# Grifolin induces apoptosis and promotes cell cycle arrest in the A2780 human ovarian cancer cell line via inactivation of the ERK1/2 and Akt pathways

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**Abstract.** Grifolin, a secondary metabolic product isolated from the mushroom *Albatrellus confluence*, has been demonstrated to possess antitumor activities in a variety of malignant cells. However, the signaling pathways and the molecular mechanisms underlying the anticancer effects of the agent in human ovarian cancer remain to be elucidated. The aim of the present study was to examine the effect of grifolin treatment on the human ovarian cancer cell line, A2780. MTT and flow cytometry analysis were used to analyze the viability of A2780 cells following treatment with grifolin. Western blotting was used analyze the expression of apoptosis-associated and cell cycle arrest-associated proteins. The results of MTT assays and flow cytometry analysis revealed that grifolin suppressed cell viability, induced apoptosis and triggered cell cycle arrest. Western blotting revealed that grifolin treatment resulted in inactivation of protein kinase B (Akt) and extracellular signal-related kinase 1/2 (ERK1/2), accompanied by upregulation of Bcl-2 associated X, apoptosis regulator, cleaved-caspase-3 and cleaved-poly (ADP-ribose) polymerase, and downregulation of B cell lymphoma-2, cyclin dependent kinase 4 and cyclinD1. The results of the present study indicated that grifolin had significant anti-cancer effects on the human ovarian cancer A2780 cells, which occurred via the Akt and ERK1/2 signaling pathways to at least a certain extent. These results demonstrate the therapeutic potential of grifolin as a treatment for ovarian cancer.

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## Introduction

Ovarian cancer is a gynecological malignancy with one of the highest mortality rates worldwide (1). Even though the incidence of cervical cancer and endometrial cancer are higher at present (2), ovarian cancer ranks fifth as a cause of cancer-associated mortality among women (3,4). Ovarian cancer is not typically diagnosed at an early stage, with the majority of women already at advanced stages (International Federation of Gynecology and Obstetrics stage) when diagnosed, which presents a severe challenge for resection and recovery (5). Based on this situation, a combination of surgery and chemotherapy are the conventional methods of treatment. However, despite the efficacy of chemotherapy, its side effects are inevitable. Thus, a novel therapeutic agent against ovarian cancer is required.

At present, the use of natural agents for cancer prevention and therapy is attracting increased attention. Grifolin, which originates from the edible fruiting bodies of the mushroom *AIbatrellus confluence*, has been reported to possess anti-oxidant and antitumor activities (6). However, the effect of grifolin on ovarian cancer cells remains to be investigated. Therefore, the aim of the present study was to examine the effect of grifolin on the human ovarian cancer cell line, A2780, *in vitro*.

The present study revealed that grifolin effectively suppressed viability and induced apoptosis of human ovarian cancer A2780 cells. The present study also researched the exact targets and molecular mechanisms underlying grifolin-induced anticancer activity in A2780 cells, and revealed that inactivation of protein kinase B (Akt) and extracellular signal-related kinase (ERK1/2) is necessary for grifolin-induced cell cycle arrest and cell apoptosis. Grifolin may therefore be a promising anti-tumor agent for the treatment of ovarian cancer.

## Materials and methods

*Cell culture*. The A2780 cell line was cultured in RPMI-1640 medium with 10% fetal bovine serum. Cells were maintained in a humidified incubator with 5% $CO_2$ at 37˚C. The cell culture media was obtained from HyClone (GE Healthcare Life Sciences, Logan, UT, USA), the fetal bovine serum was provided by Cell Signaling Technology, Inc., (Danvers, MA, USA).

*Drugs and chemicals*. Grifolin was provided by the Kunming Institute of Botany; Chinese Academy of Sciences (Kunming, China) with the following structure: 2-trans, trans-farnesyl-5-methylresorcinol (purity, >99%; high performance liquid chromatography analysis). Grifolin was prepared at a concentration of 100 mmol/l stock solution in dimethyl sulfoxide (DMSO) and stored under -20˚C so that the final concentration of DMSO was <0.1% in all assays it was used in. DMSO was provided by (Shanghai Biyuntian Bio-Technology Company, Ltd., Shanghai, China).

*Cell proliferation assay*. The effect of grifolin on A2780 cell viability was evaluated by MTT assay. Cells were seeded at a density of 3,000-3,500 cells/well in 96-well plates, which permitted logarithmic growth during the 72 h assay, and cultured overnight at 37˚C with 5% $CO_2$. Adherent cells were subsequently exposed to grifolin at varying concentrations (0, 20, 40, 60, 80 and 100 $\mu$M) for 24, 48 and 72 h. The cells were then washed with PBS twice and incubated in 20 $\mu$l MTT (HyClone; GE Healthcare Life Sciences) for 4 h. The cell suspension was discarded and the cells were supplemented with 100 $\mu$l DMSO. At the MTT endpoint, an infinite M200PRO microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure cell viability at 490 or 550 nm absorbance. The cell viability trend and the half maximal inhibitory concentrations (IC50) were analyzed using SPSS Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA). Finally, GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to draw up the bar graph.

*Clonogenic assay*. A2780 cells ($5x10^2$) were seeded on 6-well plates and exposed to grifolin (0, 25, 50 or 75 $\mu$M) for 48 h with 5% $CO_2$ at 37˚C. The cells were then washed with PBS twice and RPMI-1640 culture medium was added into the plates for ~10 days. The cells were washed with PBS twice, fixed with 4% paraformaldehyde solution for ~15 min, and then stained with 1 ml 0.2% crystal violet (HK Jimei Biology Science and Technology Company, Ltd., Beijing, China) for 30 min. Finally, the numbers of clones were counted and analyzed by using the DP71 fluorescence microscope (Olympus Corporation, Tokyo, Japan) with x200 magnification 400-600 cells per field of view assessed.

*Flow cytometry assay*. Flow cytometry was used to detect the cell cycle and apoptosis distribution. Cells ($1x10^6$) were treated with 0, 25, 50 or 75 $\mu$M grifolin for 24 h, collected and washed with ice-cold PBS twice, fixed in 75% ethanol overnight at 4˚C and stained with 20 $\mu$l Rnase A (Cell Signaling Technology, Inc., Danvers, MA, USA) and 400 $\mu$l propidium iodide (PI) at room temperature for 30 min. Apoptosis analysis was also performed using annexin V-fluorescein isothiocyanate and PI, following the manufacturers protocol. The apoptosis kit used in flow cytometry assay was provided by the (#50T; Jiehui Biology Science and Biotechnology Company, Ltd., Beijing, China,). The number of cells was calculated by FACScan flow cytometry (Bio-Rad Laboratories). The data was analyzed by WinMDI v2.9 software (The Scripps Research Institute, San Diego, CA, USA).

*Western blot analysis*. A2780 cells at a density of $4x10^5$ were seeded per well and were incubated with grifolin at concentrations of 0, 25, 50 and 75 uM for 24 h, then harvested and homogenized in 100 $\mu$l RIPA lysis buffer for ~30 min on ice. The cells were centrifuged for 15 min 4˚C at the speed 15,000 x g. The protein samples (20 $\mu$g/lane) were separated on 10 or 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes. Following blocking using 5% non-fat milk at room temperature for ~2 h, the membranes were incubated with primary antibodies against phosphorylated (p-) ERK1/2 (#4370; 1:1,000; Cell Signaling Technology, Inc.), ERK1/2 (#4060; 1:1,000; Cell Signaling Technology, Inc., MA, USA), p-Akt (#4691; 1:1,000; Cell Signaling Technology, Inc.), Akt (#4691; 1:1,000; Cell Signaling Technology, Inc.), cleaved-poly (ADP-ribose) polymerase (PARP; #5625; 1:1,000; Cell Signaling Technology, Inc.), BCL2 associated X, apoptosis regulator (Bax; #ab32503; 1:1,000; Abcam, Cambridge, UK), B cell lymphoma-2 (Bcl-2; #ab32124; 1:1,000; Abcam), cleaved-Caspase-3 (#ab2302; 1:1,000; Abcam), CyclinD1 (#2922; 1:1000; Cell Signaling Technology, Inc.), cyclin dependent kinase 4 (CDK4; #12790; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (#8457; 1:10,000; Cell Signaling Technology, Inc.) at 4˚C overnight. The blots were washed with TBST (1,000 ml TBST with 1 ml Tween-20) three times and incubated with respective secondary antibodies (Goat anti-rabbit IgG Alexa Fluor® 488; #ab150077; 1:10,000; Cell Signaling Technology, Inc.) at 4˚C for 1-2 h. Following three washes in TBST, the ImageQuant LAS 4000 (Immobilon®; EMD Millipore, Billerica, MA, USA) was used to visualize the blots and the expression levels of the proteins were analyzed using the NIH Image J system 1.48v (National Institutes of Health, Bethesda, MD, USA). Statistical significance was calculated using SPSS 18.0 software (SPSS, Inc.) and the histograms were obtained using the Graph Pad system v5.0 (GraphPad Software, Inc.).

*Statistical analysis*. A student t-test and one-way analysis of variance was used to compare the means of different treatments, using SPSS 18.0 software (SPSS, Inc.). Date were expressed as the mean ± standard deviation, and $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Grifolin decreases cell viability in the human ovarian cancer cell line, A2780*. The effect of grifolin on human ovarian cancer cell viability was analyzed using the MTT assay, clonogenic assay and flow cytometry. For this purpose, A2780 cells were incubated with grifolin (0, 25, 50, 75 and 100 $\mu$M) for 24, 48 and 72 h. The results revealed a significant decrease in the viability of A2780 cells, in a dose-dependent

and time-dependent manner (P<0.05; Fig. 1). A2780 cells ($5x10^2$) were seeded on 6-well culture plates and treated with grifolin at concentrations of 0, 25, 50 and 75 $\mu$M. The results revealed that grifolin suppressed A2780 cell viability significantly (P<0.05; Fig. 2). Flow cytometry analysis was used to determine the stage that A2780 cells were suspended in, and grifolin was demonstrated to result in a cell cycle block at the G1 phase (P<0.05; Fig. 3).

*Grifolin induced apoptosis in human A2780 ovarian cancer cells*. Flow cytometry analysis was used to determine whether grifolin induced apoptosis in A2780 cells. Grifolin-treated A2780 cells were stained with annexin V-FITC and PI following treatment with grifolin (0, 25, 50, and 75 uM) for 24 h. Grifolin-treated A2780 cells were demonstrated to have undergone apoptosis in a dose-dependent manner, and these results corroborated the data from the MTT assay (P<0.05; Fig. 4).

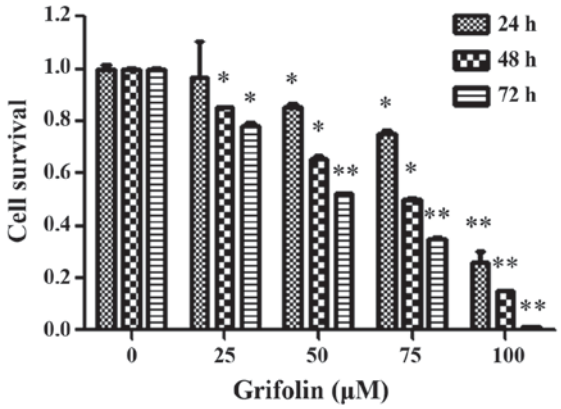


Figure 1. Effect of grifolin on A2780 human ovarian cancer cell viability. Cells were treated with various concentrations of grifolin and cell viability was analyzed using the MTT assay. The results are representative of three independent experiments. *P<0.05 vs. 0 $\mu$M control.

*Grifolin treatment affected the expression of cell cycle proteins and apoptosis family proteins*. Next, the protein expressions of cell cycle proteins (cyclinD1 and CDK4) and apoptosis associated proteins (Bcl-2, Bax, cleaved-caspase-3 and cleaved-PARP) were analyzed to further assess the potential mechanisms underlying grifolin-induced apoptosis and inhibition of cell viability. Western blot analysis was used to assess the expression of cyclinD1 and CDK4, which were visibly decreased in cells treated with grifolin compared with the control (P<0.05; Fig. 5) Western blot analysis also revealed that Bcl-2 expression was visibly decreased, while the expression of Bax was visibly increased compared with untreated cells (Fig. 5). In addition, the expression of cleaved caspase-3 and cleaved-PARP was significantly increased compared with untreated cells (P<0.05; Fig. 5). These results demonstrated that grifolin treatment downregulated cell cycle proteins and upregulated the Bax/Bcl-2 ratio, cleaved caspase-3 and cleaved-PARP expression.

*Grifolin inactivated Akt and ERK1/2 in A2780 cells*. Activation of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways has been demonstrated to be associated with the expression of apoptosis-associated proteins. Thus, western blotting was used to evaluate the expression of Akt and ERK1/2 in grifolin-treated A2780 cells, and to determine whether the Akt and ERK1/2 pathways are potential signaling mechanisms underlying grifolin-induced apoptosis. The expression levels of p-Akt and p-ERK1/2 were significantly decreased in a dose-dependent manner in grifolin-treated cells compared with the untreated control (P<0.05; Fig. 6). These results suggested that inactivation of Akt and ERK1/2 may be the key mechanism underlying grifolin-induced apoptosis.

## Discussion

At present, there has been growing interest in the function of grifolin, in particular concerning its anticancer effects. Grifolin, a secondary metabolite product isolated from the mushroom *A. confluence*, exhibits anti-cancer and therapeutic properties. Multiple studies have revealed that grifolin inhibits cell growth in certain types of cancer (7,8). Grifolin has been demonstrated to induce cell-cycle arrest via the ERK1/2 pathway in human nasopharyngeal carcinoma cells (7) and to induce apoptosis through inhibition of the Akt pathway in human osteosarcoma cells (8). However, there is little research concerning the effect of grifolin on ovarian cancer cells to date. In the present study, A2780 ovarian cancer cells were treated with different concentrations of grifolin in order to elucidate the underlying signaling pathways and molecular mechanisms. The results revealed that grifolin triggered apoptosis and cell cycle arrest in a dose-and time-dependent fashion, and decreased the phosphorylation of Akt and ERK1/2 in A2780 ovarian cancer cells *in vitro*. The results indicated that grifolin may inhibit cell viability and induce apoptosis in human ovarian cancer cells via two pathways; the Akt and the ERK1/2 pathways.

Multiple studies have demonstrated that mammalian cell cycle progression is closely associated with cyclins and CDKs (9,10). Uncontrolled cell cycle progression is an identified characteristic of multiple types of cancer (9). A previous study demonstrated that grifolin significantly induced cell cycle arrest at the G1 phase in human nasopharyngeal carcinoma cell line CNE1 (7). Verifying this finding, the data obtained from flow cytometry analysis in the present study indicated that grifolin induced G1 cell cycle arrest in A2780 cells. Thus, attention was directed towards the underlying molecular mechanisms of cell cycle arrest in A2780 cells treated with grifolin. CyclinD1 has been demonstrated to be a mitogenic sensor that amplifies extracellular growth signals (10). Overexpression of different cyclins and CDKs provides cancer cells with the advantage of rapid cyclical growth (11). The cyclinD1/CDK4 complex promotes DNA synthesis and cell cycle progression from the G1 to the S phase (12). In accordance, downregulation of cyclinD1 and CDK4 may be a promising treatment for cancer. As expected, the present study demonstrated that grifolin treatment resulted in a dose-dependent decrease in the expression of cyclinD1 and CDK4. Thus, the data obtained by flow cytometry were further verified by the results of the western blot assay.

Members of the caspase family participate in physiological activity through pathways including the mitochondrial and death receptor pathway (13). Activation of distinct caspase

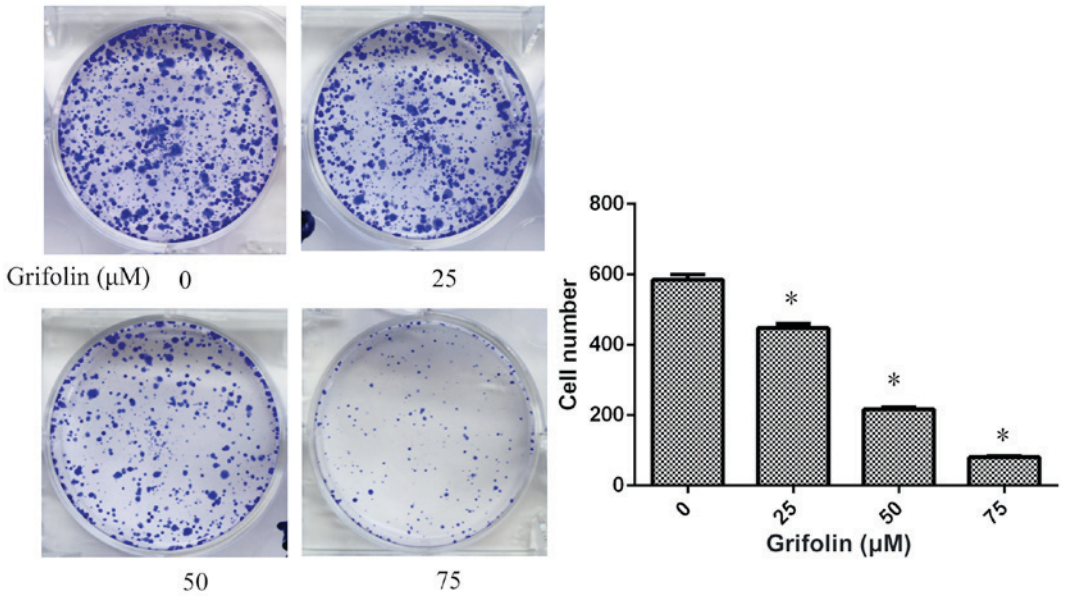


Figure 2. A2780 cells were treated with different concentrations of grifolin, and assessed by clonogenic assay. Data were expressed as the mean ± standard deviation, and the experiments were repeated three times. *P<0.05 vs. 0 $\mu$M control.

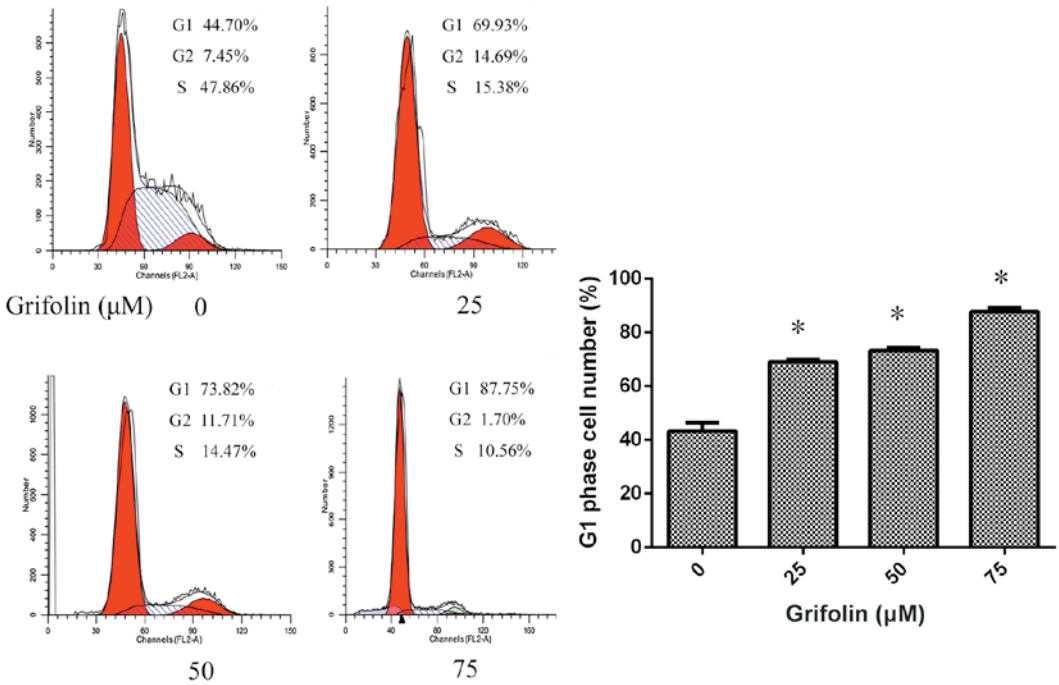


Figure 3. Effects of grifolin on the cell cycle stage of A2780 human ovarian cancer cells, as assessed by flow cytometry. Data are representative of three independent experiments. *P<0.05 vs. 0 $\mu$M control.

cascades is known to be involved in activating and cleaving certain proteins that are crucial to cellular activities and functions (14). In general, caspase-3 is recognized as the key apoptotic mediator (15). A previous study demonstrated that one of the caspase family substrates, PARP, which possesses a wide range of physiological functions, the most prominent of which is recovering damaged DNA (16), is cleaved by caspase-3 into two fragments (24 and 89 kDa, respectively), with different functions. The 24 kDa PARP fragment weakens DNA repair by inhibiting DNA repair enzymes, thereby contributing to cell death (17). In the present study, treatment with grifolin resulted in the upregulation of cleaved caspase-3 and cleaved PARP in A2780 cells, further demonstrating that the anti-cancer activity is regulated by caspase-dependent cell apoptosis. As well as investigating the activation of caspase-3 and PARP, the expression of Bcl-2 family members, which have been identified as upstream regulators of the caspase cascade (18,19), were assessed. It is possible to subdivide the

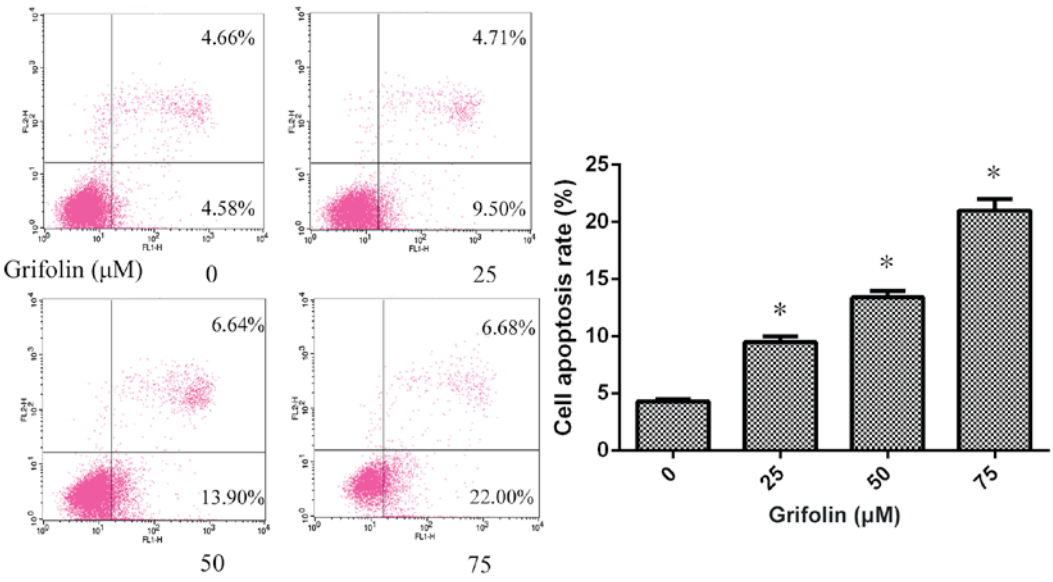


Figure 4. Pro-apoptotic activity of grifolin on A2780 human ovarian cancer cells. The proportion of early apoptotic A2780 cells were measured by flow cytometry. Data are representative of three independent experiments. *P<0.05 vs. 0 $\mu$M control.

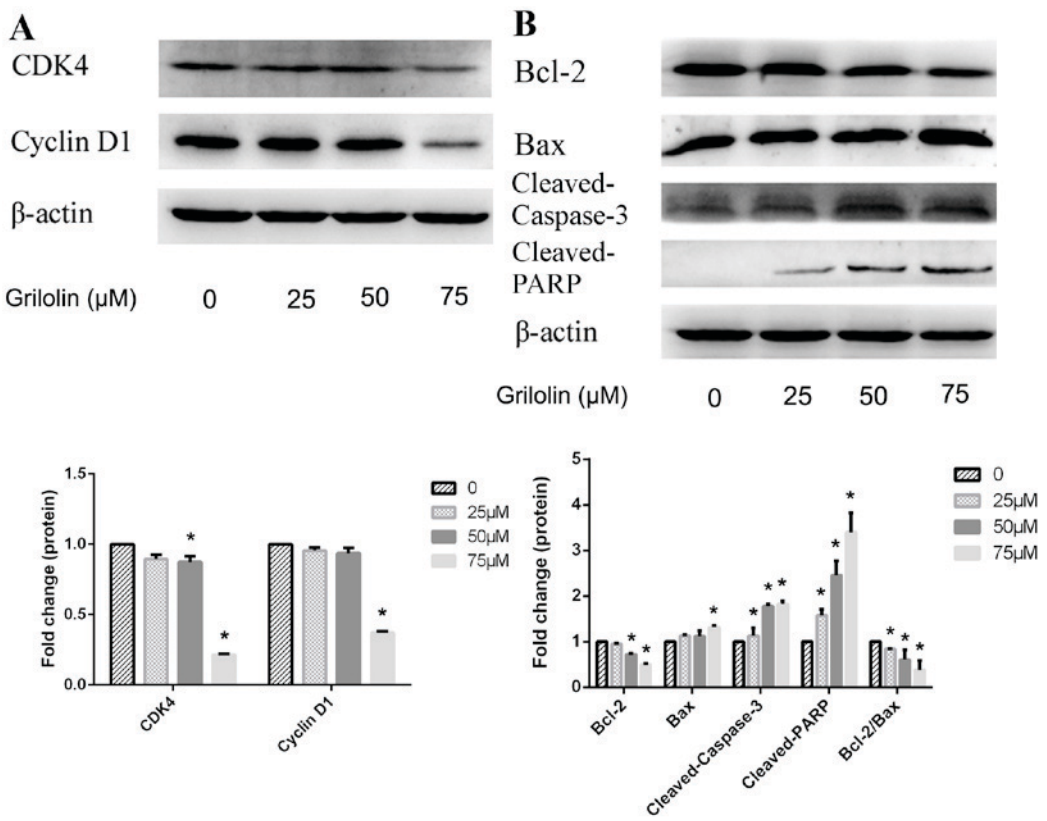


Figure 5. (A) Western blot analysis of cell cycle proteins of A2780 human ovarian cancer cells, following 24 h treatment with 0, 25, 50 or 75 $\mu$M grifolin. (B) Western blot analysis of apoptosis-associated proteins following 24 h treatment with 0, 25, 50 or 75 $\mu$M grifolin. Equal loading and transfer were demonstrated by repeat probing with β-actin. CDK4, cyclin dependent kinase 4; Bcl-2, B cell lymphoma-2; Bax, Bcl-2 associated X, apoptosis regulator; PARP, poly (ADP-ribose) polymerase. Data are representative of 3 independent experiments. *P<0.05 vs. 0 $\mu$M control.

Bcl-2 protein family into pro-apoptotic components, including Bax and Bcl-2 associated agonist of cell death, and anti-apoptotic components, including Bcl-2 and B cell lymphoma-extra large (20). The present study demonstrated that Bcl-2 protein levels were decreased, as demonstrated by western blotting, while the expression of Bax was slightly altered. However, the Bcl-2/Bax ratio was significantly lowered. It has been confirmed that the Bcl-2/Bax ratio is crucial for regulating cellular apoptosis (21). Alteration of this ratio may increase the rate of apoptosis of A2780 cells exposed to grifolin.

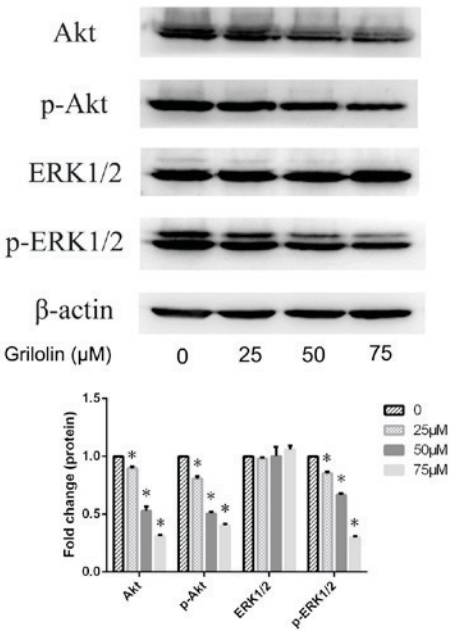


Figure 6. Western blot analysis of p-Akt, Akt, p-ERK1/2 and ERK1/2 expression in A2780 human ovarian cancer cells treated with 0, 25, 50 or 75 $\mu$M grifolin for 24 h. Equal loading and transfer were demonstrated by repeat probing with β-actin. p-, phosphorylated; Akt, protein kinase B; ERK1/2, extracellular signal-related kinase. Data are representative of 3 independent experiments. *P<0.05 vs. 0 $\mu$M control.

Cellular signaling pathways are known to include diverse proteins, and are complex communication networks that control basic biological functions. Several cellular signaling pathways are known to regulate cell proliferation and apoptosis, including nuclear factor-κB, PI3K/Akt, MAPK and p53 signaling pathways. The aim of the present study was to define the pathways participating in grifolin-induced anti-cancer effects. The majority of previous studies have confirmed that activation of the ERK1/2 or Akt pathway is associated with the anticancer effect of grifolin (7,8) Nevertheless, to date, knowledge concerning the effect of grifolin in human ovarian cancer cells and underlying mechanisms is lacking. Thus, the present study aimed to explore the signaling pathways underlying the pro-apoptotic action of grifolin on A2780 cells. Accumulating evidence indicates that the Akt pathway is a complex signal transduction pathway participating in rigorous regulation of cell differentiation, proliferation and apoptosis (22,23). Akt phosphorylation promotes cell survival and proliferation, and activation of Akt may contribute to tumorigenesis and tumor progression (24). The results of the present study demonstrated that the activation of Akt in A2780 cells was decreased by grifolin in a concentration-dependent manner. MAPK members are classified into three major subfamilies: p38MAP, ERK1/2 and c-Jun N-terminal Kinase, and phosphorylation of ERK1/2 lead to progressing cell cycle and inhibiting cell apoptosis (25). Furthermore, in the present study, the phosphorylation level of ERK1/2 was evaluated and demonstrated to be markedly decreased by grifolin. Hence, it is possible to conclude that grifolin induced apoptosis and arrested cell cycle progression at the G1 phase by downregulating phosphorylated ERK1/2 and Akt levels, which resulted in altered expression of caspase-3, Bax, CDK4 and cyclinD1.

In conclusion, grifolin, which has been known for several decades to demonstrate various pharmacological and physiological effects and to inhibit the growth of multiple cancer cell lines, also had an anticancer effect against the human ovarian cancer cell line, A2780. To the best of our knowledge, the present study is the first report to explore the activity of grifolin on human ovarian cancer cells. The results demonstrated that inactivation of Akt and ERK1/2 was the mechanism underlying grifolin-induced cell cycle arrest and cell apoptosis. Grifolin may therefore be a promising antitumor agent for the treatment of ovarian cancer.

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