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Resveratrol suppresses gastric cancer cell proliferation and survival through inhibition of PIM-1 kinase activity

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ABSTRACT

The proviral integration site for Moloney murine leukemia virus (PIM) family of serine/threonine-specific kinases consist of three isoforms, that regulate proliferation, apoptosis, metabolism, invasion, and metastasis of cancer cells. Among these, abnormally elevated kinase activity of PIM-1 contributes to the progression of gastric cancer and predicts poor prognosis and a low survival rate in gastric cancer patients. In the present study, we found that resveratrol, one of the representative chemopreventive and anticarcinogenic phytochemicals, directly binds to PIM-1 and thereby inhibits its catalytic activity in human gastric cancer SNU-601 cells. This resulted in suppression of phosphorylation of the proapoptotic Bad, a known substrate of PIM-1. Resveratrol, by inactivating PIM-1, also inhibited anchorage-independent growth and proliferation of SNU-601 cells. To understand the molecular interaction between resveratrol and PIM-1, we conducted docking simulation and found that resveratrol directly binds to the PIM-1 at the ATP-binding pocket. In conclusion, the proapototic and anti-proliferative effects of resveratrol in gastric cancer cells are likely to be mediated through suppression of PIM-1 kinase activity, which may represent a novel mechanism underlying its chemopreventive and anticarcinogenic actions.

1. Introduction

Gastric cancer is the fourth most common malignancy, substantially contributing to cancer-related mortality in many countries [1]. In Korea, gastric cancer was the most commonly diagnosed cancer in 2016 [2], and is predicted to be the second prevalent in men in 2019 [3]. As molecular and genetic alterations are complex in the pathogenesis of gastric cancer, it is still difficult to make an early diagnosis, 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 in reducing the mortality of this malignancy [4].

Over the past decades, there has been increasing interest in identifying phytochemicals that can prevent or halt carcinogenic processes. As conventional cancer therapy causes 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 [5]. It has been reported that intake of dietary chemopreventives contributes to a reduction in the cancer incidence by suppressing multiple oncogenic events [6,7]. Resveratrol (*trans*-3,4′,5-trihydroxystilbene) was first isolated from the roots of *Veratrum grandiflorum* O. Loes (white hellebore) and is abundant in red wine, grapes, berries, peanuts, and more than 70 other plant species [8]. Resveratrol has been extensively investigated for its chemopreventive and chemotherapeutic potential [9,10]. This

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phytochemical is well known for its inhibitory effects on the growth and proliferation of many different types of cancer cells [11–14].

Recently, the serine/threonine family of proviral integration site for Moloney murine leukemia virus (PIM) kinases have emerged as new targets for cancer therapy. PIM kinases are evolutionarily conserved and composed of PIM-1, PIM-2 and PIM-3 isoforms. Of these, PIM-1 is overexpressed in hematologic and solid tumors and implicated in their pathogenesis.

PIM-1 is involved in cancer cell cycle regulation [15], survival [16], drug resistance [17–19], and Myc-related tumorigenicity [20,21]. Most representative cellular substrates of PIM-1 include $p21^{WAF1}$ [22,23], Bad [24], and mouse double minutes 2 homolog (Mdm2) [25] which are regulators of apoptosis and cell cycle progression. One of the mediators of cytokine signaling responsible for regulating PIM-1 expression is STAT3. STAT3 can bind directly to the ISFR/GAS-sequence of the PIM-1 promoter [26]. Overexpression of PIM-1 has been associated with poor survival in many different types of malignancies including gastric cancer [27], suggesting its potential as a prognostic and therapeutic target [28,29].

In the present study, we investigated the effects of resveratrol on proliferation, invasiveness, and survival of gastric cancer cells with PIM-1 as a potential target.

2. Materials and methods

2.1. 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 and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). Primary antibodies for PIM-1 and β-actin were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for PIM-1, caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), Bcl-2-associated death promoter (Bad), phospho (Ser112)-Bad (P-Bad) and cleaved PARP were supplied by Cell Signaling Technology (Danvers, MA, USA). *PIM-1* siRNA (5′- CCGGTGCAAGATCTCTTCGAC TTCA -3′) and RNAi negative control duplexes were purchased from Genolution Pharmaceuticals, Inc. (Seoul, South Korea). MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The 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). Adenosine 5′-triphosphate (ATP) was supplied by New England Biolabs (Ipswich, MA, USA). RNAi-MAX and Lipofectamine 3000 were products of Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

SNU-216, SNU-484, SNU-601, SNU-638, and SNU-668 cell lines were purchased from the Korean Cell Line Bank (http://cellbank.snu.ac.kr). The cells were cultured in monolayers by using RPMI 1640 supplemented with 10% FBS and 1% antibiotic-antimycotic. Cells were maintained in a 37 ˚C in humidified incubator ventilated with 5% CO_2 and 95% air.

2.3. Transient transfection with small interfering RNA (siRNA) or overexpression vector

Cells at a confluence of 60–70% were transfected with control siRNA (40 nM) or *PIM-1* siRNA (40 nM) with Lipofectamine RNAi-MAX reagent according to the manufacturer's instructions. For overexpression of PIM-1, cells were transfected with a plasmid carrying cDNA for *PIM-1* using Lipofectamine reagent. The transfection was conducted according to the manufacturer's instructions. An empty vector was used as a control.

2.4. Cell proliferation assay

Cells were cultured for 96 h in the presence of various concentrations of resveratrol dissolved in DMSO. Cells were then treated with the MTT reagent dissolved in phosphate-buffered saline (PBS) at a final concentration of 0.5 mg/ml for additional 4 h. After medium was removed, DMSO was added to dissolve the formazan crystal, and the absorbance at 570 nm was read.

2.5. Anchorage-independent growth assay

The anchorage-independent cell transformation assay 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 concentrations 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).

2.6. Clonogenic assay

Cells were plated (5 x 10^2 cells/well) in 6-well culture plates. 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.

2.7. Western blot analysis

Cells were gently washed with cold PBS, scraped, and centrifuged at 15,000 *g* for 5 min. Pellets were suspended in lysis buffer and subjected to repeated freezing and thawing three times, followed by centrifugation at 20,000 *g* for 15 min. Supernatant was collected as a whole cell lysate. The protein concentration of whole cell lysate was determined by using a 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 h 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 antibody in 3% fat-free dry milk-TBST for 1 h 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.

2.8. 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 Moloney murine leukemia virus reverse transcriptase (Promega; Madison, WI, USA) for 50 min at 42 ˚C and again for 15 min at 72 ˚C. One μl of cDNA was amplified in sequential reactions using Maxtime PCR PreMix Kit (iNtRON Biotechnology; Seongnam, South Korea). The mRNA expression of *PIM-1* (28 cycles of 94 ˚C for 30 s, 48 ˚C for 30 s, and 72 ˚C for 30 s) and *GAPDH* (20 cycles of 94 ˚C for 30 s, 55 ˚C for 30 s, and 72 ˚C for 30 s) was measured. These PCR cycles were followed by final extension for 7 min at 72 ˚C. 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 2.0% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

2.9. Annexin V and propidium iodide (PI) staining

Cells were washed with cold PBS and centrifuged at 1,500 *g* for 4 min three times. Supernatant was aspirated, and the cells were resuspended in 1 ml of 1x Annexin V binding buffer. Five μl of Annexin V-FITC and 5 μl of PI were added to the cells and incubated for 15 min at room temperature in the dark. After addition of 1x binding buffer, the cells were analyzed by FACS caliber.

2.10. 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% bovine serum albumin (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, the cells were incubated with FITC conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody diluted at 1:1,000 for 1 h and examined under a fluorescence microscope.

2.11. Affinity chromatography and pull-down assay

CNBr-activated Sepharose™ 4B was obtained from GE Healthcare (Little Chalfont, UK). Resveratrol-conjugated Sepharose 4B beads were prepared according to the protocol described by Urusova et al. [30]. 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 *g*, the resulting supernatant (500 μg) was mixed with 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 BSA, 0.02 mM PMSF, and 1x protease inhibitor mixture]. After overnight incubation in a rotator at 4 ˚C, the samples were rinsed 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 subjected to Western blot analysis. Proteins bound to the beads were analyzed by immunoblotting with anti-PIM-1 antibody.

2.12. Kinase assay

The catalytic activity of PIM-1 was measured by using PIM-1 KINOMEscan™ platform which is a comprehensive high-throughput system. For most assays, kinase-tagged T7 phage strains were prepared in an *E. coli* host derived from the BL21 strain. *E. coli* were grown to log-phase, infected with T7 phage, and incubated with shaking at 32 ˚C until lysis. The lysates were centrifuged and filtered to remove cell debris. The remaining kinase was produced in HEK-293 cells and subsequently tagged with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated resveratrol for 30 min at room temperature to generate affinity resins for the kinase assay. The liganded beads were blocked with excess biotin and washed with SEA BLOCK blocking buffer (1% BSA, 0.05% Tween 20, and 1 mM DTT) to reduce nonspecific binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20% SeaBlock, 0.17x PBS, 0.05% Tween 20, 6 mM DTT). All reactions were performed in polystyrene 96-well plates in a final volume of 0.135 ml. The assay plates were incubated at room temperature with shaking for 1 h, and the affinity beads were rinsed with wash buffer (1x PBS, 0.05% Tween 20). The beads were then resuspended in elution buffer (1x PBS, 0.05% Tween 20, 0.5 μM non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 min. The kinase concentration in the eluates was measured by qPCR.

2.13. Docking simulation

AutoDock Vina program [31] (The Scripps Research Institute; La Jolla, CA, USA) was used in the docking studies. Initial structures of ligand-free PIM-1 and AMPPNP-bound PIM-1 [32] were obtained from the Protein Data Bank (PDB) (PDB ID: 1XQZ and PDB ID: 1XR1, respectively), and coordinates for the resveratrol were generated using the GlycoBioChem PRODRG2 Server (http://davapc1.bioch.dundee.ac.uk/prodrg/). The grid maps for docking studies were centered on the ATP binding pocket and comprised 60 X 60 X 60 points with 1.0 Å spacing after AMPPNP was removed from the complex structure. The AutoDock Vina program was run with eight-way multithreading and the other parameters were default settings in the AutoDock Vina program.

2.14. 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 $***p < 0.001$.

3. Results

3.1. PIM-1 is overexpressed in gastric cancer patients and cell lines

We first examined the prognostic significance of the expression level of each of three PIM kinase isoforms using the public dataset. Kaplan-Meier plotter analysis revealed the correlation between expression levels of PIM-1 and prognosis of gastric cancer. The probabilities of overall survival among patients with gastric cancer were significantly reduced with an increased *PIM-1* transcript level (Fig. 1A left panel). Higher PIM-1 expression was also associated with a shorter time to first progression in gastric cancer patients (Fig. 1A right panel). There was less prominent association between PIM-2 expression and overall survival or time to first progression (Fig. 1B). For PIM-3, the high transcript levels had opposite effects, compared with PIM-1 (Fig. 1C). Based on these observations, we focused on PIM-1 in the subsequent studies.

Next, we checked the expression levels of PIM-1 in various gastric cancer cell lines. We examined the PIM-1 expression in five different SNU gastric cancer cell lines which were established from Korean cancer patients. SNU-216 and SNU-601 express relatively higher levels of *PIM-1* than the other three (SNU-484, SNU-638 and SNU-668) cell lines (Fig. 2A). The SNU-601 cell line was selected to evaluate the subcellular localization of PIM-1 by immunocytochemistry. As illustrated in Fig. 2B, PIM-1 was found to be predominantly localized in nucleus, which is consistent with the previous observation [33]. Ectopic overexpression of PIM-1 in SNU-668 cells that exhibit a relatively low basal level of PIM-1 expression resulted in a significant increase in the proliferation of gastric cancer cells (Fig. 2C). In contrast, knockdown of *PIM-1* markedly reduced the cell viability compared with control (Fig. 2D). *PIM-1* silencing in SNU-601 cells also suppressed colony formation as determined by the clonogenic assay (Fig. 2E). We also assessed the effect of *PIM-1* knockdown on apoptosis by differential staining of cells with Annexin V and PI followed by flow cytometric analysis. The proportion of apoptotic cells increased markedly (Fig. 2F), as reflected by cleavage of caspase-3 and PARP, hallmarks of apoptosis (Fig. 2G). Thus, it is evident that PIM-1 contributes cell proliferation and survival of SNU-601 cells.

3.2. Resveratrol suppresses gastric cancer cell proliferation

Treatment of SNU-216, SNU-601 and SNU-668 cells with resveratrol (25, 50 and 100 μM) resulted in decreased cell proliferation in a

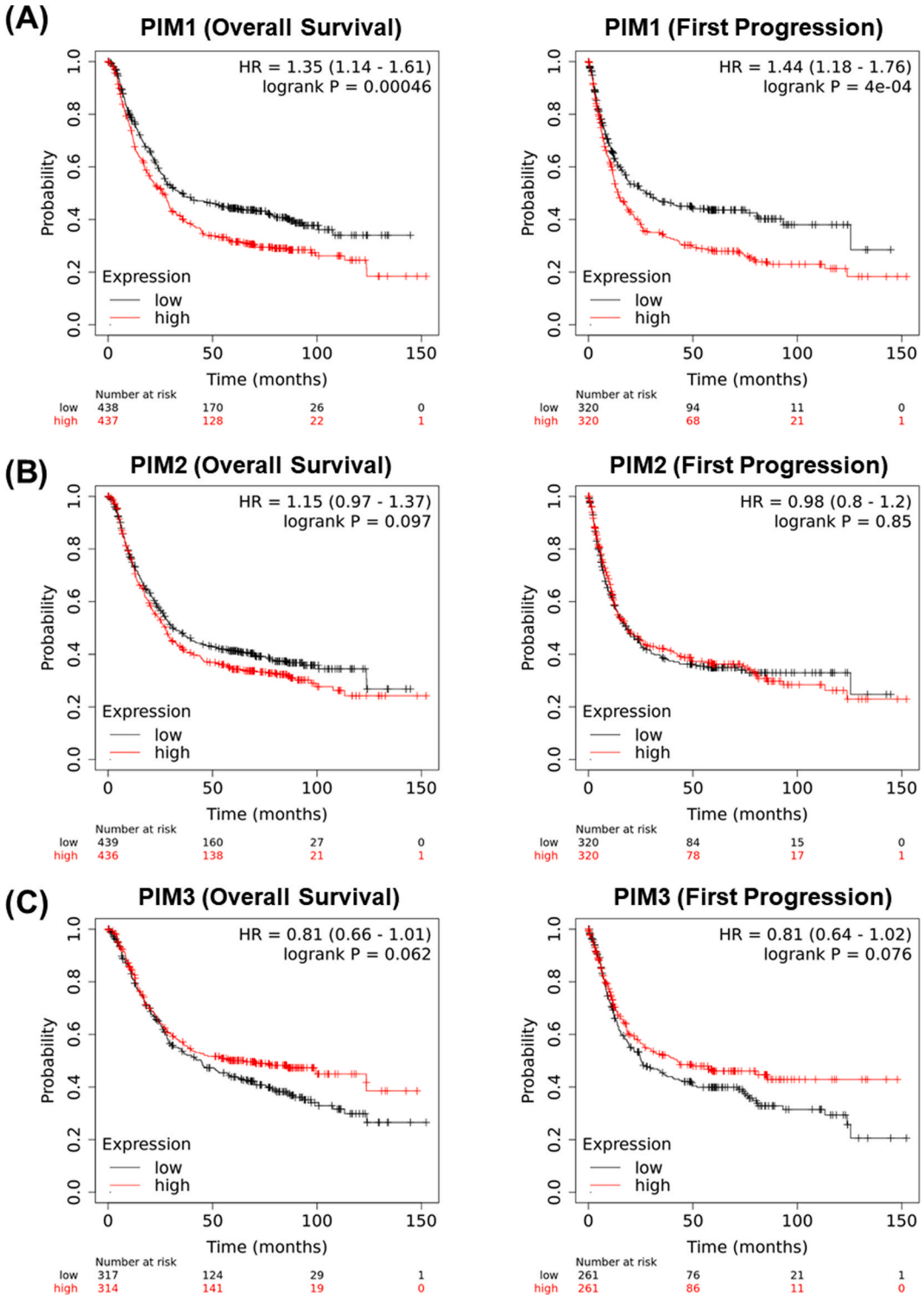

Fig. 1. Overexpression of *PIM-1* in human gastric cancer and its implication for tumor growth and progression. Kaplan-Meier analysis of median overall survival time (left) and time to first progression (right) of patients with gastric cancer, stratified by the mRNA expression levels of three PIM isoforms based on 209193_at (A), 204269_at (B) and 224739_at (C) probes. Source: Kaplan-Meier plotter database (http://www.kmplot.com).

concentration-dependent manner. The IC_{50} values of resveratrol in inhibiting the proliferation of these cell lines were 55.41 μM, 30.14 μM and 87.17 μM, respectively. Interestingly, SNU-668 gastric cancer cells with low basal PIM-1 expression were less susceptible to the anti-proliferative effect of resveratrol (Fig. 3A), compared with the other two cell lines expressing higher level of PIM-1. This finding suggests that the anti-cancer effect of resveratrol can be mediated, at least in part, by targeting PIM-1.

Resveratrol also caused concentration-dependent inhibition of colony formation (Fig. 3B) and anchorage-independent growth (Fig. 3C) of SNU-601 cells. Moreover, resveratrol treatment resulted in the increased proportion of apoptotic cells (Fig. 4A) and cleavage of caspase-3 and PARP (Fig. 4B). Bad, a prototypic pro-apoptotic protein, is one of the major substrates of PIM-1. Under the same experimental

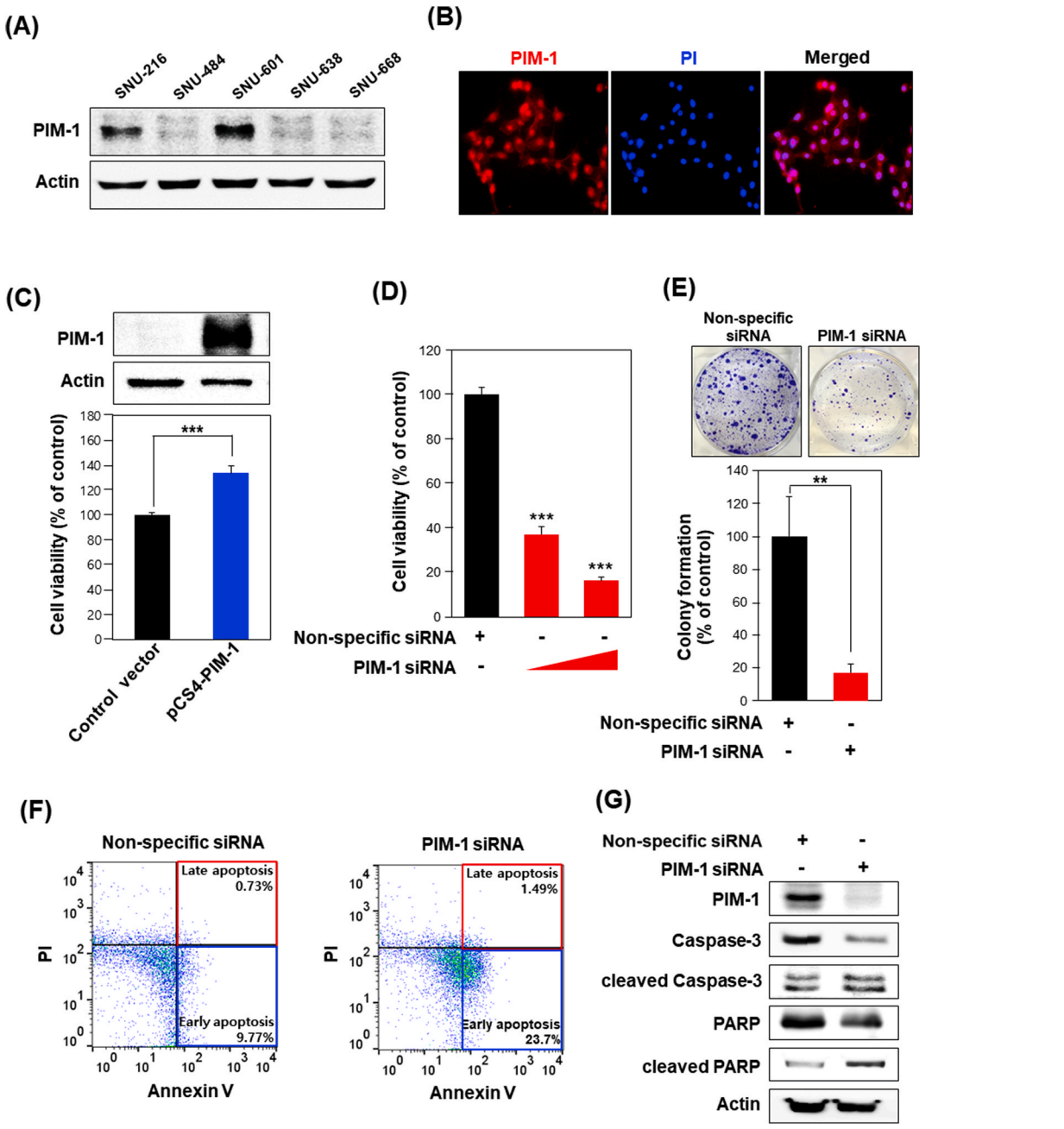

Fig. 2. Effects of PIM-1 on viability, survival, and invasiveness of human gastric cancer cells. (A) Basal levels of PIM-1 protein expression were measured by Western blot analysis in different human gastric cancer cell lines. (B) Subcellular localization of PIM-1 in the SNU-601 cell line determined by immunocytochemistry. (C) After 24 h of seeding in a 48-well plate, SNU-668 cells were transfected with empty pCS4 plasmid (Control) or pCS-PIM-1. Cell viability was measured by the MTT assay 96 h after transfection. Data represent mean ± SD (n = 3); ***Significantly different from the control ($p < 0.001$). (D) SNU-601 cells were seeded into 48-well plates. After 24 h of incubation, cells were transfected with *PIM-1* siRNA (5 nM and 40 nM) or non-specific siRNA, and the cell viability was measured by the MTT assay. Data represent mean ± SD (n = 3); ***Significantly different from the control ($p < 0.001$). (E) SNU-601 cells were seeded into 6-well plates at a density of 0.5×10^4 per well. Next day, *PIM-1* siRNA and non-specific siRNA were transfected. After 2 weeks, cells were stained by 0.5% crystal violet. Data represent mean ± SD (n = 3); **Significantly different from the control ($p < 0.01$). (F) SNU-601 cells were transfected with *PIM-1* siRNA for 72 h and harvested. Representative flow cytometric dot plots represent changes in the proportion of apototic cells. (G) Immunoblotting was conducted using apoptotic marker antibodies in *PIM-1* silenced and control cells.

conditions, the inactivation of Bad through phosphorylation at Ser112 was repressed (Fig. 4C). Taken together, these data suggest that resveratrol inhibits proliferation of SNU-601 gastric cancer cells, possibly by targeting PIM-1.

3.3. Resveratrol inhibits catalytic activity, but not expression of PIM-1

In the next experiment, we examined the effect of resveratrol on PIM-1 expression. The mRNA (Fig. 4D, F) and protein (Fig. 4E, G) levels of PIM-1 did not show marked differences with and without resveratrol treatment in SNU-601 cells. Then, we determined the effects of resveratrol on catalytic activity of PIM-1 based on a competition assay. The principle of this assay is schematically represented in Fig. 5A. As illustrated in Fig. 5B, resveratrol inhibited the kinase activity of PIM-1 in a concentration-dependent manner. We speculated that resveratrol may inactivate PIM-1 through structural modification. To verify

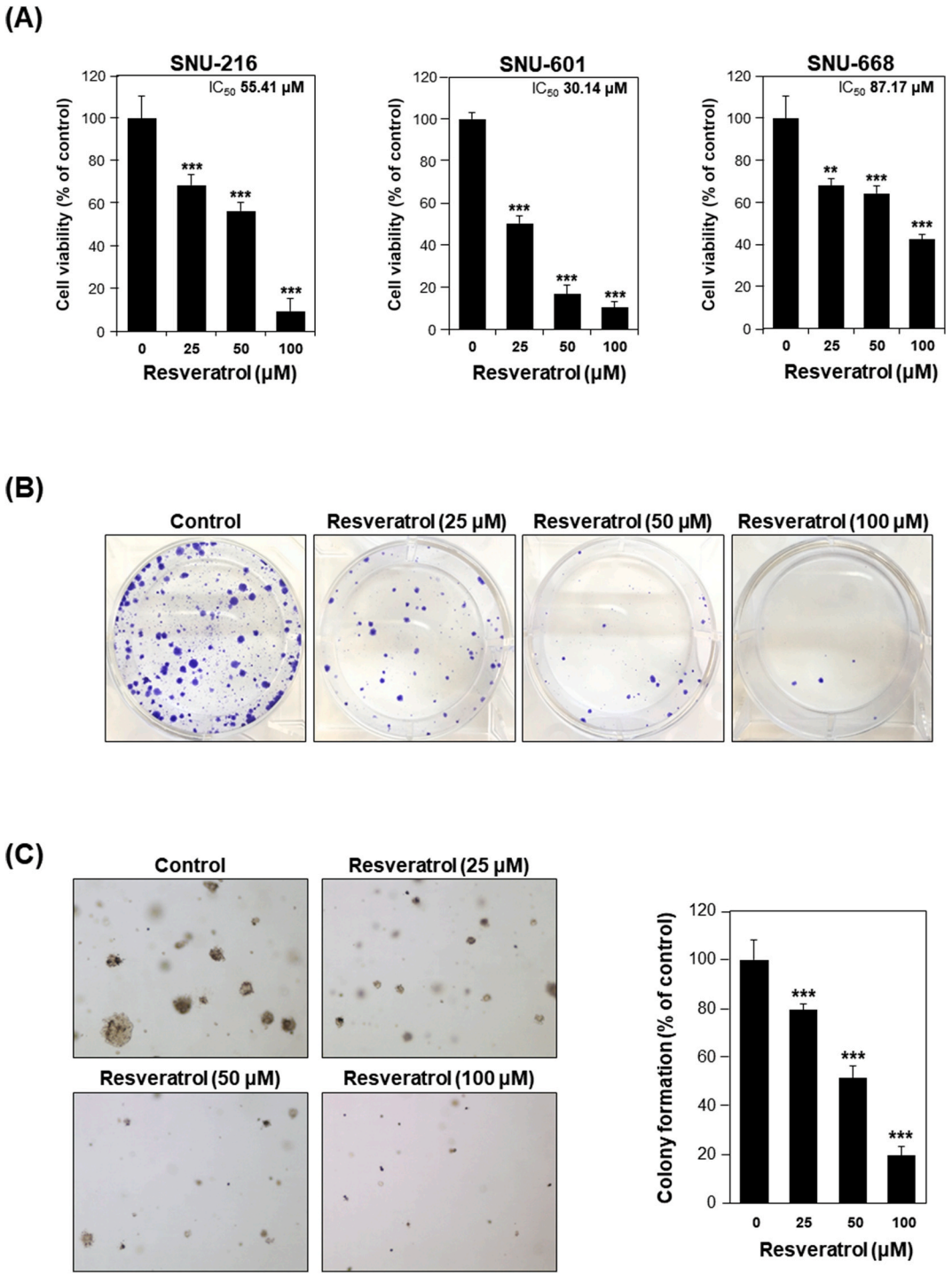

Fig. 3. Resveratrol inhibits cell proliferation and growth of human gastric cancer cells. (A) SNU-216, SNU-601 and SNU-668 cells were seeded in 96-well plates. After 24 h of incubation, cells were treated with resveratrol (0, 25, 50 and 100 μM) for 96 h. Cell viability was measured by the MTT assay. The 50% inhibitory concentration of cell proliferation in SNU-216, SNU-601 and SNU-668 were 55.41 μM, 30.14 μM, 87.17 μM, respectively. The asterisks indicate differences between untreated and resveratrol treated groups. Data represent mean ± SD (n = 3) (**$p < 0.01$ and ***$p < 0.001$). (B) SNU-601 cells were seeded and after 24 h, cells were treated with resveratrol (0, 25, 50 and 100 μM) for 2 weeks. (C) 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 the density of 8×10^3 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. Data represent mean ± SD (n = 3). ***Significantly different from the control ($p < 0.001$).

physical interaction between PIM-1 and resveratrol in SNU-601 cancer cells, the Sepharose 4B assay was performed (Fig. 5C). Whole lysate of SNU-601 cells was precipitated with resveratrol-conjugated Sepharose 4B beads, suggesting that resveratrol directly binds to PIM-1 (Fig. 5D).

3.4. 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 identification of the binding site of resveratrol. The structures of ligand-free PIM-1 and AMPPNP-bound PIM-1 were selected as docking

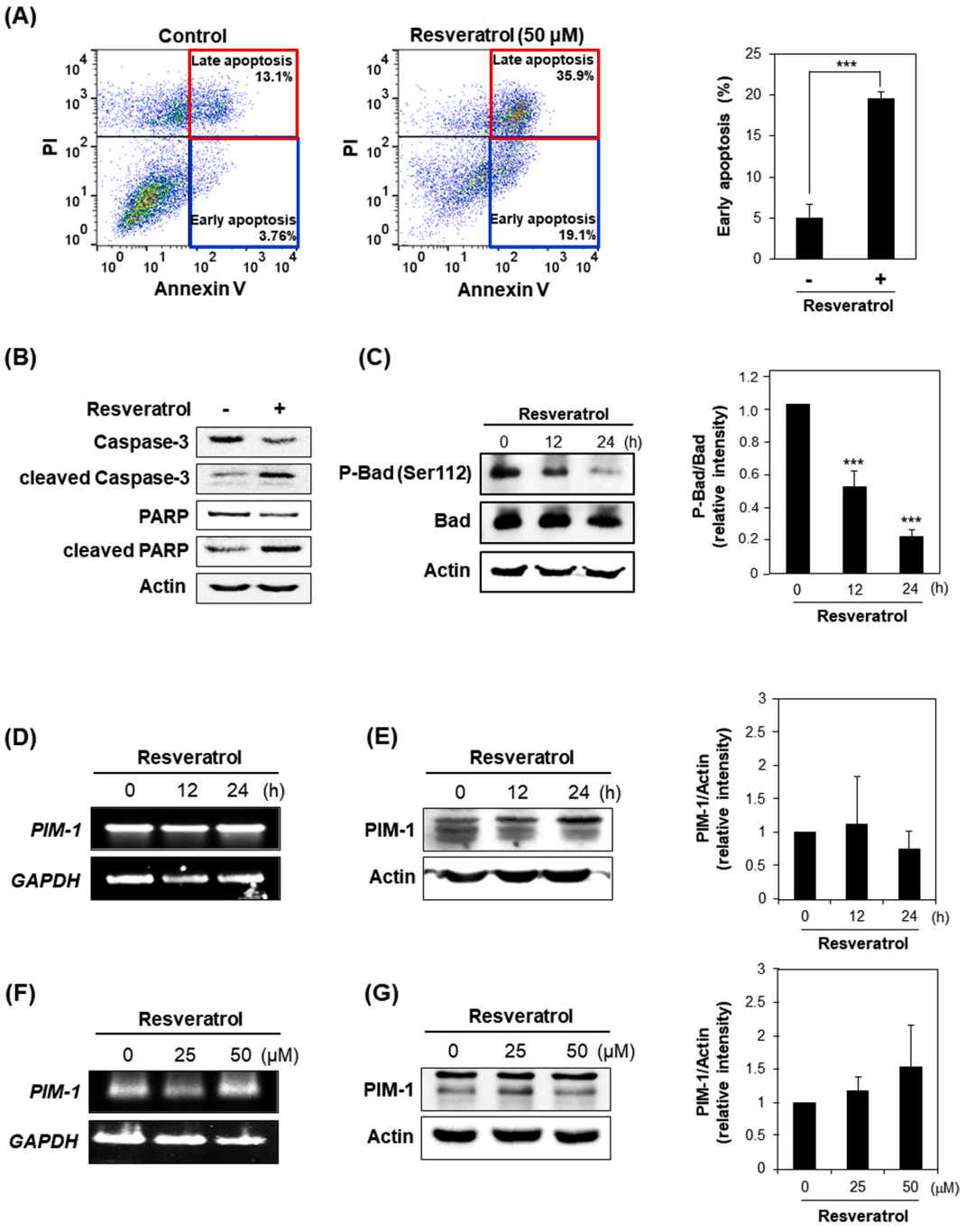

Fig. 4. Resveratrol induces apoptosis and activates apoptotic signaling in gastric cancer cells. (A) SNU-601 cells were seeded into 6-well plates and treated with resveratrol (50 μM) for 48 h. After staining with PI and Annexin, cells were analyzed by the FACS Calibur Flow Cytometer. Red box and blue boxes represent late and early apoptotic cells, respectively. Data represent mean ± SD (n = 3) (***$p < 0.001$). (B) Whole lysates from SNU-601 cells treated with resveratrol (50 μM) for 48 h were subjected to Western blot analysis. Data represent mean ± SD (n = 3). ***Significantly different from the control ($p < 0.001$). (C) After treatment with 50 μM of resveratrol, the expression levels of Bad and its phosphorylated form (P-Bad) in SNU-601 cells were measured by Western blot analysis. Data represent mean ± SD (n = 3). ***Significantly different from the control ($p < 0.001$). (D and E) SNU-601 cells were treated with 50 μM of resveratrol for 12 h and 24 h. The expression of *PIM-1* mRNA (D) and its protein product (E) was determined by semi-quantitative RT-PCR and Western blot analyses, respectively. (F and G) After treatment with resveratrol (25 or 50 μM) for 24 h, mRNA (F) and protein (G) expression of PIM-1 was measured as described above. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

templates because their crystal structures showed a significant difference in a loop enveloping the active site (Fig. 6A). Control docking experiments were implemented with AMPPNP to the crystal structures of PIM-1 in complex with AMPPNP (PDB ID: 1XR1) after removal of the AMPPNP, which exhibited the correct binding mode of AMPPNP at the ATP-binding pocket of PIM-1 with stabilization energy of −7.0 kcal/mol (data not shown). The docking experiments executed with resveratrol revealed that the molecule occupies the ATP-binding pockets of both ligand-free PIM-1 and AMPPNP-bound PIM-1 with stabilization energies of −7.9 kcal/mol and −7.1 kcal/mol, respectively (Fig. 6Ba and b). These data suggest that resveratrol is capable of occupying the PIM-1 ATP-binding pocket.

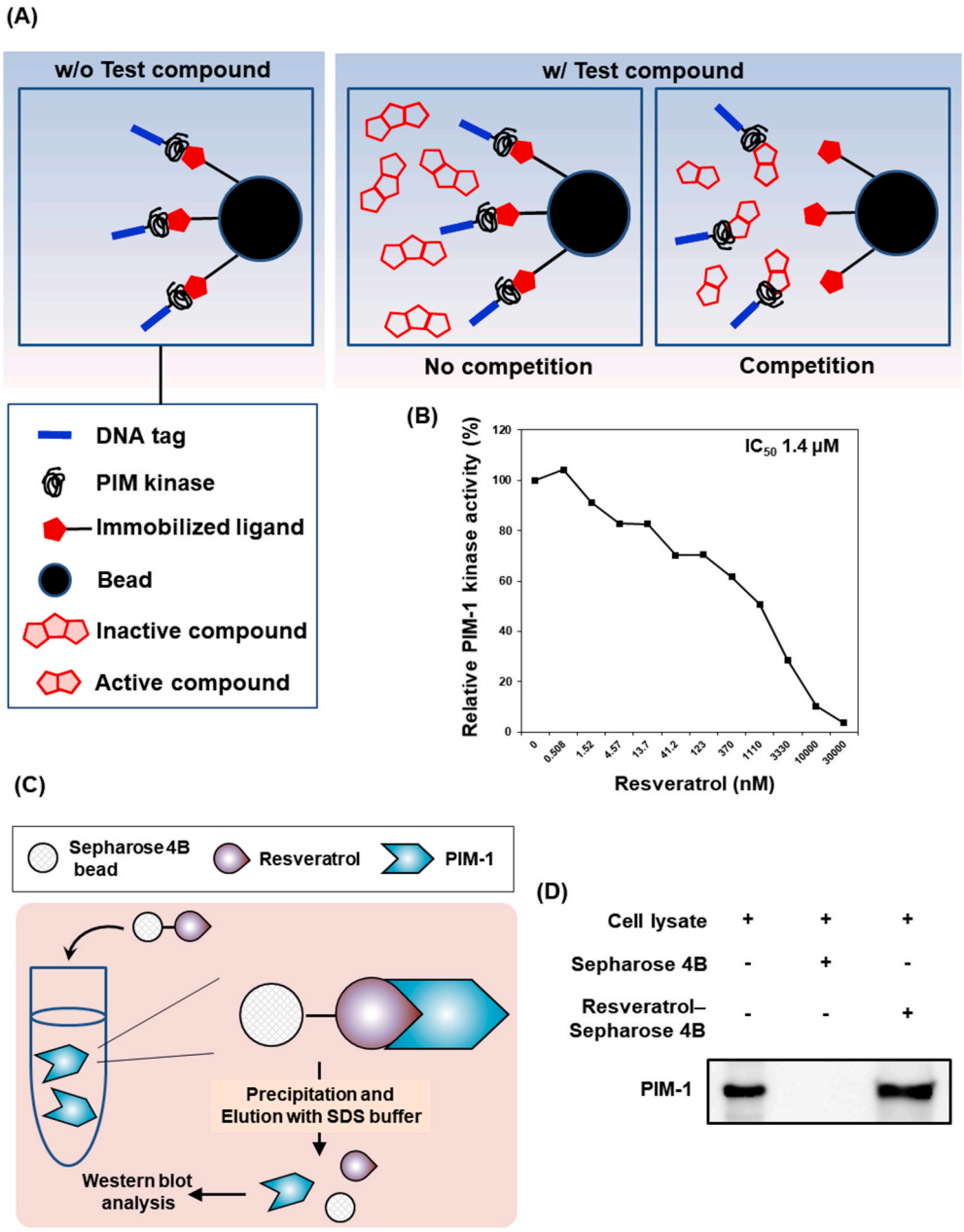

Fig. 5. Resveratrol covalently binds to PIM-1 and inhibits its kinase activity. (A) The catalytic activity of PIM-1 was determined based on a competition assay (DiscoveRX screen system) that quantitatively measures the ability of a compound to compete with an immobilized, active-site directed ligand. The assay system consists of three components: DNA-tagged kinase, immobilized ligand, and a test compound. The ability of the test compound to compete with the immobilized ligand was measured via quantitative PCR of the DNA tag. (B) Effects of resveratrol on PIM-1 kinase activity. Data represent the average value of two independent experiments, and the half maximal inhibitory concentration of resveratrol was calculated as 1.4 μM. (C) Whole-cell lysates from SNU-601 cells were incubated with resveratrol-Sepharose 4B beads. The PIM-1 bound to the resveratrol conjugated Sepharose 4B beads was pulled down by centrifugation and analyzed by Western blot analysis using antibody against PIM-1. (D) Lane 1 represents the input control and lane 2, a negative control in which the cell lysates were precipitated with Sepharose 4B beads only. In lane 3, SNU-601 cell lysates were precipitated using resveratrol conjugated to Sepharose 4B beads.

4. Discussion

Kinases have been considered as a potential target for anti-cancer therapy as they are frequently mutated in many different types of human tumors [34,35]. PIM-1 has attracted much attention for the anti-cancer drug development. In a wide range of human tumors of both hematopoietic and epithelial origin, PIM-1 kinase is frequently over-expressed. Especially, constitutive overexpression of PIM-1 in gastric tumor is associated with poor prognosis and a low survival rate of cancer patients [36,37]. Therefore, many attempts have been made to discover molecular inhibitors of this enzyme. Nevertheless, some obstacles, such as drug toxicity and safety, have hampered the development of the effective PIM-1 inhibitors for use in cancer therapy [33]. In this study, we found that the catalytic activity of PIM-1 was inhibited by resveratrol treatment. Notably, resveratrol inhibited proliferation and survival of gastric cancer cells more efficiently in PIM-1 overexpressing cells. These effects of resveratrol mimick those achieved by PIM-1 silencing.

Resveratrol has been extensively investigated with regards to its suppressive effect on cancer and inflammation. Resveratrol inhibits Src and STAT3 signaling in malignant cells, resulting in cell cycle arrest and loss of viability [38]. It also suppresses constitutively active NF-κB through inhibition of IκBα kinase, phosphorylation of IκBα and p65, etc [13]. In another study, resveratrol blocked the ubiquitination of NEMO

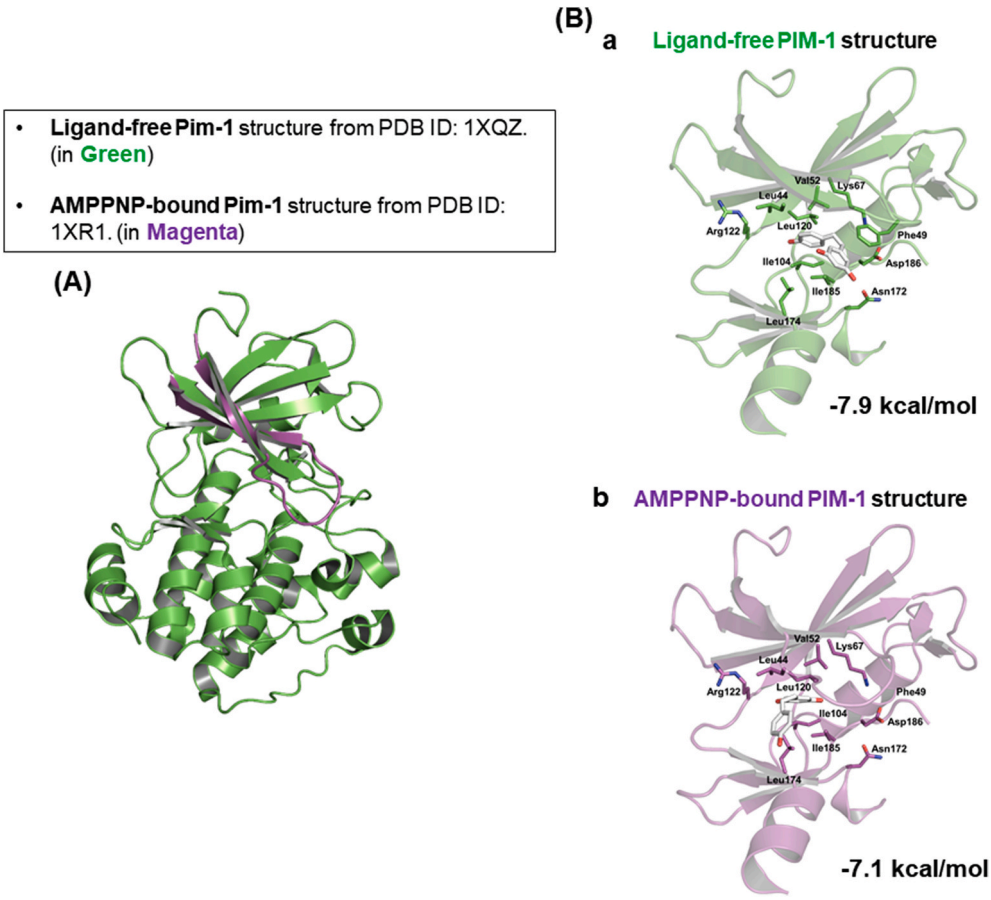

Fig. 6. Resveratrol binds to the PIM-1 ATP-binding pocket. (A) Cartoon representation of ligand-free PIM-1 (green) and a loop region of AMPPNP-bound PIM-1 showing a conformational change upon AMPPNP binding (magenta). (B) Cartoon representations showing results of docking simulation of resveratrol to ligand-free PIM-1 with stabilization energy of −7.9 kcal/mol (Ba) and AMPPNP-bound PIM-1 with stabilization energy of −7.1 kcal/mol (Bb). Resveratrol bound to PIM-1 is illustrated as a stick model with carbon and oxygen atoms in white and red, respectively. Amino acid residues at the ATP-binding pocket are shown in stick representations with nitrogen and oxygen atoms coloured in blue and red, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and inhibited IκB kinase β-mediated NF-κB activation [14]. We speculated that suppression of upstream signaling which regulates PIM-1 expression and direct inhibition of PIM-1 may provoke a synergistic effect on growth and progression of gastric cancer. However, PIM-1 expression was unchanged by resveratrol despite its inhibitory effects on STAT3 and NF-κB signaling.

PIM-1 utilizes many kinds of cellular substrates which are involved in regulating cell cycle, apoptosis, cell metabolism, etc. Examples are c-Myc, $p21^{WAF1/CIP1}$, Mdm2, and Bad [39]. PIM-1 forms a complex with Myc and Myc-associated factor X (MAX), and phosphorylates Ser10 of histone H3 on the nucleosome. This increases transcriptional activity of Myc [20,21]. PIM-1 also phosphorylates Thr145 of $p21^{WAF1/CIP1}$, thereby decreasing stability and nuclear translocation of this cell cycle inhibitory protein [22,23]. Furthermore, phosphorylation of Mdm2 by PIM-1 at Ser166 and Ser186 increases the ability of Mdm2 to interact with its inhibitor, ARF. This blocks the degradation of Mdm2, leading to accumulation of the tumor suppressor p53 [25]. As a result, senescence-associated β-galactosidase accumulates [25]. Phosphorylation of Bad at Ser112 facilitates proteasomal degradation of this pro-apoptotic protein [24]. Because Bad is directly phosphorylated by PIM-1, we measured the phosphorylation level of Bad in the absence and presence of resveratrol. Resveratrol treatment inhibited the phosphorylation of Bad without altering the expression level of total Bad in SNU-601 gastric cancer cells.

Because PIM-1 is overexpressed in many cancers and involved in cancer specific pathways, it represents a potential target for anti-cancer drug development [33]. 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 thereby it reduces cancer cell viability and their chemo-resistance [39,40]. Nevertheless, the clinical development of PIM-1 inhibitors has been failed due to side effects, such as cardiac toxicity [41]. Thus, natural compounds are preferred to synthesized pharmaceuticals because of their relatively low side effects and toxicity. In this context, resveratrol derived from red wine, grapes, berries and peanuts may have a potential as a clinically relevant PIM-1 inhibitor.

In summary, our study provides new insights into the molecular mechanisms responsible for the anti-tumor effect of resveratrol (Fig. 7). Resveratrol inhibits PIM-1 activity through direct binding, thereby regulating the functional activities of PIM-1 substrate molecules. This account for its inhibitory effects on gastric cancer cell growth and proliferation. Multiple sequence alignment of PIM-1, PIM-2, and PIM-3 using Clustal Omega displayed with ESPript 3.0 (http://espript.ibcp.fr) shows that PIM-1, PIM-2, and PIM-3 share high sequence identity (53.75% between PIM-1 and PIM-2, and 66.77% between PIM-1 and PIM-3) (Supplementary Fig. 1). Indeed, the structures of ligand-free PIM-1 and 3 YR-bound PIM-2 were highly similar over 217 equivalent C_α atoms with a root-mean-square deviation (RMSD) of 1.02 Å.

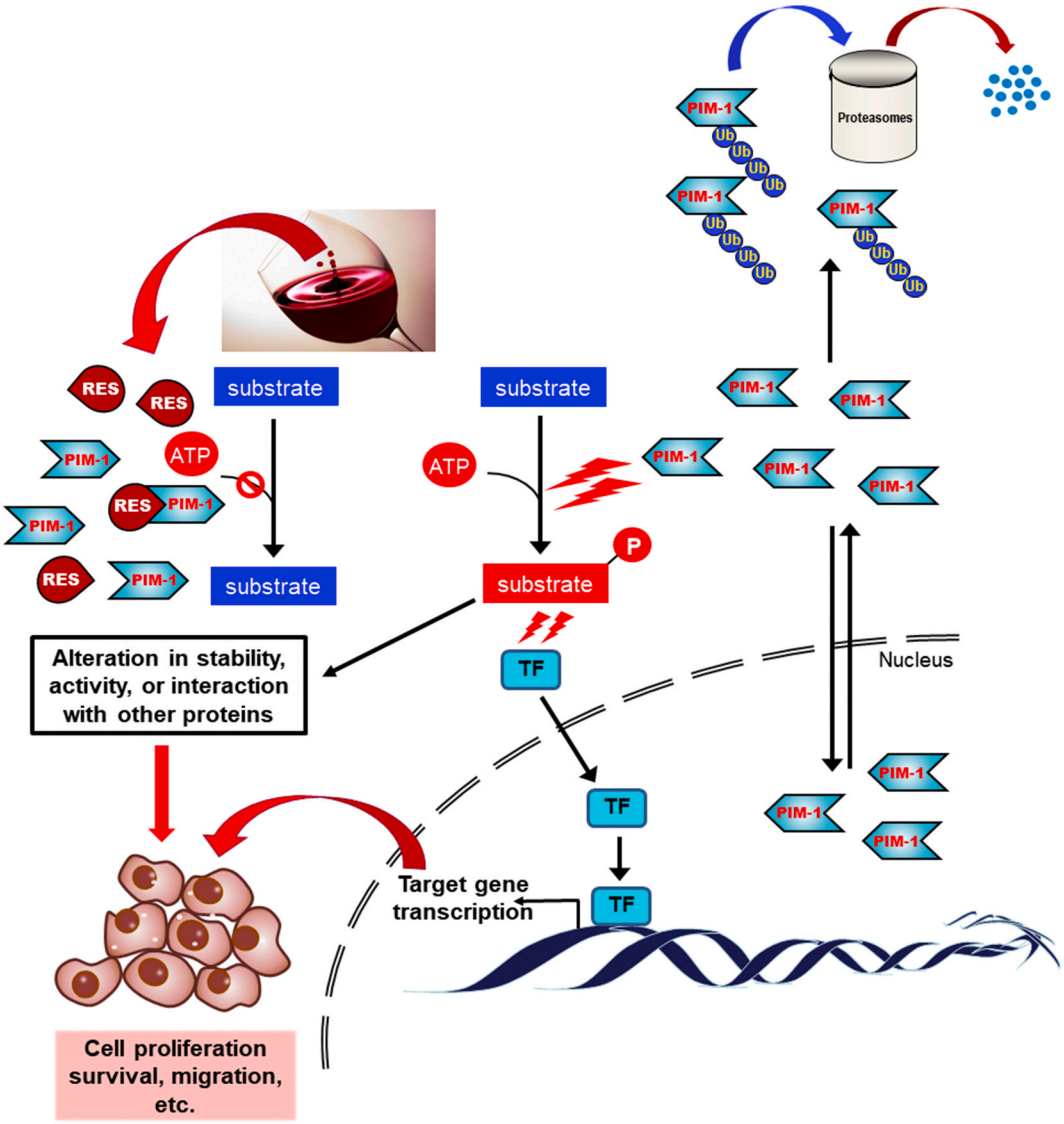

Fig. 7. Schematic representation of resveratrol targeting PIM-1 kinase in gastric cancer cells. PIM-1 is upregulated in some gastric cancer cells, and stimulates their growth through phosphorylation of substrate proteins with subsequent overactivation of oncogenic transcription factors (TF). This leads to altered transcription of genes involved in cell cycle, migration, and survival. Alternatively, PIM-1-mediated phosphorylation of some proteins can alter their stability, activity, or interaction with other proteins. Resveratrol (RES) binds and structurally alters PIM-1 which may hamper ATP binding to its catalytic site. This leads to suppression of cancer cell proliferation, survival, and migration.

Although the structure of PIM-3 is not available, we presume that its structure would be similar to the that of PIM-1, considering the high sequence similarity. Therefore, we do not exclude the possibility of resveratrol binding to these two other PIM isoforms, thereby inhibiting their catalytic activities, especially by occupying the ATP binding pocket. Results from our preliminary test tube kinase assay revealed that resveratrol inhibits PIM-2 kinase activity to the extent equivalent to that observed with PIM-1. However, the effect on PIM-3 activity was much weaker (data not shown), Further studies will be necessary to more precisely assess the comparative effects of resveratrol on the kinase activities of PIM isozymes.

Declaration of competing interest

No potential conflict of interest was reported by the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2020.108413.

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