Abstract. A previous study reported that a novel dammarane-type triterpene saponin, ginsenoside-Rg18, derived from the root of Panax ginseng, displayed hydroxyl radical scavenging, anti-bacterial and cytotoxic activities. However, the underlying molecular mechanisms of its anti-proliferative effect on non-small cell lung cancer (NSCLC) A549 cells remains unclear. In the present study, it was determined that Rg18 inhibited the proliferation of A549 cells with a half-maximal inhibitory concentration of 150 µM. Flow cytometry analysis indicated that cell cycle progression was blocked by Rg18 at G1 phase in A549 cells, which was accompanied by downregulation of cyclin-dependent kinase 2 (CDK2), CDK4, CDK6, cyclin D1, cyclin D2, cyclin E and phosphorylated retinoblastoma protein expression at the protein level. In addition, the CDK inhibitors (CDKNs), CDKN1A and CDKN1B, were upregulated following Rg18 treatment. Furthermore, Rg18 treatment resulted in the intracellular accumulation of reactive oxygen species (ROS), and a dose-dependent inhibition of p38 mitogen activated protein kinase (p38), c-Jun N-terminal kinase (JNK) and nuclear factor-κB phosphorylation. Taken together, Rg18-mediated G1 phase arrest was closely associated with the generation of intracellular ROS, and p38, JNK and NF-κB/p65 inhibition in A549 human NSCLC cells.

Introduction

Lung cancer is one of the leading causes of cancer-associated mortality worldwide (1). Non-small-cell lung cancer (NSCLC) accounts for ~80% all cases of lung cancer and has a 5-year survival rate of 15% (2). Resistance can be developed to contemporary chemotherapy regimens for NSCLC and various adverse side effects can be invoked (3). Therefore, the identification and development of more effective, less toxic antitumor NSCLC therapies is urgently required.

The cell cycle is a highly regulated mechanism of controlling cell growth, proliferation and survival. Cancer cells exhibit dysregulated cell cycles, with the overexpression of positive regulators and inhibition of negative regulators, resulting in unlimited replication potential (4). Therefore, the development of agents targeting the dysregulated cell cycle has been considered to be a suitable strategy for cancer therapy (3). Cell cycle progression depends on cyclin-dependent kinase (CDK) activation status, which act consecutively in G1 phase to initiate S phase and, in G2 phase, to initiate mitosis. With mitogenic stimulation, cyclin D is activated, in turn activating CDK4 and 6. Cyclin D-dependent kinases phosphorylate retinoblastoma proteins (Rb), which relieves transcription factor E2F1 from inhibition and allows for the expression of specific E2F1-target genes (5). The cyclin E-CDK2 complex concludes Rb phosphorylation and permits the activation of E2F1-responsive genes (6). Cyclin-CDK complexes are inhibited by two CDK inhibitor (CDKN) families: Inhibitors of CDKN4 (INK4) (p15INK4B, p16INK4A, p18INK4C and p19INK4D) and CDKN1A (p21CIP1/WAF1), 1B (p27(KIP1)) and 1C (p57(KIP2)) (7). The CDKNs also perform central cell cycle-regulating functions, coordinating internal and external signals that modulate cell cycle progression (7). Thus, the cell cycle is tightly controlled in response to external and internal factors. In abnormal conditions, including DNA damage, cell cycle progression is inhibited to prevent harmful progression.

Reactive oxygen species (ROS) are chemically reactive molecules, including the superoxide anion, hydrogen peroxide and the hydroxyl radical. ROS are endogenously
produced during metabolic activities of the cell, including the oxidative phosphorylation of mitochondria (8). ROS may also arise from reactions with exogenous sources, including xenobiotic compounds (9). Cellular function requires moderate ROS levels for normal cell signaling. However, ROS overproduction can be toxic to cells owing to their peroxidative activity (10). One proposed anticancer strategy is the use of exogenous ROS-stressing agents or inhibition of endogenous antioxidants to elevate intracellular ROS levels to toxic amounts, triggering cell cycle arrest in the target cancer cell (11).

Ginsenosides are triterpene saponins and the major pharmacologically active components extracted from the roots and rhizomes of various Panax species. Ginsenosides are responsible for the majority of functions of ginseng, including angiogenesis, vasorelaxation and antioxidation (8). These molecules also possess anti-inflammatory and antitumor properties (12-14). In a previous study, four dammarane-type triterpene saponins isolated from the Panax ginseng root exhibited effective hydroxyl radical scavenging ability, as well as antibacterial and cytotoxic activities (15). However, to the best of our knowledge, there has been no research into the anti-proliferative effects of these compounds or their underlying molecular mechanisms. As part of an ongoing screening program for the evaluation of the anti-proliferative potential of natural compounds, the mechanisms underlying the cell cycle-arresting activities of Rg18 in NSCLC A549 cells were investigated in the present study.

Materials and methods

Chemicals and reagents. Rg18 and Rs11 (Fig. 1A) were kindly provided by Dr. Kyung-Tack Kim (Korea Food Research Institute, Wanju-gun, South Korea), and its purity of >96% was determined by high-performance liquid chromatography-mass spectrometry analyses (15). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were all obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). MTT, phenylmethylsulfonyl fluoride (PMSF), -acetylcysteine (NAC), 2',7'-dichlorofluorescin diacetate (DCFH-DA), bisacrylamide and sodium dodecyl sulfate were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). CDK2 (sc-163), CDK4 (sc-260), CDK6 (sc-7961), cyclin D1 (sc-8396), cyclin D2 (sc-593), cyclin E (sc-198), p21WAF1/CIP1 (sc-397), p27KIP1 (sc-1641), Rb (sc-102), p38 mitogen activated protein kinase (p38, sc-535), c-Jun N-terminal kinase (JNK, sc-7345), extracellular signal-regulated kinase (ERK, sc-94), p65 (sc-72), p-JNK (sc-12882), p-ERK (sc-7383) and -actin (sc-81178) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Phosphorylated (p)-Rb (no. 9307), p-p38 (no. 9215), p-p65 (Ser536, no. 3031) primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Proteinase K, ribonuclease A, and TEMED were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Cell culture. The human lung adenocarcinoma A549 cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). A549 cells were grown at 37°C in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin sulfate, in a humidified atmosphere of 5% CO2.

MTT assay. Cell viability was assessed by MTT assay, as described previously (16). A total of 5x104 cells/ml were seeded in wells containing 100 µl complete medium in a 96-well plate. After 24 h, Rg18 or Rs11 was added at concentrations of 0, 6.25, 12.5, 25, 50, 100 and 200 µM and cells were incubated for 48 h. A total of 50 µl MTT (stock solution, 5 mg/ml in PBS) was added, and the plates were incubated for an additional 4 h. The medium was disposed and the formazan blue was dissolved using 100 µl DMSO/well. The optical density was measured at 540 nm.

Cell cycle analysis. The cell cycle distribution was analyzed using propidium iodide staining as described previously (17). A total of 1x106 cells were collected from each experimental group, washed twice with PBS (4°C), fixed and permeabilized with 70% ethanol at 4°C for 1 h. The cells were washed once more with PBS and resuspended in a solution containing propidium iodide (50 µg/ml) and RNase A (250 µg/ml) for 30 min at room temperature. Fluorescence-activated cell sorting (FACS) was then performed to determine the cell cycle stage of each cell, using the CytomicsTM FC 500 flow cytometry and CXP cytometer software (version 2.0; Beckman Coulter, Inc., Indianapolis, IN, USA).

Protein extraction and western blot analysis. Cells were collected by centrifugation at 200 x g for 10 min at 4°C, washed twice with PBS at 4°C, and centrifuged at 200 x g for 5 min. The resulting cell pellet was resuspended in 1X protein lysis buffer (Intron Biotechnology, Inc., Seongnam, Korea). The protein concentration was determined using the protein lysis buffer (Intron Biotechnology, Inc., Seongnam, Korea). The protein concentration was determined using the manufacturer's instruction. Equal amounts of cell lysates (5-15 µl) were separated by 8-12% SDS-PAGE and transferred into nitrocellulose membranes. The membranes were incubated with the aforementioned primary antibodies. The membranes were then incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated Goat anti-mouse IgG (no. 31430), Goat anti-rabbit IgG (no. 31460), or Rabbit anti-Goat IgG (no. 81-1620) (Thermo Fisher Scientific Inc.) and visualized using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), according to the manufacturer's protocol. -actin was used as a loading control and densitometric analysis was performed using Quantity One® Software (version 4.6.3; Bio-Rad Laboratories, Inc.).

ROS detection (DCFH-DA assay). Intracellular ROS levels were detected using a DCFH-DA assay, as described previously (18). Cells (5x104 cells/ml) were pretreated with and without 10 mM NAC for 1 h and then treated with 150 µM Rg18 for 24 h. The cells were collected and suspended in pre-warmed PBS (37°C) prior to the addition of 20 µM DCFH-DA and incubated for 30 min at 37°C. When transported into the cells, DCFH-DA formsDCFH by deacetylation, and upon oxidation, fluorescent 2',7'-dichlorofluorescin is formed. Fluorescence intensity was analyzed by Cytomics™ FC 500 flow cytometry and CXP cytometer software (version 2.0; Beckman Coulter, Inc., Indianapolis, IN, USA).
Statistical analysis. All data are presented as the mean ± standard deviation of the results from three independent experiments. One-way analysis of variance followed by Dunnett’s post hoc test was performed to identify differences between groups using GraphPad Prism software (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Rg18 inhibits cell proliferation and induces cell cycle arrest in A549 cells. The anti-proliferative effects of Rg18 and Rs11 were examined in A549 cells using an MTT assay (Fig. 1B) and half-maximal inhibitory concentration (IC₅₀) values. The A549 cells were treated with different concentrations of Rg18 and Rs11 for 48 h, and it was found that Rg18 significantly decreases the viability of A549 cells, and these decreases are concentration-dependent (IC₅₀=140.09 µM). However, Rs11 did not show obvious cytotoxic effects up to 100 µM and it decreased cell viability at 200 µM (cell viability: 51.78 %). The following experiments in A549 cells were conducted using Rg18 owing to their greater sensitivity to this ginsenoside.

To investigate the mechanism of the anti-proliferative effect of Rg18, flow cytometry was used to identify changes in cell cycle distribution following treatment with Rg18. A549 cells were treated with various concentrations of Rg18 (0, 50, 100 and 150 µM) for 48 h, and the percentage of A549 cells in G₁ phase increased from 63.3±1.5% (control group) to 68.6±2.1, 69.8±1.4 and 71.1±2.5% upon treatment with 50, 100 and 150 µM Rg18, respectively (Fig. 1C).
Effect of Rg18 on protein expression levels of various G1 phase cell cycle regulators in A549 cells. Rg18-induced G1 arrest in A549 cells (Fig. 1C), therefore, the protein expression levels of cell cycle regulatory molecules were investigated following 50, 100 or 150 µM Rg18 treatment. Rg18 treatment significantly reduced the protein expression level of CDK2, CDK4, CDK6, cyclin D1, cyclin D2 and cyclin E in A549 cells. Rg18 treatment also increased the protein expression levels of p21^CIP1/WAF1 and p27^KIP1 after a 48-h treatment. These proteins modulate CDK activity in various phases of the cell cycle, including G1. As Rb phosphorylation is influential in the transit from G1 to S phase (19), the phosphorylation levels of Rb were also examined. Following treatment with 150 µM Rg18 for 48 h, the degree of phosphorylation and expression levels of Rb significantly decreased (Fig. 2).

Rg18 treatment results in upregulation of intracellular ROS levels and downregulates p38, JNK and nuclear factor-κB (NF-κB) activation in A549 cells. ROS generation is an early sign of cell cycle arrest (20). To analyze ROS generation induced by Rg18 treatment, a DCFH-DA assay was performed. Following treatment with 150 µM Rg18 for 2 h, the generation of ROS increased significantly, an effect that was blocked by pretreatment with the antioxidant NAC (Fig. 3A). To investigate the molecular mechanism of Rg18-induced G1 arrest, p38, ERK, JNK, and NF-κB (p65) phosphorylation were analyzed in Rg18-treated A549 cells. These molecules serve notable roles in cell cycle regulation (21,22). Rg18 treatment resulted in a concentration-dependent decrease in p38, JNK and NF-κB/p65 phosphorylation, but there was no change to ERK phosphorylation levels (Fig. 3B).

**Discussion**

Previous studies have demonstrated that ginsenosides isolated from the root of *P. ginseng* C. A. Meyer could inhibit cancer cell growth in vitro and in vivo via cell cycle arrest (14,23-25).
In a previous study, it was demonstrated that four novel ginsenosides isolated from the *P. ginseng* root exhibited hydroxyl radical scavenging, anti-bacterial and cytotoxic activities (15). The aim of the present study was to determine whether Rg18 exerted an anti-proliferative effect on A549 cells and to characterize the molecular mechanism involved. The results demonstrated that Rg18 inhibited the proliferation of A549 cells and flow cytometric assays indicated that treatment with Rg18 lead to G<sub>1</sub> arrest in A549 cells.

Cell cycle progression is highly controlled by interactions of various regulators, including the cyclins and their catalytic partners, CDKs (6). CDK complexes are formed and activated at specific cell cycle phases; their activities are necessary for progression through distinct cell cycle phases (7). Progressing through the G<sub>1</sub> phase requires either CDK4 or CDK6 activity, followed by the activation of CDK2. The cyclin-CDK complex formed during G<sub>1</sub> phase catalyzes the phosphorylation of the dominant inhibitors of G<sub>1</sub>/S phase cell cycle progression, the Rb family of tumor suppressor proteins, thereby allowing progression to S phase (26,27). Cyclin-CDK complexes can bind p21<sup>CIP1/WAF1</sup> and p27<sup>KIP1</sup>, which inhibit kinase activities and prevent cell cycle progression (28). Western blot analysis demonstrated that Rg18 decreased the expression levels of cyclin D1, cyclin D2, cyclin E, CDK4, CDK6 and CDK2 in A549 cells. Furthermore, decreased CDK expression has been demonstrated to be associated with Rb under-phosphorylation, which is known to result in the sequestering of E2F, and thereby inhibition of the cell cycle progression (29).

Figure 3. Rg18 upregulates the level of intracellular ROS and regulates the activation of p38, JNK and NF-κB in A549 cells. (A) DCFH-DA was added to stain the A549 cells following treatment with 150 µM Rg18 for 2 h. Flow cytometry was used to analyze the level of ROS in the cells. ***P<0.001 vs. the control group; #P<0.01 vs. Rg18 treatment group. (B) Western blot analysis was performed with antibodies against p-p38, p38, p-JNK, JNK, p-ERK, ERK, p-p65, p65 and β-actin. The histograms represent the mean fluorescence intensity of samples analyzed ***P<0.001 vs. Rg18 treatment group. Data presented are the mean ± standard deviation of the results from three independent experiments. ROS, reactive oxygen species; p-p38, phosphorylated p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; DCFH-DA, 2',7'-dichlorofluorescin diacetate; ERK, extracellular signal-regulated kinase; NAC, N-acetylcysteine.
The results indicate that Rg18 influences cell cycle progression via the upregulation of p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{KIP1} protein expression in A549 cells. It was apparent that strong CKI upregulation mediated Rg18-induced G\textsubscript{i} phase arrest and the inhibition of cell growth. Overall, the G\textsubscript{i} phase blockade in A549 cells appeared to be mediated by the downregulation of CDK activity associated with CKI induction, such as by p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{KIP1}.

ROS are involved in multiple types of chemically induced cell cycle arrest; evidence indicates that increased oxidative stress is associated with cell cycle arrest induced by certain anticancer agents (11,30). Among the protopanaxadiols, ginsenoside-Rb\textsubscript{1}, has been demonstrated to significantly increase the expression of genes encoding antioxidant enzymes, including superoxide dismutase and catalase \textit{in vitro} (31). The present study demonstrated that Rg18 treatment increased intracellular ROS levels, which led to cell cycle arrest.

The mitogen-activated protein kinases (MAPKs) are also involved in cell cycle regulation (21), and three pathways, ERK, JNK and p38, are closely associated with the progression of a number of malignant types of cancer, including breast and ovarian cancer, and NSCLC (32,33). JNK and p38 function in stress reactions and the induction of cell cycle arrest (34). The anticancer activity of 20(S)-protopanaxadiol in colon cancer cells is mediated by downregulation of the ERK, JNK and NF-\kappa B signaling pathways (35). Additionally, compound K significantly inhibited phorbol 12-myristate 13-acetate-induced matrix metalloproteinase 9 protein expression and secretion via suppression of DNA-binding and activator protein-1 transcriptional activities, downstream of the p38, ERK and JNK pathways (36). However, it has been established that selenite-induced ROS arrest the cell cycle of NB4 cells at the G\textsubscript{i} phase by inhibiting the JNK activating transcription factor 2 \textit{axis in vitro} and \textit{in vivo} (37). In the present study, it was demonstrated that Rg18 treatment suppressed the phosphorylation of JNK and p38 in A549 cells.

Data from previous studies indicated that blocking the activation of NF-\kappa B could be a critical target for the regulation of cell proliferation and antioxidant behaviors (38-40). Ginsenoside Rg\textsubscript{3} has been reported to inhibit NF-\kappa B, induce G\textsubscript{i} arrest and enhance susceptibility to docetaxel and other chemicals in prostate cancer cells (41). Furthermore, the ginsenoside Rd has been demonstrated to elevate intracellular glutathione levels by increasing \gamma-glutamyl cysteine ligase activation in rat hepatocyte H4IIE cells through NF-\kappa B-DNA binding (42). This result indicates that NF-\kappa B serves as a cellular marker for cell cycle arrest in HL-60 cells. In the present study, Rg18 treatment inhibited the phosphorylation of NF-\kappa B/p65 in A549 cells. However, the exact mechanism of this effect, and whether it took place at the transcriptional and/or translational levels, requires further investigation.

In summary, Rg18 was found to inhibit the proliferation of A549 cells by arresting the cell cycle at the G\textsubscript{i} phase by downregulating CDK2, CDK4 and CDK6 expression, in association with the induction of p21\textsuperscript{CIP1/WAF1} and p27\textsuperscript{KIP1}. The results indicated that this was, at least in part, due to intracellular ROS production and the downregulation of multiple signaling pathways, including JNK, p38 and NF-\kappa B/p65. Further research is required to dissect the underlying mechanisms of these pathway changes. However, these results illustrate the potential use of Rg18 in cancer treatment, either alone or in combination with other anticancer agents.

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**Availability of data and materials**

All data generated and/or analyzed during this study are included in this published article.

**Authors’ contributions**

DGL and JSS designed experiments, analyzed the data and statistics, and drafted the manuscript. KTK, SYC, and MHL. did isolation, structural analysis, and a purity analysis of Rg18 and Rg11. KTL designed the study, coordinated the project and gave final approval of publication. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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