

Germacrone induces lung cancer cell apoptosis and cell cycle arrest via the Akt/MDM2/p53 signaling pathway

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Abstract. Germacrone (GM) displays a wide range of anti-tumor, antioxidant and anti-inflammatory effects; however, to the best of our knowledge, the effects of GM on lung cancer cell apoptosis and cell cycle arrest have not been previously reported. The aim of the present study was to investigate the effects of GM on the apoptosis and cycle arrest of lung cancer cells. Cell viability, proliferation and apoptosis were assessed by performing Cell Counting Kit-8, colony formation and TUNEL assays, respectively. Western blotting was performed to detect the expression levels of apoptosis-, cell cycle- and Akt/MDM2 proto-oncogene (MDM2)/p53 signaling pathway-related proteins. Compared with the control group, 50, 100 and 200 μ M GM significantly inhibited lung cancer cell proliferation, but significantly induced cell apoptosis and G₁/S cell cycle arrest. GM also significantly altered the expression levels of Akt/MDM2/p53 signaling pathway-related proteins compared with the control group. Administration of Akt activator SC79 significantly reversed GM-mediated anti-proliferative, proapoptotic and pro-cell cycle arrest effects in lung cancer cells. Therefore, the results of the present study demonstrated that GM induced lung cancer cell apoptosis and cell cycle arrest via the Akt/MDM2/p53 signaling pathway.

Introduction

Lung cancer is the most common malignant tumor worldwide. With increased industrialization, air quality around the

world has been reduced to varying degrees (1). The incidence of lung cancer remains high, with a 5-year survival rate of 16-18% worldwide (2). Lung cancer can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC), which accounts for 85% of all lung cancer cases, according to pathological morphology and the degree of differentiation (3). Therefore, investigating the pathogenesis of NSCLC and identifying novel therapeutic drugs and targets for the disease is important.

Germacrone (GM), a natural product isolated from the traditional Chinese medicine zedoary, displays a wide range of pharmacological functions, including antitumor (4), antioxidant (5) and anti-inflammation activities (6). In addition, it has been reported that GM displays antitumor activity *in vitro* (7). GM induces prostate cancer cell apoptosis and autophagy by inhibiting the Akt/mTOR signaling pathway (8). GM also serves an anticancer role by inducing G₂/M phase cell cycle arrest and promoting apoptosis in gastric cancer cells (4). Furthermore, GM inhibits breast cancer cell line proliferation by inducing cell cycle arrest at the G₀/G₁ and G₂/M phases, and promoting cell apoptosis (9). An *et al* (10) demonstrated that GM displays a significant protective effect against lipopolysaccharide-induced acute lung injury in neonatal rats. However, to the best of our knowledge, the effect of GM on lung cancer has not yet been reported.

A previous study revealed that zedoary inhibits tumor cell proliferation, invasion and migration by inhibiting the Akt signaling pathway, suggesting a potential antitumor mechanism (11). The Akt/MDM2 proto-oncogene (MDM2)/p53 signaling pathway serves an important role in the regulation of apoptosis and proliferation (12,13). The Akt signaling pathway is a classic intracellular signaling pathway that causes tumorigenesis via a range of mechanisms (14,15). The malignancy of numerous different types of cancer, including lung cancer, is associated with increased abnormal activity of the Akt signaling pathway (16-18). Akt phosphorylates the p53 suppressor MDM2 and promotes MDM2 to translocate to the nucleus, thereby inhibiting the function of p53 (19).

Therefore, the present study investigated the role of GM in lung cancer cells and its underlying molecular mechanism, with the aim of providing a theoretical basis for the treatment of lung cancer.

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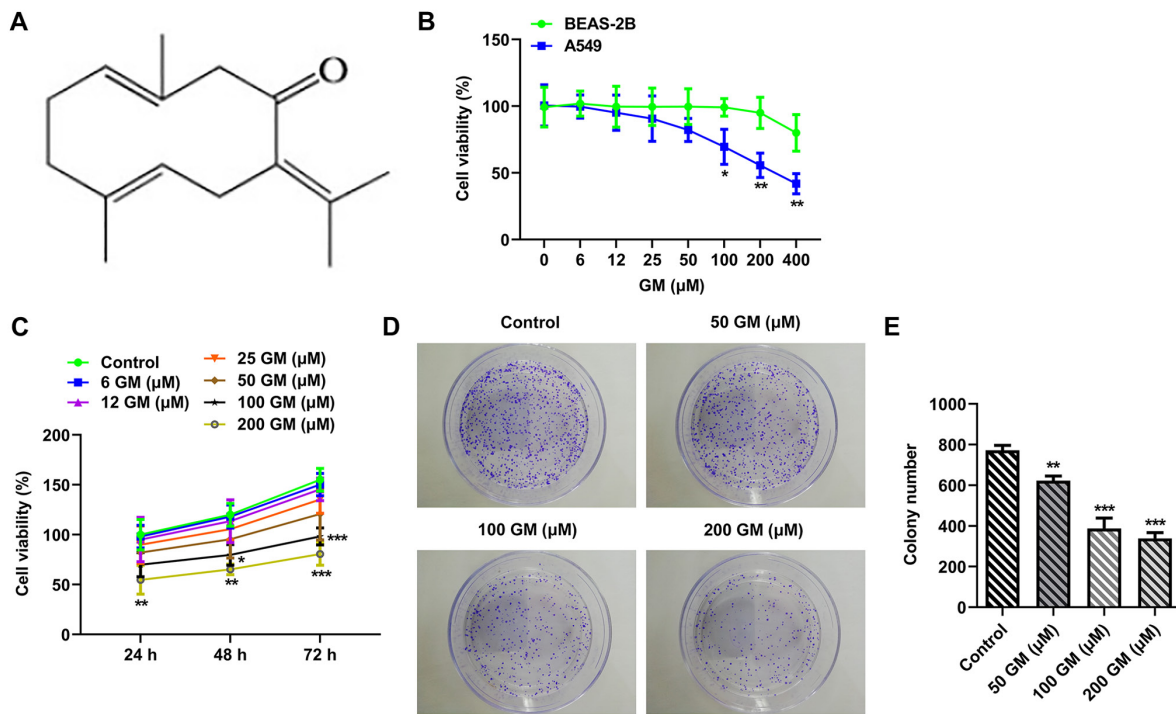


Figure 1. GM inhibits lung cancer cell proliferation. (A) Chemical structure of GM. (B) Effect of different concentrations of GM on BEAS-2B and A549 cell viability (n=5). * $P<0.05$ and ** $P<0.001$ vs. BEAS-2B. (C) Effect of different concentrations of GM on A549 cell viability at 24, 48 and 72 h (n=5). * $P<0.05$, ** $P<0.001$ and *** $P<0.001$ vs. Control. (D) Cell proliferation was detected by performing colony formation assays (magnification, x10). (E) Statistical analysis of D (n=3). ** $P<0.001$ and *** $P<0.001$ vs. control. GM, germacrone.

Materials and methods

Cell culture. The human bronchial epithelial cell line (BEAS-2B) and lung cancer cell line (A549) were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO_2 . Cells were cultured with different concentrations (6, 12, 25, 50, 100, 200 and 400 μM) of GM for 48 h at 37°C (purity >98%; Shanghai YuanYe Biotechnology Co., Ltd.). Cells in the control group were treated with equal concentrations of DMEM.

Cell Counting Kit-8 (CCK-8) assay. A549 cell viability was assessed by performing the CCK-8 assay (Beyotime Institute of Biotechnology). Following treatment, 10 μl CCK-8 solution was added to each well and incubated for 4 h at 37°C. Absorbance was measured at a wavelength of 450 nm using a microplate reader.

Colony formation assay. Cells were seeded (5×10^2 cells/well) into 6-well plates. Following incubation for 15 days at 37°C, visible colonies were fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature and stained with crystal violet for 5 min at room temperature. Stained colonies (≥ 50 cells) were visualized using a light microscope (magnification, x100, Olympus Corporation) equipped with a ColorView II Soft Imaging System digital camera (Olympus Corporation).

TUNEL assay. Cell apoptosis was assessed by performing the Click-iT® TUNEL Alexa Fluor® Imaging assay (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were fixed with

4% paraformaldehyde for 30 min at room temperature and then washed with PBS. Subsequently, 0.3% Triton X-100 in PBS was added and incubated for 5 min at room temperature. Cells were stained with DAPI at room temperature for 10 min (Thermo Fisher Scientific, Inc.). A total of 50 μl TUNEL reaction mixture was added for 1 h at 37°C. Cells were sealed with anti-fluorescence quenched sealing solution and FITC-labeled TUNEL-positive cells were visualized in three randomly selected fields of view using an IX70 confocal microscope (Olympus Corporation) at x20 magnification. To calculate the proportion of apoptotic cells, the number of apoptotic cells and the number of total cells were counted.

Western blotting. The effect of GM treatment on the expression levels of apoptosis-related proteins was determined via western blotting. Briefly, total protein was extracted from cells using RIPA buffer (Nantong Chem-Base Co., Ltd.). Following centrifugation at 300 x g at 4°C for 5 min, protein concentrations were determined using a BCA kit (Nantong Chem-Base Co., Ltd.). Proteins (40 μg /lane) were separated via 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). Following blocking with 5% skimmed milk for 2 h at 37°C, the membranes were incubated overnight at 4°C with the following primary antibodies (all Abcam): Anti-Bcl-2 (1:1,000; cat. no. ab32124), anti-Bax (1:1,000; cat. no. ab182733), anti-cleaved (C)-caspase 3 (1:1,000; cat. no. ab32042), anti-caspase 3 (1:1,000; cat. no. ab13847), anti-C-poly(ADP-ribose) polymerase (PARP; 1:1,000; cat. no. ab32064), anti-PARP (1:1,000; cat. no. ab191217), anti-Cyclin D1 (1:1,000; cat. no. ab16663), anti-CDK4 (1:1,000; cat. no. ab108357), anti-CDK6 (1:1,000;

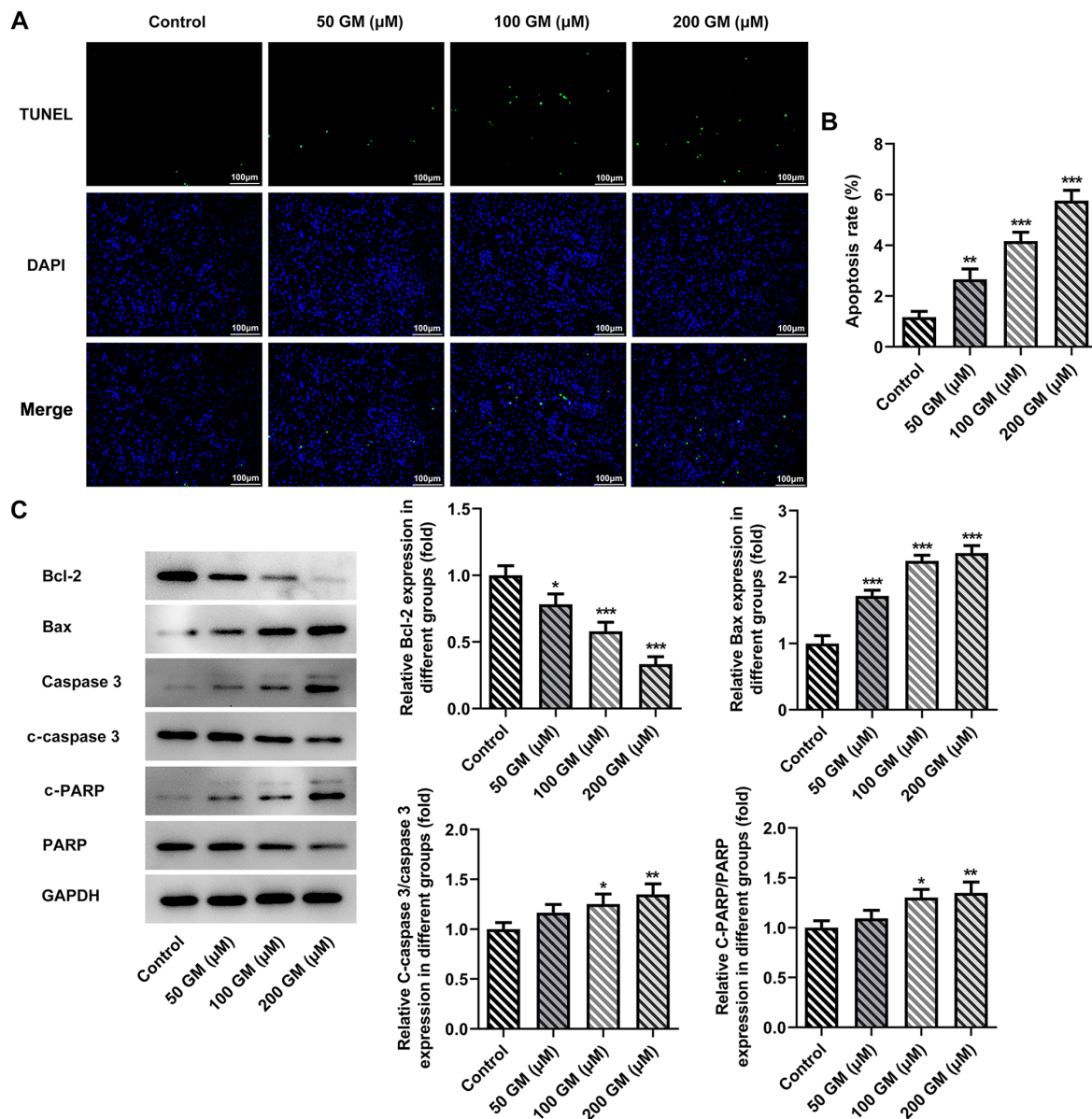


Figure 2. GM induces lung cancer cell apoptosis. A549 cell apoptosis was (A) detected by performing TUNEL assays and (B) quantified (n=3). (C) Expression levels of apoptosis-related proteins were detected via western blotting (n=3). *P<0.05, **P<0.001 and ***P<0.001 vs. control. GM, garmacrone; C, cleaved; PARP, poly(ADP-ribose) polymerase.

cat. no. ab124821), anti-p-Akt (1:1,000; cat. no. ab38449), anti-Akt (1:1,000; cat. no. ab8805), anti-p-MDM2 (1:1,000; cat. no. ab22710), anti-MDM2 (1:1,000; cat. no. ab16895), anti-p53 (1:1,000; cat. no. ab26) and anti-GAPDH (1:1,000; cat. no. ab9485). Subsequently, the membranes were incubated with the corresponding secondary antibody (1:5,000; cat. no. ab150077; Abcam) for 2 h at 37°C. Subsequently, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibodies (1:5,000; cat. no. ab150077; Abcam). Protein bands were visualized using the HRP-ECL system (Nantong Chem-Base Co., Ltd.). ImageJ software (version 146; National Institutes of Health) was used to analyze the fold change in protein levels.

Flow cytometry. Cell cycle was detected by EzCell™ Cell Cycle Analysis kit (cat. no. K920-100; BioVision, Inc.). A549 cells (5×10^2 cells/well) were synchronized in serum-free

medium for 24 h. Subsequently, cells were treated with 8 μ g/ml SC79 (cat. no. ab146428; Abcam) or GM (50, 100 or 200 μ M) at 37°C for 24 h. Cells in the control group were treated with equal concentrations of DMEM. Following washing twice with PBS, cells were trypsinized and collected by centrifugation at 300 x g at 4°C for 5 min, followed by fixation with 70% ethanol at 4°C for 24 h. After washing with PBS, cells were stained with PI in staining solution supplemented with RNase A for 30 min at room temperature. The cell cycle was assessed using the FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (version 7.6.1; Flow Jo LLC.).

Statistical analysis. Statistical analyses were performed using SPSS software (version 13.0; SPSS, Inc.). Data are presented as the mean \pm standard deviation. Comparisons among multiple groups were analyzed using one-way ANOVA followed by

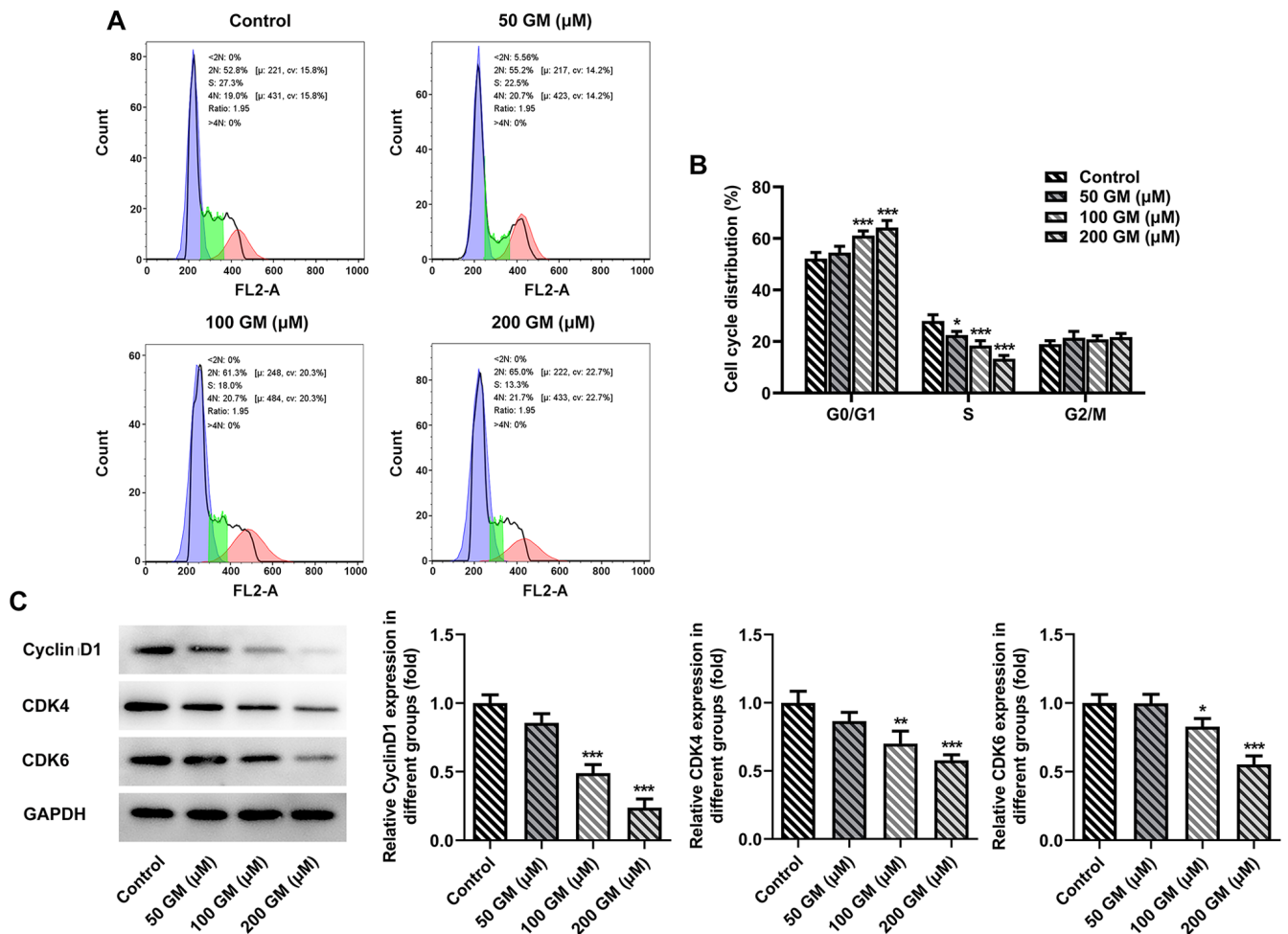


Figure 3. GM induces lung cancer cell G_1/S phase cell cycle arrest. Cell cycle distribution was (A) determined via flow cytometry and (B) quantified ($n=3$). (C) Expression levels of cell cycle-related proteins were determined via western blotting ($n=3$). * $P<0.05$, ** $P<0.001$ and *** $P<0.001$ vs. control. GM, germacrone.

Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GM inhibits lung cancer cell proliferation. The chemical formula of GM is presented in Fig. 1A. The CCK-8 assay was performed to evaluate the effect of different concentrations of GM (0, 6, 12, 25, 50, 100, 200 and 400 μM) on cell viability (20,21). Cell viability was markedly decreased by GM treatment in a concentration-dependent manner, with the effects of GM on A549 cell viability being more obvious compared with BEAS-2B cell viability (Fig. 1B). Subsequently, A549 cells were treated with different concentrations of GM for 24, 48 or 72 h (Fig. 1C). The results demonstrated that A549 cell proliferation was significantly decreased by GM treatment (200 μM) at 24 h compared with the control group. Therefore, cells were treated with 50, 100 or 200 μM GM for 24 h for subsequent experiments. The colony formation assay results demonstrated that cell proliferation was significantly decreased by GM treatment in concentration-dependent manner compared with the control group (Fig. 1D and E).

GM induces lung cancer cell apoptosis and G_1/S cycle arrest. Cell apoptosis was detected by performing TUNEL assays.

The results demonstrated that cell apoptosis was significantly increased by GM treatment in a concentration-dependent manner compared with the control group (Fig. 2A and B). Moreover, GM-treated cells (100 and 200 μM) displayed significantly increased expression levels of apoptosis-related proteins, including Bax, c-caspase 3 and c-PARP, and significantly decreased expression levels of Bcl-2 compared with the control group (Fig. 2C). The cell cycle was detected via flow cytometry. The results indicated that the proportion of GM-induced lung cancer cells (50, 100 and 200 μM) arrested in the G_1/S cell cycle phase was significantly increased compared with the control group (Fig. 3A and B). Subsequently, western blotting was performed to detect the expression levels of cell cycle-related proteins, including cyclin D1, CDK4 and CDK6. Compared with the control group, the expression levels of cyclin D1, CDK4 and CDK6 following treatment with 100 or 200 μM GM were significantly downregulated in a concentration-dependent manner (Fig. 3C). Collectively, the aforementioned results indicated that GM induced lung cancer cell apoptosis and G_1/S phase cell cycle arrest.

GM alters the Akt/MDM2/p53 signaling pathway. Compared with the control group, the expression levels of phosphorylated (p)-Akt and p-MDM2 were significantly decreased,

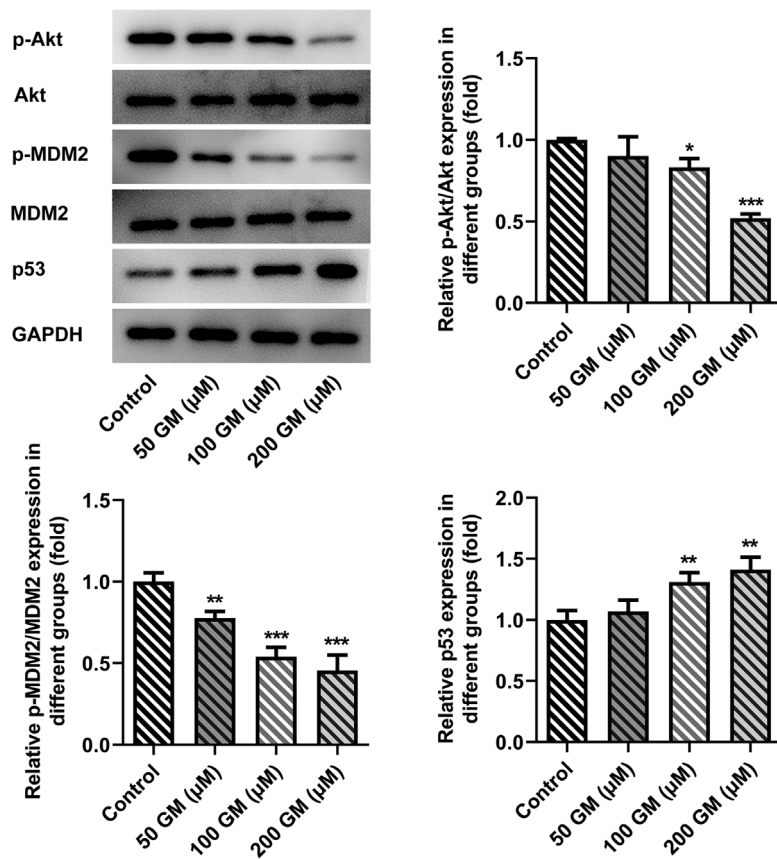


Figure 4. GM alters the Akt/MDM2/p53 signaling pathway. Expression levels of Akt/MDM2/p53 signaling pathway-related proteins were detected via western blotting (n=3). *P<0.05, **P<0.001 and ***P<0.001 vs. control. GM, germacrone; MDM2, MDM2 proto-oncogene; p, phosphorylated.

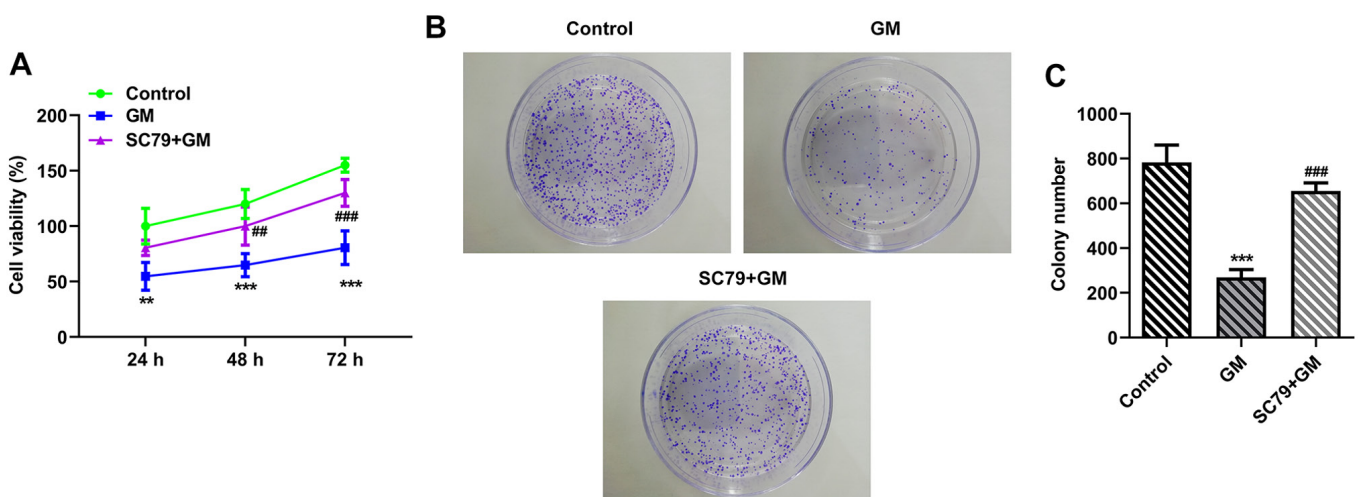


Figure 5. SC79 reverses the antiproliferative effects of GM on lung cancer cells. (A) Effect of SC79 on GM-mediated inhibition of A549 cell viability at 24, 48 or 72 h (n=5). Cell proliferation was (B) detected by performing colony formation assays (magnification, x10) and (C) quantified (n=3). **P<0.01, ***P<0.001 vs. control; ##P<0.01, ###P<0.001 vs. GM. GM, germacrone.

and the expression levels of p53 were significantly increased following 100 and 200 μM GM administration (Fig. 4). Therefore, the results indicated that GM altered the Akt/MDM2/p53 signaling pathway. The most notable effects on protein expression were observed following treatment with 200 μM GM, so this concentration was selected for subsequent experiments.

SC79 reverses the antiproliferative, proapoptotic and pro-cell cycle arrest effects of GM on lung cancer cells. To further verify the aforementioned results, cells were treated with Akt activator SC79. Cells were divided into the following two groups: i) GM; and ii) SC79+GM. The CCK-8 (Fig. 5A) and colony formation (Fig. 5B and C) assay results demonstrated that cell viability (at 48 and 72 h)

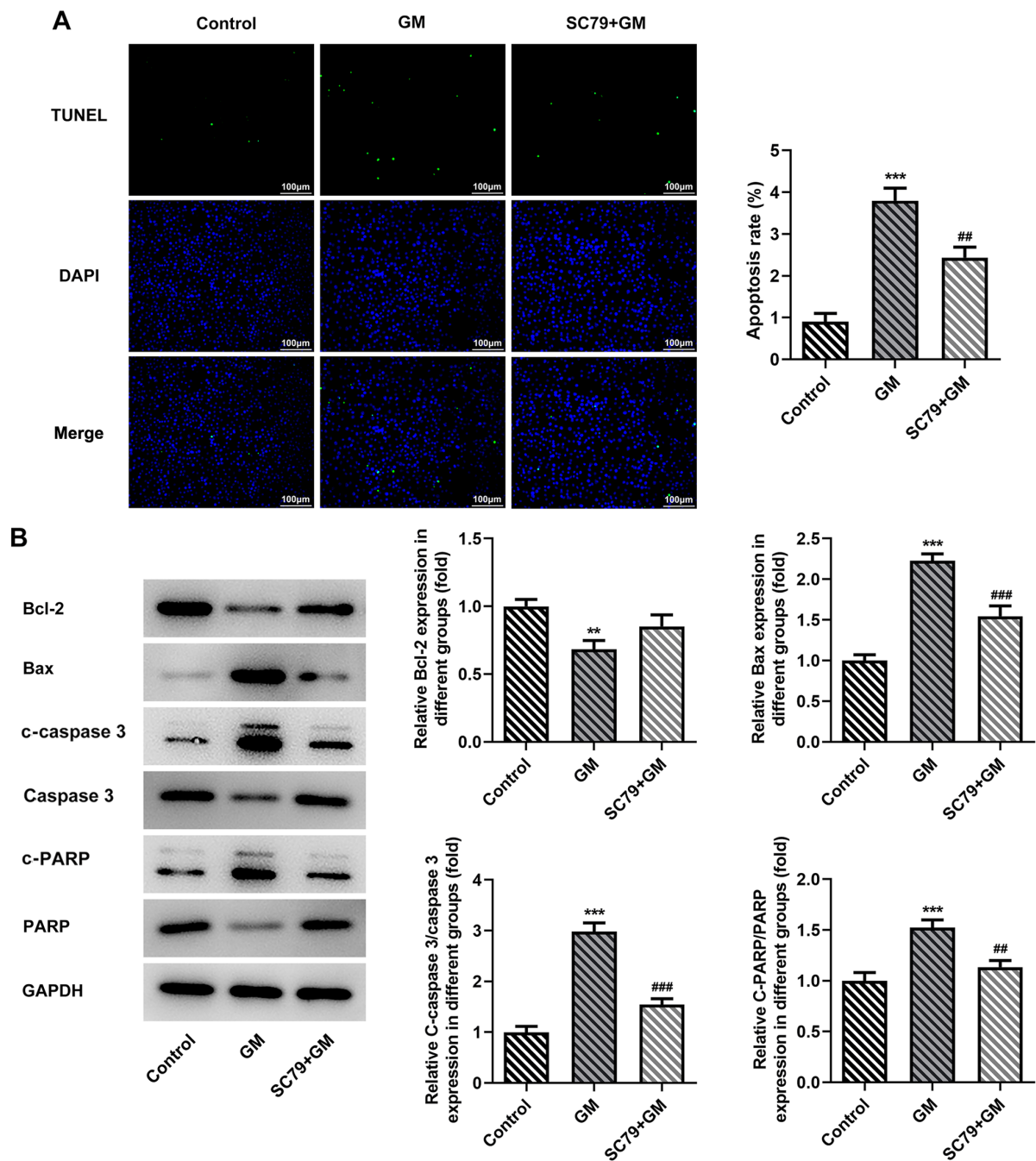


Figure 6. SC79 reverses the proapoptotic effects of GM on lung cancer cells. A549 cell apoptosis was (A) detected by performing TUNEL assays and quantified (n=3). (B) Expression levels of apoptosis-related proteins were detected via western blotting (n=3). **P<0.01 and ***P<0.001 vs. control. ##P<0.01 and ###P<0.001 vs. GM. GM, germacrone; C, cleaved; PARP, poly(ADP-ribose) polymerase.

and proliferation were significantly increased in the SC79+GM group compared with the GM group, respectively. Cell apoptosis was detected by performing TUNEL assays. Compared with the GM group, the apoptosis rate of the SC79+GM group was significantly decreased (Fig. 6A). Furthermore, compared with the GM group, the SC79+GM group displayed significantly decreased expression levels of Bax, C-Caspase 3 and c-PARP, and slightly increased expression levels of Bcl-2 (Fig. 6B). The flow cytometry results demonstrated that SC79 significantly reversed GM-induced G₁/S phase cell cycle arrest (Fig. 7A and B). Subsequently, western blotting was performed to detect the

expression levels of cyclin D1, CDK4 and CDK6. Compared with the GM group, the expression levels of cyclin D1, CDK4 and CDK6 in the SC79+GM group were significantly increased (Fig. 7C). In addition, the expression levels of Akt/MDM2/p53 signaling pathway-related proteins were detected. The results demonstrated that p-Akt and p-MDM2 expression levels were significantly upregulated, and p53 expression levels were significantly downregulated in the SC79+GM group compared with the GM group (Fig. 8). Collectively, the results demonstrated that SC79 reversed the antiproliferative, proapoptotic and pro-cell cycle arrest effects of GM on lung cancer cells.

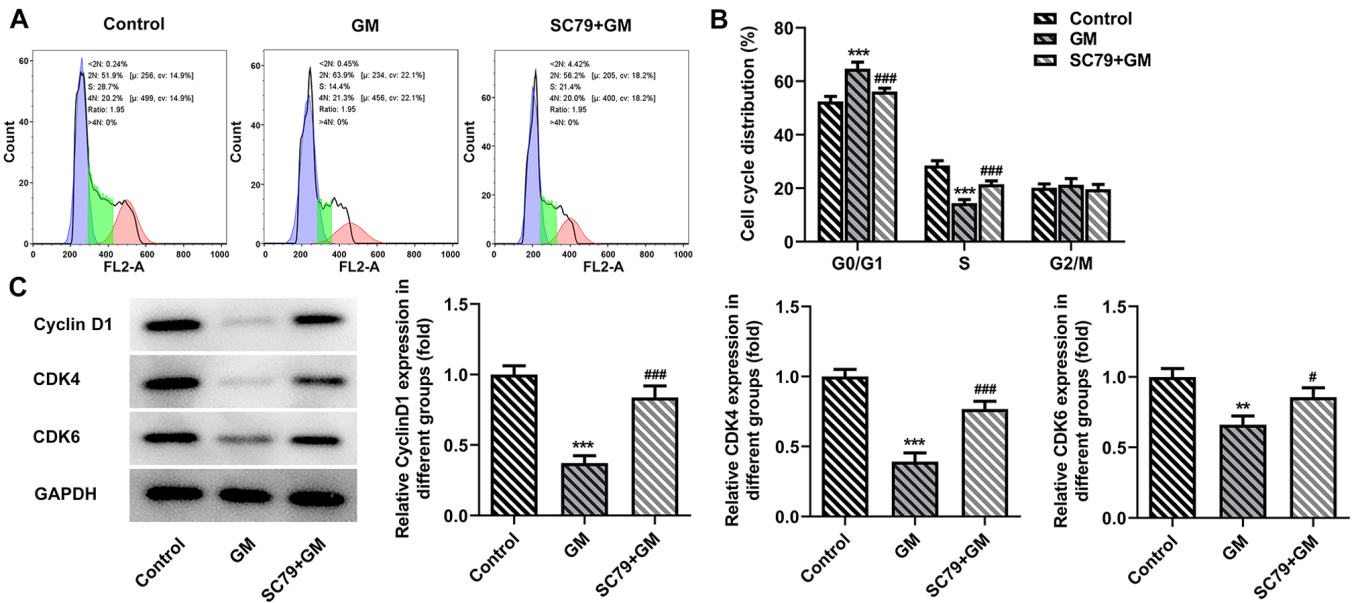


Figure 7. SC79 reverses the pro-cell cycle arrest effects of GM on lung cancer cells. Cell cycle distribution was (A) measured via flow cytometry and (B) quantified (n=3). (C) Expression levels of cell cycle-related proteins were determined via western blotting (n=3). **P<0.01 and ***P<0.001 vs. control; #P<0.05 and ###P<0.001 vs. GM. GM, germacrone.

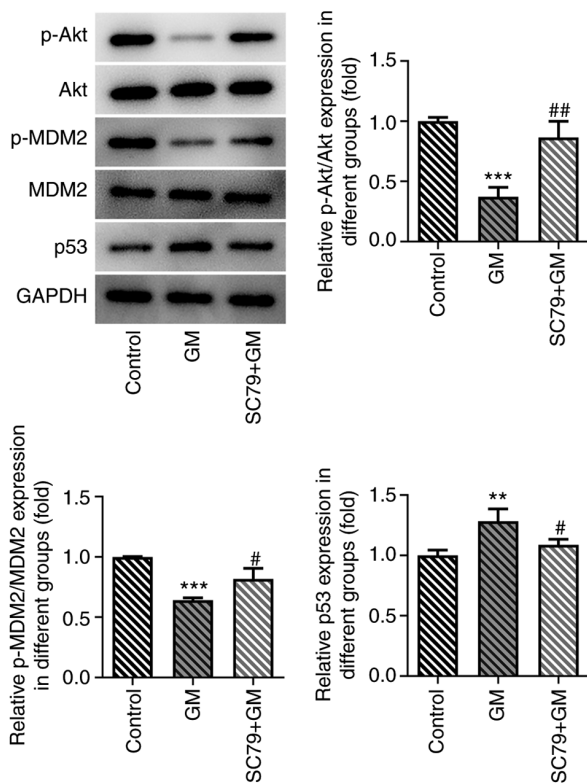


Figure 8. Effect of SC79 on the Akt/MDM2/p53 signaling pathway. Expression levels of Akt/MDM2/p53 signaling pathway-related proteins were determined via western blotting. **P<0.01 and ***P<0.001 vs. control; #P<0.05 and ##P<0.01 vs. GM. MDM2, MDM2 proto-oncogene; GM, germacrone; p, phosphorylated.

Discussion

GM displays antiviral, antioxidant, anti-inflammatory and immunomodulatory pharmacological effects (20,22).

Pharmacological studies have demonstrated that GM also displays antitumor effects on gastric cancer (4), glioma (21) and breast cancer (9) by inducing cell cycle arrest and promoting cell apoptosis. Cai *et al* (23) reported that curcuminol extracted from zedoary enhanced celecoxib-induced growth inhibition and apoptosis in NSCLC. However, to the best of our knowledge, the effects of GM isolated from zedoary in lung cancer have not been previously reported. Therefore, the present study investigated the effects of GM on lung cancer cells. The results of the present study demonstrated that GM induced lung cancer cell apoptosis and cell cycle arrest, thereby inhibiting the development of lung cancer.

Following further investigation into the effects of GM on lung cancer cell apoptosis and cell cycle arrest and its molecular mechanism, the present study demonstrated that the expression levels of p-Akt and p-MDM2 were significantly downregulated, whereas the expression levels of p53 were significantly upregulated by different concentrations of GM (100 and 200 μ M) compared with the control group. Malignancy in various different types of cancer cells (such as breast, ovarian epithelial, prostate and gastric cancer) is associated with increased abnormal Akt signaling (24). Akt phosphorylates the p53 suppressor MDM2, promoting nuclear translocation of MDM2, thereby inhibiting p53 (25). As a classical tumor suppressor gene, p53 mutations occur in >50% of all malignant tumors (26). The protein encoded by p53 is a transcription factor that controls the initiation of the cell cycle, and regulates cell proliferation and apoptosis via a complex molecular network (27). It has been previously reported that the expression levels of p-Akt and p-MDM2 are upregulated in prostate cancer cells, which downregulates p53 expression and induces cell cycle arrest (28). Similarly, genistein inhibited esophageal cancer cell proliferation by inhibiting activation of the Akt/MDM2/p53 signaling pathway (13).

Furthermore, GM induces prostate cancer cell apoptosis and autophagy by inhibiting the Akt/mTOR signaling pathway (8). GM can serve a role in breast cancer cells by targeting the estrogen receptor, MAPK, AKT and other signaling pathways (29,30). The aforementioned studies suggested that GM could target the Akt signaling pathway. Following administration of SC79, the antiproliferative, proapoptotic and pro-cell cycle arrest effects of GM on lung cancer cells were significantly reversed. Collectively, the results of the present study demonstrated that GM induced lung cancer cell apoptosis and cell cycle arrest via the Akt/MDM2/p53 signaling pathway. However, it has been previously reported that GM can decrease the expression levels of p-Akt in cerebral ischemia-reperfusion injury model rats, which was inconsistent with the results of the present study; therefore, future studies should investigate this inconsistency further (31).

A key limitation of the present study was that the therapeutic effect of GM on lung cancer was not studied in a mouse tumor model. Therefore, future studies should investigate the therapeutic effect of GM on lung cancer *in vivo*.

In conclusion, the present study demonstrated that GM promoted lung cancer cell apoptosis and cell cycle arrest by inhibiting the Akt/MDM2/p53 signaling pathway. The results of the present study provided a theoretical basis for identifying potential drugs and targets for the treatment of lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and JC contributed to the conception and design of the present study, analyzed and interpreted the data, and critically revised the manuscript for important intellectual content. KS, HL and JD contributed to designing the study, analyzed the data, and drafted and revised the manuscript. DH, ZL and WW substantially contributed to the conception and design of the study, acquired, analyzed and interpreted the data, and drafted and critically revised the manuscript for important intellectual content. YZ and JC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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