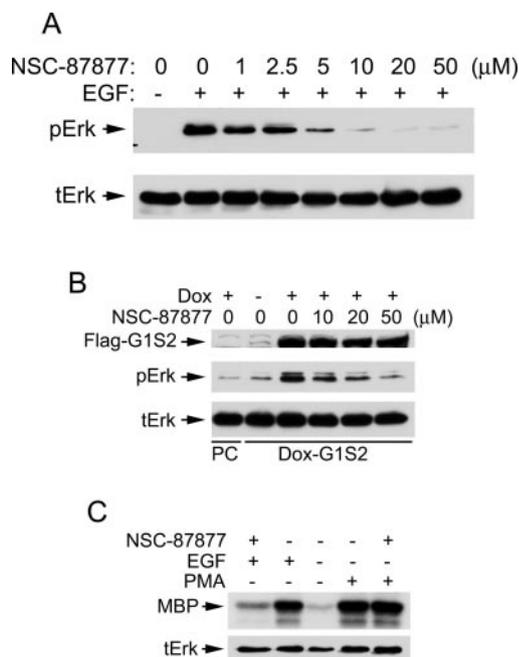


Fig. 4. NSC-87877 inhibits Shp2-dependent Erk1/2 activation. A, serum-starved HEK293 cells were pretreated with various concentrations of NSC-87877 (0–50 μ M) for 3 h and then stimulated with EGF (1 ng/ml, 5 min). Erk1/2 activation was analyzed by immunoblotting of cell lysate supernatants (20 μ g/each) with antibodies to phosphorylated, active Erk1/2 (pErk) or total Erk2 (tErk). B, Flp-In-T-Rex-293 cell line containing doxycycline-inducible Gab1PH-Shp2ΔN (Dox-G1S2) was incubated in serum-free medium with or without doxycycline (2 μ g/ml) in the presence of indicated concentration of NSC-87877 for 3 h. Cell lysates were analyzed by immunoblotting with antibodies to Flag-tag (for detection of Gab1PH-Shp2ΔN), active Erk1/2 (pErk), or total Erk2 (tErk). PC, parental Flp-In-T-Rex-293 cells. Flag-G1S2, Flag-Gab1PH-Shp2ΔN cells. C, HEK293 cells were pretreated with or without NSC-87877 (20 μ M, 3 h) and then stimulated with EGF (1 ng/ml, 5 min) or PMA (100 nM, 10 min). Erk1/2 was immunoprecipitated from cell lysate supernatants (100 μ g/each) and Erk1/2 kinase activity was determined by phosphorylating myelin basic protein (MBP) with [γ -³²P]ATP. After the kinase reaction, reaction mixtures were separated on a SDS-polyacrylamide gel, transferred onto a nitrocellulose filter, and subjected to autoradiography. After autoradiography, the filter was used for immunoblotting analysis with an antibody to Erk2 (tErk) to examine the amounts of Erk2 in immunoprecipitates.



Fig. 5. NSC-87877 has no apparent effect on signaling steps before Shp2 activation in EGF-stimulated cells. HEK293 cells were serum-starved for 18 h, preincubated with or without NSC-87877 (20 μ M, 3 h), and then treated with EGF (5 ng/ml, 5 min) or bovine serum albumin (–). Gab1 was immunoprecipitated from cell lysate supernatants. Immunoprecipitates were analyzed by immunoblotting with antibodies to phosphotyrosine (pY), Gab1, or Shp2. An aliquot of each cell lysate supernatant (30 μ g) was also examined by immunoblotting with an antibody to Shp2 (bottom). pY-Gab1, tyrosine-phosphorylated Gab1.

NSC-87877 on EGF signaling in MDA-MB-468 cells that express both Shp2 and Shp1. Near confluent MDA-MB-468 cells were serum-starved, pretreated with NSC-87877 or solvent, and then stimulated with EGF. Shp2 and Shp1 were immunoprecipitated from cell lysates and their PTP activities were determined by the immune complex PTP assay. Similar to that observed in HEK293 cells, EGF induced Shp2 activation in MDA-MB-468 cells and NSC-87877 inhibited the EGF-induced Shp2 PTP activity in these cells (Fig. 6A). Shp1 immunoprecipitated from serum-starved MDA-MB-468 cells had low basal PTP activity similar to that of Shp2. It is noteworthy that EGF did not induce Shp1 activation in re-

peated experiments (Fig. 6B). Figure 6C shows that incubation of Shp2 immunoprecipitated from EGF-stimulated MDA-MB-468 cells with 2 μ M NSC-87877 in vitro inhibited the Shp2 PTP activity by $85 \pm 9\%$, which is similar to the 93% inhibition predicted from the dose-dependent inhibition curves of Shp2-PTP domain and Shp2E76K mutant (see Fig. 7). This result demonstrates that NSC-87877 can directly inhibit Shp2 isolated from EGF-stimulated MDA-MB-468 cells.

Paxillin is a physiological substrate of Shp2 (Ren et al., 2004). As shown in Fig. 6D, EGF-induced paxillin dephosphorylation in MDA-MB-468 cells, which was inhibited by

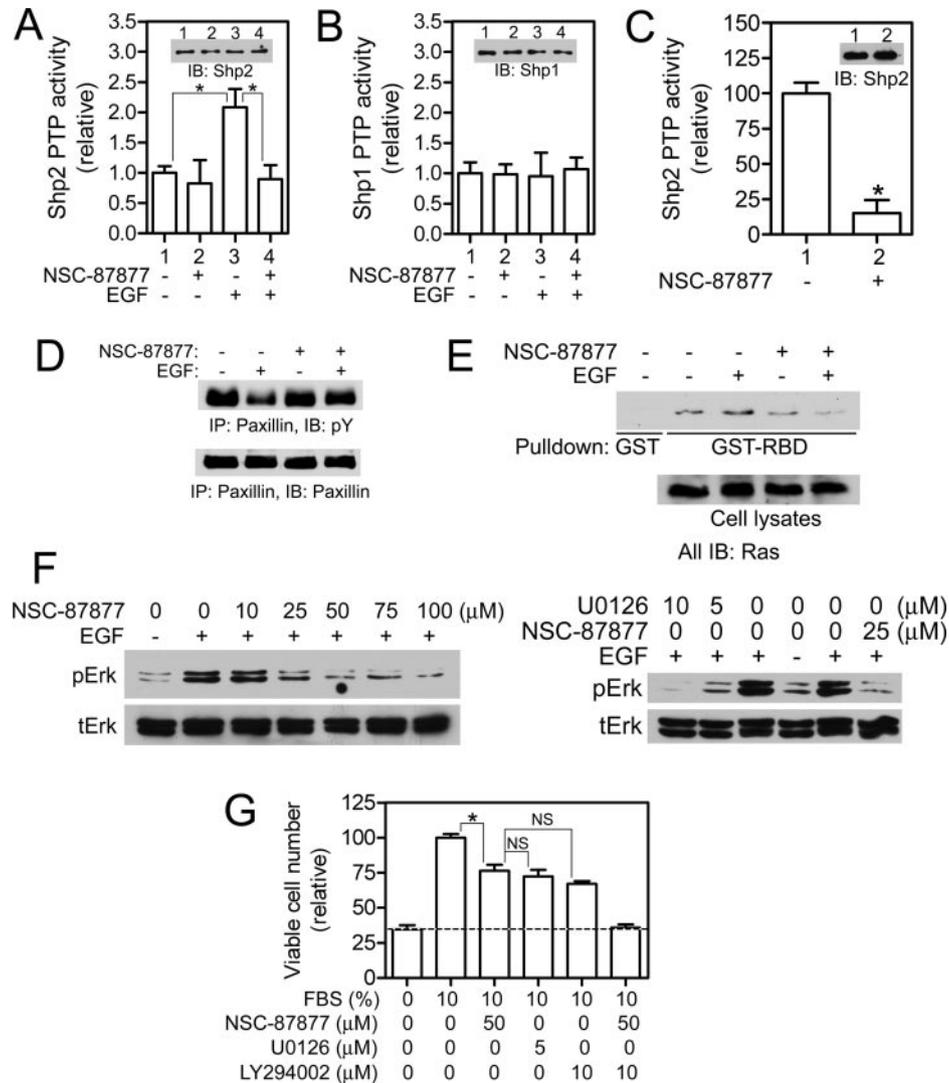


Fig. 6. Inhibitory effects of NSC-87877 in MDA-MB-468 cells. A and B, serum-starved MDA-MB-468 cells were treated with NSC-87877 and EGF as indicated, and Shp2 (A) and Shp1 (B) PTP activities were determined by the immune complex PTP assay (see *Materials and Methods*). The relative PTP activities are shown ($n = 4$). C, Shp2 was immunoprecipitated from EGF-stimulated MDA-MB-468 cells. PTP activity was then determined in the absence or presence of 2 μ M NSC-87877. Data were from two independent experiments performed in duplicate. Background signal from immunoprecipitates without anti-Shp2 antibody were subtracted. After determination of PTP activities, an equal portion of each immunoprecipitate was analyzed by immunoblotting with antibodies to Shp1 or Shp2 (A–C, insets). D, paxillin was immunoprecipitated from serum-starved MDA-MB-468 cells treated with NSC-87877 and EGF as indicated. Paxillin immunoprecipitates was analyzed by immunoblotting with antibodies to phosphotyrosine (top) or paxillin (bottom). E, serum-starved MDA-MB-468 cells were treated with NSC-87877 and EGF as indicated. Cleared cell lysates (0.2 mg of protein/each) were incubated with GST-agarose (control) or GST-RBD-agarose to pull down active-Ras-GTP, which was visualized by immunoblotting with an anti-Ras antibody (top). Bottom, immunoblotting analysis of an equal amount of cell lysates (100 μ g/each) with an anti-Ras antibody. F, serum-starved MDA-MB-468 cells were pretreated for 3 h with NSC-87877 or 30 min with U0126 at indicated concentrations and then stimulated with EGF (2 ng/ml, 10 min) or mock-treated. Cell lysates (20 μ g/each) were analyzed by immunoblotting with antibodies to phospho-Erk1/2 or total Erk1/2. G, MDA-MB-468 cells were cultured in DMEM with or without 10% fetal calf serum in the presence or absence of NSC-87877, U0126, LY294002, or a combination of LY294002 and NSC-87877 as indicated. Cell viability was measured after 4 days of drug treatments. The data were from two independent experiments performed in triplicate and quintuplicate ($n = 8$). *, $p < 0.05$. NS, not statistically significant.

NSC-87877. Thus, inhibition of Shp2 PTP activity by NSC-87877 in MDA-MB-468 cells blocked dephosphorylation of its protein substrate in these cells, demonstrating a direct functional consequence of Shp2 inhibition. Shp2 functions upstream of Ras in the Ras-Erk1/2 MAP kinase pathway (Neel et al., 2003). To determine whether NSC-87877 could inhibit Ras activation, EGF-induced Ras activation in MDA-MB-468 cells was analyzed by the GST-RBD pulldown assay. As shown in Fig. 6E, more active Ras was pulled down by GST-RBD in EGF-stimulated cells than in serum-starved cells, indicating that Ras was activated in EGF-stimulated cells. However, if cells were pretreated with NSC-87877, EGF-induced Ras activation was blocked (Fig. 6E). EGF-induced Erk1/2 activation was consistently inhibited by NSC-87877 in MDA-MB-468 cells (Fig. 6F). Thus, NSC-87877 can inhibit EGF-stimulated Erk1/2 activation in MDA-MB-468 cells that coexpress Shp2 and Shp1.

Assessment of Cytotoxicity of NSC-87877 and the Effect of NSC-87877 on Cell Viability. To assess the potential cytotoxicity of NSC-87877, MDA-MB-468 cells were incubated in DMEM, DMEM/1% FBS, or DMEM/5% FBS and treated with 50 or 100 μ M NSC-87877 for 24 h. Lactate dehydrogenase release was then used as a measure of cytotoxicity. The highest cytotoxicity value ($2.9 \pm 2.5\%$) was detected in cells treated with 100 μ M NSC-87877 in DMEM/5% FBS, but it was not statistically significant ($p = 0.24$). Likewise, no cytotoxicity effect of NSC-87877 was observed in HEK293 cells under the same conditions (the highest value was $3.1 \pm 2.7\%$, $p = 0.99$).

To determine whether NSC-87877 can affect cell viability, MDA-MB-468 cells cultured in DMEM/10% FBS were treated with NSC-87877 (50 μ M), the Mek inhibitor U0126 (5 μ M), the phosphoinositide 3-kinase inhibitor LY294002 (10 μ M), and a combination of NSC-87877 and LY294002 for 4 days and the relative viable cell numbers were determined.

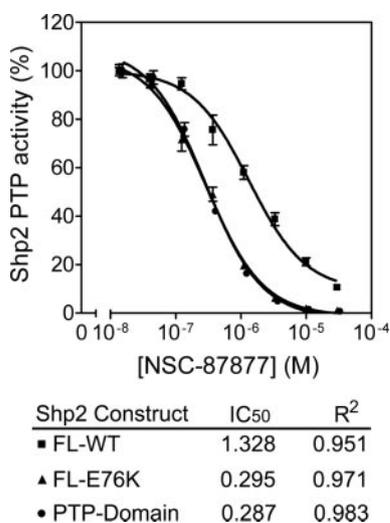


Fig. 7. The wild-type full-length Shp2 is less sensitive to NSC-87877 inhibition than the E76K mutant and the PTP domain constructs. PTP activity of FL-Shp2 and FL-E76K was determined in the absence or presence of various concentrations of NSC-87877 (one-third dilution starting at 30 μ M). Data from three independent experiments performed in triplicate or duplicate were combined for fitting the dose-response curves. PTP inhibition data of the Shp2 PTP-domain were obtained from three separated experiments (a subset of data from Table 1) in which the NSC-87877 starting concentration was 33 μ M. Points represent mean \pm S.E.M.

As shown in Fig. 6G, NSC-87877, U0126, and LY294002 treatment alone significantly reduced cell viability, which corresponded to 36, 42, and 50% inhibition of 10% FBS-stimulated cell proliferation. The differences among inhibitory effects of NSC-87877, U0126, and LY294002 at the tested concentrations were not statistically significant. FBS-stimulated growth was completely inhibited by a combination of NSC-87877 and LY294002 treatment (Fig. 6G). Thus, NSC-87877 can significantly reduce MDA-MB-468 cell viability/proliferation, especially in combination with a phosphoinositide 3-kinase inhibitor.

Comparison of the Potency of NSC-87877 on Full-Length Shp2, Shp2 E76K Mutant, and Shp2 PTP Domain. Before activation, the N-SH2 domain of Shp2 interacts with the PTP domain. It has been suggested that the interaction is disrupted by gain-of-function Shp2 mutations that occur in the N-SH2 domain or the PTP domain (Tartaglia et al., 2003; Keilhack et al., 2005). The E76K mutation, located within the N-SH2 domain, is one of the most frequent mutations found in JMML (Tartaglia et al., 2003). We prepared GST fusion proteins of the full-length, wild-type Shp2 (FL-Shp2) and the Shp2 E76K mutant (FL-E76K) and compared the sensitivity to NSC-87877 of these Shp2 constructs. Figure 7 shows that NSC-87877 inhibited FL-E76K with an IC₅₀ (0.295 μ M) that is similar to that for the Shp2 PTP domain construct. However, the IC₅₀ of NSC-87877 for FL-Shp2 was 4.5-fold higher (1.328 μ M) (Fig. 7). Thus, the wild-type full-length Shp2 is less sensitive to NSC-87877 than the E76K mutant or the PTP domain constructs, suggesting that the N-SH2-PTP domain interaction could interfere with NSC-87877 binding to Shp2 and that NSC-87877 preferentially inhibits the activated Shp2.

Discussion

Although molecular biology and genetic evidence has suggested that Shp2 plays important roles in growth factor and cytokine signaling and that Shp2 mutations are linked to human diseases, chemical biology interrogation of Shp2 function and signaling mechanisms had not been possible because of the lack of a suitable Shp2 inhibitor. In this study, we identified NSC-87877 as a potent Shp2 inhibitor and demonstrated that it could inhibit Shp2 PTP activity and Shp2-mediated Erk1/2 activation in intact cell models. To our knowledge, this represents the first successful identification of a Shp2 PTP inhibitor that was effective in inhibiting Shp2-dependent Erk1/2 activation in the cells. Our study also provided the first pharmacological evidence of the involvement of Shp2 PTP in growth factor-stimulated Erk1/2 activation. This important discovery opens a new avenue in Shp2 research by providing a novel tool for chemical biology exploration of Shp2 function and signaling mechanisms.

Our *in vitro* PTP assays showed that NSC-87877 inhibited Shp2 selectively over PTP1B, HePTP, DEP1, CD45, and LAR, but it inhibited Shp2 and Shp1 with a similar potency. Development of a Shp2-specific inhibitor that does not cross-inhibit Shp1 is an ultimate goal. Nevertheless, our study here illustrated that a Shp2 PTP inhibitor without selectivity between Shp2 and Shp1 could still be a useful reagent for studying Shp2 in cell cultures. Unlike Shp2, which is ubiquitously expressed, Shp1 expression is limited to hematopoietic cells and certain epithelial cells. Furthermore, Shp1 is

epigenetically silenced in some leukemias and lymphomas (Oka et al., 2002). Shp1 PTP inhibition activity becomes irrelevant in cells that do not express Shp1 or contain a minimal amount of Shp1. Although Shp1 negatively regulates cytokine and immune receptor signaling in hematopoietic cells, the role of Shp1 in epithelial cells is less clear. Expression of exogenous wild-type or PTP-inactive Shp1 in HEK293 cells was found to have little effect on the Erk1/2-dependent Elk-1 activation by EGF (Bennett et al., 1996). It is noteworthy that our data show that EGF activated Shp2 but not Shp1 in the MDA-MB-468 human breast carcinoma cells. Thus, even in certain cells where Shp1 is present, it may not play a significant role in a particular signaling pathway. Under this condition, a Shp2 inhibitor without selectivity between Shp1 and Shp2 could be used to analyze Shp2 function.

In the resting state, the N-SH2 domain of Shp2 is known to bind to the catalytic domain, which results in autoinhibition of the Shp2 PTP activity (Hof et al., 1998). We found that the Shp2 E76K mutant and the Shp2 PTP domain constructs were equally sensitive to NSC-87877 inhibition, whereas the full-length, wild-type Shp2 was approximately 4.5-fold less sensitive to NSC-87877. This finding is significant. First, it gives another piece of evidence that NSC-87877 binds to the catalytic cleft of the Shp2 PTP domain. Second, it provides the first chemical biology evidence to support the model that the N-SH2 domain-PTP domain interaction is disrupted in the JMML-associated E76K mutant (Tartaglia et al., 2003; Keilhack et al., 2005). Third, it suggests that NSC-87877 preferentially inhibited activated Shp2, which can result either from activating mutations such as E76K or from Shp2 binding to Shp2 SH2 domain docking proteins in activated cells.

NSC-87877 contains two arylsulfonic acid groups, one as naphthylsulfonic acid and the other as quinolinesulfonic acid. The arylsulfonic acid moiety has been identified as a pharmacophore of PTP inhibitors (Huang et al., 2003; McCain et al., 2004). In fact, suramin, a hexasulfonated polyaromatic naphthylurea, and several polysulfonic derivatives of suramin that contain multiple naphthylsulfonic acid groups or phenylsulfonic acid groups, are PTP inhibitors in vitro (McCain et al., 2004). Although negatively charged arylsulfonic acids were thought to be unfavorable for cellular uptake, compounds containing multiple arylsulfonyl acid groups capable of entering cells are not without precedent. For instance, suramin, which has six arylsulfonic acid groups, can enter cells through an active process (Stein, 1993). Whereas the means by which NSC-87877 enters cells remains to be studied, we noticed that it was necessary to incubate cells with NSC-87877 for 2 to 3 h to observe the inhibitory effects of NSC-87877 in the cells, suggesting that NSC-87877 cannot rapidly get into cells.

Because of the similarity between Shp1 and Shp2, development of a Shp2-specific inhibitor will be challenging but not impossible. Selectivity between Shp1 and Shp2 (either toward Shp1 or toward Shp2) was observed among several hits from the NCI Diversity Set chemical library (our unpublished data). Shp1 and Shp2 catalytic domains are known to have different substrate specificity (O'Reilly and Neel, 1998), suggesting that the catalytic cleft is not identical between Shp1 and Shp2. Furthermore, the surface electrostatic potential of the catalytic cleft is much more positive in human

Shp2 than in human Shp1 (Yang et al., 1998). The PTP catalytic cleft consists of a base and four sides in the 3D structures (Hof et al., 1998; Yang et al., 2003). Although amino acid residues present at the base of Shp1 and Shp2 PTP catalytic clefts are identical, all four sides of the catalytic cleft contain one or more residues that are different between Shp1 and Shp2. These differences will provide the basis for developing a Shp2-specific PTP inhibitor in our continuing effort.

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Address correspondence to: Dr. Jie Wu, Molecular Oncology Program, SRB-3, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612. E-mail: wu@moffitt.usf.edu
