M S . Thesis

The expression and role of Src, a non-receptor protein tyrosine kinase, in HSC-3 human oral cancer cells

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A Thesis Submitted for Master Degree

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In addition, I would like to express my special thanks to my husband, who has supported me financially and spiritually and has always encouraged me throughout this journey. His contribution to my achievements is enormous.

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1. Introduction

1.1. Background of The Study:

Oral squamous cell carcinoma (OSCC) comprises more than 90% of the upper airodigestive tract, and most evidence of malignancy was found in the head and neck (1,2,3). Moreover, OSCC is a high metastasis tumor in the oral cavity and is counted as one of the ten most common cancers in the world (4,5). OSCC can be a reason for the mortality of patients and modifications in the facial area as well as disfigurement (1,4,5). Until now, surgery, radiotherapy, and limited chemotherapeutic drugs (cisplatin, 5-fluorouracil, doxorubicin) are the treatment modalities for oral cancers that can diminish their metastasis and recurrence. However, even though there are comprehensive research designs for treating oral cancer, the global number of patients with OSCC has increased (6). Continuous findings of new and effective therapeutic drugs and molecular targets are still required for a better approach to treating and combating OSCC (7).

The proto-oncogene *Src* encodes Src, a non-receptor tyrosine kinase involved in cancer cell proliferation, growth, survival, and migration due to their association with malignant transformation and oncogenesis (8,9,10). Studies have shown that Src is highly phosphorylated (activated) in many human solid cancers and cancer cells, including prostate, breast, colon, and hematologic malignancies (11,12,13), and that inhibition of Src by its pharmacological inhibitor or gene silencing causes the reduced tumor mass and growth suppression of cancer cells (14,15,16). These

previous findings point out that Src activation and inhibitor could be a crucial therapeutic target and regimen for treating cancers in which Src hyperactivation plays oncogenic roles. A wealth of information illustrates that multiple growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF), can induce the phosphorylation and activation of Src, which can further regulate a cascade of downstream targets such as phosphatidylinositide-3 kinases (PI3K), protein kinase B (PKB, also named a serine/threonine kinase (Akt)), mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT-3), and extracellular signal-regulated kinases 1/2 (ERK-1/2) to control cell proliferation, cycle, and migration in the colon and other cancer (17,18, 19, 20). Recent evidence demonstrates that Src is overexpressed in OSCC and the overexpression of Src is closely linked to the development of OSCC (21). However, up to date, little is known about the expression phosphorylation (activation), and role of Src in the growth of human oral cancer cells.

PP1,4-Amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo [3,4-d]-pyrimidine is a selective inhibitor of Src (22,23). Previous studies have demonstrated that PP1 has strong anti-tumor effects on hematological and solid tumors, such as Ras-associated cancers, rat basophilic leukemia cells, and renal cancer cells (24,25,26). However, the regulatory effect and mechanism of PP1 on the growth of human oral cancer cells are also not fully understood.

1.2. Aims of The Study:

1. To check whether Src is expressed and phosphorylated in HSC-3 cells, a tumorigenic human oral (tongue) cancer cell line.

2. To confirm the role of Src in the growth of HSC-3 cells by using gene silencing.

3. To explore whether PP1, the selective Src inhibitor, suppresses the growth and induces the apoptosis of HSC-3 cells.

4. To elucidate molecular and signaling mechanisms by which PP1 inhibits growth and induces the apoptosis of HSC-3 human oral cancer cells.

2. Materials and Methods

2.1. Chemicals and Antibodies:

PP1 was bought from Calbiochem (Madison, WI, USA). Cell culture media and reagents, including Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and phosphate-buffered saline (PBS) were purchased from Welgene company (Daegu, Korea). 3-(4,5-dimethylthiazol-2-y)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was obtained from Promega company (Madison, WI, USA). Bradford's reagent was purchased from Bio-Rad (Hercules, California, USA). A protease inhibitor cocktail (PIC, 100X) was bought from Calbiochem (Madison, WI, USA). Control siRNA (sc-37007) and Src siRNA (sc-29228) were obtained from Santa Cruz Biotechnology (Delaware, CA, USA). Enzyme-linked chemiluminescence (ECL) Western detection reagents were bought from ThermoScientific (Waltham, MA, USA). LY294002 and PD98059 were obtained from BiomolResearch Lab (Plymouth Meeting, PA, USA).Cell culture plastic wares were purchased from SPL Life Sciences (Gyeonggi-do,Korea). A detailed list of antibodies used in this study is included in Table 1.

2.2. Cell Culture:

HSC-3 human oral cancer cell line, normal human gingival fibroblast (HGF)-1 cell line, and A549 human lung cancer cell line were bought from Japanese Cancer Research Resources Bank (Tokyo, Japan). HSC-3

cells and HGF-1 cells were grown in DMEM-high glucose (Sigma-Aldrich, StLouis, MO, USA), A549 cells were grown in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37ºC in a humidified atmosphere with 5% CO_2.

2.3. Cell Viability and Survival Assay:

HSC-3 or HGF-1 cells were seeded at 1 × 10^4cells/100 µL/well in a 96-well plate for cell viability assay. After overnight, cells were treated with vehicle control (0.01% DMSO) or PP1, the Src inhibitor at different concentrations (0, 0.1, 0.5, 1, 5, and 10 µM) for 24 hours. Cells were then washed twice with PBS, followed by measurement of the cell viability using an MTS reagent according to the manufacturer's protocol. Shortly, 80 µl of the culture media (DMEM) and 20 µL of MTS solution were added to each well, and the plate was incubated at 37℃ for 1 hour. The density at 490 nm of the microplate reader (SPECTRA max 340PC; Molecular Devices, LLC) was used to measure each well's absorbance. For cell survival assay, HSC-3 and HGF-1 cells were seeded at 2 × 10^4cells/500 µL/well and 2.5 × 10^4cells/500 µL/well in a 24-well plate, respectively, for cell viability assay. After overnight, cells were treated with vehicle control (0.01% DMSO) or PP1 at different concentrations (0, 0.1, 0.5, 1, 5, and 10 µM) for 24 hours. Then cells were washed twice with PBS. The number of surviving cells which are not stained with trypan blue dye was counted using a phase-contrast microscope. The cell count assay was carried out in triplicate. Data are mean ±standard error (SE) of three independent experiments.

2.4. DNA Fragmentation Assay:

Analysis of DNA fragmentation was described in detail in a previous study (27). The intact or fragmented DNA was extracted and measured through electrophoresis at 50 V on a 1.8% agarose gel containing Gel Red nucleus (Cat. No. 41003, Biotium, Fremont, CA, USA) for 40 minutes. The intact or fragmented DNA was then visualized and photographed under UV illumination after staining with ethidium bromide (0.1 μg/mL) by a Gel documentation system (Gel Doc-XR, Bio-rad, Hercules, CA, USA).

2.5. Preparation of Whole-Cell Lysate:

HSC-3 cells (2 × 105 cells/mL) were grown in 6-well plates. After overnight, cells were treated with control, PP1, and/or other reagents for the indicated times. At each time point, the conditioned HSC-3 cells were washed, collected, and lysed in a RIPA buffer (Sigma-Aldrich; Merck; St. Louis, MO, USA) containing PIC (1X). The whole-cell lysates were centrifuged at 1.2074 × 104× *g* for 20 min at 4° C. The resultant supernatant was recovered, and its protein concentration was defined using Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford,IL, USA).

2.6. Immunoblot Analysis:

Proteins (40 μg) were saperated by 10% or 12% of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene

difluoride membrane (PVDF, Millipore, Bedford, MA, USA). The membranes were blocked with 5% (*w*/*v*) skim milk containing TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween 20 (TBST) for 2 h and incubated with respective antibody of target proteins listed in Table 1 at 4 ° C. The membranes were washed with TBST three times and further incubated with horseradish peroxidase (HRP)-conjugated anti-goat immunoglobulin (IgG) or anti-mouse IgG or anti-rabbit IgG for 2 h at RT. Next, the membranes were rinsed with TBST three times and developed with enhanced chemiluminescence (ECL) reagents. Actin expression levels were used as an equal protein loading control.

2.7. siRNA Transfection:

HSC-3 cells were transfected with 100 pM of control siRNA (sc-37007) or Src siRNA (sc-29228) using Lipofectamine RNAiMAX (Invitrogen) for 48 hours. The control or Src siRNA-transfected cells were lysed, and the expression levels of Src and other proteins were determined by immunoblot analysis.

2.8. Statistical Analysis:

Data are expressed as mean ± standard error (SE) of three independent experiments. Statistical significance between various categories was designated by one-way ANOVA, followed by Dunnett's post hoc test, using SPSS 11.5 software (SPSS,Inc.). The difference was considered statistically significant for the value of $p < .05$.

Table 1. Antibodies Used for Western Blot Analysis

Antibodies	Dilution used	Source	Catalog no.
Primary antibodies			
p-Src (T416)	1:2,000	Cell Signalling	#2101
Src	1:2,000	Cell Signalling	#2108
p-c-Met (Y1234/1235	1:2,000	Cell Signalling	#3077
c-Met	1:2,000	Cell Signalling	#3127
p-EGFR (Y1068)	1:2,000	Cell Signalling	#2234
EGFR	1:2,000	Cell Signalling	#2645
p-JAK2 (Y1007/1008)	1:2,000	Santa Cruz Biotechnology	sc-21870

JAK2	1:2,000	Santa Cruz Biotechnology	sc-278
p-STAT-3 (Y705)	1:2,000	Santa Cruz Biotechnology	sc-8059
STAT-3	1:2,000	Santa Cruz Biotechnology	sc-8019
p-PKB (S473)	1:2,000	Cell Signalling	#9271
PKB	1:2,000	Cell Signalling	#9272
p-ERK1/2 (T202/Y204)	1:2,000	Cell Signalling	#9101
ERK1/2	1:2,000	Cell Signalling	#9102
Procaspase-9	1:2,000	Enzo Life Sciences	ADI-AAM-39#9746
Procaspase-3	1:2,000	Enzo Life Sciences	ADI-AAP-113

Procaspase-8	1:2,000	Cell Signalling	#9746
PARP	1:2,000	Santa Cruz Biotechnology	sc-53643
p-eIF-2α (S51)	1:2,000	Abcam	Ab32157
eIF-2α	1:2,000	Cell Signalling	#9722
p-S6 (S235/236)	1:2,000	Cell Signalling	#2211
S6	1:2,000	Cell Signalling	#2317
ATF-4	1:2,000	Santa Cruz Biotechnology	sc-200
B-Actin	1:10,000	Sigma	A5441
Secondary antibody			
Goat anti-rabbit IgG-HRP	1:5,000	Jackson ImmunoResearch	111-035-045

		Laboratories	
Goat anti-mouse IgG-HRP	1:5,000	Jackson ImmunoResearch Laboratories	111-035-062

3. Results

3.1. Src was expressed and highly phosphorylated in HSC-3 human oral cancer cells:

Firstly, I investigated the endogenous expression and phosphorylation levels of Src in HSC-3, HGF-1, and A549 cells that were grown in the culture media containing 10% FBS for 8 h by using Western blot analysis. As shown in Figure 1A, there were substantial and similar total expression levels of Src in HSC-3, A549, and HGF-1 cells (low panel). However, of note, there were much higher levels of phosphorylated Src in HSC-3 cells than in A549 and HGF-1 cells. These results suggest that HSC-3 cells have a unique regulatory mechanism of Src hyperphosphorylation. Aforementioned, that the phosphorylation of Src is induced by growth factors in cancer cells (17). Given that FBS used in the culture media for the growth of HSC-3 cells herein contains various growth factors and mitogens, I next probed whether the hyperphosphorylation of Src seen in HSC-3 cells is due to FBS. To this end, HSC-3 cells were grown in the culture media containing no FBS or 10% FBS overtime. At each time point, I measured the phosphorylation and expression levels of Src in HSC-3 cells grown in the absence and presence of 10% FBS. Apparently, while there were low levels of phosphorylated Src in HSC-3 cells grown in the absence of 10% FBS, there were much higher levels of that in cells grown in the presence of 10% FBS at times tested (Figure 1B). These results advocate that the hyperphosphorylation of Src detected in HSC-3 cells is FBS-dependent.

3.2. siRNA-based Src knockdown caused a significant growth inhibition of HSC-3 cells:

To directly see the role of Src expression (and phosphorylation) in the growth of HSC-3 cells, I next carried out siRNA transfection experiments in which cells were transfected with 100 pM of control or Src siRNA for 48 h, followed measurement of the expression levels of Src and the cell survival. As shown in Figure 2A, results of immunoblot assay showed strong reduction of Src protein expression in the Src siRNA-transfected HSC-3 cells compared with that in the control siRNA-transfected cells, which support the Src siRNA transfection efficacy herein. Of importance, data of cell count analysis (Figure 2B) and microscopic observation (Figure 2C) illustrated that Src knockdown significantly reduced the survival and number of HSC-3 cells compared with those in the siCon-transfected cells, which strongly advocates the positive role of Src expression (and phosphorylation) in the growth of HSC-3 cells.

3.3. Treatment of the Src kinase inhibitor PP1 led to significant reduction of the survival (growth) of HSC-3 cells:

Aforementioned, the small molecule PP1 (Figure 3A) is a selective pharmacological inhibitor of Src (22). To next have an insight of PP1 application as a new anti-oral cancer candidate drug and Src as a potential molecular target in oral cancer (cells), I treated HSC-3 cells with PP1 at different concentrations (0.1, 0.5, 1, 5, or 10 μM)

for 24 h, followed measurement of the cell proliferation and survival by using MTS and cell count assay, respectively. To compare and see the specificity, normal HGF-1 cells were also exposed to PP1 at the same concentrations for 24 h, followed measurement of the cell proliferation and survival. There was no significant alteration in the proliferation between these two different cell lines after 24 h treatment with PP1 at doses applied (Figure 3B). However, as shown in Figure 3C, treatment with PP1 particularly at 5 and 10 μM for 24 h led to a significant reduction of the survival of HSC-3 cells. However, there was no or little reduction in the survival of HGF-1 cells at concentrations tested. Moreover, as shown in Figure 3D, results of microscopic observation further illustrated that PP1 treatment at 5 and 10 μM for 24 h caused strong reduction of the numbers of HSC-3 cells (upper panels), while there was no remarkable change in those of HGF-1 cells by the 24 h exposure of PP1 at doses applied (lower panels). These results point out that PP1 selectively reduces the growth of HSC-3 cells by a unique regulatory mechanism and the role of Src kinase in the proliferation and survival (growth) of HSC-3 and HGF-1 cells.

3.4. PP1 treatment caused the reduced phosphorylation levels of not only its target Src kinase but also c-Met, EGFR, JAK2, STAT-3, PKB, and ERK-1/2 in HSC-3 cells:

Next, to molecularly understand the PP1's anti-survival effect on HSC-3 cells, I investigated the treatment effect of PP1 at 1 or 10 μM on the phosphorylationand expression levels of Src kinase as well

as other cell growth-related signaling proteins in HSC-3 cells over time. As depicted in Figure 4A, data of kinetic studies clearly showed that treatment with PP1 resulted in a dose and time-dependent decrease in the phosphorylation levels of Src kinase, but it did not affect its total expression levels in HSC-3 cells at doses and times tested, supporting the drug's efficacy to inhibit its target. Moreover, as shown in Figure 4B, treatment with PP1 at 10 μM caused the reduced phosphorylation levels of c-Met without influencing its total protein expression levels in HSC-3 cells at 8 and 24 h. In addition, PP1 treatmentat 1or 10 μM had abilities to lower the phosphorylation levels of EGFR, PKB, and ERK-1/2 without changing their total protein expression levels in these cells at 2, 8, or 24 h. Distinctly, while PP1 treatment at 1 or 10 μM for 2 or 8 h had no or enhancing effect on the protein phosphorylation and expression levels of STAT-3, the drug treatment for 24 h led to a dose-dependent decrease in the protein's phosphorylation and total expression levels in HSC-3 cells. Results of subsequent triplicate experiments further demonstrated the ability of PP1 treatment with 10 μM for 8 h to vastly reduce the protein phosphorylation levels of Src without altering its total expression levels in HSC-3 cells (Figure 4C).The densitometry data of Figure 4C for the phosphorylation levels of Src normalized by the protein total expression levels in HSC-3 cells is given in Figure 4D.

3.5. Treatment with Erlotinib (an inhibitor of EGFR), AG490 (an inhibitor of JAKs/STATs), LY294002 (an inhibitor of PI3K/PKB), orPD98059 (an inhibitor of MEK-1/2 and ERK-1/2) resulted in strong decrease in the

survival of HSC-3 cells:

Given that PP1 treatment that suppressed the survival of HSC-3 cells (Figure 3C) also lowered the phosphorylation (activation) of not only its target Src kinase but also c-Met, EGFR, JAK2, STAT-3, PKB, and ERK-1/2 in these cells (Figure 4A-D), it was reasonable to think a role of the reduced phosphorylation of each of these proteins in the PP1's growth suppressive effect on HSC-3 cells. To indirectly prove this, I next investigated the effect of Erlotinib (an inhibitor of EGFR), AG490 (an inhibitor of JAKs/STATs), LY294002 (an inhibitor of PI3K/PKB), or PD98059 (an inhibitor of MEK-1/2 and ERK-1/2) on the survival of HSC-3 cells. To this end, HSC-3 cells were treated without or with respective pharmacological inhibitors at different concentrations for 24 h, followed measurement of any change of the cell survival. As shown in Figure 5A-D, of interest, treatment with Erlotinib, AG490, LY294002, and PD98059 led to a dose-dependent decrease in the survival of HSC-3 cells, respectively. Microscopic observation also revealed a concentration-dependent reduction of the cell number by treatment with Erlotinib, AG490, LY294002, or PD98059 (Figure 5E).

3.6. PP1 treatment induced the apoptosis of HSC-3 cells along with the activation of caspase-9/8 and the production of cleaved PARP:

Inhibition of cancer cell growth is associated with induction of

apoptosis, also named programmed cell death (3). Appearance of fragmented nuclear DNA is a typical hallmark of cells undergoing apoptosis. Therefore, I explored whether PP1 treatment could induce the apoptosis of HSC-3 cells by using DNA fragmentation assay. Strikingly, treatment with PP1 at 1, 5, or 10 μM for 24 h resulted in generation of fragmented genomic DNA in HSC-3 cells (Figure 6A), assuring the drug's ability to induce the apoptosis of HSC-3 cells. Activation of caspases including caspase-9 and caspase-8 is a principal feature for the execution of programmed cell death by various apoptotic stimuli in human cancer cells (28). Most caspases are expressed as precursor and inactive forms with high molecular weight (MW) in cells (29). However, upon cells are exposed to apoptotic stimuli, they are processed to become active caspases with low MW (30). This led me to further test whether PP1 treatment influences the expression and activation levels of (pro)caspase-9 and (pro)caspase-8 in HSC-3 cells. As shown in Figure 6B, of note, treatment with PP1 at 1 or 10 μM for 2 h in triplicate experiments decreased the expression levels of procaspase-9 and procaspase-8 while increasing those of cleaved (active) caspase-9 and caspase-8 in HSC-3 cells. Active caspase-9 and caspase-8 involve in the cleavage of many intracellular proteins, including poly (ADP-ribose) polymerase (PARP), whose expressions are crucial for the proliferation and growth of cells (31). Interestingly, treatment with PP1 at 1 or 10 μM for 2 h in triplicate experiments further caused high accumulation of cleaved PARP in HSC-3 cells. The densitometry data of Figure 6B for each of the expression levels of procaspase-9 and procaspase-8 and cleaved PARP normalized by those of control actin protein expression levels in HSC-3 cells are given in Figure 6C.

3.7. PP1 treatment also altered the phosphorylation and expression levels of several ER stress and translation-related markers in HSC-3 cells:

Inhibition of cancer cell growth and/or induction of apoptosis is further influenced by an alteration of the phosphorylation and expression levels of ER stress markers, such as eukaryotic initiation factor 2-α (eIF-2α) and activating transcription factor (ATF4), and of translation regulatory proteins including ribosomal protein S6 (also called rpS6 or S6), a component of the 40S ribosomal subunit (32,33,34). This led me to additionally investigate the effect of PP1 on the phosphorylation and expression levels of eIF-2α, ATF4, and S6 in HSC-3 cells. As indicated in Figure 7, results of time course experiment demonstrated that treatment with PP1 at 1 or 10 μM for 8 or 24 h led to a time and dose-dependent slight increase in the phosphorylation levels of eIF-2α without affecting its total expression levels in HSC-3 cells. Furthermore, PP1 treatment particularly at 10 μM for 8 h caused an elevation of the expression levels of ATF4 in HSC-3 cells. Strikingly, while treatment with PP1 at 1 or 10 μM for 2 h and at 1 μM for 8 h had no effects on the phosphorylation and total expression levels of S6 in HSC-3 cells, that with PP1 at 10 μM for 8 h and at 1 or 10 μM for 24 h led to a marked decline of the phosphorylation levels of S6 without altering its total expression levels in these cells. caused an elevation of the expression levels of ATF4 in HSC-3 cells. Control actin protein expression levels remained constant under these experimental conditions.

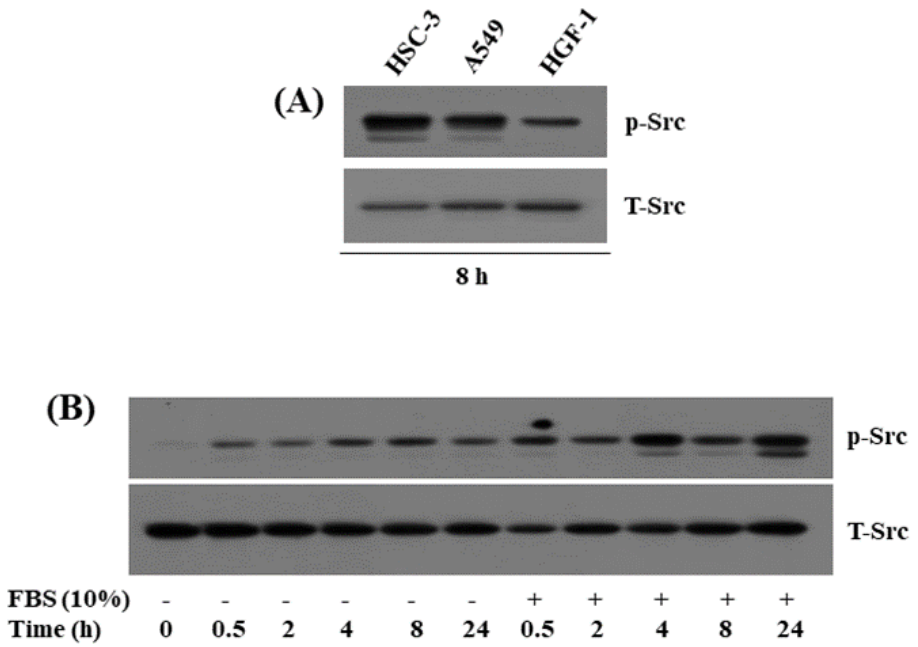

Figure 1. Confirmation of high phosphorylation and expression levels of Src in HSC-3 human oral cancer cells. (A) HSC-3 human oral cancer cells, A549 human lung cancer cells, and HGF-1 normal human gingival fibroblasts were grown in complete media containing 10% FBS for 8 h. Whole-cell lysates were prepared and analyzed by Western blotting. (B) HSC-3 cells were grown in complete media containing no FBS or 10% FBS for the designated times. At each time point, whole-cell lysates were prepared and analyzed for Western blotting.

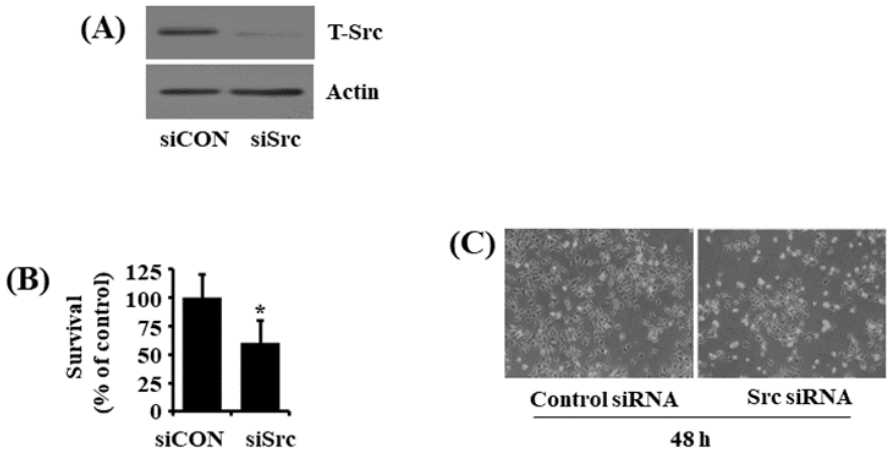

Figure 2. Suppressive effects of Src knockdown on the growth of HSC-3 human oral cancer cells. (A) HSC-3 cells were transfected with 100 pM of control siRNA or Src siRNA for 48 h. Whole-cell lysates were collected and proceed for Western blotting. (B) HSC-3 cells were transfected with 100 pM of control siRNA or Src siRNA for 48 h, followed by cell count assay to measure the number of survived cells. The cell count assay was performed in triplicate. Data are mean ± SE of three independent experiments.*$p<0.05$ compared with vehicle control. (C) Images of the cells transfected with the control siRNA and Src siRNA were obtained by phase contrast microscopy, 100 ×.

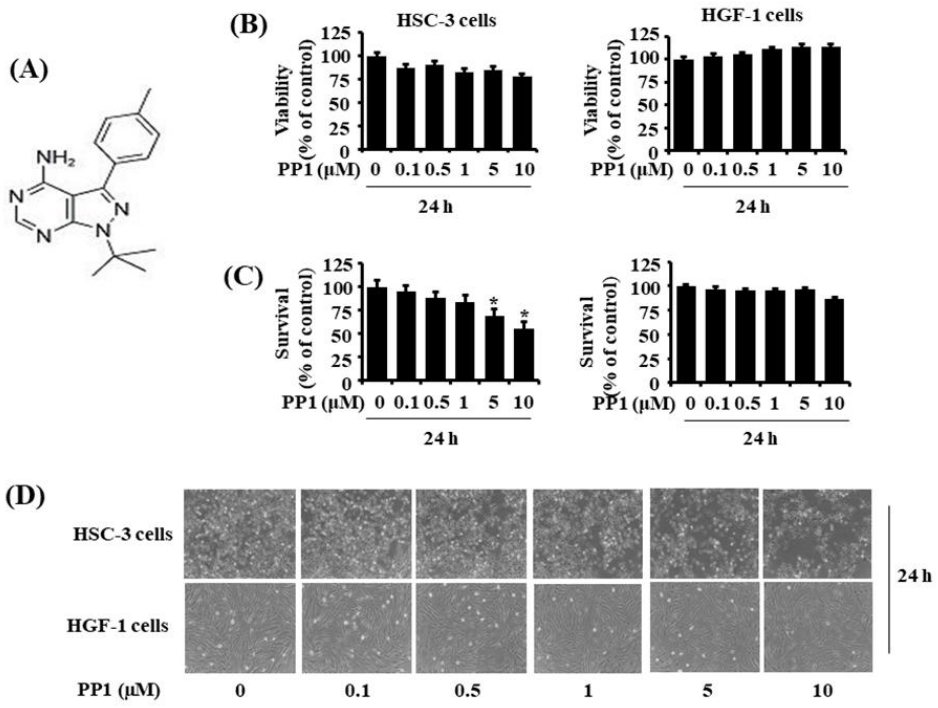

Figure 3. Suppressive effects of PP1, a selective inhibitor of Src, on the survival of HSC-3 human oral cancer cells. (A) The chemical structure of PP1. (B) HSC-3 cells were treated with vehicle control (DMSO) and different concentrations of PP1 (0, 0.1, 0.5, 1, 5, and 10 μM) for 24 h, followed measurement of the cell viability (proliferation) by using MTS assay at 24 h. (C) HSC-3 cells were treated with vehicle control (DMSO) and PP1 at indicated concentrations for 24 h, followed measurement ofthe cell survival by using cell count analysis. (D) Images of the conditioned cells were obtained by phase contrast microscopy, 100 ×. Each image is a representative of three independent

experiments. Data are mean ± SE of three independent experiments. *$p<0.05$ compared with the values of control (0.1% DMSO).

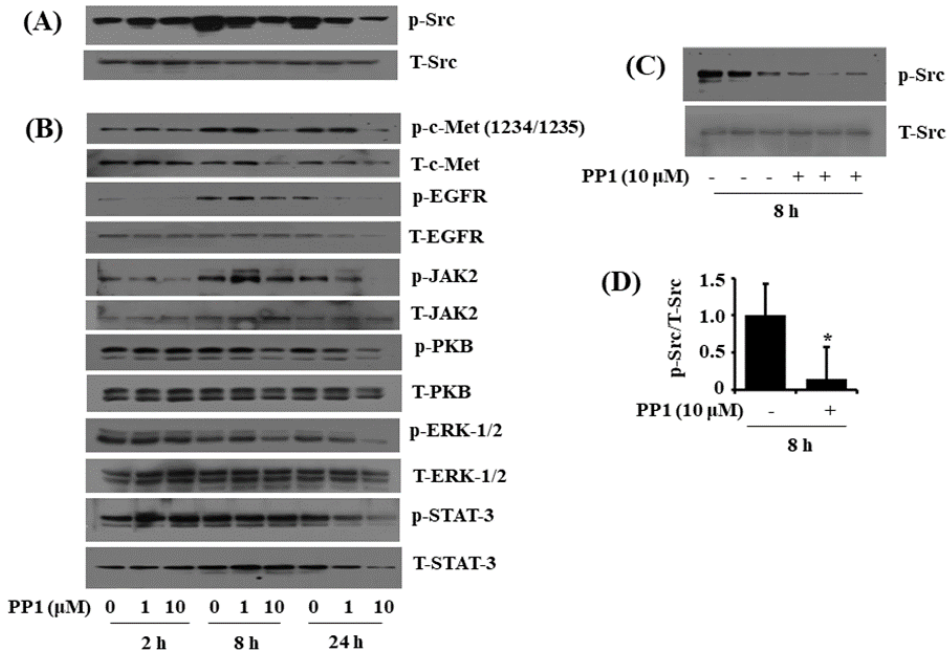

Figure 4. Inhibitory effects of PP1 on the phosphorylation and expression levels of not only Src but also other signaling proteins in HSC-3 human oral cancer cells. (A, B) HSC-3 cells were treated with vehicle control (0.1% DMSO) and PP1 (1 or 10 μM) for the indicated times. At each time point, whole-cell lysates were prepared and analyzed by Western blotting. (C) HSC-3 cells were treated with vehicle control (0.1% DMSO) and PP1 (10 μM) in triplicate for 8 h. Whole-cell lysates were prepared and analyzed for Western blotting. (D) The densitometry data of (C). *$p<0.05$ compared with control at the designated time.

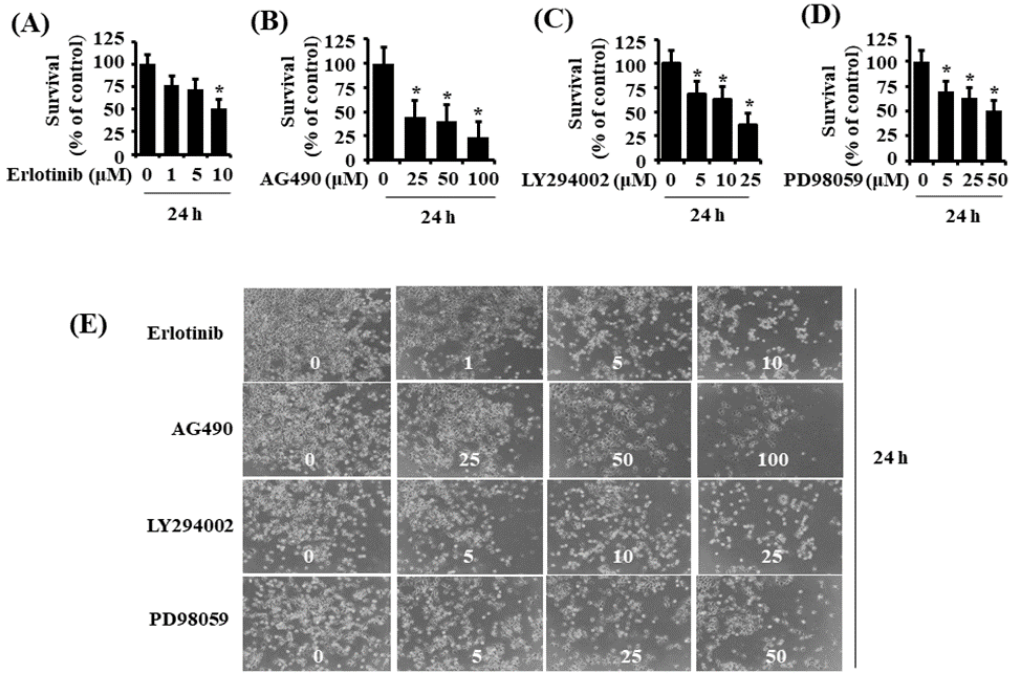

Figure 5. Inhibitory effects of Erlotinib, AG490, LY294002, or PD98059 on the growth of HSC-3 human oral cancer cells. (A-D) HSC-3 cells were treated with Erlotinib (A), AG490 (B), LY294002(C), and PD98059 (D) at the indicated concentrations for 24 h, followed measurement of the cell survival by using cell count analysis. Data are mean ±SE of three independent experiments. *$p<0.05$ compared with vehicle control. (E) Images of the conditioned cells of A-D were obtained by phase-contrast microscope, 100 ×.

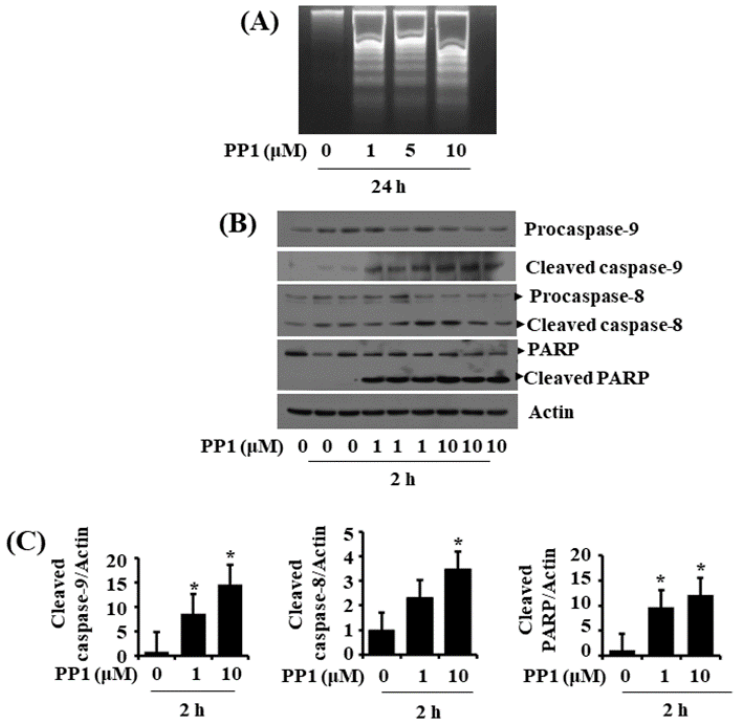

Figure 6. Effects of PP1 on the apoptosis and expression levels of cell apoptosis-related markers in HSC-3 human oral cancer cells. (A) HSC-3 cells were treated with vehicle control (DMSO) and PP1 (1, 5, and 10 μM) for 24 h. Genomic DNA from the conditioned cells was extracted and analyzed on a 1.8% agarose gel. (B) HSC-3 cells were treated with vehicle control (DMSO) and PP1 (1 or 10 μM) in triplicates for 2 h. Whole-cell lysates were prepared and analyzed for Western blotting. (C) The densitometry data of (B).

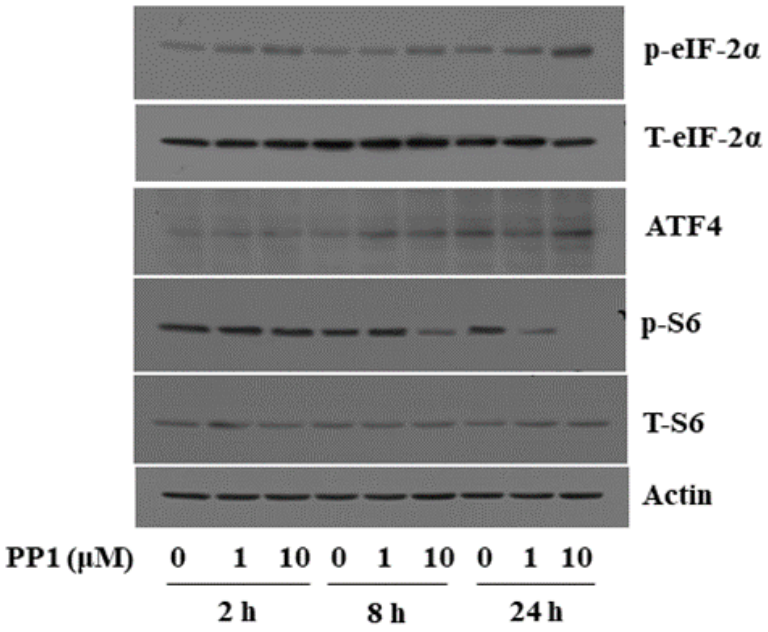

Figure 7. Effects of PP1 on the expression and phosphorylation levels of ER stress or translation-related markers in HSC-3 human oral cancer cells. HSC-3 cells were treated with vehicle control (DMSO) or PP1 (1 and 10 μM) for the designated times. At each time point, whole-cell lysates were prepared and analyzed for Western blotting.

4. Discussion

Previously, it has been reported that Src is highly phosphorylated (activated) in several types of human cancers including tongue, ductal carcinoma, and breast, and the hyperactivation of Src is associated with the development of these tumors (3,35,36). However, up to date, the expression, phosphorylation, and role of Src in the growth of human oral cancer cells are poorly understood. The present study demonstrated that there was a FBS-dependent strong phosphorylation of Src in HSC-3 cells, and inhibition of Src by PP1, a Src pharmacological inhibitor or gene silencing caused the apoptosis of HSC-3 cells and the reduced cell survival. The present study further provided experimental evidence that the PP1's pro-apoptotic and anti-survival effects on HSC-3 cells are mediated through control of the phosphorylation and expression levels of multi-targets, including Src, c-Met, EGFR, JAK2, STAT-3, PKB,ERK-1/2, eIF-2α,ATF-4, S6, caspase-9/8, and PARP.

Through initial experiments, I herein found that Src is substantially expressed in three different cell lines, including HSC-3 oral cancer cells, HGF-1 normal gingival cells, and A549 lung cancer cells, under normal cell culture media containing 10% FBS. Strikingly, I further observed that there were much higher levels of phosphorylated (active) forms of Src in HSC-3 cells than other two cell lines under the same experimental conditions. Aforementioned, the phosphorylationof Src is influenced by growth factors in cancer cells (17). Assuming that FBS contains growth factors and HSC-3 cells grown in the presence of 10% FBS expressed much higher levels of phosphorylated Src than in cells grown in the absence of FBS in this

study, it is likely to be that FBS (and its components like growth factors) could be a key clue to induce Src hyperphosphorylation in HSC-3 cells. Collectively, these results suggest that HSC-3 cells have a unique regulatory mechanism of Src hyperphosphorylation in a FBS-dependent manner. Until now, the role of Src in the growth of HSC-3 oral cancer cells is not fully understood. Interestingly, I investigated that knockdown of Src caused substantial growth suppression of HSC-3 cells, advocating that PP1's growth-suppressive effect on these cells might be mediated through Src inhibition.

The Src kinase inhibitor PP1 has been used to analyze the role of Src in different types of cancer cells such as rat basophilic leukemia cells and pancreatic endocrine tumor cells (25,37). However, the regulatory effect and mechanism of PP1 on the growth of HSC-3 oral cancer cells remain unclear. This led me to promptly investigate whether PP1 affects the growth of HSC-3 oral cancer cells. Here I found the ability of PP1 to markedly reduce the survival of HSC-3 oral cells without influencing that of normal HGF-1 cells, supporting the drug's selectivity to target HSC-3 oral cancer cell growth. In the present study, PP1 was further shown to induce a dose-dependent suppression of Src phosphorylation in HSC-3 cells, highlighting the drug's efficiency to inhibit its target kinase. Mounting evidence suggest Sr can upstream activator and downstream mediator of EGFR and the PP1-induced apoptosis by inhibition of EGFR phosphorylation in non-small cell lung cancer (38,39). One of the well-known Src functions is an adaptor-protein signaling cascade of c-Met, in which activation of Src is related to the increase of aggressiveness of many cancers (40). Furthermore, the role of Src in regulating PKB, ERK-1/2 signaling pathway in melanoma oncogenesis has been reported (41,42). For these reasons, in this study, I tested the effect of PP1

on the phosphorylation and expression levels of c-Met, EGFR, PKB, and ERK-1/2 in HSC-3 oral cancer cells, and found that PP1 had abilities to substantially reduce the phosphorylation levels of c-Met, EGFR, PKB, and ERK-1/2 in HSC-3 cells. Given that c-Met, EGFR, PKB, and ERK-1/2 favors the growth of many cancer cells, it is thus speculative that the PP1's growth suppressive effect on HSC-3 cells is partially due to the inhibition of c-Met, EGFR, PKB, and ERK-1/2. Indeed, results of pharmacological inhibition studies supported the notion, as shown by the ability of respective pharmacological inhibitor of c-Met, EGFR, PKB, and ERK-1/2 to substantially lower the survival of HSC-3 cells.

The JAK2/STAT3 signaling pathway is essential for EMT (epithelial-to-mesenchymal transition), apoptosis, proliferation, and drug resistance of different cancers and participated in the stemness of OSCC cells (43,44). Activation of JAKs triggers the phosphorylationof STAT proteins, including STAT-3, which are vital for expressed genes and serve as a marker of proliferation, survival, and apoptosis in cancer cells (45,46). This finding suggests that STAT-3 also could be one key point of treatment for human oral cancers overexpressing STAT-3 (47,48,49). The regulation effect of PP1 on the STAT-3 protein expression in human oral cancer cells is unknown. Of note, inthis study, PP1 completely inhibits the phosphorylation of JAK2 without affecting its total expression. Meanwhile, it decreased phosphorylation levels of STAT-3 and slightly its total expression in HSC-3 cells. Given that pharmacological inhibition of JAK2 and STAT-3 by AG490 (a JAK/STAT inhibitor) led to reduced survival of HSC-3 cells, it suggests that JAK2 and STAT-3 may also contribute to the HSC-3 cell survival.

The apoptosis is mainly induced through the intrinsic (mitochondrial-responsible) and/or extrinsic (DR-responsible) pathways in different cell lines (50). It is well documented that the caspases are responsible protein markers for apoptosis in cancer cells (51). Herein, PP1 at 1 and 10 μM induced the HSC-3 cell apoptosis by increasing proteolytically cleaved caspase-9, caspase-8, and cleaved PARP, as well as diminished procaspase-3 followed by DNA fragmentation. A limitation of this study is that we did not profoundly detect the role of DR-related extrinsic and mitochondrial-related intrinsic pathways in PP1-mediated apoptosis. Therefore, these pathways in oral cancer cell apoptosis remain to be further confirmed.

ER stress participates in apoptosis induced by anti-cancer drugs and/or agents in oral squamous cell carcinoma (52). ER stress initiates when cells undergo protein synthesis inhibition and misfolded or unfolded proteins are accumulated in the ER (53) and upregulation the expression of ATF-4, a transcription factor, and phosphorylated eIF-2α, a translation-responsible protein (54). The high phosphorylation levels of eIF-2α show its inactive form and inhibition of global translation (55). ATF-4, a transcription regulatory factor, plays an essential role in recovery from ER stress via arranging genes that participate in recapturing ER stress (56). Notably, the phosphorylation of S6, a translation-related ribosomal protein, is attended in the activation of global translation (34). Since PP1 increased the phosphorylation levels of eIF-2α and the expression of ATF-4 while decreasing the phosphorylation levels of S6 in HSC-3 cells, ER stress induction and inhibition of global translation in HSC-3 cells may be the effects of the drug as a growth-suppressiveand pro-apoptotic.

5. Summary

It is concluded that Src is highly expression and phosphorylated in HSC-3 human oral cancer cells in a FBS-dependent manner, and its high expression and phosphorylation are crucial for the growth of HSC-3 cells. PP1, a pharmacological inhibitor of Src, has strong anti-survival and pro-apoptotic effects on HSC-3 cells, mediated by regulating the phosphorylation and expression levels of not only Src but also other multiple signaling factors. This study shows that Src and its inhibitor as a potential therapeutic target and regimen for treating oral cancers.

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The expression and role of Src, a non-receptor protein tyrosine kinase, in HSC-3 human cancer cells.

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(Abstract)

Src phosphorylation (activation) is linked to the proliferation and survival of many cancer cells. Little is known about the phosphorylation and expression of Src and its pharmacological inhibition by PP1, an Src inhibitor, in the growth of oral cancer cells. Here we demonstrated that Src was expressed and highly phosphorylated in HSC-3 human oral cancer cells, compared with normal human gingival fibroblasts. Importantly, siRNA based Src knockdown led to a significant reduction of HSC-3 cellsurvival, addressing that Src is crucial for HSC-3 cell survival. Of note, treatment with PP1 at 1 and 10 μM markedly reduced growth and induced apoptosis of HSC-3 cells. Moreover, PP1 treatment down-regulated the phosphorylation

levels of both Src and c-Met, EGFR, JAK2, STAT-3, PKB, and ERK1/2 in HSC-3 cells. Results of additional pharmacological inhibition study revealed that activities of EGFR, JAK2, STAT-3, PKB, and ERK1/2 also favored the growth of HSC-3 cells. Furthermore, treatment with PP1 caused high cleaved caspase-9, caspase-8, and PARP expressions in HSC-3 cells. PP1 treatment further increased eIF-2α phosphorylation levels while decreasing S6 phosphorylation levels in HSC-3 cells. These results indicate that Src is expressed and highly phosphorylated in HSC-3 cells, and inhibition of Src by PP1 led to strong anti-survival and pro-apoptotic effects on HSC-3 cells, which are mediated by controlling multiple targets' phosphorylation and expression levels.

HSC-3 인간 구강암세포에서 비수용체 티로신 단백질 인산화 효소인Src의 발현 및 역할

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(초록)

Src 인산화(활성화)는 많은 암세포의 증식과 생존에 관련이 있습니다. 구강 암세포의 성장에서Src의인산화 및 발현과Src 억제제인PP1에의한 약리학적 저해에 대해서는 거의 알려져 있지 않습니다. 여기서 우리는 정상적인 인간잇몸 섬유이 세포와 비교하여Src가HSC-3 인간구강 암 세포에서 발현되고 고도로 인산화 된다는 것을 입증했습니다. 중요한 것은, siRNA 기반의 Src 녹다운은HSC-3 세포생존의 상당한 감소를 이끌었고, 이는Src가HSC-3 세포 생존에 중요하다는 것을 의미합니다. 주목할만한 점은PP1을1 μM과10 μM으로 처리하면 성장이 현저하게 감소하고HSC-3 세포의 세포자살이 유도된다는 것입니다. 또한, PP1 처리는HSC-3 세포에서Src 및c-Met, EGFR, JAK2, STAT-3, PKB 및 ERK1/2의 인산화 수준을 하향 조절했습니다. 추가적인 약리학적 억제 연구의 결과는 EGFR, JAK2, STAT-3, PKB 및 ERK1/2의 활성또한 HSC-3 세포의 성장에 도움이 된다는 것을보여주었습니다. 또한,

PP1을 이용한 치료는HSC-3 세포에서 높은절단 력을 가지는caspase-9, caspase-8 및PARP 발현을 유발했습니다. PP1 처리는HSC-3 세포의S6 인산화 수준을 감소시키면서eIF-2α 인산화 수준을 더욱 증가시켰습니다. 이러한 결과는Src가HSC-3 세포에서 발현되고 고도로 인산화 된다는 것을나타내며, PP1에 의한Src의 억제는 여러표적의 인산화 및발현 수준을 제어함으로써 매개되는HSC-3 세포에 대한강력한anti-survival 및pro-apoptotic 효과를 초래한다는 것을 나타냅니다.