

# Heat-processed neoginseng, KG-135, down-regulates G1 Cyclin-dependent kinase through the proteasome-mediated pathway in HeLa cells

WON-HEE LEE<sup>1</sup>, JOON-SEOK CHOI<sup>1,2</sup>, HYUN YOUNG KIM<sup>3</sup>, JEONG-HILL PARK<sup>3</sup>,  
SEUNG-KI LEE<sup>1,3</sup> and YOUNG-JOON SURH<sup>3</sup>

<sup>1</sup>Division of Pharmaceutical Biosciences, <sup>2</sup>BK21 Applied Pharmaceutical Sciences Research Division, <sup>3</sup>The Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

Received July 15, 2008; Accepted September 5, 2008

DOI: 10.3892/or\_00000246

**Abstract.** High temperature heat treatment of ginseng (*Panax ginseng*, C.A. Meyer) generates KG-135 (heat-processed neoginseng) which contains a mixture of three major ginseng saponins, ginsenosides Rk1, Rg3 and Rg5. Ginsenosides, particularly of the diol-type including Rk1, Rg3 and Rg5, have been shown to induce cell growth arrest in various cell types of human cancer. Herein, we report that KG-135 is able to arrest the cell cycle in human cervix adenocarcinoma HeLa cells. KG-135 arrests cells at the G1 phase of the cell cycle with an IC<sub>50</sub> value of 69 µg/ml. The G1 phase arrest is associated with down-regulation of Cyclin D1/Cdk4 and Cyclin B1/Cdc2 activities in cells after treatment with KG-135. Furthermore, down-regulation of G1 Cyclin-dependent kinase activities is kinetically well related to the decreased intracellular protein levels of these kinases. In addition, the decrease in the levels of Cyclin D1/Cdk4 and Cyclin B1, but not of Cdc2, is similarly prevented by co-treatment of cells with MG-132, a potent proteasome inhibitor. Thus, the KG-135-induced arrest of the cell cycle at G1 phase in HeLa cells represents a novel mechanism that involves proteasome-mediated degradation of the Cyclins (Cyclin D1 and B1) and Cdk4 proteins.

## Introduction

Progression of cell cycle in mammalian cells is driven by sequential activation of different members of the Cyclin and Cyclin-dependent kinase (Cdk) family. In human cancers, the activities of Cyclin/Cdk kinases are universally over-activated.

Thus, cancer cells can be forced to undergo apoptotic cell death by arresting the cell cycle through Cdk inhibition (1). Progression of G1 is mainly controlled by Cyclin D/Cdk4 and Cyclin E/Cdk2 complexes and transition from G1 to S phase is governed by Cyclin A/Cdk2, while G2/M phase transition is mediated by the Cyclin B/Cdc2 complex. These Cyclin/Cdk activities are down-regulated by binding of Cdk inhibitor proteins (CKIs), p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> (2-4). Thus, CKIs are thought to be good target molecules for controlling the cell cycle of cancer cells, and many investigators have been working to develop specific Cdk inhibitors and small peptide inhibitors from endogenous Cdk inhibitors of Cyclin/Cdk complexes (5).

Ginseng (*Panax ginseng*, C.A. Meyer) is one of the most widely used herbal medicines in Asian countries. There exists evidence supporting that some ginseng saponins (ginsenosides) have cytostatic or cytotoxic activities against various types of tumor cells in culture (6-9), including human hepatocarcinoma and cholangiocarcinoma cells (10,11), human breast cancer cells (12), lung adenocarcinoma A549 cells (13), cervix cancer cells (14) and monocytic leukemia cells (15). In addition, chronic administration of ginseng has been reported to reduce the incidence of some cancers, such as esophageal, gastric, and colorectal, and pulmonary tumors (16,17). Red ginseng is heat-processed ginseng and contains several specific ginsenosides that are not detected in white ginseng which is air-dried. These red-ginseng-specific ginsenosides have been shown to have more potent cytostatic or cytotoxic activity than other ginsenosides against various cancer cells (18,19). In addition, 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced epidermal ornithine decarboxylase activity (ODC) and ODC mRNA expression were shown to be down-regulated by pretreatment of female ICR mice with a methanol extract of red ginseng (20). Moreover, TPA-induced production of tumor necrosis factor in the mouse skin was inhibited by pre-treatment with the same extract. Heat treatment of ginseng at a temperature higher than that applied to the conventional preparation of red ginseng generates KG-135, a mixture of ginsenosides that constitute about equal amounts of three ginsenosides, Rk1, Rg3 and Rg5. KG-135 has also been suggested to potently inhibit tumor promotion, an activity

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**Correspondence to:** Dr Y.-J. Surh, Division of Pharmaceutical Biosciences, College of Pharmacy, Seoul National University, Kwanak-gu, Seoul 151-742, Republic of Korea  
E-mail: surh@plaza.snu.ac.kr

**Key words:** ginsenosides, G1 phase, Cyclin B1/Cdc2

which appeared to be associated with its ability to suppress the inflammatory response in the human breast epithelial cell line, MCF-10A (21). The ginsenoside Rg3 was shown to inhibit TPA-induced cyclooxygenase-2 expression, NF- $\kappa$ B activation and tumor promotion (22). In addition, ginsenoside Rg5 was suggested to down-regulate Cyclin E/ Cdk2 activity by a mechanism that decreases the intracellular levels of Cyclin E in SK-Hep1 cells (23). In this study, we investigated whether KG-135 specifically interfered with progression of the cell cycle through mechanisms that down-regulate Cyclin-dependent kinase activities in HeLa cells. Herein, we show that the cell cycle of HeLa cells is specifically arrested at G1 phase after treatment with KG-135. The KG-135-induced G1 arrest is associated with specific inhibition of activities of Cyclin D/Cdk4 and Cyclin B/Cdc2 kinases by a mechanism that involves degradation of Cyclin/Cdk complexes by proteasomes.

## Materials and methods

**Materials.** A KG-135 stock solution of 75 mg/ml in DMSO was provided by Ginseng Science (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY, USA) and [ $\gamma$ - $^{32}$ P]-labeled ATP was purchased from NEN<sup>TM</sup> Life Science Products, Inc. (Boston, MA, USA). Anti-p21 antibody was from Millipore (Billerica, MA, USA), and the other antibodies were from SantaCruz Biotechnology (SantaCruz, CA, USA). Easy-spin<sup>TM</sup> total RNA isolation kit and Maxime RT PreMix (Oligo(dT)15 Primer) were purchased from Intron Biotechnology (Gyeonggi Province, Korea). All other general drugs and chemicals were purchased from Sigma Chemical Co.

**Cell culture and drug treatment.** HeLa cells, derived from a squamous cell carcinoma of the cervix, were maintained as a monolayer culture in DMEM supplemented with 5% (by volume) heat-inactivated fetal bovine serum (FBS) and 1% antibiotics and antimycotics mix, at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, and were fed every 3 days. Cells (7x10<sup>5</sup>) (30% confluency) were seeded on 100-mm diameter tissue culture plates (Nalge Nunc International, Denmark) and incubated. Twenty-four hours from seeding, KG-135 was introduced to HeLa cells. Floating and adherent cells were harvested using a cell scraper for the immunoblot analyses and histone H1 kinase assays. In co-treatment of KG-135 and MG-132, the cell culture was pre-treated with MG-132 for 3 h before KG-135 treatment.

**MTT assay.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to assess time- and dose-dependent cell growth inhibitory activity of KG-135. Cells were plated in 96-well plates and incubated for 24 h using the conditions for cell culture described above, then cells were treated with various concentrations of KG-135 for 12, 24 and 36 h, respectively. After treatment, 15  $\mu$ l of 5 mg/ml MTT stock was added to each well, and the cells were incubated for 4 h. MTT formazan was dissolved in 100  $\mu$ l of DMSO, and the absorbance of each well was measured at 570 nm of wavelength.

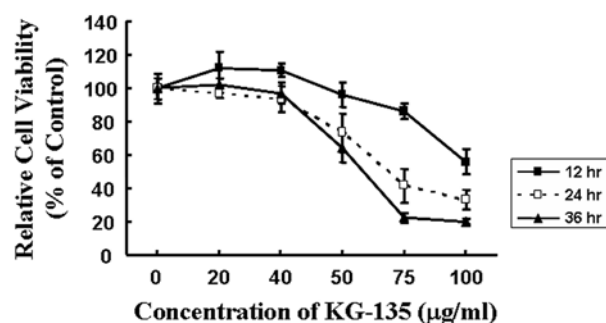


Figure 1. KG-135 shows growth inhibitory effect on HeLa cells. HeLa cells were incubated with KG-135 in DMEM containing 5% FBS for intervals of 12 h for a period of 36 h. Relative cell viability was determined by MTT assay as described in Materials and methods. Data are presented as the mean  $\pm$  standard deviation of individual triplicate experiments.

**Flow cytometric analysis.** HeLa cells were plated at a density of cells (7x10<sup>5</sup>) per 100-mm dish, cultured in DMEM containing 5% FBS, and treated with 75  $\mu$ g/ml KG-135 in DMEM for intervals of 6 h. Adherent and floating cells were trypsinized and fixed with 70% ethanol and stored at 4°C. Fixed cells were rehydrated with phosphate-buffered saline and suspended in staining solution containing 50  $\mu$ g/ml propidium iodide and 100  $\mu$ g/ml RNase A. Cell cycle analysis was performed using a flow cytometer (FACS calibur, BD). The percentage of cells in each phase of the cell cycle was analyzed by CellQuest<sup>®</sup> Pro software.

**Immunoprecipitation and H1 kinase assay.** An aliquot (200  $\mu$ g) of protein from each cell extract was pre-cleared with protein A-agarose beads (Millipore, Billerica, MA, USA), and the supernatant was incubated with shaking for 4 h in an ice bath with polyclonal rabbit antibodies for each Cdk. Immune-complexes associated with each Cdk were collected and washed 3 times with lysis buffer and twice with kinase assay buffer containing 50 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM EGTA, 50 mM  $\beta$ -glycerol phosphate, 25 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 tablet/50 ml complete protease inhibitor cocktail (Roche) and 1 mM phenylmethyl sulfonyl fluoride (PMSF). The immune-complexes were then incubated for 20 min at 30°C in 50  $\mu$ l of the kinase assay buffer supplemented with 1  $\mu$ g of histone H1 (Millipore).

**Immunoblot analysis.** Floating and adherent cells were harvested and washed with ice-cold PBS. Harvested cells were then solubilized with lysis buffer containing 20 mM Tris (pH 7.5), 0.5% Triton<sup>®</sup> X-100 (Bio-Rad Laboratories, Hercules, CA, USA), 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 1 mM EGTA, 50 mM  $\beta$ -glycerophosphate, 25 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2  $\mu$ g/ml leupeptin, 2  $\mu$ g/ml pepstatin A, 2  $\mu$ g/ml antipain and 1 mM PMSF. After incubation on ice for 1 h, insoluble materials were removed by centrifugation at 12,000 x g for 15 min, and protein concentrations were determined using the BCA<sup>TM</sup> protein assay reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Each sample was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The

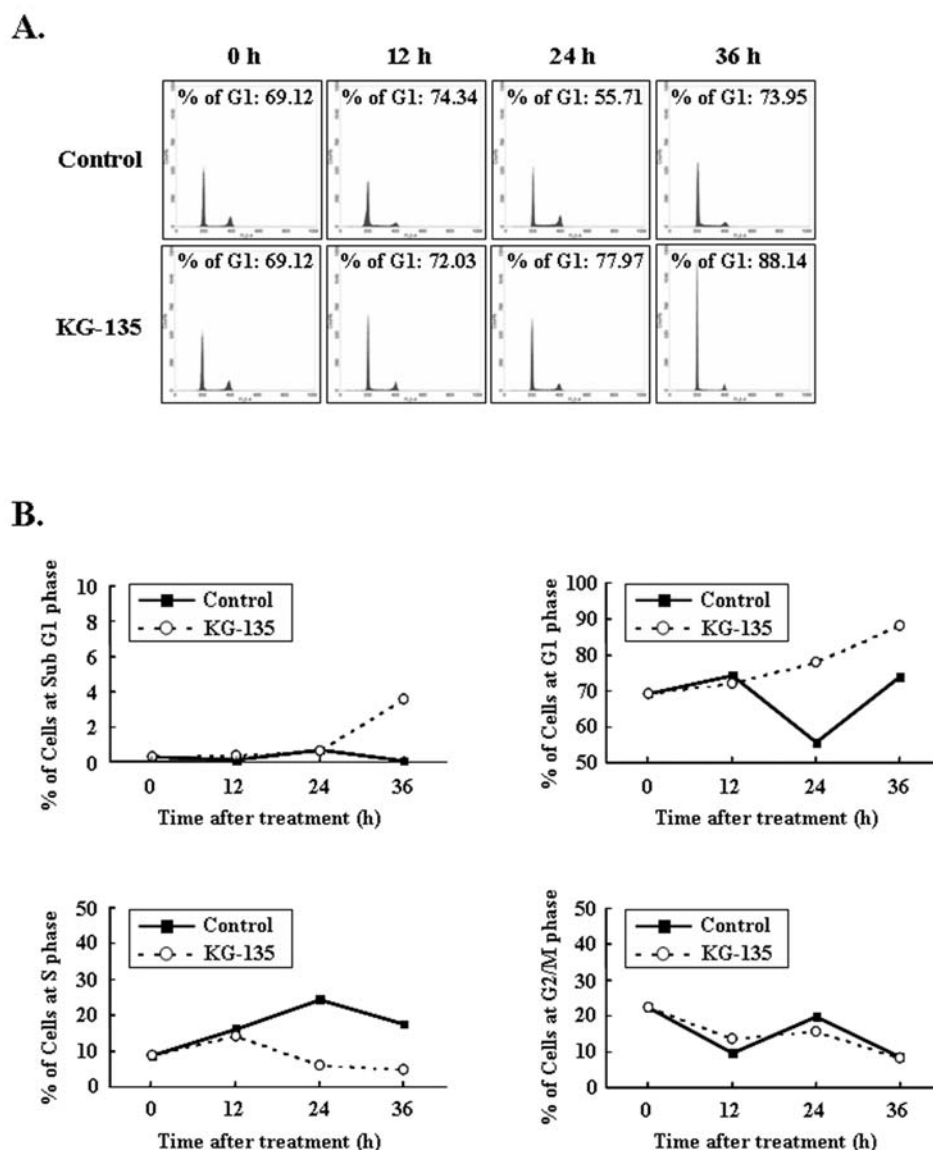


Figure 2. KG-135 treatment arrests cell cycle at G1 phase. (A) HeLa cells were plated at  $7 \times 10^5$  cells per 100-mm dish and cultured in DMEM containing 5% FBS, and were treated with or without  $75 \mu\text{g/ml}$  KG-135 in DMEM. At 12 h intervals of KG-135 treatment, cells were trypsinized and fixed with 70% ethanol. Flow cytometric analysis was performed as described in Materials and methods. Percentages of cells in G1 phase were calculated and typed in a set of FACS plots. (B) Percentages of cells in each phase were calculated and plotted as a function of time duration of KG-135 treatment.

blotted membranes were blocked with 5% non-fat dry milk (Carnation®, Glendale, CA, USA) in PBS with 0.1% Tween-20 (PBST) at room temperature for 1 h and probed with specific antibodies. The probed blots were washed and incubated with a horseradish peroxidase-coupled anti-rabbit IgG (Pierce), and then visualized by ECL™ (GE Healthcare, UK).

**Preparation of total RNA and reverse transcription-polymerase chain reaction (RT-PCR) analysis.** HeLa cells were plated at a density of  $7 \times 10^5$  cells per 100 mm dish and cultured in DMEM containing 5% FBS, and treated with  $75 \mu\text{g/ml}$  of KG-135 in DMEM for intervals of 6 for 36 h. The medium was then removed by aspiration and 1 ml easy-Blue™ (Intron Biotechnology, Korea) reagent was added to each dish to lyse the cells. Total RNA extraction and reverse-transcription were performed as described by the manufacturer.

The cDNA samples were amplified by polymerase chain reactions:  $94^\circ\text{C}$  for 1 min,  $56^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 1 min, for 30 cycles. Amplified products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and analyzed by GelDoc imaging system (Bio-RAD) under UV light.

## Results

**KG-135 arrests cells at the G1 phase of the cell cycle in HeLa cells.** We first assessed whether KG-135 interfered with the proliferation of HeLa cells by performing the MTT assay. When HeLa cells were treated with various concentrations of KG-135 for 12, 24 and 36 h, the cell viability was decreased in a time- and dose-dependent manner with an approximate  $\text{IC}_{50}$  value of  $69 \mu\text{g/ml}$  (Fig. 1).

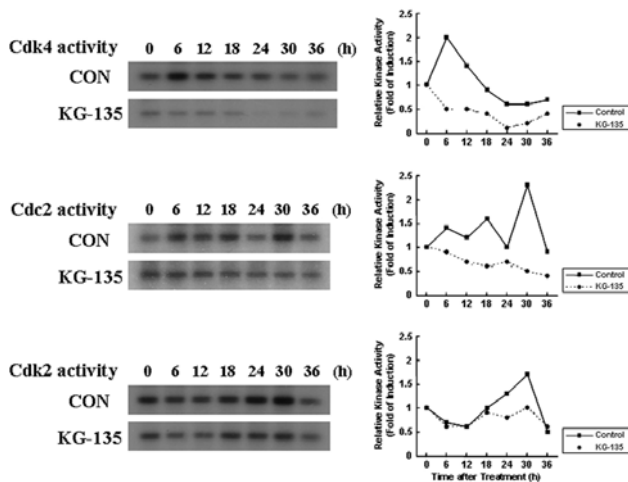


Figure 3. KG-135 treatment selectively inhibits Cdk4 and Cdc2 activities. (A) HeLa cells were incubated with or without 75  $\mu$ g/ml KG-135 in DMEM for 36 h, and cell lysates were prepared at 6-h intervals. Histone H1 kinase activity associated with immune-complexes precipitated by anti-Cdk4, Cdc2, and Cdk2 was determined as described in Materials and methods. Relative activities of each kinase were calculated by calibrated densitometer and plotted as a fold of induction.

To examine whether this decrease in cell viability was a consequence of cell cycle arrest at a specific phase or a consequence of cell death, we analyzed by flow cytometry the cell cycle of HeLa cells after treatment with KG-135. The results showed that cell growth was arrested at the G1 phase by treatment with 75  $\mu$ g/ml KG-135. The proportion of cells in the G1 phase gradually increased >88% of the total cells after 36 h of KG-135 treatment, while that in control cells

changed in a cell cycle-dependent fashion from 55 to 74% (Fig. 2A). The results indicated that the cell populations in S phase and G2/M phase decreased by 4.77 and 8.09%, respectively, after 36 h of KG-135 treatment (Fig. 2B). In addition, when cells were treated with KG-135 for 36 h, caspase-3 activity was not detectably activated (data not shown), although the sub G1 fraction was minimally elevated by 3.56%, as compared to that of the untreated cells (Fig. 2B). These results indicate that KG-135 arrests the cells at the G1 phase of the cell cycle in HeLa cells.

*Cdk4 and Cdc2 kinase activities are down-regulated in KG-135-treated HeLa cells.* Since cell cycle arrest was induced at G1 phase in KG-135-treated cells, we examined whether KG-135 could induce the down-regulation of Cyclin/Cdk activities that are responsible for cell cycle progression of G1 phase. The results indicated that Cdk4 and Cdc2 kinase activities were markedly down-regulated after 6 h of treatment with 75  $\mu$ g/ml KG-135, while Cdk2 activity was decreased after 24 h, as compared with the corresponding Cdk kinases activity in untreated control cells (Fig. 3).

These data indicate that KG-135-induced arrest of the cell cycle at G1 phase is associated with down-regulated Cdk4, Cdc2 and Cdk2 kinase activity in HeLa cells.

*Down-regulation of Cdk activity is associated with reduced protein levels of Cyclins and Cdk inhibitors and also with increased protein levels of Cdk inhibitors, p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> in KG-135-treated HeLa cells.* To investigate the possible mechanisms involved in the down-regulation of G1 Cyclin-dependent kinases activity in KG-135-treated HeLa cells, we first tested whether the down-regulated Cdk activities were temporally associated with altered protein levels of Cdk

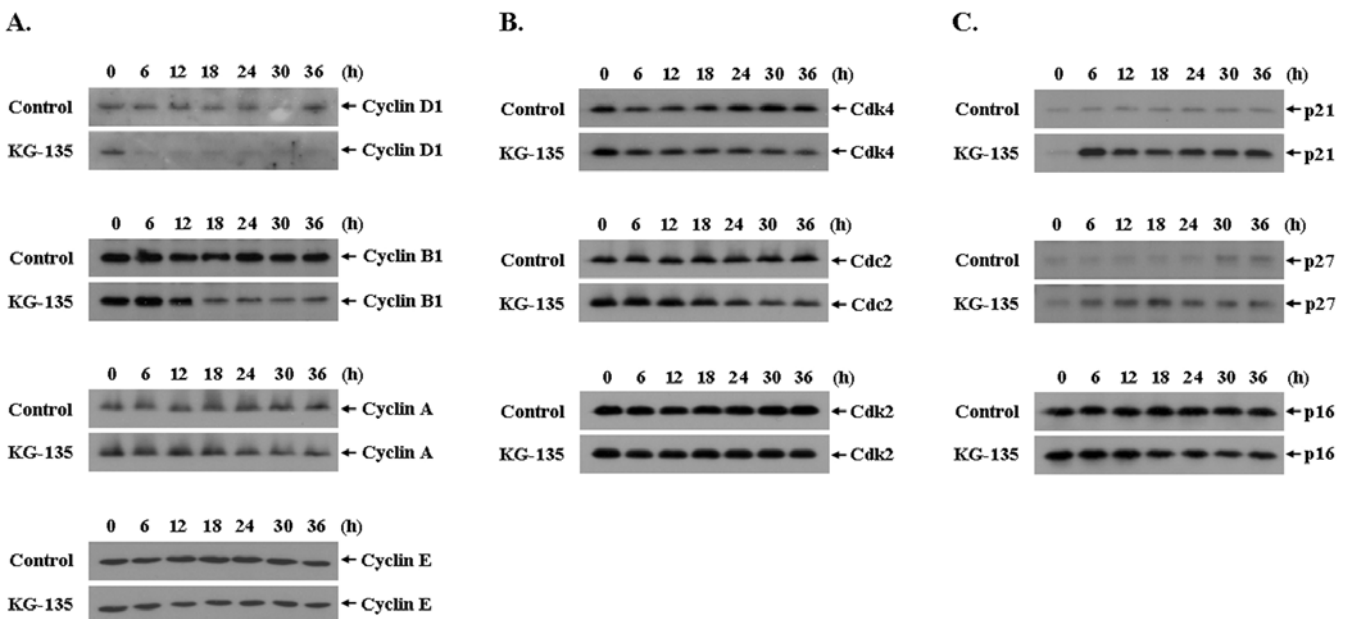


Figure 4. Protein levels of Cyclins and Cyclin-dependent protein kinases are decreased by KG-135 treatment in a time-dependent manner. (A) HeLa cells were treated with 75  $\mu$ g/ml KG-135 in DMEM for 36 h, and cell lysates were prepared at 6 h intervals. Levels of Cyclin D1, B1, A and E in total cell lysate were determined by immunoblot analysis using anti-Cyclin D1, B1, A and E antibodies. (B) Levels of Cdk4, Cdc2 and Cdk2 were determined by immunoblot analysis using anti-Cdk4, Cdc2 and Cdk2 antibodies. (C) Levels of p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and p16<sup>Ink4</sup> were determined by immunoblot analysis using anti-p21<sup>Waf1/Cip1</sup>, -p27<sup>Kip1</sup> and -p16<sup>Ink4</sup> antibodies.



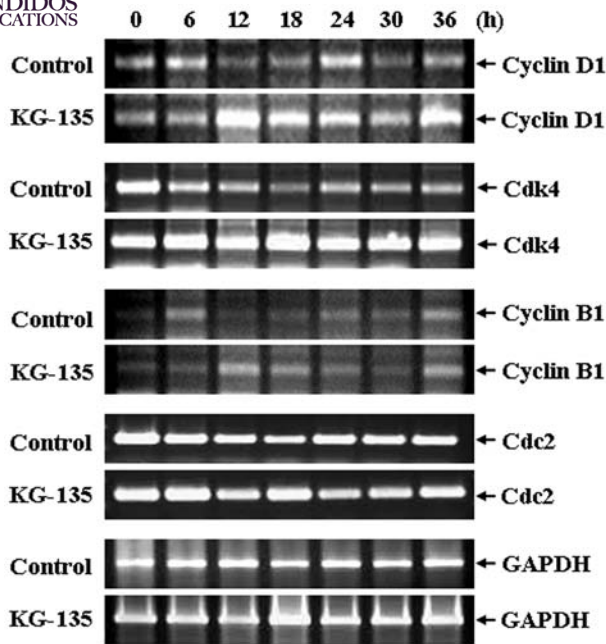


Figure 5. mRNA levels of Cyclin D1/Cdk4 and Cyclin B1/Cdc2 are not altered by KG-135 treatment. HeLa cells treated with 75  $\mu$ g/ml KG-135 for 36 h, and total mRNA was extracted from at 6-h intervals. cDNAs of each sample were prepared by reverse-transcription reaction, and 1  $\mu$ g/ml of cDNAs were amplified by polymerase chain reaction (PCR) with specific primers for Cyclin D1, Cdk4, Cyclin B1 and Cdc2, as described in Materials and methods. PCR products of each gene were electrophoresed on a 1.5% agarose gel, and images of the gel were obtained by GelDoc imaging system.

complexes in cells after treatment with KG-135 (Fig. 4). The results from immunoblot analyses indicated that the intracellular protein levels of Cyclins D1 and B1 were markedly decreased after 6-12 h with KG-135, while the protein levels of Cyclins A and E were minimally altered (Fig. 4A). Moreover, the protein levels of Cdk4 and Cdc2 were also decreased after 6 and 18 h of treatment respectively, while the Cdk2 protein level remained unaltered (Fig. 4A and B). We also examined the intracellular protein levels of endogenous Cdk inhibitors, p21<sup>Waf1/Cip1</sup>, p27<sup>Kip1</sup> and p16<sup>Ink4</sup> in KG-135-treated cells. The results indicated that the protein levels of p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> were dramatically increased over time from 6 to 36 h of treatment with KG-135, while the p16<sup>Ink4</sup> protein level was not changed during the time course tested (Fig. 4C). We then investigated whether these decreases in the protein levels of Cyclins and Cdks might also occur at the transcriptional level in KG-135-treated cells. The results from reverse transcription-polymerase chain reaction (RT-PCR) indicated that mRNA levels of Cyclin D1, Cdk4, Cyclin B1 and Cdc2 varied as the cells underwent cell cycle progression in untreated control cells (Fig. 5). In contrast, mRNA levels of Cyclin D1 and Cdk4 were markedly elevated and sustained throughout the time course in cells treated with KG-135, as compared to the mRNA levels of untreated control cells. The results showed that the mRNA levels of Cyclins D1 and B1, and Cdk4 and Cyclin B1 maximally increased by 48% (12 h), 65% (12 h) and 55% (18 h), respectively, while the mRNA level of Cdc2 decreased by 46% after 24 h of treatment.

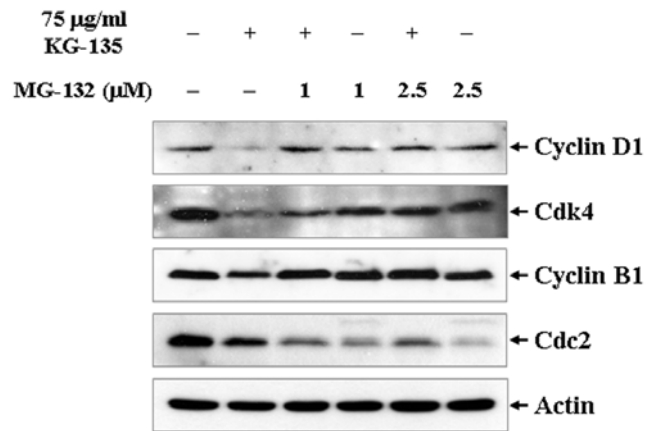


Figure 6. The proteasome inhibitor, MG-132, blocks KG-135-induced down-regulation of Cyclin D1, Cdk4 and Cyclin B1 protein in HeLa cells. HeLa cells were pre-treated with 1 or 2.5  $\mu$ M MG-132, and after 3 h cells were washed with growth media. Cells were then treated with 75  $\mu$ g/ml KG-135 for 24 h. Thereafter, whole-cell extracts were prepared, and 50  $\mu$ g of protein was resolved on 12% SDS-PAGE, electrotransferred, and probed with anti-Cyclin D1, -Cdk4, -Cyclin B1, -Cdc2 and -actin as described in Materials and methods.

However, these changes in intracellular mRNA levels did not temporally correlate to the reduced intracellular protein levels of corresponding proteins in cells induced by treatment with KG-135.

These results indicate that KG-135-induced down-regulation of Cdk4, Cdc2 and Cdk2 kinase activities can be primarily attributed to respective decreases in intracellular protein levels and also, in part, to increased protein levels of endogenous Cdk inhibitors, p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>. The results also indicated that decreases in protein levels of Cyclin D1, Cdk4, Cyclin B1 and Cdc2 in KG-135-treated cells may occur at the post-translational level.

*Decreases in protein levels of Cyclin D1/Cdk4 and Cyclin B1 are efficiently blocked by co-treatment with MG-132, a proteasome inhibitor.* We then investigated whether the KG-135-induced reduction of intracellular levels of Cyclin D1/Cdk4 and Cyclin B1/Cdc2 was mediated by the proteasome pathway, by using MG-132, a potent, selective and reversible proteasome inhibitor. Pre-treatment of cells with MG-132 markedly prevented the KG-135-induced decreases in protein levels of Cyclin D1, Cdk4 and Cyclin B1 in a dose-dependent manner (Fig. 6). In contrast, the reduced protein level of Cdc2 in KG-135-treated cells was not prevented by pre-treatment of cells with MG-132. The result also indicated that treatment of cells with MG-132 alone minimally affected the intracellular levels of these proteins. These data suggest that KG-135-induced decreases in the protein levels of Cyclin D1, Cdk4 and Cyclin B1, but not of Cdc2, are mediated by proteasome-mediated degradation in the KG-135 treated cells.

## Discussion

The present study was aimed to investigate the mechanism by which KG-135, heat processed neoginseng, exerts its anti-

proliferative effects in human cancer HeLa cells. Our results show that KG-135 induces cell cycle arrest at the G1 phase of HeLa cells by a mechanism that involves down-regulation of Cdk4 and Cdc2 kinase activities through the proteasomal degradation of Cyclin D1, Cdk4 and Cyclin B1.

There is evidence to suggest that the activity of Cyclin-dependent kinases is universally over-activated in human cancers. Thus, Cyclin-dependent kinases are thought to be good target molecules for developing anti-cancer drugs for various types of human cancers. Since it has been suggested that ginsenosides exert cytostatic or cytotoxic activities against various cancer cell types in culture and also in animal cancer models, it is important to understand how these agents arrest cell proliferation and consequently lead to apoptosis in cancer cells.

In our earlier studies, we showed that ginsenosides of the diol type, including Rg5, Rs3, Rs4, panaxadiol and Rh2 induce cell cycle arrest at lower doses, and apoptosis at higher doses (11,14,23-25). These diol-type ginsenosides share a common dammarane skeletal structure with -OH groups attached at C-3 and C-12, but they play different functional roles in cell cultures (26). KG-135 is a heat-processed neoginseng produced to enrich the contents of diol-type ginsenosides which constitute roughly equal amounts of three ginsenosides, Rk1, Rg3 and Rg5. Earlier studies suggested that KG-135 exerts much higher cytostatic and cytotoxic effects against human cancer cells in cultures than do conventional red ginseng and white ginseng preparations. Rg5 has been shown to increase intracellular p21<sup>Waf1/Cip1</sup> protein levels and also to down-regulate Cyclin E-dependent kinase activity, while Rs3 and Rs4 were shown to elevate protein levels of p53 and p21<sup>Waf1/Cip1</sup> and also to down-regulate Cyclins E- and A-dependent kinases activity in cell cultures (11,23,24). Panaxadiol also selectively inhibits Cyclin A-dependent kinase activity by a mechanism associated with increased intracellular p21<sup>Waf1/Cip1</sup> levels, while Rh2 induces cell cycle arrest at the G1/S boundary by down-regulating Cyclin E-dependent kinase activity, which may be associated with increased intracellular levels of p27<sup>Kip1</sup> (14,25). When taken together with the results of other groups (12,13,27), our results suggest that diol-type ginsenosides inhibit cell proliferation by arresting the cell cycle at G1 or G1/S phase by down-regulating Cyclin-dependent kinase activities. However, the underlying mechanism of this down-regulation in cells treated with ginsenosides is still unknown. In this study, our results indicate that KG-135 induces cell cycle arrest at G1 phase in human cervix carcinoma HeLa cells. The cell population at G1 phase gradually increased to 88.14% of the total cells after 36 h of treatment, while those in S and G2/M phase decreased by 8.09 and 4.77%, respectively (Fig. 2A and B). After 36 h, KG-135 treatment increased the number of cells in subG1 phase by 3.56%, but it did not activate caspase-3 activity in HeLa cells (data not shown). Thus, under these experimental conditions, KG-135 does not induce apoptotic cell death, but potently arrests the cells at the G1 phase of the cell cycle. The cell cycle arresting effect at the G1 phase is kinetically well correlated with down-regulation of the Cyclin-dependent kinase activities of Cyclin D1/Cdk4 and Cyclin B1/Cdc2 (Fig. 3). Cyclin E/Cdk2 activity was also down-regulated in the same cells, but the down-regulation kinetics did not

correlate with cell cycle arrest at G1 phase, as the kinase activity was down-regulated after 24 h of KG-135 treatment. We found that the KG-135-induced down-regulation of these Cyclin-dependent kinases activity correlated well with the time-dependent decreases in protein levels of Cyclin D1, Cdk4, Cyclin B1 and Cdc2 in cells treated with KG-135 (Fig. 4A and B). It is interesting to note that the down-regulation of Cyclin D1/Cdk4 and Cyclin B1/Cdc2 activity, but not Cyclin E/Cdk2 activity, also kinetically correlates with increased levels of p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> proteins in cells treated with KG-135 (Fig. 4C). Thus, the KG-135-induced down-regulation of Cdk4 and Cdc2 activity is likely to be due to the reduced intracellular levels of Cyclin D1/Cdk4 and Cyclin B1/Cdc2 complexes and also to the elevated protein levels of inhibitors p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>.

These results support earlier studies suggesting that the anti-proliferating effect of ginsenoside Rg5 is related to down-regulation of Cyclin E-dependent kinase activity by increased protein levels of p21<sup>Waf1/Cip1</sup> and to decreased protein levels of Cyclin E, Cdk2, and Cdc25A in human hepatocarcinoma cells (23). Moreover, ginsenoside Rg1, a diol-type ginsenoside, has been shown to block the cell cycle at G1 phase by a mechanism that is possibly associated with the down-regulation of Cyclin D1 levels and the up-regulation of intracellular levels of p27<sup>Kip1</sup>, p21<sup>Waf1/Cip1</sup> and p53 in human arterial vascular smooth muscle cells (28). These results indicate that diol-type ginsenosides, such as Rg5, Rg1, and Rg3, are able to block cells at the G1 phase of the cell cycle by down-regulating G1 Cyclin-dependent kinase activities. In addition, these diol-type ginsenosides are commonly able to down-regulate G1-Cyclin/Cdk activity by reducing the intracellular levels of the Cyclin-Cdk complexes and/or by increasing the levels of the Cyclin-Cdk inhibitor proteins, p27<sup>Kip1</sup> and p21<sup>Waf1/Cip1</sup>. However, diol-type ginsenosides appear to differentially influence target molecules and their intracellular levels in cells. Since diol-type ginsenosides are able to down-regulate G1-Cyclin/Cdk activity by mechanisms that involve different target molecules, it has been suggested that a mixture of diol-type ginsenosides, such as KG-135 which contains roughly equal amounts of Rk1, Rg3, and Rg5, would be more effective in inhibiting the growth of cancer cells (23,29-31). It is noteworthy that KG-135 down-regulates G1 Cyclin/Cdk activity through multiple mechanisms that mediate decreases in the levels of Cyclins and Cdks and increases in the levels of Cyclin-Cdk inhibitor proteins, p27<sup>Kip1</sup> and p21<sup>Waf1/Cip1</sup>. The results indicate that diol-type ginsenosides in KG-135 exert additive effects, and down-regulate G1 Cyclin/Cdk activity through multiple combined mechanisms as described above.

We found that KG-135-induced degradation of Cyclins D1 and B1, and Cdk4 is effectively prevented by co-treatment with MG-132, a proteasome inhibitor, while the degradation of Cdc2 protein is not rescued by MG-132, indicating that the down-regulation of Cdc2 kinase activity is not associated with the proteasome pathway in KG-135-treated cells. In addition, the down-regulated Cdk2 activity does not correlate with proteasome-mediated degradation of Cyclin E and Cdk2 proteins, or with the inhibition kinetics of the cell cycle, since Cdk2 activity is down-regulated after 24 h of KG-135 treatment. Thus, KG-135 is able to arrest cells at the G1



the cell cycle by down-regulating the Cyclin activity that is mediated through the proteasome-mediated pathway. This is the first study to show that ginsenoside can induce the degradation of Cdk4, Cyclin B1 and Cyclin D1 via the proteasome-mediated pathway. Many studies have suggested that proteolysis of cell cycle regulators by the proteasome is essential for cell cycle progression or arrest in mammalian cells. For example, curcumin, a well-known chemopreventive agent, has been shown to suppress proliferation of various cancer cells through proteasome-mediated down-regulation of cyclin E (32), and rapamycin causes a G1 arrest in HER-2-overexpressing breast cancer cells that is associated with proteasome-mediated degradation of Cyclin D3 (33).

Our results also indicate that KG-135 induces elevation of the intracellular protein levels of p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>. This KG-135-induced expression of CDK inhibitors correlates well in a time-dependent fashion with the down-regulated activity of Cdk4 and Cdc2. Thus, it is very likely that KG-135-induced down-regulation of the G1-Cyclin/Cdk kinases activity is also significantly mediated by increased expression of the CDK inhibitors. Down-regulation of Cdc2 kinase activity in KG-135-treated cells is also likely to be associated with the elevated levels of the Cdk inhibitors in the cells.

Many investigators have invested substantial efforts to develop Cdk inhibitors as anti-cancer drugs on the basis of the fact that progression of many human cancers is associated with abnormal activation of Cdk activity. Since it has been difficult to develop selective Cdk inhibitors that inhibit a specific Cdk kinase activity, other attempts have been made to develop a pan Cdk inhibitor that could inhibit Cdk-activating kinases activity or increase the expression of endogenous Cdk inhibitor proteins, such as p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>. We suggest that KG-135, a mixture of three diol-type ginsenosides, is a useful candidate for the treatment of human cancers, since it exhibits selective down-regulation of G1-Cyclin/Cdk kinases activity through the proteasome pathway and induces the expression of Cdk inhibitor proteins.

## Acknowledgements

We thank Ginseng Science Corporation (Seoul, Korea) for donation of KG-135 and this work was supported by grant A04-0021-AK1101-07A4-00040B (2007) from fund M10104000129-02J0000-05910 from the Ministry of Health and Welfare, Korea (to S.K.L.).

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