약학석사학위논문

Resveratrol Suppresses Gastric Cancer Cell Growth and Proliferation through Inhibition PIM-1 Kinase Activity

Resveratrol 의 PIM-1 효소 활성 감소를 통한

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김 수 진

Abstract

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The PIM kinases are serine/threonine kinases composed of three members PIM-1, PIM-2 and PIM-3, and regulate cell cycle progression, apoptosis, cell metabolism, invasion and metastasis. Among these, abnormally elevated kinase activity of PIM-1 contributes to the progression of gastric cancer which is responsible for poor prognosis and low survival. In the present study, a kinase assay was conducted to identify a new molecular target of resveratrol, one of the representative chemopreventive and anticarcinogenic phytochemicals. PIM-1 and PIM-2 kinase activities were markedly inhibited by a relatively low concentration of resveratrol. Using SNU gastric cancer cell lines which were established from Korean gastric cancer patients, a PIM-1 overexpressing cancer cell line (SNU-601) was selected. In a SNU-601 gastric cancer cell line, resveratrol physically binds to the PIM-1 kinase and reduces phosphorylation of Bad which is a well-known substrate of PIM-1. Moreover, resveratrol barely exerts inhibitory effects on the transcription and expression of PIM-1. Resveratrol by inhibiting PIM-1 activity suppresses anchorage-dependent and -independent growth and neoplastic transformation of SNU-601 cell. Likewise, proliferative and colony forming ability of

SNU-601 cells were prominently reduced by knockdown of PIM-1, and the proportion of apoptotic cells was also increased. In conclusion, the inhibitory effects of resveratrol on gastric cancer cell growth and proliferation are likely to be mediated through suppression of PIM-1 kinase activity.

Keywords:

Resveratrol, PIM-1, PIM-2, kinase activity, Gastric cancer

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Introduction

Gastric cancer is the fourth most common malignancy, substantially contributing to cancer-related mortality in many countries (Forman and Burley 2006). In Korea, the incidence of gastric cancer was the second highest next to lung cancer in 2012 (Jung, Won et al. 2015), and is predicted to be the most prevalent in men in 2015 (Jung, Won et al. 2015). As molecular and genetic alterations are complex in the pathogenesis of gastric cancer, it is still difficult to make an early diagnosis for this malignancy, and most of the patients are diagnosed at advanced stages. Despite the improvement in the treatment of gastric cancer, such as surgery, chemotherapy and radiotherapy, these conventional therapies have limited efficacy to reduce the high mortality of gastric cancer (Wu, Nie et al. 2009). Therefore, identification of novel therapeutic targets for the development of new effective anticancer therapies is needed.

Over the past decades, there has been increasing interest in identifying phytochemicals that can halt carcinogenic processes. As conventional cancer therapy has caused substantial adverse effects, one of the most promising approaches to reduce the burden of gastric cancer is considered to be the use of chemopreventive agents (Bertuccio, Chatenoud et al. 2009). It has been reported that intake of chemopreventive agents from diet contributes to a decreased incidence of cancer (Thomasset, Berry et al. 2007, Key 2011). Results from clinical applications also suggest that some bioactive chemopreventive agents have a potential ability to suppress multiple oncogenic steps. Resveratrol (*trans*-3,4',5-trihydroxystilbene) was first isolated from the roots of *Veratrum grandiflorum* O. Loes (white hellebore) and has been found to be abundant in red wine, grapes, berries, peanuts, and in more than 70 other plant species (Baur and Sinclair 2006). Resveratrol has been extensively investigated for its chemopreventive and chemotherapeutic potential as anti-oxidant, anti-inflammatory, anti-cancer, anti-aging, anti-viral, cardiovascular and neuroprotective effects (Kundu and Surh 2008). This phytochemical is well known to exert inhibitory effects on the growth and proliferation of many different types of cancer cells (Cui, Jin et al. 2010, Bjorklund,

Roos et al. 2011). However, the mechanism underlying inhibitory effects of resveratrol on the progression of gastric cancer is not clear.

Recently, a family of PIM kinases have emerged as new targets for cancer therapy. PIM-1 was originally identified by cloning the retroviral integration sites in MMLV-induced lymphomas(Cuypers, Selten et al. 1984, Cuypers, Selten et al. 1986). PIM kinases are short-lived serine/threonine kinases which are composed of PIM-1, PIM-2 and PIM-3 and their functions are overlapping. Among these, PIM-1 has been implicated in pathogenesis of many cancers. PIM-1 is overexpressed in hematological malignancies and solid tumors which is associated with poor prognosis. Hence, PIM-1 is considered as a potential cancer diagnostic marker (Nawijn, Alendar et al. 2011). PIM-1 also appears to be involved in cell cycle regulation (Bachmann, Hennemann et al. 2004), cell survival (Kim, Baird et al. 2005), drug resistance (Xie, Xu et al. 2006, Xie, Xu et al. 2008) and Myc-related tumorigenicity (Zippo, De Robertis et al. 2007, Wang, Anderson et al. 2012). Most representative cellular substrates of the PIM-1 include p21wafl (Wang, Bhattacharya et al. 2002, Zhang, Wang et al. 2007), Bad (Aho, Sandholm et al. 2004) and MDM2 (Hogan, Hutchison et al. 2008) which are regulators of apoptosis and cell cycle progression. One of the mediators of cytokine signaling essential for PIM-1 expression is STAT3. STAT3 can bind directly to the PIM-1 promoter at the ISFR/GAS-sequence and regulates the PIM-1 gene expression (Heinrich, Behrmann et al. 2003). Recently, PIM-1 expression has been reported to correlate with prognosis of gastric cancer, but its potential as a candidate therapeutic target has not been explored in detail.

In the present study, I demonstrated that resveratrol could inhibit PIM-1 kinase activity through direct binding to PIM-1, thereby inhibiting proliferation, and anchorage-independent growth of human gastric cancer cells and inducing apoptosis of these cells, suggesting a therapeutic potential of this phytochemical in the management of gastric cancer.

Materials and Methods

Reagents and antibodies

Resveratrol (5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Roswell Park Memorial Institute (RPMI) 1640 Medium, fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). Primary antibodies for PIM-1, PIM-2 and β-actin were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for PIM-1, caspase-3, cleaved caspase-3, PARP, Bad, phosphor-Bad (Ser112) and cleaved PARP were supplied by Cell Signaling Technology (Danvers, MA, USA). The PIM-1 siRNA (5'- CCGGTGCAAGATCTCTTCGACTTCA -3') and RNAi negative control duplexes were purchased from Genolution Pharmaceuticals, Inc (Seoul, Korea). MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)] was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bicinchoninic acid (BCA) protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL, USA). Polyvinylidene difluoride (PVDF) membrane was supplied from Gelman Laboratory (Ann Arbor, MI, USA). RNAi-MAX and Lipofectamine 3000 were products of Invitrogen (Carlsbad, CA, USA). Crystal violet was obtained from AMRESCO (Solon, OH, USA).

Cell line

Human gastric cancer cell lines (SNU-216, SNU-484, SNU-601, SNU-638 and SNU-668) were purchased from the Korean Cell Line Bank and cultured in monolayers at 37°C, 5% CO_2 by using RPMI 1640 supplemented with 10% FBS and 1% antibiotic-antimycotic. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Transient transfection of small interfering RNA (siRNA) and expression vectors

Cells at a confluence of 60-70% were transfected with control siRNA (40 μM) or PIM-1 siRNA (40 μM) with lipofectamine RNAi-MAX reagent according to the manufacturer's instructions. After 96-hour transfection, cell lysates were prepared as described in Western bolt analysis.

Cell-based resveratrol competition assays

CNBr-activated Sepharose™ 4B was obtained from GE Healthcare (Little Chalfont, United Kingdom). Cells were lysed by using lysis buffer [50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.01% Nonidet P-40, 0.02 mM phenylmethylsufonylfluoride (PMSF), and 1x protease inhibitor mixture]. After centrifugation at 15,000 x *g*, the resulting supernatant (500μg) was incubated with 100 μL resveratrol-Sepharose 4B beads or Sepharose 4B beads alone as a control in reaction buffer [50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 mg/mL bovine serum albumin (BSA), 0.02 mM PMSF, and 1x protease inhibitor mixture]. These samples were washed five times with washing buffer [50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF] and detected by Western blotting.

Cell proliferation assay

Cell were cultured for 96 hours in the presence of various concentrations of resveratrol dissolved in DMSO. After 96-hour incubation, MTT dissolved in PBS was treated at a final concentration of 0.5 mg/mL for 4 hours. Medium was removed, and DMSO was added to fuse formazan crystal. The absorbance was read at 570 nm.

Western blot analysis

Cells were gently washed with cold PBS, scraped and centrifuged at 15,000 x *g* for 5 min. Pellets were suspended in lysis buffer and freezing and thawing were repeated three times using liquid nitrogen, followed by centrifugation at 21,000 x *g* for 15 min. Supernatant was collected as whole cell lysate. The protein concentration of whole cell lysates was determined by using the BCA protein assay kit. Protein samples from whole

cell lysates were mixed with sodium dodecyl sulfate (SDS) sample loading dye and boiled at 99°C for 5 min. Protein samples were electrophoresed on SDS-polyacrylamide gel and transferred to PVDF membranes. The blots were then blocked with 5% fat-free dry milk- TBST (Tris-based saline containing 0.1% Tween-20) buffer for 1 hour at room temperature. The blots were incubated with primary antibodies in TBST. Following three washes with TBST, the blots were incubated with rabbit or mouse secondary antibodies in 3% fat-free dry milk-TBST for 1 hour at room temperature. The blots were rinsed again three times with TBST, and the transferred proteins were incubated with the ECL substrate detection reagent for 1 min according to the manufacturer's instructions and visualized with LAS 4000.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To generate cDNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) for 50 min at 42 ℃ and again for 15 min at 72 ℃. One μl of cDNA was amplified in sequential reactions using Maxtime PCR PreMix Kit (iNtRON Biotechnology). The mRNA expression of PIM-1 (28 cycles of 94 ℃ for 30 sec, 48 ℃ for 30sec, and 72 ℃ for 30 sec) gapdh (20 cycles of 94 ℃ for 30 sec, 55 ℃ for 30 sec, and 72 ℃ for 30 sec) was examined. These PCR cycles were followed by final extension for 7-min at 72 ℃. The primers used for each RT-PCR reactions are as follows (forward and reverse, respectively):

PIM-1, 5'-ATC CTT ATC GAC CTC AAT CG-3' and 5'-TGA TGA TCT CTT CGT CAT GC -3'; gapdh, 5'-CCG AGA TGG GGT TGA TAA TG -3' and 5'-ACA GTG GCC ACC TAC AAA GG -3'. Amplification products were resolved by 2 % agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

Annexin Ⅴ and propidium iodide (PI) staining

Cells were washed with cold PBS and centrifuged at 1,500 x *g* for 4 min three times. Supernatant was aspirated and cells were re-suspended in 1 mL of 1x annexin Ⅴ binding buffer. 5 μL of Annexin Ⅴ-FITC and 5 μL of PI were added to the cells and incubated 15 min at room temperature in the dark. The 1x binding buffer was added and analyzed by FACS caliber.

Anchorage-independent growth assay

Anchorage-independent cell transformation was performed in SNU-601 cells. Briefly, cells (8×10^3 cells/well) suspended in RPMI1640 media containing 10% FBS were added to 0.3% agar with or without indicated doses of resveratrol. The cultures were maintained at 37°C in a 5% CO_2 incubator for 2 weeks, and the cell colonies were scored using an ECLIPSE Ti inverted microscope and the NIS-Elements AR (V. 4.0) computer software program (NIKON Instruments Korea, Seoul, Korea) as described previously (Colburn, Wendel et al. 1981).

Clonogenic assay

Cells were counted and plated 500 cells per dish in 6-mm cell culture dishes. After 7–10 days, the cells were fixed in cold ethanol for 30 min and stained by 0.5% crystal violet for 30 min. Stained cells were rinsed by tap water and dried in room temperature.

Immunocytochemistry

Cells were rinsed with PBS, and fixed for 10 min at 4 °C with 5% acetic acid in methanol, followed by blocking with 5% BSA in PBS for 30 min in room temperature. Cells were incubated with PIM-1 antibody diluted 1:200 in blocking buffer at 4°C. After washing with PBS, cells were incubated with FITC conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody diluted at 1:1000 for 1 hour and examined under a fluorescent microscope.

Statistical analysis

When necessary, data were expressed as means of ± SD of at least three independent experiments, and statistical analysis for single comparison was performed using the Student's *t*-test. The criterion for statistical significance was * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

PIM-1 and PIM-2 kinases activities are inhibited by resveratrol

To verify the possible targets of resveratrol for its chemopreventive and anti-carcinogenic effects, cancer-related 395 kinase profiles provided by DiscoveRX were screened with a single concentration (20 μM) of resveratrol treatment. The activities of PIM-1 and PIM-2 were significantly inhibited compared with other kinases (data are not shown). The half maximal inhibitory concentrations (IC_{50}) of PIM-1 and PIM-2 were determined. PIM-1 and PIM-2 kinases activities were markedly reduced in the presence of resveratrol in a dose-dependent manner. The IC_{50} values of resveratrol to inhibit PIM-1 kinase and PIM-2 kinase were 1.4 μM and 0.5 μM, respectively **(Fig. 1A)**. Two independent experiments of the kinase assay were conducted, and each point represents an average value.

PIM-1 is overexpressed at cytoplasm in gastric cancer cell lines

To check the expression levels of PIM-1 and PIM-2 in various gastric cancer cell lines, five different SNU gastric cancer cell lines were selected which were established from Korean cancer patients. SNU-216 and SNU-601 had relatively higher levels of PIM-1 than SNU-484, SNU-638 and SNU-668, and PIM-2 kinase was not detected in these cell lines **(Fig. 2A)**. It has been reported that PIM-1 is mainly expressed in nucleus and cell membranes (Merkel, Meggers et al. 2012). SNU-601 cells were selected to assess the subcellular localization of PIM-1 kinase by immunocytochemistry. Interestingly, PIM-1 kinase was considerably overexpressed not only in nucleus and cell membrane but also in cytoplasm.

PIM-1 kinase silencing strongly inhibits cell proliferation and induces apoptosis in SNU-601 cells

As PIM-1 is known to play a role in the phosphorylation of many cell cycle regulators and apoptosis-related proteins (Fedorov, Muller et al. 2010), its effects on proliferation and apoptosis of gastric cancer cells were checked. For this purpose, the

MTT assay was conducted in SNU-601 cells transfected with siRNA against PIM-1. As a result, cell viability was significantly reduced compared with control in 96 hours **(Fig. 3A)**. SNU-601 cells were transfected with PIM-1 siRNA (40 nM) for the clonogenic assay. Knockdown of PIM-1 significantly suppressed anchorage-dependent cell proliferation and colony formation **(Fig. 3B)**. The effect of PIM-1 knockdown on apoptosis was determined by Annexin V and propidium iodide (PI) staining followed by flow cytometry. The proportion of apoptotic cells increased markedly **(Fig. 3C)**, and level of apoptotic markers, such as cleaved caspase-3 and poly ADP ribose polymerase (PARP) were significantly elevated **(Fig. 3D)**. Thus, it is evident that PIM-1 also contributes to cell proliferation and survival of SNU-601 cells.

Resveratrol suppresses gastric cancer cell growth

As shown in **Fig. 4**, SNU-216, SNU-601 and SNU-668 cells were treated with resveratrol (25 μM, 50 μM and 100 μM) exhibited decreased cell proliferation. The IC_{50} values of resveratrol in inhibiting the proliferation of these cell lines were 55.41 μM in SNU-216, 30.14 μM in SNU-601 and 87.17 μM in SNU-668. Interestingly, PIM-1 deficient SNU-668 gastric cancer cells were resistant to the inhibitory effect of resveratrol **(Fig. 4C)**. This finding suggests that the anti-cancer effect of resveratrol can be partially dependent on PIM-1 expression.

To further investigate the anti-carcinogenic activity of resveratrol, we measured the effect of resveratrol on anchorage-independent and anchorage-dependent growth in SNU-601 gastric cancer cells which overexpress PIM-1. Cells were treated with different concentration of resveratrol (25 μM, 50 μM and 100 μM). Resveratrol inhibited the growth of SNU-601 cells as revealed by the reduced number of colonies **(Fig. 5A and 5B)**. The number of colonies was counted under a microscope at six independent spots per well. Treatment of SNU-601 cells with 50 μM of resveratrol markedly enhanced levels of apoptosis markers, cleaved caspase-3 and cleaved PARP **(Fig. 6A)** and also increased the proportion of apoptotic cells **(Fig. 6B)**. Taken together, these data indicate that resveratrol exerts inhibitory effects on gastric cancer cell growth and proliferation through induction of apoptosis that mimic the effect of PIM-1 silencing.

Resveratrol binds to PIM-1 and inhibits phosphorylation of Bad at Serine 112

To determine whether resveratrol inhibits PIM-1 kinase activity and/or expression SNU-601 cells were treated with resveratrol (25 μM and 50 μM) for 12 or 24 hours. The mRNA and protein levels of PIM-1 did not show marked differences, and quantification data of Western blot analysis also show no statistical significance in SNU-601 cells **(Fig.7A – D)**. To verify physical binding between PIM-1 kinase and resveratrol in SNU-601 cancer cells, the Sepharose 4B assay was performed. Whole lysate of SNU-601 cells was precipitated with resveratrol-conjugated Sepharose 4B beads or Sepharose 4B beads alone. PIM-1 directly binds to resveratrol-conjugated Sepharose 4B beads and not to resveratrol-unconjugated 4B beads **(Fig. 7E)**. Thus, it is conceivable that PIM-1 and resveratrol physically interact each other in SNU-601 cells. Bad is known to be a direct phosphorylation target of PIM-1, especially at serine 112(Aho, Sandholm et al. 2004). The whole lysate of SNU-601 treated with a single dose (50 μM) of resveratrol showed reduced phosphorylation of Bad at serine 112 **(Fig. 7F)** which accounts for functional activation of this proapoptotic protein.

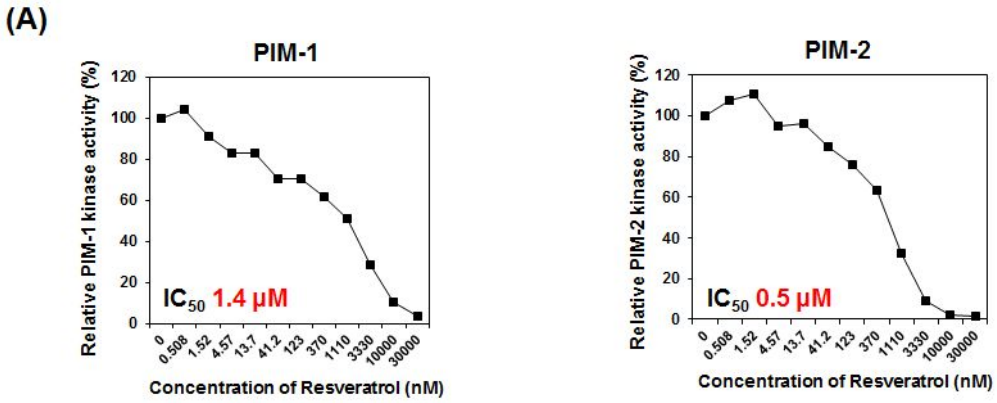

Figure 1. Resveratrol inhibits PIM kinase activity. (A) Data represent the average value of two independent experiments and the half maximal inhibitory concentration (1.4 µM) of resveratrol to inhibit PIM-1 kinase activity. (B) Resveratrol inhibits PIM-2 kinase activity in a concentration-dependent manner with the maximal inhibitory concentration of 0.5 µM.

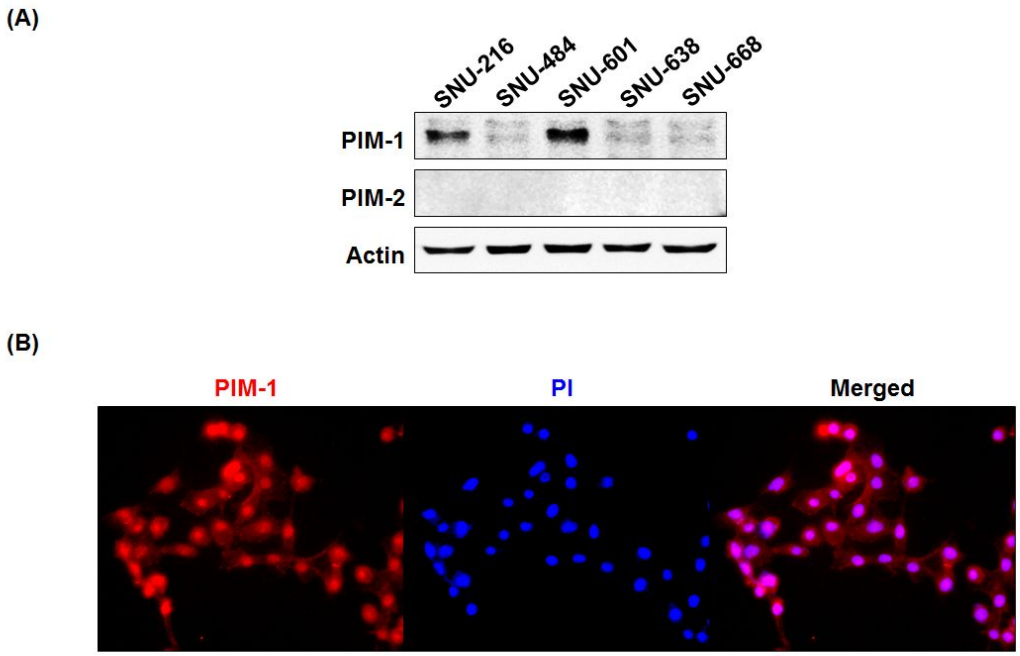

Figure 2. Expression level of PIM-1 and its subcellular localization in the SNU-601 human gastric cancer cell line. (A) Basal levels of PIM-1 and PIM-2 were evaluated in protein lysates from human gastric cancer cell lines; SNU-216, SNU-484, SNU-601, SNU-638 and SNU-668. (B) Subcellular localization of PIM-1 kinase in SNU-601 cell line was assessed by immunocytochemistry.

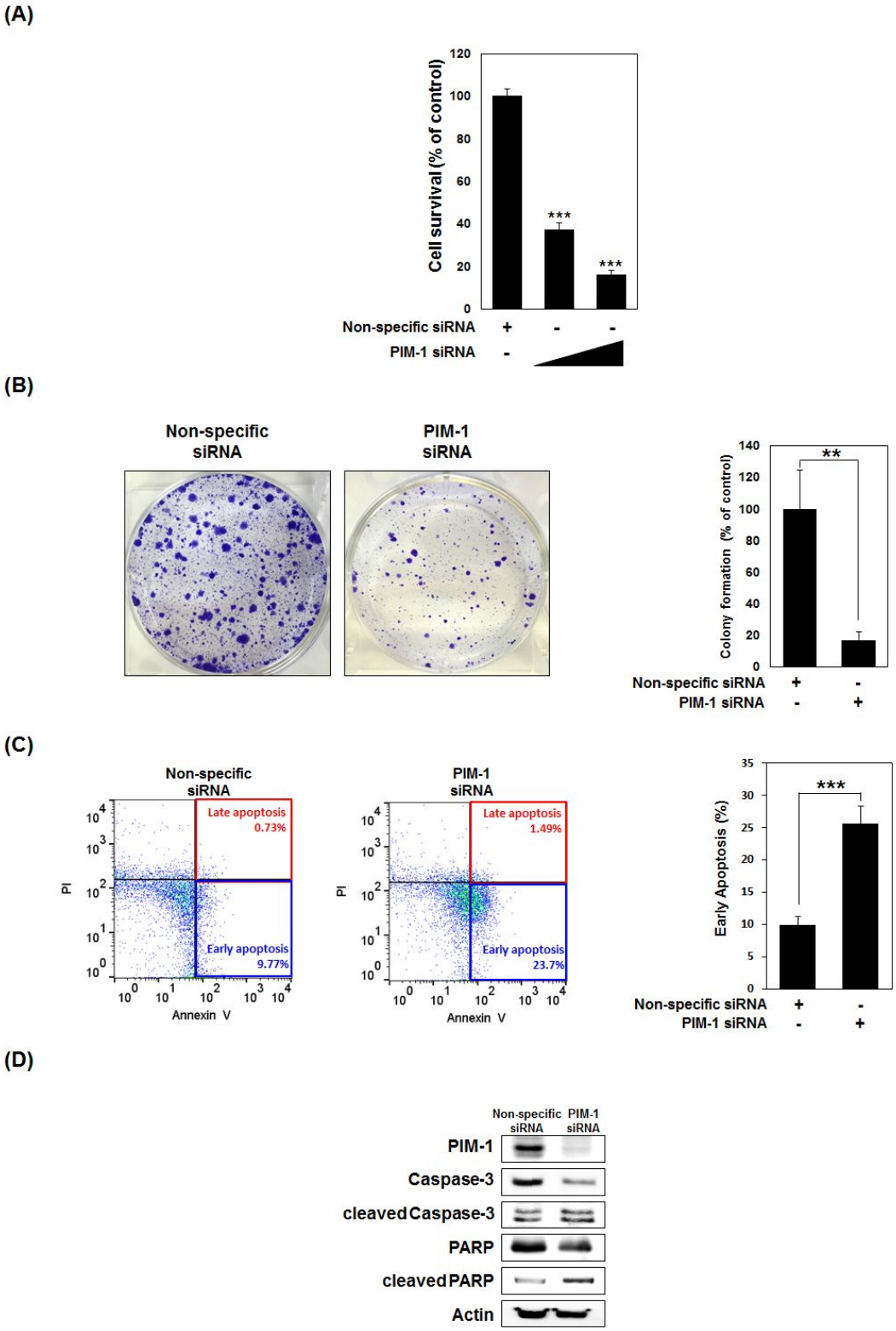

Figure 3. Knockdown of PIM-1 inhibits cell proliferation and induces apoptosis in SNU-601 cells. (A) After SNU-601 cells were seeded into 48-well plates and after 24 hours, they were transfected with PIM-1 siRNA (5 nM and 40 nM) or non-specific siRNA according to RNAi-MAX manufacturer's instructions. (B) SNU-601 cells were seeded into 6-well plates at a density of 0.5 x 10^4 per well. On the next day, PIM-1

siRNA and non-specific siRNA were transfected. After 2 weeks, cells were stained with 0.5% crystal violet. (C) Flow cytometric dot plots represent changes in the proportion of apototic cells. Knockdown of PIM-1 enhances expression of apoptosis-related proteins in the SNU-601 cancer cell line. Cells were transfected with PIM-1 siRNA for 72 hours and harvested. (D) Immunoblotting was conducted using apoptotic marker antibodies. The asterisks show differences between contorl and PIM-1 silenced groups. All data represent means ± SD (n=3) (**, $P < 0.01$; ***, $P < 0.001$)

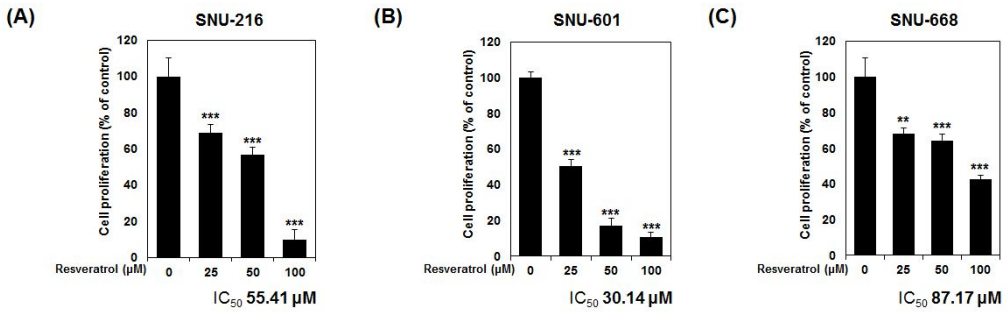

Figure 4. Resveratrol inhibits proliferation of the human gastric cancer cells. SNU-216 (A), SNU-601 (B) and SNU-668 (C) cells were seeded in 96-well plate. After 24 hours, cells were treated with resveratrol (0 μM, 25 μM, 50 μM and 100 μM) for 96 hours. Cell viablity was measured by the MTT assay. The 50% inhibitory concentration of cell proliferatrion in SNU-216, SNU-601 and SNU-668 were 55.41 μM, 30.14 μM, and 87.17 μM, respectively. The asterisks show differences between untreated and resveratrol treated groups. All data represent means ± SD (n=3) (**, $P < 0.01$; ***, $P < 0.001$)

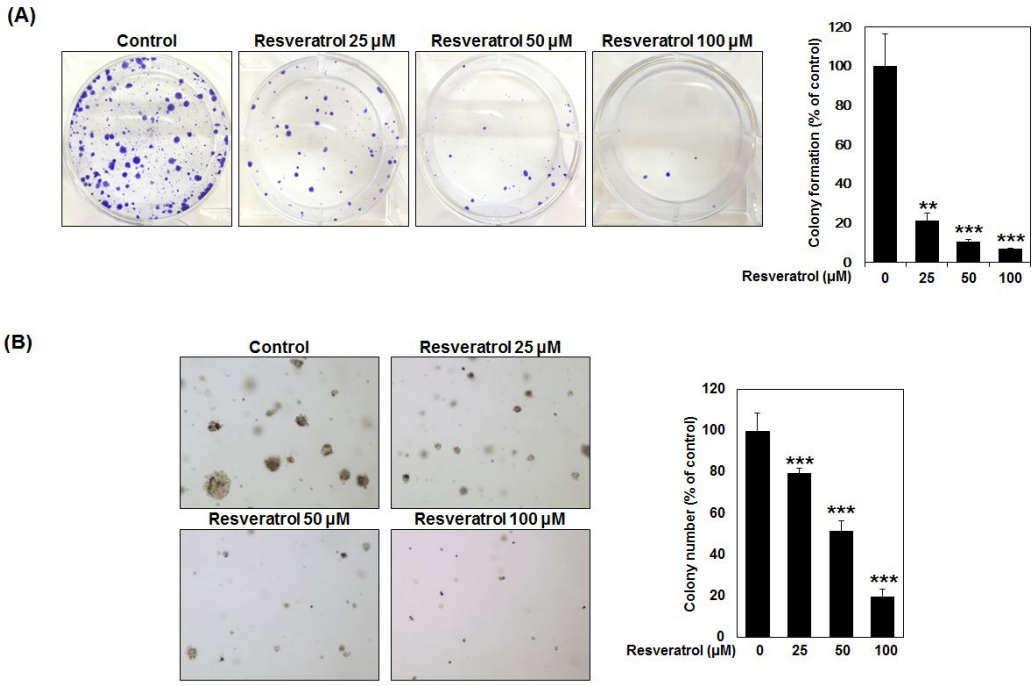

Figure 5. Resveratrol suppresses anchorage-dependent and anchorage-independent growth of SNU-601 cells. (A) SNU-601 cells were seeded and after 24 hours, resveratrol was treated at 0 μM, 25 μM, 50 μM and 100 μM for 2 weaks. (B) The 6-well plate was coated with 0.3% bottom agar with resveratrol and SNU-601 cells were suspended with 0.3% top agar at 8 x 10^3 cells per well. After 2 weeks, the cell colonies were scored using an ECLIPSE Ti inverted microscope and the NIS-Elements AR (V. 4.0) computer software program. The asterisks showed differences between untreated and resveratrol treated groups. All data represent means ± SD (n=3) (**, $P < 0.01$; ***, $P < 0.001$)

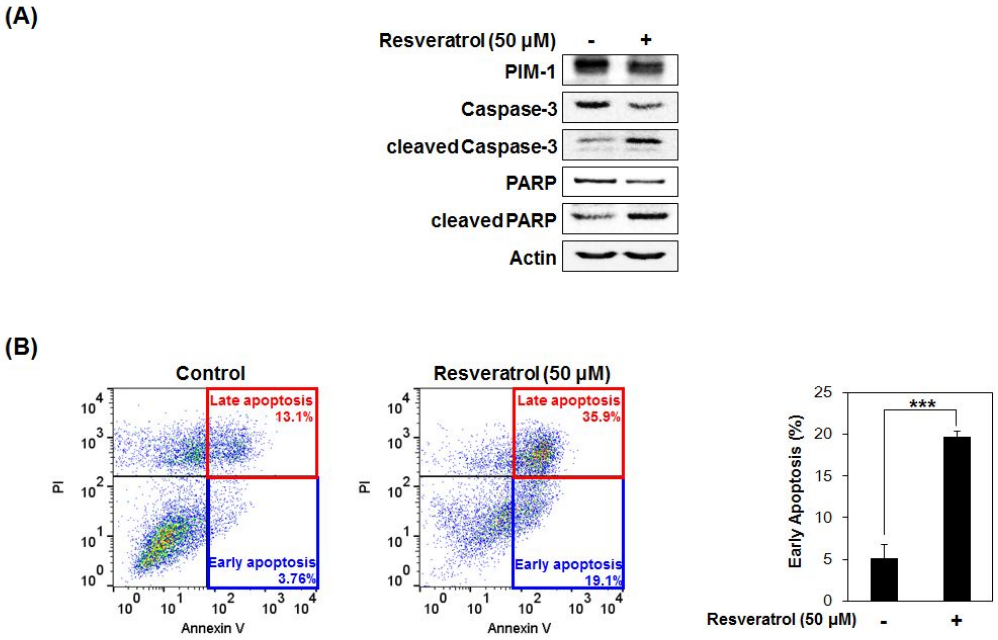

Figure 6. Resveratrol induces apoptosis and activates apoptotic signaling in SNU-601 gastric cancer cells. (A) Whole lysates from SNU-601 cells treated with resveratrol 50 μM were subjected to Western blot analysis. (B) SNU-601 cells were seeded into 6-well plates and treated with resveratrol (50 μM) for 48 hours and harvested. After propidium iodide (PI) and Annexin Ⅴ staining, cells were analyzed by the FACS Calibur Flow Cytometer. Red box represents late apoptotic cells and blue box early apoptotic cells. The asterisks denote differences between untreated and resveratrol treated groups. All data represent means ± SD (n=3) (***, $P < 0.001$)

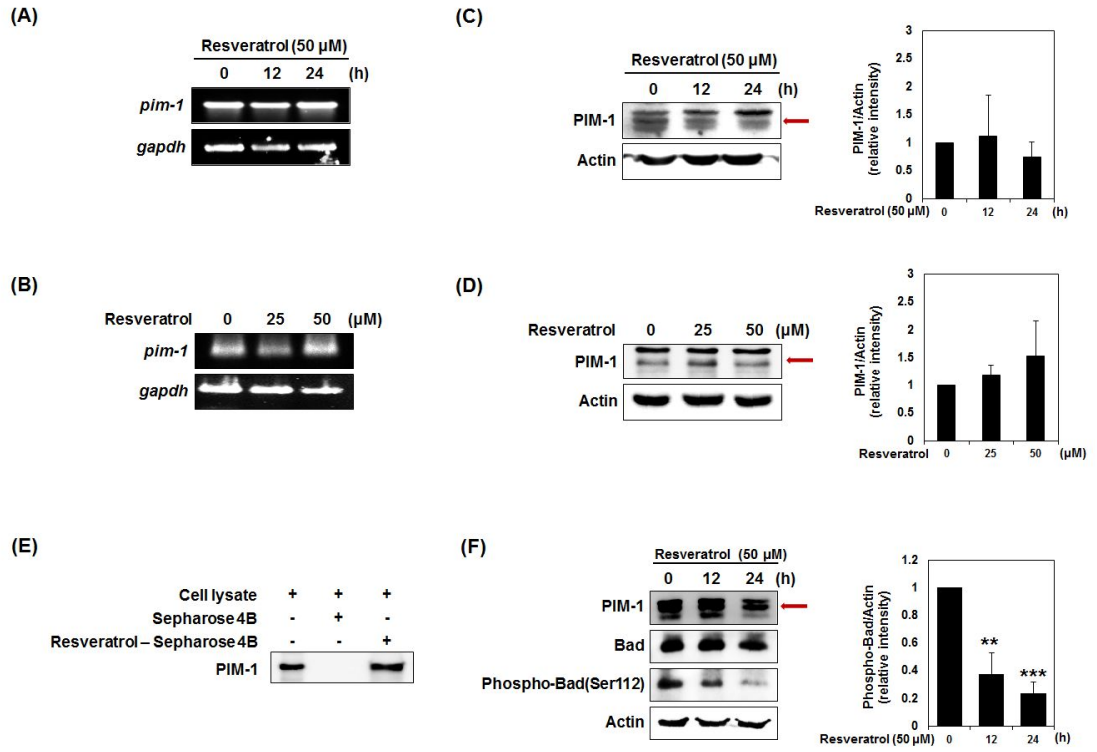

Figure 7. Resveratrol inhibits PIM-1 activity with direct binding. SNU-601 cells were treated with 50 μM of resveratrol for 12 hours and 24 hours (A) and with resveratrol at 25 μM and 50 μM for 24 hours (B). The expression of PIM-1 was determined by semi-quantitative RT-PCR. GADPH was measured to ensure equal amounts of cDNA loaded. (C and D) The protein level of Pim-1 was determined by immunoblotting analysis. Actin was used as an equal loading control for normalization. Both quantification graphs did not show stastical significance between contol and resveratrol treated groups. (E) Resveratrol directly bound to PIM-1 as determined by immunoblotting with an antibody against PIM-1 in the SNU-601 gastric cancer cell line. Lane 1 represents the input control and lane 2 negative control cell lysates precipitated with Sepharose 4B beads only. In lane 3, SNU-601 cell lysates were precipitated using resveratrol-Sepharose 4B beads. (F) Resveratrol (50 μM) inhibits Bad phosphorylation in SNU-601 cells which is one of the major substrates of Pim-1 kinase. All data represent means ± SD (n=3) (**, $P < 0.01$; ***, $P < 0.001$)

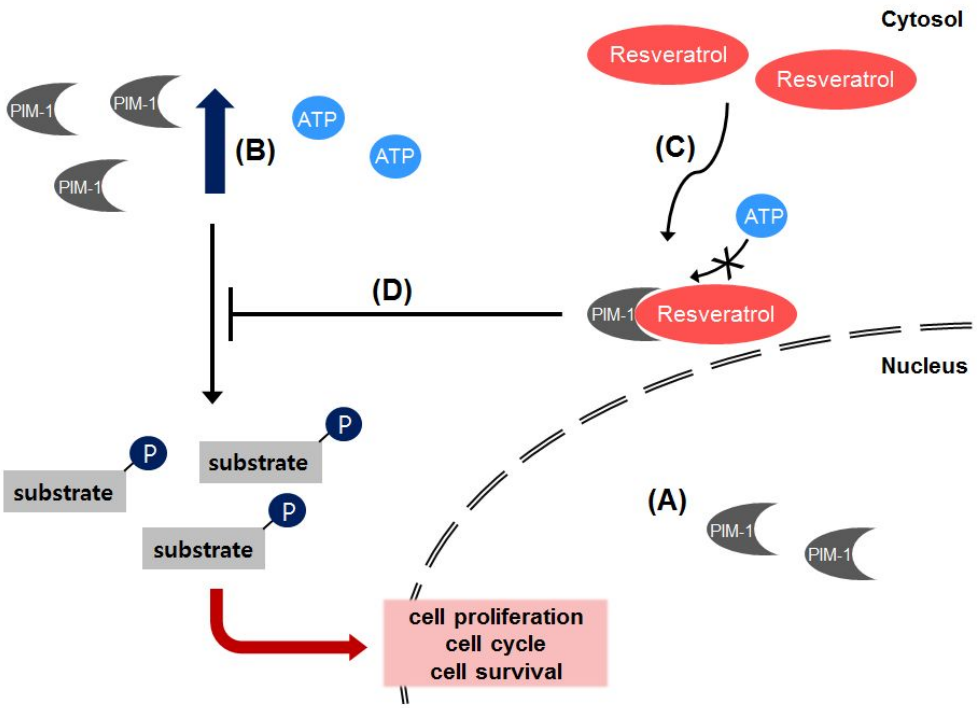

Figure 8. Schematic representation of resveratrol targeting PIM-1 kinase in gastric cancer cells. (A) In the normal cell, PIM-1 kinase is located in nucleus and cell membrane. (B) PIM-1 kinase is overexpressed in cancer cells such as the SNU-601 cell line, and regulate cancer cell growth through phosphorylation of substrates related with cell cycle and apoptosis such as Bad. (C) Resveratrol binds to the PIM-1 active site and hinders ATP binding. (D) Eventually, resveratrol inhibits cell growth by reducing the PIM-1 kinase activity.

Discussion

Kinases have been considered as potential targets for anti-cancer therapy as they are frequently mutated in many different types of human tumors (Fedorov, Muller et al. 2010, Knight, Lin et al. 2010). PIM-1 is one such kinase that has attracted much attention for the anti-cancer drug development. In a wide range of human tumors of both hematopoietic and epithelial origins, PIM-1 kinase is frequently overexpressed. Especially, overexpression of PIM-1 in gastric tumor is responsible for poor prognosis and a low survival rate (Warnecke-Eberz, Bollschweiler et al. 2009, Yan, Yau et al. 2012). Although many molecular inhibitors of this enzyme have been discovered, there are some obstacles such as drug toxicity and safety which hamper their clinical use for cancer therapy (Merkel, Meggers et al. 2012). In this study, I found that the kinase activities of PIM-1 and PIM-2 were decreased by resveratrol treatment. Resveratrol inhibited proliferation of several gastric cancer cell lines. Moreover, resveratrol significantly suppressed survival, proliferation and colony formation in the PIM-1 overexpressing SNU-601 gastric cancer cell line. In addition, inhibition of PIM-1 by resveratrol shows a similar pattern to that for inhibition of PIM-1 by siRNA.

Resveratrol has been extensively studied with regards to its suppressive effect on cancer and inflammation. Resveratrol inhibits Src and STAT3 signaling in malignant cells, resulting in cycle arrest and loss of viability (Kotha, Sekharam et al. 2006) and suppresses constitutively active NF-κB through inhibition of IκBα kinase and phosphorylation of IκBα and p65 (Bhardwaj, Sethi et al. 2007). In another study, resveratrol blocked the ubiquitination of NEMO and inhibited IκB kinase β-mediated NF-κB activation (Ren, Wang et al. 2013). I speculated that suppression of upper signaling which regulates PIM-1 expression and direct inhibition of PIM-1 kinase may provoke a synergistic effect in inhibition of PIM-1 kinase.

PIM-1 utilizes many kinds of cellular substrates which are involved in regulating cell cycle, apoptosis, cell metabolism, etc. Well known substrates are p21waf1, Bcl-2-associated death promoter (Bad), Mouse double minute 2 homolog (MDM2), and Myc (Blanco-Aparicio and Carnero 2013). A recent study has shown that PIM-1 kinase has an effect on tumorigenicity of Myc. PIM-1 forms a complex with Myc and MAX and

phosphorylates Ser10 of histone H3 on the nucleosome. This increases transcriptional activity of Myc (Zippo, De Robertis et al. 2007, Wang, Anderson et al. 2012). PIM-1 also phosphorylates Thr145 of p21waf1, thereby decreasing stability and nuclear translocation of the cell cycle inhibitory protein (Wang, Bhattacharya et al. 2002, Zhang, Wang et al. 2007). Furthermore, phosphorylation of MDM2 at Ser116 and Ser186 hampers its degradation, leading to accumulation of p53 (Hogan, Hutchison et al. 2008). In addition, phosphorylation of Bad at Ser112 induces proteasomal degradation of this pro-apoptotic protein and cell survival (Aho, Sandholm et al. 2004). As a result, this enhances senescence-associated β-galactosidase levels, which is associated with the induction of senescence (Hogan, Hutchison et al. 2008). Because Bad is directly phosphorylated by PIM-1, I measured the phosphorylation level of Bad, which is representative of PIM-1 activity. Resveratrol treatment inhibited the phosphorylation of Bad and did not alter the expression level of Bad in SNU-601 gastric cancer cells. The further elucidation of other PIM-1 substrates will help confirm the effect of resveratrol in gastric cancer.

Because PIM-1 is overexpressed in many cancers and involved in cancer specific pathways, it represents a potential target for anti-cancer drug development. Indeed, many PIM-1 kinase inhibitors have been developed targeting ATP competitive binding (Merkel, Meggers et al. 2012). The most prominent inhibitor of PIM-1 is SGI-1776 which suppresses all PIM kinase family members at low concentrations by occupying the ATP binding site. SGI-1776 induces G1 arrest and apoptosis and reduces cell viability and chemo resistant (Chen, Redkar et al. 2011, Blanco-Aparicio and Carnero 2013). Nevertheless, the clinical development of the compound was failed due to cardiac toxicity during a phase I trial (Batra, Maris et al. 2012). Thus, natural compounds are preferred to chemical compound because of the relatively low toxicity and side effects. In this contexts, resveratrol derived from the food resources like red wine, grapes, berries and peanuts is of particular interest.

In summary, the results from this study provide new insights into the biological and molecular mechanisms responsible for the antitumor effect of resveratrol. Resveratrol inhibits PIM-1 activity through direct binding to this kinase, thereby regulating the

functional activities of PIM-1 substrates involved in gastric cancer cell growth and proliferation.

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국문초록

PIM-1 인산화 효소는 Serine/threonine 인산화 효소로서 PIM-1, PIM-2 그리고 PIM-3 의 세가지 종류가 있으며, 세포주기의 진행과 세포사멸, 대사, 암의 침윤과 전이를 조절한다. 이 중에서 향상된 PIM-1 인산화 효소의 활성은 인체 위암의 진행에 기여하며 암 환자들의 나쁜 예후와 낮은 생존율에 영향을 주는 것으로 알려져 있다. 본 연구에서는 식물화학물질(phytochemical) 중에서 암 예방과 항암 효과를 가지는 레스베라트롤(resveratrol)의 새로운 표적을 인산화 효소 스크리닝을 통하여 밝혀내었다. 그 결과 PIM-1 과 PIM-2 의 효소 활성이 낮은 농도의 레스베라트롤 처리에 의에 현저히 감소하였다. 이에 한국인 위암 환자 세포에서 수립된 SNU 위암 세포 주를 사용하여 PIM-1 과 PIM-2 의 발현 정도를 확인하였으며, 그 중 SNU-601 세포주에서 PIM-1 의 발현이 현저하게 증가되어 있는 것을 관찰하였다. 이 세포주에서 레스베라트롤은 PIM-1 의 발현과 전사에는 영향을 주지 않으나 PIM-1 인산화 효소의 잘 알려진 기질인 Bad 단백질의 인산화를 억제 함을 관찰하였다. 레스베라트롤은 PIM-1 인산화 효소를 저해함으로써 SNU-601 세포주의 부착의존적 성장과 부착비의존적 성장을 저해하고 세포사멸을 촉진함으로써 종양으로의 변화를 억제하였다. 또한 레스베라트롤의 처리시 이 세포주의 증식과 콜로니(colony) 형성능력이 PIM-1 발현의 저해로 현저히 억제되었고, caspase-3 와 PARP 의 활성화를 통해 아폽토시스에 의한 세포 사멸 비율이 증가됨을 관찰하였다. 결론적으로, 인체 위암세포 주에서의 레스베라트롤의 성장 억제 및 세포사멸 효과는 PIM-1 인산화 효소의 활성 저해와 관련 있으며 이를 레스베라트롤의 암 예방과 항암 효과의 새로운 기전으로 제시하고자 한다.

주요어: 레스베라트롤, PIM-1, PIM-2, 효소 활성, 위암

학 번: 2014-24813

Appendix

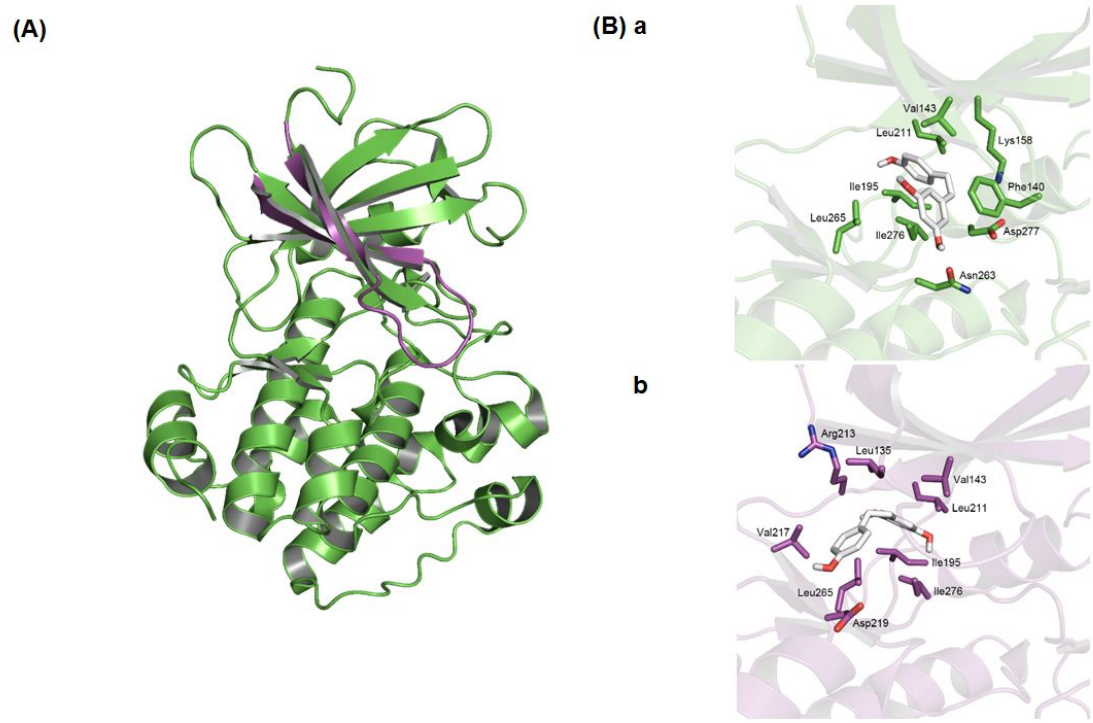
Result

Resveratrol interacts with PIM-1 kinase at the ATP-binding site.

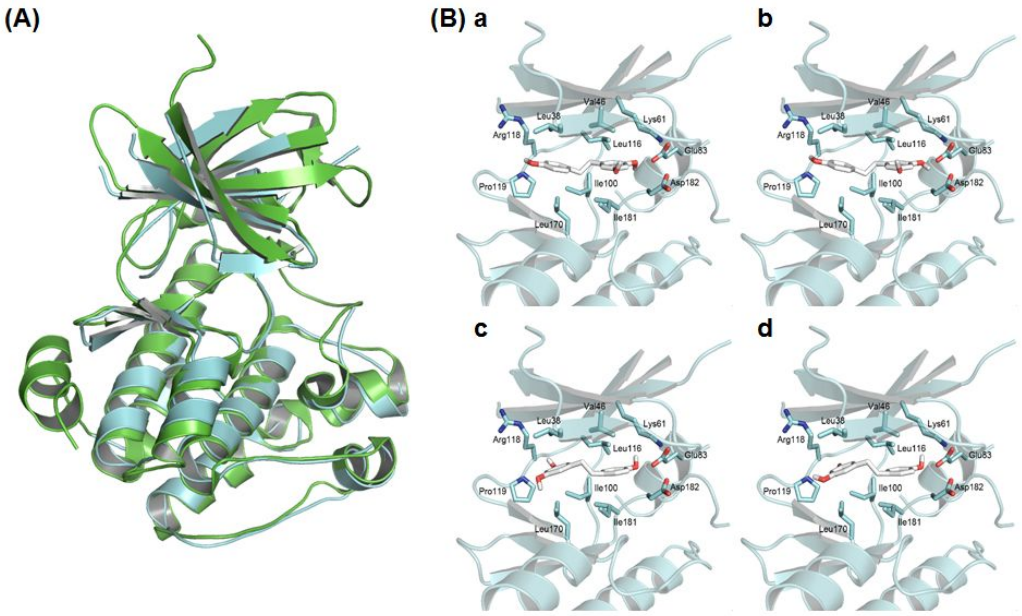
To gain further insights into the molecular interaction between resveratrol and PIM-1, docking experiments were implemented with focus on the active site of the protein. The structure of PIM-1 alone and in complex with AMPPNP were selected as a docking template because their crystal structures showed a significant difference in a loop enveloping the active site **(Appendix Fig. 1A)**. Control docking experiments were executed with AMPPNP to the crystal structures of PIM-1 after AMPPNP was removed from the complex structure. It was found from docking experiments that the ligand resveratrol was located in the active site **(Appendix Fig. 1Ba and b)** with stabilization energy lower than that of AMPPNP. The stabilization energies of predicted resveratrol binding modes on apo-PIM-1 and AMPPNP-bound PIM-1 were calculated to be -7.9 and -7.1 kcal/mol, respectively, while the value for AMPPNP was -7.0 kcal/mol. These data suggest that resveratrol binds to the PIM-1 ATP-binding pocket.

Resveratrol directly binds to the PIM-2 ATP-binding pocket.

Docking experiments were also performed to determine interaction between PIM-2 and resveratrol. Apo-PIM-1 (green) and 3YR-bound PIM-2 (cyan) have very similar structures as illustrated in **Appendix Fig. 2A**. The control docking experiment was conducted and the result showed that the 3YR molecule took position in original binding site of PIM-2 **(Appendix Fig. 2B)**. Binding affinity of 3YR to PIM-2 from the control docking experiment is -10.3 kcal/mol. There were four binding sites of resveratrol to PIM-2 in the order of decreasing binding affinity (Appendix Fig. 2B a, -8.0 kcal/mol ; b, -8.0 kcal/mol ; c, -7.9 kcal/mol ; d, -7.8 kcal/mol) Resveratrol was located in the ATP-binding pocket of PIM-2. The pocket is mainly hydrophobic, well-suited for harboring the resveratrol molecule. This finding indicates that resveratrol is a candidate therapeutic drug for PIM-2 inhibition.



Appendix Figure 1. Resveratrol binds to the PIM-1 ATP-binding pocket. (A) Stereographic representation of apo-PIM-1 alone (green) and in complex with AMPPNP (magenta). Stereo-view presentations simulating docking poses of resveratrol to apo-PIM1 (Ba) and AMPPNP-bound PIM1 (Bb). Resveratrol bond to PIM-1 as illustrated using a stick model with carbon and oxygen atoms in white and red, respectively. Amino acid residues interacting with the resveratrol are shown as a stick model with nitrogen and oxygen atoms coloured as blue and red, respectively.



Appendix Figure 2. Resveratrol directly binds to the PIM-2 ATP-binding pocket. (A) Stereographic representation of apo-PIM-1 alone (green) and 3YR-bound PIM-2 (cyan). (B)

The control docking simulation shows that the docked 3YR molecule (shown as a stick model) is located at its original binding site (shown as spheres), with virtually no difference in its original binding mode. (C) Four binding models in the order of decreasing binding affinity are shown (a-d).

Method

Docking simulation

AutoDock Vina program [1] (The Scripps Research Institute, CA, USA) was used in the docking studies. Initial structures of Apo-PIM-1 and AMPPNP-bound PIM-1 [2] were obtained from the Protein Data Bank (PDB) (PDB ID: 1XQZ and PDB ID: 1XR1, respectively) and coordinates for the resveratrol was generated using the GlycoBioChem PRODRG2 Server (http://davapc1.bioch.dundee.ac.uk/prodrg/) [3]. The grid maps for docking studies were centered on the ATP binding site and comprised 60 X 60 X 60 points with 1.0 Å spacing after AMPPNP was removed from the complex structure, AutoDock Vina program was run with eight-way multithreading and the other parameters were as default settings in AutoDock Vina program. And this assay kindly supported by Hyunggu Hahn in Structure Pharmacy Laboratory.

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