JS-K induces G_2/M phase cell cycle arrest and apoptosis in A549 and H460 cells via the p53/p21$^{WAF1/CIP1}$ and p27^{KIP1} pathways

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Abstract. Lung cancer is one of the most common malignancies worldwide, with high mortality and morbidity rates. O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) is a potent anticancer agent that acts against a subset of human non-small cell lung cancer (NSCLC) cell lines; however, the underlying mechanisms of JS-K in NSCLC remain unclear. The present study aimed to evaluate the anticancer effect of JS-K and investigate its underlying mechanisms in A549 and H460 cells. In the present study, A549 and H460 cells were treated with JS-K, and then evaluated by cell viability assay, flow cytometry and western blot analysis. JS-K markedly induced cell cycle arrest at the G_2/M phase in a concentration and time-dependent manner in both cell lines. This was associated with increased expression levels of p53, and the cell cycle inhibitors p21$^{WAF1/CIP1}$ and p27^{KIP1}, which, in turn, inhibited the expression of Cdc2, cyclin B1 and cyclin-dependent kinase 2. In addition, JS-K-induced inhibition of proliferation was revealed to be partially modulated by the upregulation of p53 and p21^{WAF1}, the ratio of Bax/Bcl-2, and the activation of both the intrinsic and extrinsic apoptotic pathways in A549 and H460 cells. These results demonstrated that JS-K could trigger cell cycle arrest at the G_2/M phase and apoptosis in A549 and H460 cells, which was likely mediated via the p53/p21$^{WAF1/CIP1}$ and p27^{KIP1} pathways. Overall, the results indicated that JS-K may be used as an anticancer agent for the treatment of NSCLC.

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Introduction

Lung cancer is the second most common type of cancer (1) and is the leading cause of cancer-associated mortality (2), with >1.38 million mortalities reported worldwide (3). At present, the majority of patients with lung cancer have a poor prognosis, with a 5-year relative survival rate of <17% (4). Lung cancer can be classified into small-cell lung cancer and non-small cell lung cancer (NSCLC), according to histological characteristics (5). More than 80% of lung cancer cases are NSCLC (6), which is composed of adenocarcinoma, squamous cell carcinoma and large cell carcinoma (7). Due to a lack of symptoms associated with early-stage lung cancer, the majority of lung cancer cases are diagnosed at an advanced stage when there are limited treatment options (8-10). The survival rate for patients with early-stage NSCLC is relatively higher following surgery (11). However, the majority of patients have a high risk of recurrence. Systemic chemotherapy is the most mainstream therapeutic option for the treatment of advanced NSCLC; however, the median survival time is only slightly >18 months from the time of diagnosis (12,13). Furthermore, it has been demonstrated that chemotherapy has notable resistance and insensitivity in the treatment of lung cancer (14). Therefore, the investigation of novel anticancer drugs with high efficiency and selectivity against lung cancer is required.

The well-known tumor suppressor gene p53 has been identified to be involved in DNA repair, transcription, genomic stability, cell cycle and apoptosis following cellular exposure to various types of stress (15-17). Both cell cycle arrest and apoptosis are important tumor-suppressive pathways that involve p53 (18,19). Cell cycle arrest is regulated by the depletion of critical cell cycle proteins. Upon p53 activation, the cyclin-dependent kinase (CDK) inhibitor p21$^{WAF1/CIP1}$ is upregulated, which leads to the inhibition of numerous cell cycle proteins, including CDK-4,

CDK-6/cyclin D, CDK2/cyclin E and cyclin B1/Cdc2 (20). Cell apoptosis is associated with complex signaling pathways. p53 mediates cell apoptosis by upregulating BH3-only proteins, including Bax, and downregulating Bcl-2 family members, including Bcl-2 (21). As an intracellular suicide program, cell apoptosis is also mediated by the activation of caspases, including caspase-3, -8 and -9, and poly(ADP-ribose) polymerase (22,23). Therefore, triggering pathways involved with apoptosis and inhibiting cell cycle progression may present new strategies for the treatment of lung cancer.

Nitric oxide (NO) serves a crucial role in regulating the activity of cancer (24). As an arylated diazeniumdiolate-based anticancer agent, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K; Fig. 1) exhibits cytotoxic and potent antitumor effects in several types of human cancer, including prostate cancer (25), hepatoma (26), acute myeloid leukemia (27), multiple myeloma (28) and bladder cancer (29). JS-K can also enhance cytotoxicity in doxorubicin-treated renal carcinoma cells by upregulating the expression of p53 (30). Notably, a number of studies have reported that JS-K was selectively cytotoxic to cancer cells and demonstrated no significant toxicity towards normal cells (25,28,31). A previous study demonstrated that a reactive oxygen species (ROS) activation mechanism contributed to JS-K-induced apoptosis in human NSCLC cells, including H1944 and H1703 cells (32). However, there is limited understanding regarding the mechanism of action of JS-K in other lung cancer cells, including A549 and H460 cells. The present study aimed to investigate the underlying antitumor mechanism of JS-K in A549 and H460 human NSCLC cells. To the best of our knowledge, the present study is the first to demonstrate that p53/$p21^{WAF1/CIP1}$ and $p27^{KIP1}$ proteins are involved in JS-K-induced apoptosis and cell cycle arrest.

Materials and methods

Chemicals. JS-K was acquired from Sigma-Aldrich; Merck KGA (Darmstadt, Germany; cat. no. J4137), and stored at -80˚C at a concentration of 20 mM in 100% dimethyl sulfoxide (DMSO). The final density of DMSO used in the present study was <0.1%. Pifithrin-α (PFT-α) was purchased from MedChemExpress (New Jersey, USA; cat. no. HY-15484) and diluted to a final concentration of 30 μM with 100% DMSO. Antibodies against caspase-3 (cat. no. 9665S), caspase-9 (cat. no. 9502S), caspase-8 (cat. no. 4790S), Bcl-2 (cat. no. 4223S), Bax (cat. no. 2772S), p53 (cat. no. 9282S), $p21^{Waf1/Cip1}$ (cat. no. 2947S), cyclin B1 (cat. no. 4138P), CDK2 (cat. no. 2546P), cyclin E1 (cat. no. 4129P), $p27^{Kip1}$ (cat. no. 3686S), Cdc2 (cat. no. 9112S), p-Cdc2 (cat. no. 4539S) and GAPDH (cat. no. 2118L) were all obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies (caspase-3, caspase-9, caspase-8, Bcl-2, Bax, p53, $p21^{Waf1/Cip1}$, cyclin B1, CDK2, cyclin E1, $p27^{Kip1}$, Cdc2, p-Cdc2 and GAPDH) were diluted at the 1:1,000 with 5% bovine serum albumin (BSA). Goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase antibody was used as the secondary antibody (Sino Biological, Inc., Beijing, China; cat. no. SSA004). The secondary antibody was diluted with 5% non-fat milk in proportion of 1:2,000. The Annexin V-FITC Apoptosis Detection kit was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan; cat. no. AD10). Cell Cycle Analysis kit (cat. no. C1052) and MTT (cat. no. ST316) were all obtained from Beyotime Institute of Biotechnology (Shanghai, China).

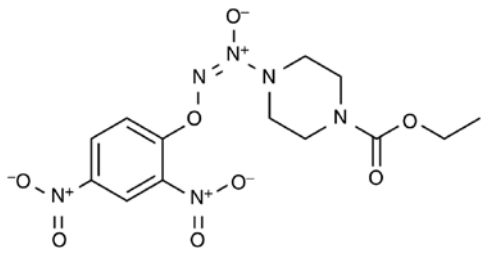

Figure 1. Chemical structures of JS-K. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

Cell culture and JS-K treatment. The human NSCLC cell lines A549 and H460 were obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology at the China Academy of Sciences (Shanghai, China). In brief, A549 and H460 cells were respectively cultured in Dulbecco's modified Eagle's medium (DMEM; cat. no. C11995500BT) and RPMI-1640 medium (cat. no. C11875500BT) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), which were supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10099141), 100 U/ml penicillin and 100 U/ml streptomycin. The cells were maintained in a humidified incubator at 37˚C with 5% CO_2. Subsequently, the A549 cells were treated with various concentrations of JS-K (0, 1, 2 or 5 μM) for 48 h. In addition, the H460 cells were treated with different concentrations of JS-K (0, 5, 10 or 15 μM) for 24 h at which point the confluency was 60-70%. Another group of A549 cells were cultured with 5 μM JS-K for 0, 12, 24 or 48 h, and H460 cells were treated with 15 μM JS-K for 0, 6, 12 and 24 h. Moreover, A549 and H460 cells were pretreated with PFT-α (40 and 10 μM, respectively) for 1 h in the presence or absence of JS-K (5 and 15 μM, respectively).

MTT assay. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the cell toxicity and growth inhibition following JS-K treatment. Briefly, A549 and H460 cells were seeded in 96-well plates at a density of 5,000 cells/well overnight for attachment and recovery. Subsequently, the cells were pretreated with 0, 1, 2, 5, 10, 15 and 20 μM JS-K for 24 or 48 h. After the indicated time, 20 μl MTT solution (5 mg/ml) was added to each well and the cells were maintained in a humidified incubator at 37˚C with 5% CO_2 for 4 h. The supernatants were removed and 150 μl DMSO was added to each well to completely dissolve the formazan crystals. Subsequently, the plates were placed on an orbital shaker for 15 min and the absorbance was then assessed at 490 nm with an automated spectrophotometric plate reader (PerkinElmer, Inc., Waltham, MA, USA). All experiments were repeated independently in triplicate. The inhibition ratio was calculated using the following equation: Inhibition ratio (%) = (OD_{490} control-OD_{490} JS-K-treated)/OD_{490} control x 100%, where OD is the optical density.

Cell cycle analysis. Cells were quantified using a Cell Cycle Analysis kit (Beyotime Institute of Biotechnology, Haimen,

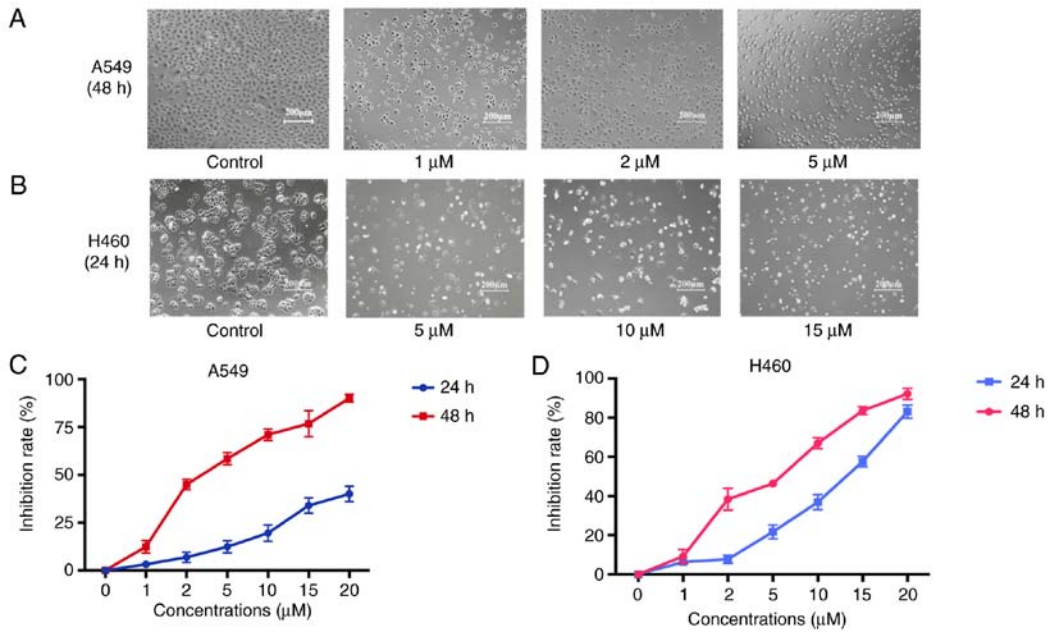

Figure 2. JS-K inhibits the viability of A549 and H460 cells. Various concentrations of JS-K induced apoptosis of (A) A549 and (B) H460 cells, which was revealed by microscopy. Scale bar, 200 μm. Quantification of MTT assay data for (C) A549 and (D) H460 cells are presented as the mean ± standard deviation (n=3). JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

China). A549 cells were pretreated with 0, 1, 2 or 5 μM JS-K for 48 h, and H460 cells were pretreated with 0, 5, 10 or 15 μM for 24 h. The cells were then washed with cold phosphate-buffered saline (PBS) and fixed with 70% ethanol overnight at 4˚C. Subsequently, the ethanol was removed and the cells were incubated with propyl iodide organism dye for 30 min at 37˚C. The dyed cells were detected with a BD Biosciences FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and then analyzed using ModFit and CellQuest software 6.1 (BD Biosciences).

Analysis of cell apoptosis. To detect JS-K-induced apoptosis of the lung cancer cells, an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining assay was performed with the Annexin V-FITC/PI kit (Sigma-Aldrich; Merck KGAA). Briefly, A549 cells were treated with 0, 1, 2 or 5 μM JS-K for 48 h, and H460 cells were treated with 0, 5, 10 or 15 μM JS-K for 24 h. The cells were then collected and resuspended in 1X binding buffer, which contained 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (pH 7.5), 2.5 mM $CaCl_2$ and 140 nM NaCl. Cells were stained in the dark with Annexin V-FITC and PI for 15 min, according to the manufacturer's protocol, prior to flow cytometric analysis. Annexin V-positive cells were regarded to be in the early stage of apoptosis, whereas Annexin V and PI-positive cells were considered to be in the late phase of apoptosis.

Western blot analysis of JS-K-regulated apoptotic proteins and cell cycle proteins. The JS-K-treated cells were collected and lysed in lysis buffer, containing 1 ml RIPA buffer and 10 μl PMSF (Beyotime Institute of Biotechnology). The samples were then centrifuged for 20 min at 16,900 x g at 4˚C to acquire the supernatants. The protein levels were quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology) and equal amounts of protein were loaded to a 10% sodium dodecyl sulfate-polyacrylamide gel. The amount of p-Cdc2 loaded per lane was 40 μg, and for the other proteins 20 μg was loaded. Following electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes, which were then blocked with 5% non-fat milk for 40 min at room temperature. The primary antibodies against cleaved-caspase-3, cleaved-caspase-9, cleaved-caspase-8, Bcl-2, Bax, p53, p21, p27, cyclin B1, CDK2, cyclin E1, Cdc2, cleaved-Cdc2 and GAPDH were incubated with the membranes at 4˚C overnight. Subsequently, anti-rabbit IgG monoclonal antibody conjugated with horseradish peroxidase was added for 1 h at room temperature. Proteins were visualized with chemiluminescent reagent and a Tanon 5200 system (Tanon Science & Technology Co., Ltd., Shanghai, China). The data were analyzed using ImageJ (v1.8.0) analysis software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data were acquired from a minimum of three independent repetitions. The results are presented as the mean ± standard deviation (SD) and the SD is indicated with an error bar in all figures. Statistical analysis was performed using SPSS software (version 17.0; IBM Corp., Armonk, NY, USA). Comparisons between groups were evaluated using one-way analysis of variance (ANOVA) (followed by Tukey's post hoc test). $P<0.05$ and $P<0.01$ were considered to indicate statistically significant differences.

Results

JS-K inhibits proliferation and induces apoptosis of A549 and H460 cells. The MTT assay with different concentrations of JS-K for 24 and 48 h demonstrated that JS-K exerted a significant inhibitory effect on the proliferation of A549 and H460 cells in a concentration- and time-dependent manner (Fig. 2). The IC_{50} value of JS-K at 48 h for A549 cells was 3.48±0.02 μM. The IC_{50} value of JS-K at 24 h for H460 cells was 11.17±0.03 μM.

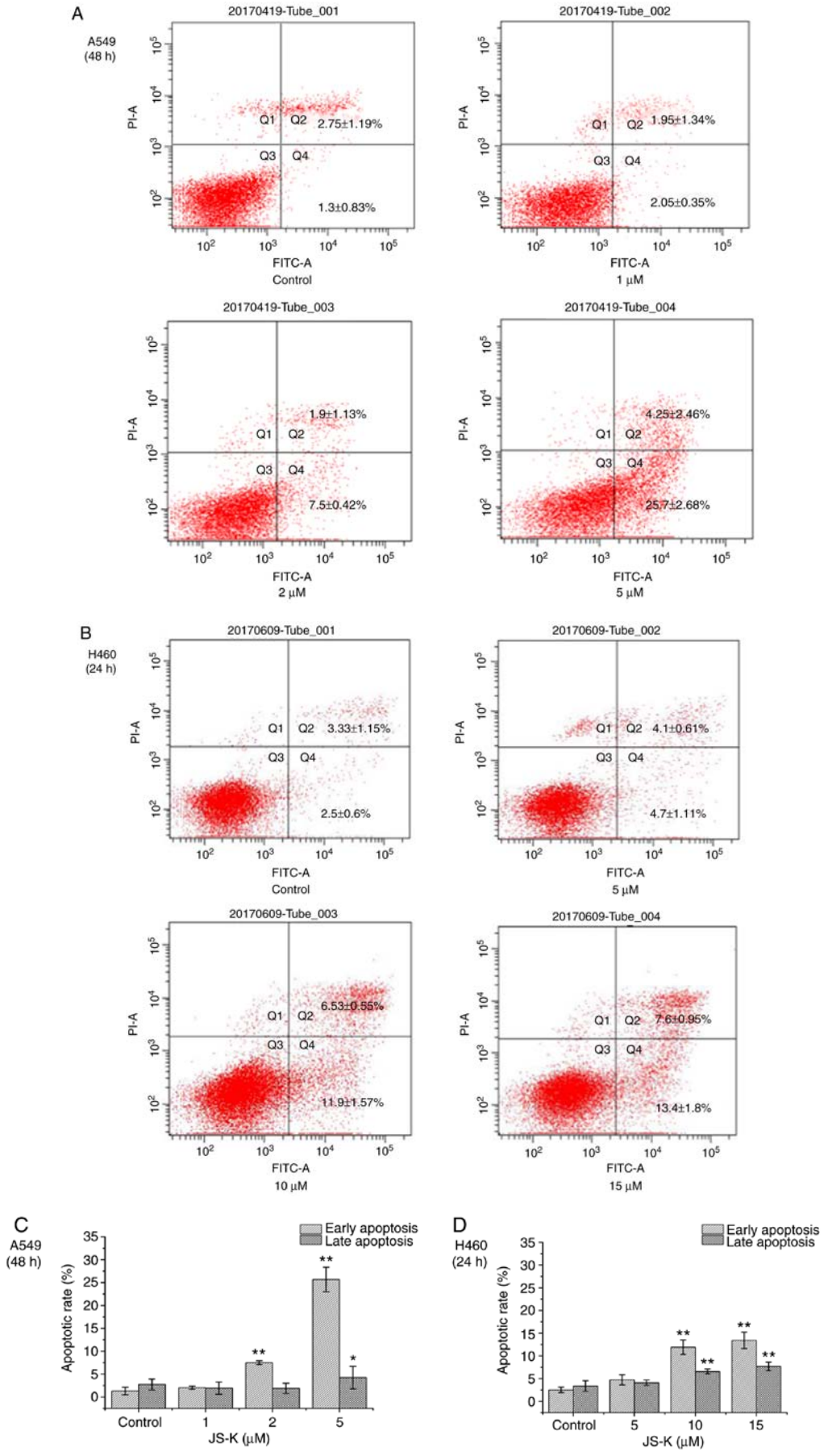

Figure 3. JS-K promotes apoptosis of A549 and H460 cells. Rates of apoptosis of (A) A549 and (B) H460 cells following treatment with various concentrations of JS-K for 48 and 24 h, respectively. Apoptotic cells were detected using flow cytometry. Quantification of the apoptosis assay data for (C) A549 and (D) H460 cells revealed the rates of early and late apoptosis. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the control group. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

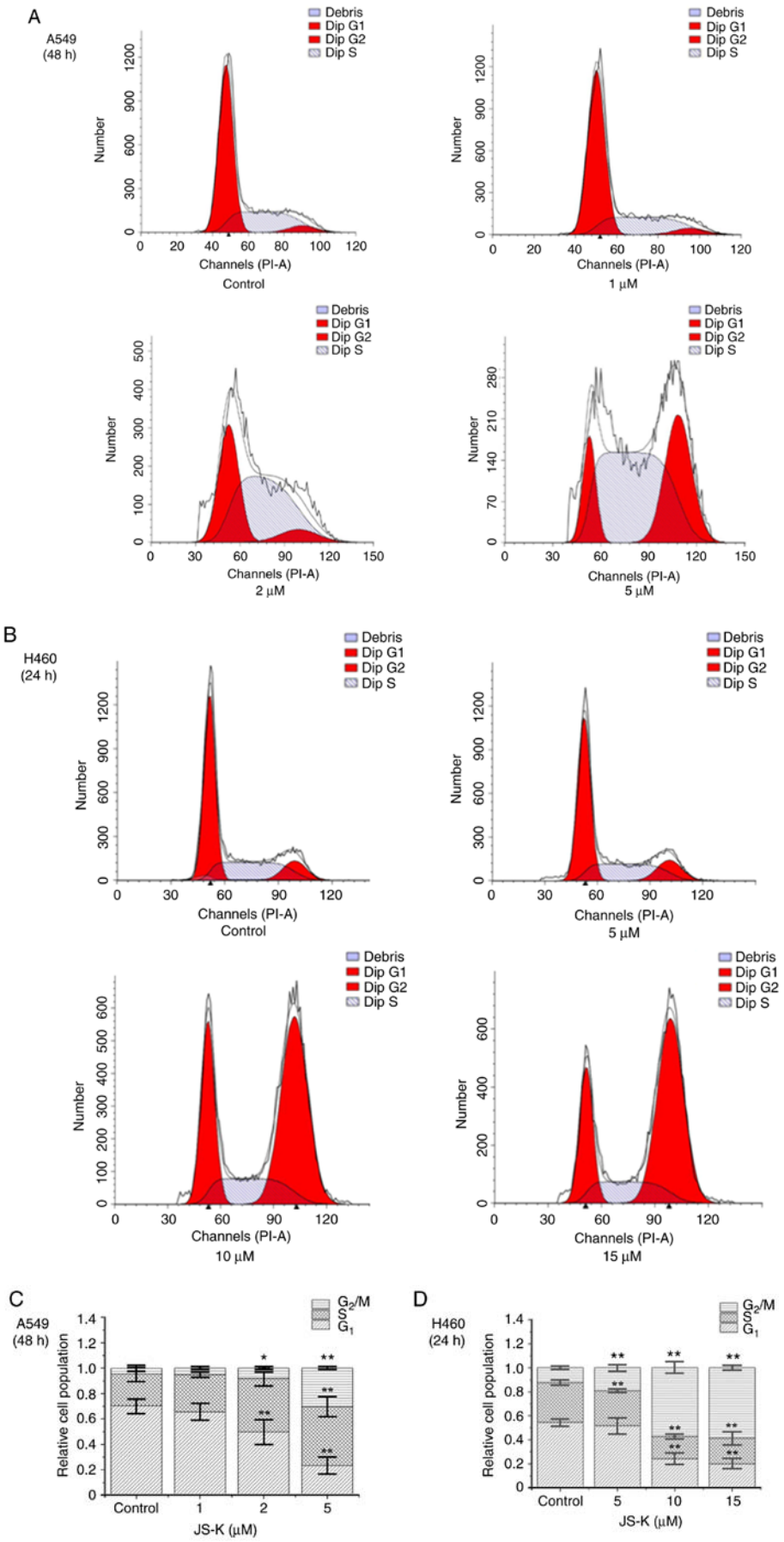

Figure 4. JS-K promotes cell cycle arrest. Flow cytometry revealed that cell cycle arrest was induced in (A) A549 and (B) H460 cells following treatment with various concentrations of JS-K for 48 and 24 h, respectively. Cell cycle distributions of (C) A549 and (D) H460 cells. The proportion of cells in the G_2/M phase increased following treatment with JS-K for the indicated time-points. *P<0.05, **P<0.01 vs. the control group. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

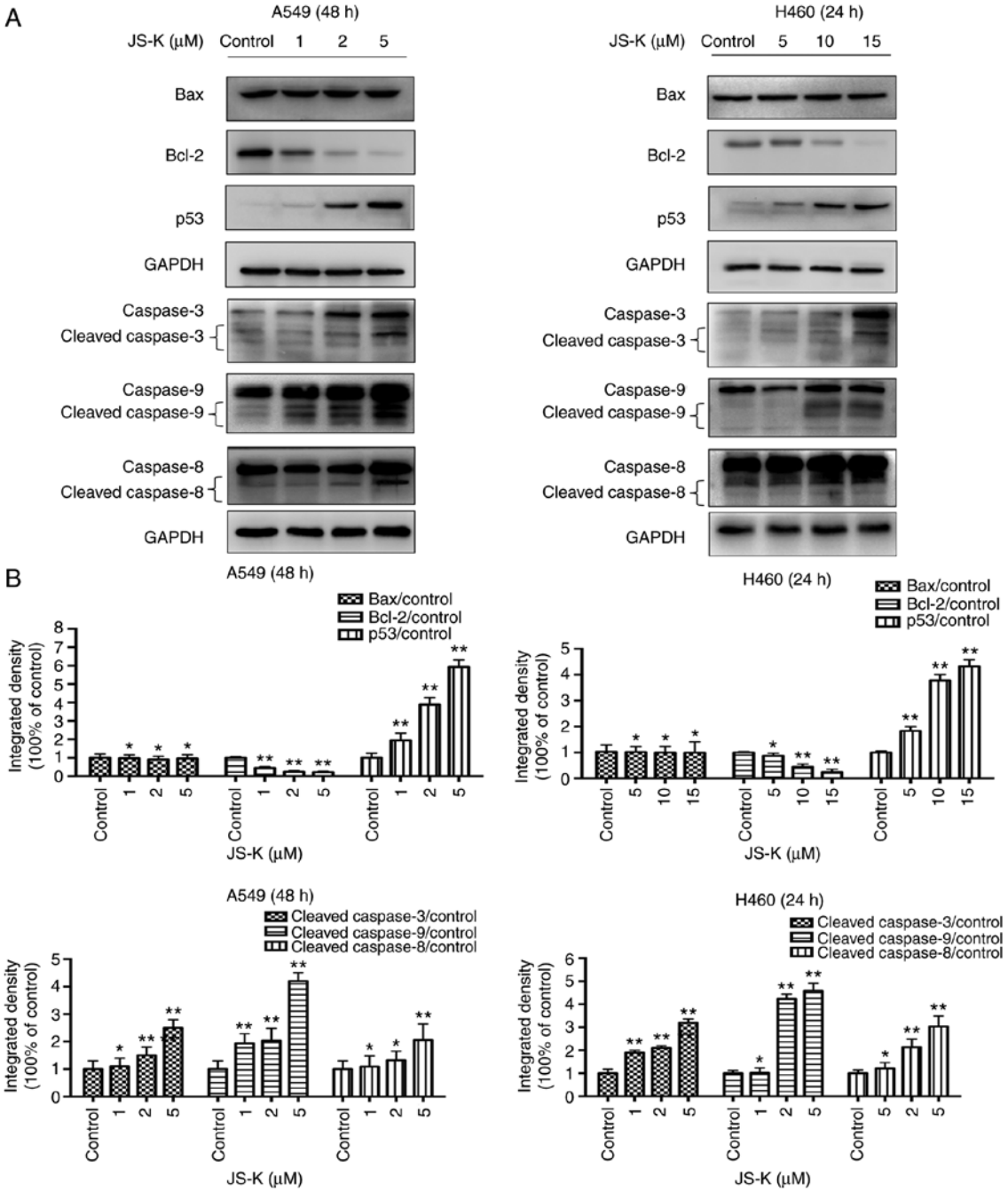

Figure 5. JS-K regulates the p53 signaling pathway. JS-K regulated the expression levels of apoptosis-associated proteins in A549 and H460 cells following treatment with various concentrations for 48 and 24 h, respectively. (A) Western blotting was used to assess the expression levels of Bax, Bcl-2, p53, caspase-3, -9 and -8. (B) Quantification of the protein expression levels. All data represent three independent experiments. Values are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the control group. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

The Annexin V-FITC/PI cell apoptosis detection kit was also used to detect apoptosis of H460 and A549 cells (Fig. 3). The results revealed a significant increase in the apoptosis rates of these cells with increasing concentrations of JS-K. In addition, the Q_2 and Q_4 cell population in A549 (4.05-29.95%) and H460 (5.83-21.0%) cells was increased compared with the control group. As demonstrated in Figs. 2 and 3, JS-K inhibited proliferation and promoted apoptosis of H460 and A549 cells in a concentration-dependent manner.

JS-K promotes cell cycle arrest. To reveal whether JS-K exhibits an effect on cell cycle arrest in human lung carcinoma cells, A549 and H460 cells were treated with various concentrations of JS-K for 48 and 24 h, respectively, and were then subjected to flow cytometric analysis following DNA staining. The population of cells in the G_2/M phase increased in A549 and H460 cells, coinciding with a reduction in cells in the G_1 phase, when compared with the control group. The results revealed that the proportion of cells in the G_2/M phase increased with increasing concentrations of JS-K following the indicated time-points (Fig. 4).

JS-K activates proteins associated with apoptosis and cell cycle arrest. The expression levels of apoptosis-associated

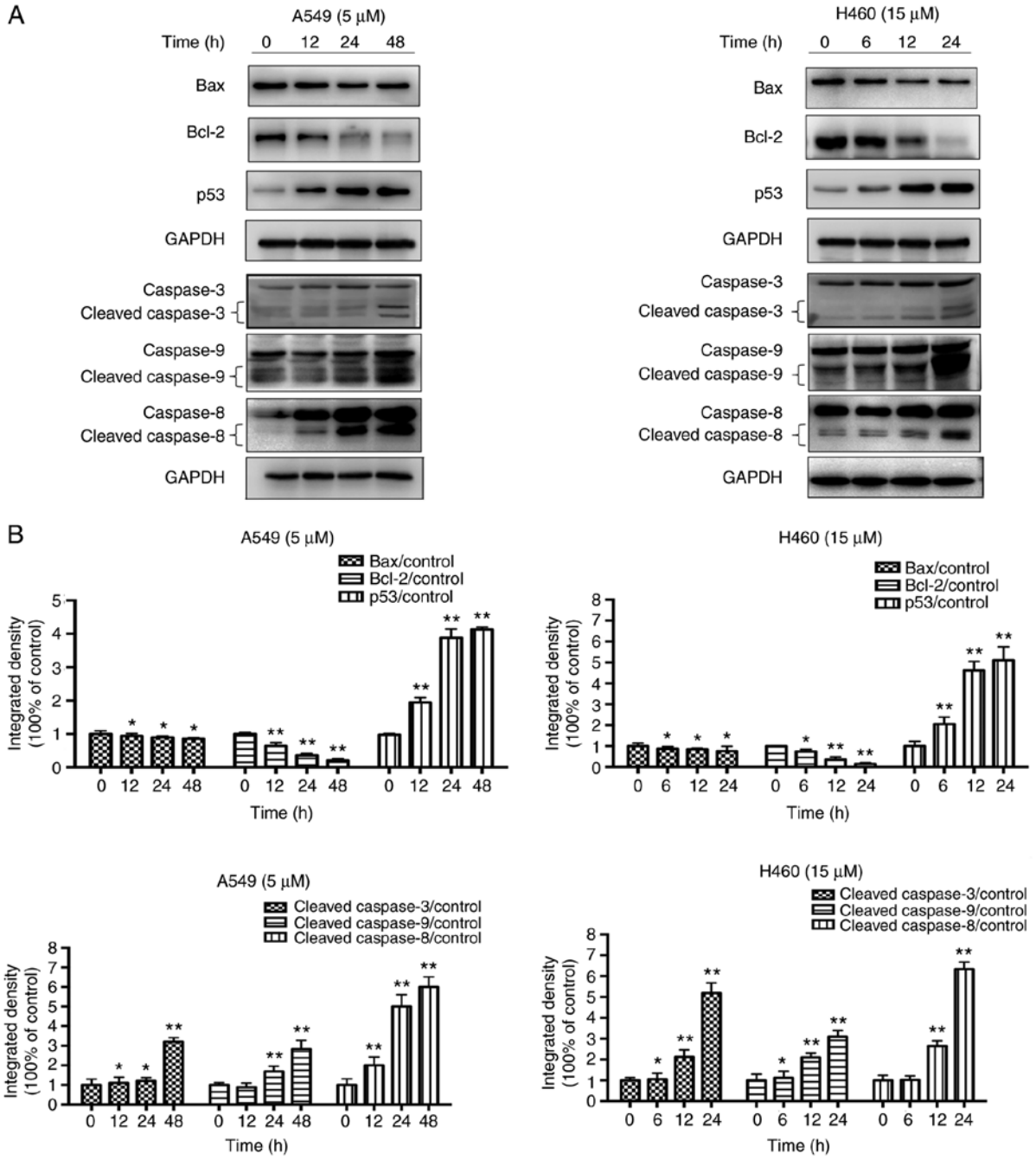

Figure 6. Treatment with JS-K affects the expression levels of apoptosis-associated proteins in a time-dependent manner. (A) Western blot analysis of the expression of apoptosis-associated proteins in A549 and H460 cells following treatment with 5 or 15 μM JS-K, respectively, for various time-points. (B) Quantification of the western blot analysis results for A549 and H460 cells. Data are representative of three independent experiments. Values are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the control group. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

proteins, including cleaved-caspase-9, cleaved-caspase-8, cleaved-caspase-3 and p53, and the ratio of Bax/Bcl-2 were identified to increase in a concentration- and time-dependent manner following treatment with JS-K compared with the control cells (Figs. 5 and 6). The amount of p-Cdc2 loaded per lane was 40 μg, and for the others 20 μg was loaded. In addition, the expression levels of proteins associated with cycle arrest, including cyclin E1, cyclin B1, p21, p27 and p-Cdc2, were revealed to increase in a concentration- and time-dependent manner following treatment with JS-K compared with the control cells. Conversely, the expression levels of CDK2, cyclin B1 and Cdc2 were revealed to decrease in a concentration- and time-dependent manner following treatment with JS-K (Figs. 7 and 8).

p53 serves a crucial role in JS-K-induced apoptosis and cell cycle arrest in A549 and H460 cells. As demonstrated in Figs. 9 and 10 the expression levels of proteins associated with apoptosis and cell cycle arrest were altered following JS-K treatment. However, it was identified that PFT-α could alleviate JS-K-induced cell apoptosis and cell cycle arrest. The results revealed that PFT-α significantly reduced the expression levels of p53, Bax, cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9, and upregulated the expression level of Bcl-2 in

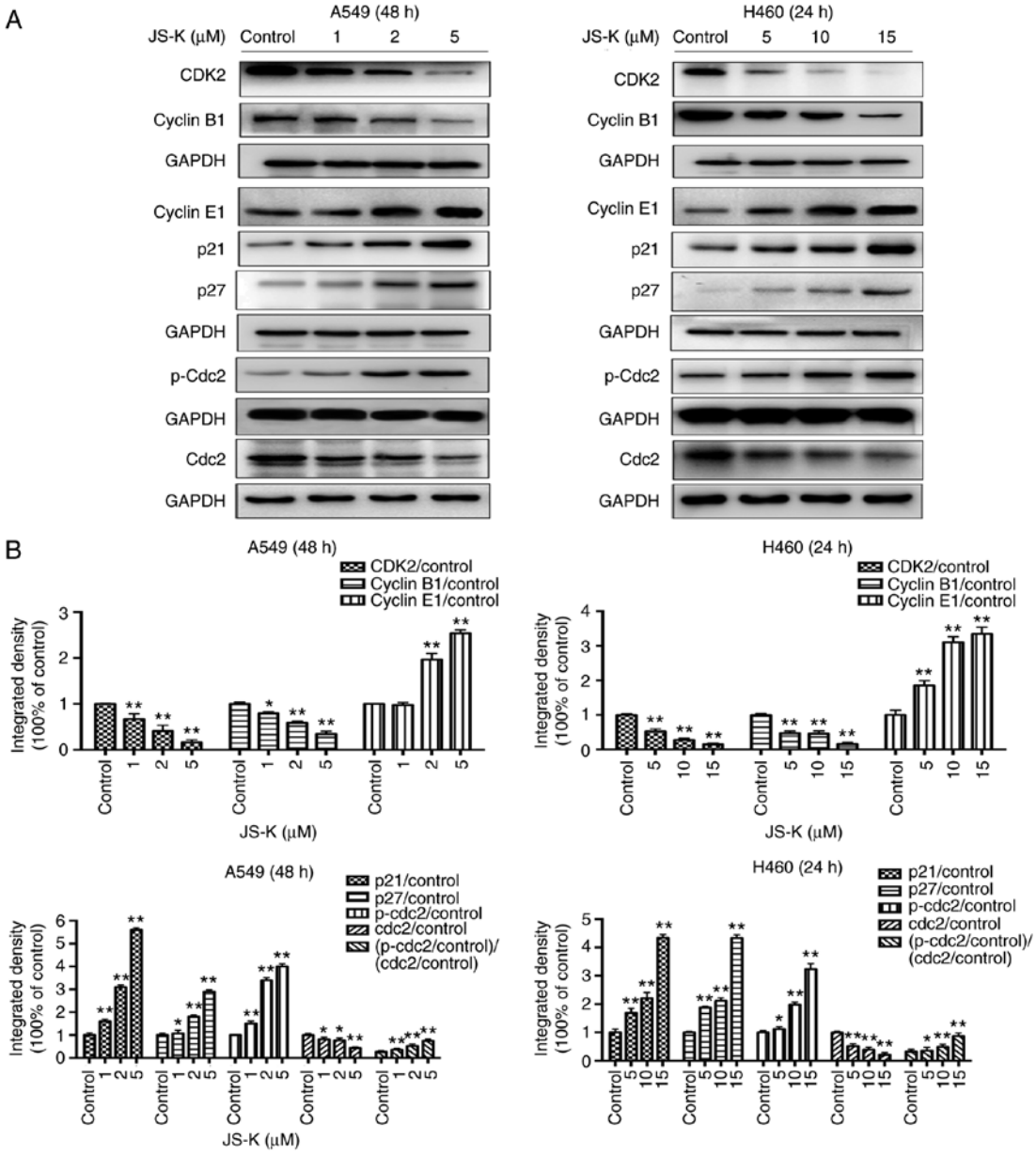

Figure 7. JS-K influences the $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ pathways. JS-K altered the expression levels of cell cycle-associated proteins in A549 and H460 cells following treatment for the indicated time-points. (A) The protein expression levels of CDK2, cyclin B1, cyclin E1, p21, p27, p-Cdc2 and Cdc2 were detected by western blot analysis. JS-K downregulated the expression of CDK2, cyclin B1 and Cdc2, and upregulated cyclin E1, p21, p27 and p-Cdc2 in a concentration-dependent manner. (B) Quantification of the western blot analysis results for A549 and H460 cells. The amount of p-Cdc2 loaded per lane was 40 μg, while for the other proteins 20 μg was loaded. Data are representative of three independent experiments. Values are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the control group. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

comparison with cells treated with JS-K alone. In addition, the expression levels of proteins associated with cell cycle arrest were altered following treatment with PFT-α. The results indicated that PFT-α significantly reduced the expression level of cyclin E1, p21, p27 and p-Cdc2. and upregulated the expression level of CDK2, cyclin B1 and Cdc2 in comparison with cells treated with JS-K alone. In summary, the results indicated a crucial role of p53 in JS-K-induced cellular apoptosis and cell cycle arrest in A549 and H460 cells.

Discussion

Despite advancements in therapeutic strategies and the development of new anticancer drugs, lung cancer is usually incurable. Therefore, investigations of novel therapeutic strategies and anticancer agents are urgently required. The antitumor effect of JS-K has attracted attention in recent years. JS-K is a diazeniumdiolate-based NO-donor prodrug, which has been widely studied due to its potential in increasing ROS levels, and the mechanisms of JS-K are largely understood. For example, JS-K has been reported to induce apoptosis of bladder cancer cells by increasing the levels of ROS (29). Although JS-K has been revealed to induce apoptosis and ROS accumulation in several types of human cancer cells, its effects on cell cycle signaling and apoptosis are poorly understood. In the present study, a potent antiproliferative effect of JS-K was observed in A549 and H460 cells. JS-K was identified to exert a marked effect on G_2/M phase arrest and evidently triggered

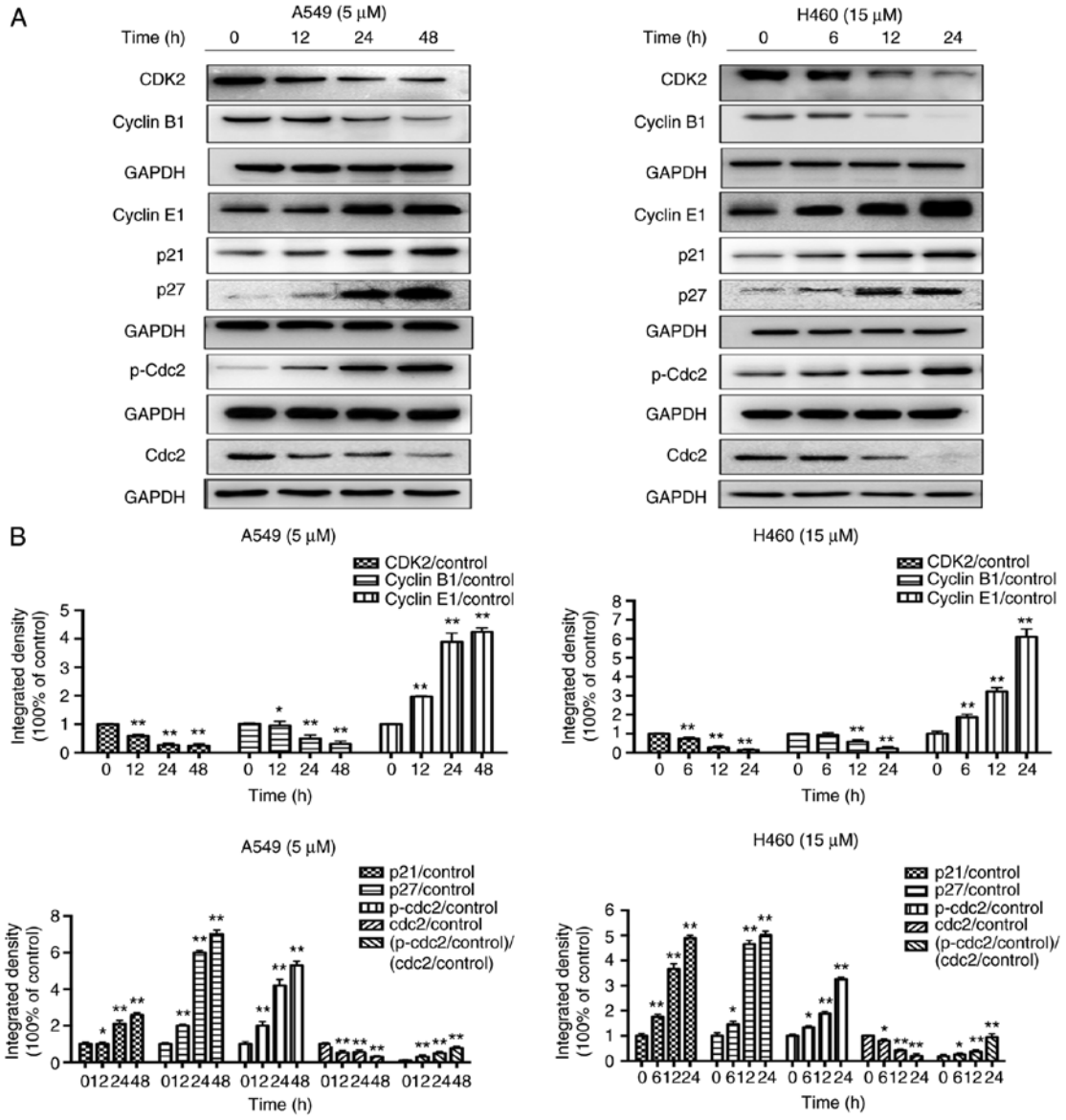

Figure 8. Treatment with JS-K affects the expression levels of cell cycle-associated proteins in a time-dependent manner. (A) Western blot analysis of the expression of cell cycle-associated proteins in A549 and H460 cells following treatment with 5 or 15 μM JS-K, respectively, for various time-points. (B) Quantification of the western blot analysis results for A549 and H460 cells. The amount of p-Cdc2 loaded per lane was 40 μg, while for the other proteins 20 μg was loaded. Data are representative of three independent experiments. Values are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. the control group. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate.

apoptosis in concentration- and time-dependent manners in both cell lines via the p53/p21$^{WAF1/CIP1}$ and p27^{KIP1} pathways.

The proliferation of tumor cells is closely associated with cell cycle arrest, which can serve as a marker for the chemopreventative or antitumor activity of drugs (33). Three checkpoints in the G_1, S and G_2/M phases are mediated by various cell cycle regulatory proteins, including cyclins, CDKs and tumor suppressor proteins, including p53, p21$^{WAF1/CIP1}$ and p27^{KIP1} (34). CDK-cyclin complexes are drivers of the cell cycle and are mediated by two families of CKIs, the CIP/KIP family, which includes p21$^{WAF1/CIP1}$ and p27^{KIP1}, and the INK4 family, which includes p15^{INK4b} and p19^{INK4d} (35). As the only CDK2-associated cyclin, cyclin E is a strong independent prognostic marker in patients with early-stage NSCLC (36). Results of the present study demonstrated that JS-K upregulated the expression level of cyclin E and downregulated the expression level of CDK2 in a concentration and time-dependent manner in A549 and H460 cells. Cdc2, also termed CDK-1, is associated with cyclin B1. The G_2/M transition is often regulated by Cdc2 in mammalian cells (23). Cdc2 serves a crucial role in the induction of mitosis. Suppressing the activity of Cdc2 resulted in cell cycle arrest at the G_2/M phase in several types of cell lines (37,38). In addition, it has been demonstrated that activation of the Cdc2/cyclin B1 complex was indispensable for the transition from the G_2 to the M phase (39). In the present study, JS-K was identified to induce cell cycle arrest at the G_2/M phase (Fig. 4). This effect was associated with a downregulation of cyclin B1 and Cdc2, an upregulation of p21$^{WAF1/CIP1}$, and phosphorylation of Cdc2 in a concentration and time-dependent manner in A549 and H460 cells (Fig. 7). For the western blot analysis, the amount of p-Cdc2 loaded per lane was 40 μg, while for the other 20 μg

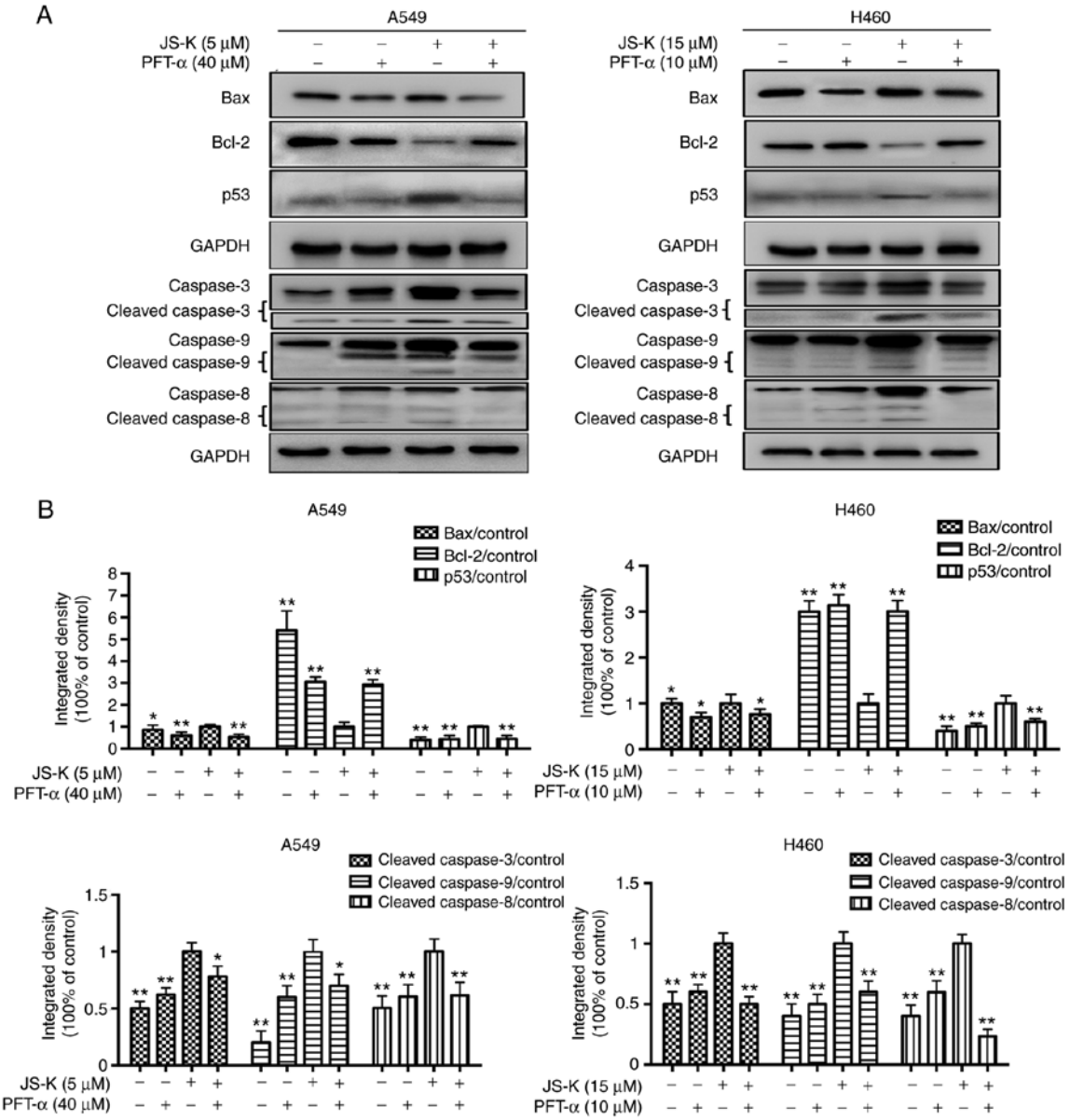

Figure 9. JS-K induces A549 and H460 cell apoptosis via the p53 signaling pathway. (A) Western blot analysis of the expression of apoptosis-associated proteins in A549 and H460 cells following co-treatment with JS-K (5 or 15 μM) and pifithrin-α (40 or 10 μM) for the indicated time-points. (B) Quantification of the western blot analysis results for A549 and H460 cells. Data are representative of three independent experiments. Values are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. JS-K alone. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate; PFT-α, pifithrin-α.

was loaded. We consider that different amounts of samples may lead to some errors in the results. After adjusting the amounts of samples, we determined that the amount of sample of each analyzed lane was uniform by exposing the internal reference protein (GAPDH). The results revealed that the expression of the reference protein (GAPDH) and the target protein (p-Cdc2) was increased after adjusting the amounts of samples. Satisfactory observation results were obtained. The trend of GAPDH expression of each analyzed lane was consistent with that before samples adjustment. Although the amount of p-Cdc2 was different from that of Cdc2, the batches of samples on the analyzed lanes were the same. In addition, three batches of samples were repeated to obtain the means and relative errors. Then the relative errors were greatly reduced. In order to determine the phosphorylation, the following was assessed in the quantification section: '(p-Cdc2/control)/(Cdc2/control)'. Therefore, the errors caused by adjusting the amounts of samples had little effect on the results of the quantification analysis.

Following binding to cyclin-CDK complexes, members of the Cip/Kip family, including $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, can prevent kinase activation and subsequently inhibit the progression of the cell cycle at the G_2/M phase (40). $p27^{KIP1}$ acts as a tumor suppressor by binding to complexes of cyclin E/CDK2 to prevent cell cycle progression from the G_1 phase to the S phase (41). A previous study revealed that a low expression level of $p27^{KIP1}$ was associated with tumor aggressiveness and poor patient survival (42). One of the functions of $p21^{WAF1/CIP1}$ is to maintain cells at G_2/M phases following DNA damage (18). In addition, $p21^{WAF1/CIP1}$ can interact with cyclin B1, which

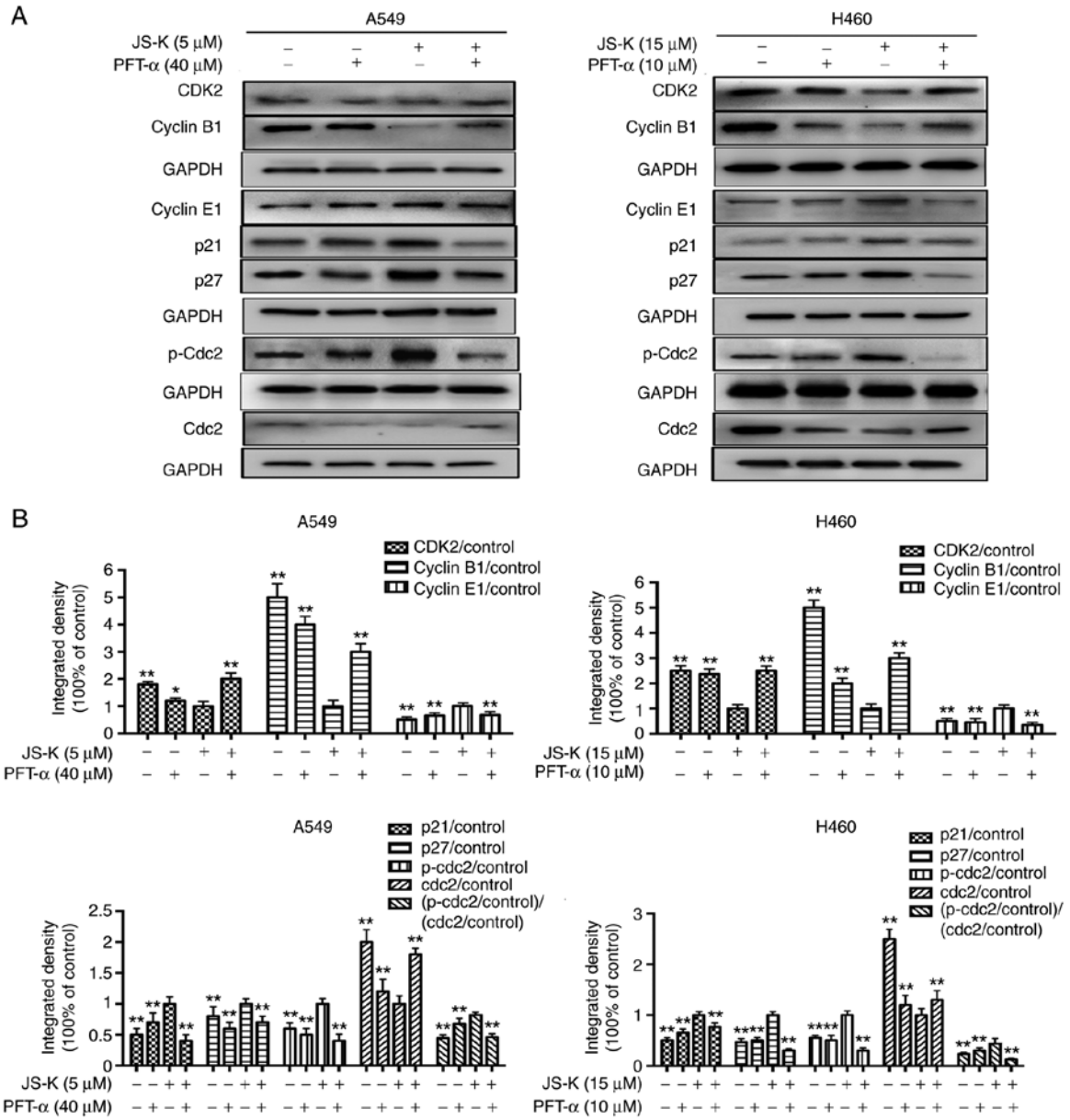

Figure 10. JS-K induces G_2/M phase cell cycle arrest in A549 and H460 cells via the $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ pathways. (A) Western blot analysis of the expression of apoptosis-associated proteins in A549 and H460 cells following co-treatment with JS-K (5 or 15 μM) and pifithrin-α (40 or 10 μM) for the indicated time-points. (B) Quantification of the western blot analysis results for A549 and H460 cells. The amount of p-Cdc2 loaded per lane was 40 μg, while for the other proteins 20 μg was loaded. Data are representative of three independent experiments. Values are presented as the mean ± tandard deviation (n=3). *P<0.05, **P<0.01 vs. JS-K alone. JS-K, O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate; PFT-α, pifithrin-α.

prevents dephosphorylation of Cdc2 by Cdc25 and inhibit the activity of CDK-1 (43). Activated p53 has been revealed to serve a role in the regulation of cell cycle progression following DNA damage. The mechanism of p53 in G_2/M phase cell cycle arrest involves the transactivation of the CDK inhibitor p21 (44). The results of the present study revealed that the expression levels of p53, $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ increased in a concentration- and time-dependent manner in A549 and H460 cells following treatment with JS-K. These findings indicated that JS-K-induced G_2/M phase cell cycle arrest may be mediated by the upregulation of p53, $p21^{WAF1}$ and $p27^{KIP1}$.

To maintain tissue homeostasis, apoptosis serves as a physiological pathway to eliminate damaged or infected cells (45). Apoptosis induction has been considered to be a useful strategy for cancer therapy. In the present study, the results of MTT assay suggested that JS-K acted as an anti-proliferation agent against A549 and H460 cells (Fig. 2). Therefore, in addition to cell cycle arrest, the present study investigated whether JS-K-induced cell growth arrest could be due to the induction of apoptosis. In the apoptosis assay, a significant increase in the apoptosis rates of A549 and H460 cells were revealed by flow cytometry following treatment with JS-K (Fig. 3).

Apoptosis can be activated via the extrinsic- or death receptor-associated pathways, as well the intrinsic- or organelle-mediated pathways (45). It is well known that p53 acts upstream of other pro-apoptotic proteins and mediates both the intrinsic and the extrinsic apoptotic pathways (46). Cysteine proteases, particularly caspases, can be classified as initiator caspases, including caspases-8 and -9, or as executioner caspases, including caspases-3, -6 and -7, based on their

mechanism of action (47). Caspase-3, an executioner caspase, can be activated by the extrinsic pathway involving the activation of caspase-8 and the intrinsic pathway involving the activation of caspase-9 (48,49). The present study revealed that JS-K treatment could trigger both the mitochondria-initiated intrinsic pathway and extrinsic pathway in A549 and H460 cells. The results demonstrated that cleaved caspase-3, -8 and -9 were significantly upregulated following treatment with JS-K, which indicated that JS-K induced apoptosis in A549 and H460 cells via the mitochondria-initiated intrinsic pathway and extrinsic pathway by the upregulation of p53 (Figs. 5 and 6).

p53 is a critical transcription factor that regulates the transcription and expression of various target genes, including Bcl-2 and Bax, which can result in cell cycle arrest and apoptosis (50,51). The anti-apoptotic ability of Bcl-2 is suppressed following binding with Bax protein (52). Overall, p53 can positively regulate the expression of the proapoptotic proteins Bax, Bad and Bak, which prevents inhibition of Bcl-2 (49). Compared with the expression level of Bcl-2 alone, the ratio of Bcl-2 to Bax has been reported to a serve a larger role in cell survival or death following an apoptotic stimulus (53,54). The present study demonstrated that JS-K markedly upregulated the expression levels of cell cycle-associated proteins, including $p21^{WAF1/CIP1}$ and p53, and also enhanced the ratio of Bax/Bcl-2 by increasing the expression level of Bax and reducing the expression level of the anti-apoptotic protein Bcl-2 in A549 and H460 cells (Fig. 5). These results indicated that p53 and Bax/Bcl-2 served an important role in JS-K-induced cell apoptosis. In addition, the results demonstrated that JS-K can be inhibited by PFT-α (Figs. 9 and 10). Following inhibition of JS-K with PFT-α, Bcl-2 suppression was markedly reversed, and $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ were significantly downregulated with a decrease in the expression level of p53. Therefore, targeting the p53/$p21^{WAF1/CIP1}$ and $p27^{KIP1}$ signaling pathway may be considered as a potential strategy to inhibit the proliferation of A549 and H460 cells.

This research was aimed at evaluating the anticancer effect of JS-K and exploring its mechanism in A549 and H460 human NSCLC cells. It is unclear whether JS-K acts on other NSCLC cells via the p53/$p21^{WAF1/CIP1}$ and $p27^{KIP1}$ pathways. In addition, *in vivo* experiments were not carried out. In order to further elucidate the mechanism of JS-K, we will use JS-K to screen NSCLC cells comprehensively, and conduct *in vivo* studies in the future. In conclusion, the present study revealed that JS-K could induce cell cycle arrest and apoptosis in A549 and H460 cells via the p53/$p21^{WAF1/CIP1}$ and $p27^{KIP1}$ pathways. These findings promote understanding regarding the antitumor effect of JS-K in NSCLC and suggest that JS-K may be a novel candidate for lung cancer therapy.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

DC, WL and RZ conceived and designed the study. DC and WL provided administrative support. ZS, RZ, XH and YY provided the materials of the study. BL, JW, ZS, CG and XH collected and assembled the data. SH, BL, JW, YY and WL performed the data analysis and interpretation. SH, CG and WL wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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