LFG-500, a newly synthesized flavonoid, induces apoptosis in human ovarian carcinoma SKOV3 cells with involvement of the reactive oxygen species-mitochondria pathway

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Abstract. Ovarian cancer is the main cause of gynecologic malignancy-related mortality in women. Therefore, the disease requires improvements in treatment options and in the potency of chemotherapeutic drugs. The study of apoptosis in tumor cells is an important field for cancer therapy and cancer molecular biology. It has recently been established that LFG-500, a new synthesized flavonoid with a piperazine and benzyl group substitution, has strong anticancer activity. However, its exact molecular mechanism is not fully understood. The present study aimed to examine the effects of LFG-500 on human ovarian cancer SKOV3 cells, as well as to identify its underlying mechanisms. The data showed that LFG-500 inhibited the growth of SKOV3 cells in a concentration-dependent manner. It was found that LFG-500 induced apoptosis in SKOV3 cells, detected by DAPI staining and an Annexin V/PI double-staining assay. Moreover, LFG-500 reduced caspase-3 protein expression and increased the Bcl-2-associated X protein/B-cell lymphoma 2 protein ratio. Further findings revealed that LFG-500 treatment resulted in reactive oxygen species (ROS) accumulation and loss of mitochondrial transmembrane potential. Collectively, these results demonstrated that LFG-500 efficiently induced apoptosis in SKOV3 cells, an event possibly associated with the trigging of the mitochondrial apoptotic pathway through ROS accumulation. Therefore, LFG-500 shows potential as a potent anticancer agent for the treatment of ovarian cancer.

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Abbreviations: ΔΨm, mitochondrial transmembrane potential; Bax, B-cell-associated X; Bcl-2, B-cell lymphoma 2; FITC, fluorescein isothiocyanate; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species

Key words: LFG-500, apoptosis, reactive oxygen species, mitochondria, SKOV3 cells

Introduction

Ovarian cancer is the most prevalent form of malignant tumor in women and is the leading cause of mortality from gynecologic malignancies (1). Due to a lack of specific and sensitive methods for early detection, 60-70% of ovarian cancer patients are diagnosed at advanced stages (2). Initial treatment for ovarian cancer patients involves cytoreductive surgery, followed by platinum-based chemotherapy (3). In spite of efforts made to improve disease diagnosis and treatment, the five-year survival rate remains low, at <40% (4,5). The acquired resistance to conventional chemotherapies, as well as the high probability of metastasis (6), makes ovarian cancer therapy more challenging. Therefore, there is a need to develop novel therapeutic agents for ovarian cancer.

The majority of the cytotoxic anticancer drugs in current use have been shown to induce apoptosis in susceptible cells. Apoptosis is regulated by an evolutionarily conserved cellular pathway that consists of the caspase family, the B-cell lymphoma 2 (Bcl-2) protein family and the adapter protein apoptotic protease activating factor 1 (7). The intrinsic pathway of apoptosis also utilizes the mitochondria, which together with the Bcl-2 family forms a signal transduction pathway. This pathway signals formation of the apoptosome (8), and enables the mitochondria to play important roles during the process of apoptosis (9). Moreover, loss of mitochondrial transmembrane potential (ΔΨm) is considered a crucial step in apoptosis (10), which in turn is mediated by reactive oxygen species (ROS) (11).

It has been proposed that high intake of fruits and non-starchy vegetables is a beneficial lifestyle factor for the prevention of cancer (12). Flavonoids are a group of compounds that are found in citrus fruits, olive oil, tea and vegetables. The antitumor effects of flavonoids have been widely recognized and studied, particularly their potent apoptotic-inducing activity (13,14). Previous studies have demonstrated that flavonoids induce apoptosis through activation of the intrinsic pathway mediator p53, along with enhancing the apoptotic effect of tumor necrosis factor-related apoptosis-inducing ligand in ovarian cancer cells (15,16).

LFG-500 is a newly synthesized flavonoid, with piperazine and benzyl group substitutions ($C_{30}H_{32}N_2O_5$; Fig. 1A), which was synthesized and supplied by Professor Zhiyu Li (China Pharmaceutical University, Nanjing, China) (17,18). Previous studies demonstrated that LFG-500 induced apoptosis in human hepatocellular carcinoma HepG2 cells (17) and inhibited the invasion of human breast cancer MDA-MB-231 cells (18). In view of the multiple anticancer effects on HepG2 and MDA-MB-231 cells, LFG-500 is a potential antitumor agent that warrants further study. However, the inhibitory effect of LFG-500 on ovarian cancer cells is not well-defined. In the present study, the apoptotic-inducing effect of LFG-500 on ovarian cancer cells and the corresponding mechanisms were investigated. The results indicate that LFG-500 induces apoptotic processes in ovarian cancer cells via intracellular ROS formation and loss of $\Delta\Psi m$. These findings provide novel evidence for the anticancer activity of LFG-500.

Materials and methods

Materials. LFG-500 (99.1% purity), supplied by Professor Qinglong Guo (China Pharmaceutical University, Nanjing, China), was dissolved in dimethyl sulfoxide (DMSO; ≥99.7% purity) as a primary stock solution. The solution was stored at -20˚C before dilution with RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) prior to each experiment. The controls were treated with an equal quantity of DMSO (0.1%) to that used in corresponding experiments. Antibodies against caspase-3 (D146; BS1518; 1:800), Bcl-2 (P65; BS1511; 1:800), B-cell-associated X (Bax) protein (S4; BS1030; 1:800) and β-actin (l102; AP0060; 1:1,000) were purchased from Bioworld Technology, Inc. (St. Louis Park MN, USA). These antibodies were diluted in antibody dilution buffer (Beyotime Institute of Biotechnology, Nantong, China) when used. The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). MTT, paraformaldehyde, formaldehyde, glycine, Triton X-100, DAPI, Tris, sodium chloride, Hepes, potassium hydroxide, potassium chloride, EDTA, NP-40, phenylmethylsulfonyl fluoride, sodium fluoride, SDS, dithiothreitol and sodium hydrogen carbonate were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

Cell culture. The human ovarian cancer cell line SKOV3 was purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). SKOV3 cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.), containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime Institute of Biotechnology). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C.

Colorimetric MTT assay. Cells (10^4/well) were seeded on 96-well plates (Corning Inc., Corning, NY, USA) for 24 h, then exposed to different concentrations of LFG-500 (2.4, 4.8, 6.9, 9.8, 14.0, 20.0, 28.6, and 40.8 μM) diluted in the medium. Following incubation for 24 h at 37˚C, the colorimetric MTT assay was performed and cell growth inhibitory rate (%) was calculated, as previously described (19). The half maximal inhibitory concentration (IC_{50}) was taken as the concentration of LFG-500 that inhibited 50% of cell growth and was calculated by the Logit method (20).

Cell morphological assessment. Cells ($2x10^5$ cells/well) were seeded in 6-well culture plates (Corning Incorporated) and exposed to LFG-500 (4, 8 and 12 μM) for 24 h at 37˚C. Following incubation, cell morphology was monitored using an inverted light microscope (CX51; Olympus Corporation, Tokyo, Japan).

DAPI staining. Cells were treated with the above stated concentrations of LFG-500 for 24 h, then fixed with 4% polyoxymethylene in phosphate-buffered saline for 20 min before being permeabilized with 0.3% Triton X-100. Following a wash with ice-cold PBS, cells were stained with DAPI (1 mg/ml) for 10 min and observed using a fluorescence microscope (IX51; Olympus Corporation) using a peak excitation wavelength of 340 nm.

Annexin V/PI double staining. Following treatment with LFG-500 (4, 8 or 12 μM) for 24 h, cells were harvested and washed with PBS. As previously described (21), apoptotic cells were identified by double supravital staining with the Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol. Briefly, the cells were resuspended in 500 μl binding buffer, and 5 μl Annexin V-FITC staining solution was added and gently mixed. Subsequently, 5 μl propidium iodide (PI) staining solution was added and mixed evenly with the cells. Finally, the cells were incubated in the dark for 15 min at room temperature, and after 1 h, the rate of cell apoptosis was evaluated and analyzed using a MACS Flow Cytometer with MACSQuantify Software (version 2.4) (both from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Early apoptotic cells were Annexin V-positive and PI-negative, whereas late apoptotic cells were Annexin V and PI-positive.

Western blot analysis. Cells were exposed to various concentrations of LFG-500 (4, 8 or 12 μM) for 24 h, then collected and lysed with RIPA buffer (Thermo Fisher Scientific, Inc.). Western blot analysis was conducted according to previously described methods (22,23). Briefly, 60 μg protein per lane was separated by 12% SDS-PAGE, transferred onto nitrocellulose membranes saturated with 1% bovine serum albumin (Sigma-Aldrich; Merck Millipore) in PBS for blocking at 37˚C for 1 h, and subsequently incubated with primary antibodies against caspase-3 (1:800), Bcl-2 (1:800) and Bax (1:800) in antibody dilution buffer overnight at 4˚C. The membranes were

washed three times with TBS containing Tween-20 buffer and incubated with the IRDye 800-conjugated secondary antibody (P/N 925-32211; 1:20,000; Rockland Immunochemicals, Inc., Pottstown, PA, USA) for 1 h, followed by washing four times with PBS. Antibody-labeled protein bands were detected with the Odyssey Infrared Imaging System (version 4.0; LI-COR Biosciences, Lincoln, NE, USA). Densities of signals on blots were evaluated using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). β-actin (1:1,000) was used as an endogenous control, and the relative expression of proteins was normalized to the control group.

Measurement of ROS level. For detecting the accumulation of intracellular ROS in SKOV3 cells, a ROS assay kit (Beyotime Institute of Biotechnology) was used according to the manufacturer's protocol. In brief, following incubation with LFG-500 (4, 8 or 12 μM) for 24 h at 37˚C, 5x10^4 cells were collected. The cells were incubated with 10 μM 2,7-dihydrodichlorofluorescein diacetate (Beyotime Institute of Biotechnology) in serum-free medium for 30 min at 37˚C and washed twice with serum-free medium. Subsequently, fluorescence intensity was measured using a MACS Flow Cytometer (Miltenyi Biotec GmbH) with MACSQuantify Software (version 2.4; Miltenyi Biotec GmbH).

ΔΨm assessment. Quantitative changes in ΔΨm at the early stage of cell apoptosis were measured using a J aggregate-forming lipophilic cation-1 (JC-1) Mitochondrial Membrane Potential Detection kit (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's instructions. Cells were treated with 4, 8, or 12 μM LFG-500 for 24 h, then digested with trypsin (Sigma-Aldrich; Merck Millipore) and centrifuged at 367 x *g* for 5 min at 4˚C. The harvested cells were washed once with PBS and incubated with 10 μM JC-1 dye in incubation buffer at 37˚C for 20 min in the dark. Subsequently, the cells were washed twice with pre-chilled buffer solution. Relative fluorescence intensities of the cells were monitored using a MACS Flow Cytometer (Miltenyi Biotec GmbH) with MACSQuantify Software (Miltenyi Biotec GmbH; software version 2.4).

Statistical analysis. The data from the different experimental groups are expressed as the mean ± standard error of the mean. The data shown were obtained from a minimum of three independent experiments. Differences between the groups were assessed by one-way analysis of variance and the Dunnett's post hoc test, which were analyzed using GRAPHPAD PRISM software (version 5.01; GraphPad Software Inc., Avenida, CA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

LFG-500 inhibits growth of SKOV3 cells. Following exposure to different concentrations of LFG-500 for 24 h, cell growth was assessed. As shown in Fig. 1B, cell growth decreased systematically with increasing concentrations of LFG-500. The IC_{50} value of LFG-500 on the SKOV3 cells was 14.01±1.2 μM. These results indicate that LFG-500 inhibits the growth of SKOV3 cells in a concentration-dependent manner.

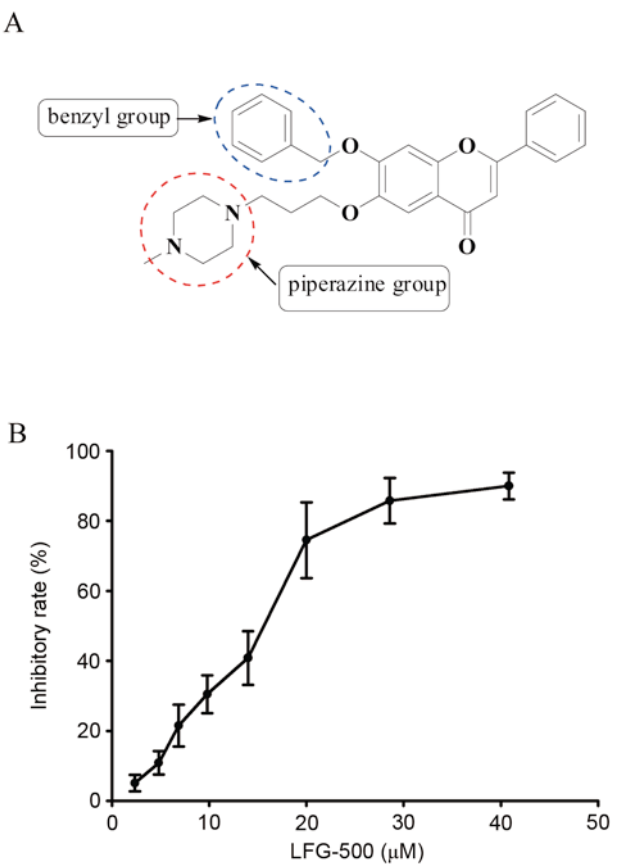

Figure 1. Chemical structure and inhibitory effects of LFG-500 on the growth of SKOV3 cells. (A) Chemical structure of LFG-500 ($C_{30}H_{32}N_2O_5$). (B) Inhibitory effects of LFG-500 on the growth of SKOV3 cells after 24 h, as measured by the MTT assay. LFG-500, synthetic flavonoid compound; SKOV3; human ovarian carcinoma cell line.

LFG-500 induces apoptosis in SKOV3 cells. Previous research has demonstrated that LFG-500 exerts apoptosis-inducing effects on human hepatocellular carcinoma HepG2 cells (17). Therefore, the present study determined whether LFG-500 could induce apoptosis in SKOV3 cells. As depicted in Fig. 2A, vehicle-treated SKOV3 cells were normal in size and shape, while the cells treated with LFG-500 (8 and 12 μM) were round and shrunken, and a small number were lysed. Moreover, the DAPI staining assay was employed to visualize the DNA damage induced by LFG-500. As shown in Fig. 2A, control cells emitted a uniform blue fluorescence, indicating that the chromatin was evenly distributed throughout the nucleus. However, cells treated with LFG-500 (8 and 12 μM) displayed morphological features of early apoptosis, in particular apoptotic bodies and nuclei pyknosis. To further confirm the apoptosis-inducing effect of LFG-500 on SKOV3 cells, Annexin V/PI double-staining was performed. As depicted in Fig. 2B, LFG-500 increased the number of early and late apoptotic cells, which was consistent with the results of the DAPI staining. The percentage of apoptotic cells in the control group was 6.1%, while the proportions of apoptotic cells reached 9.2, 22.1 and 37.6% following 24 h treatment with 4, 8 and 12 μM LFG-500, respectively (Fig. 2C). This increase was significant for the 8 μM ($P<0.05$) and 12 μM ($P<0.01$) treatments. These results indicate that apoptosis induction is involved in the growth inhibitory effect of LFG-500 on SKOV3 cells.

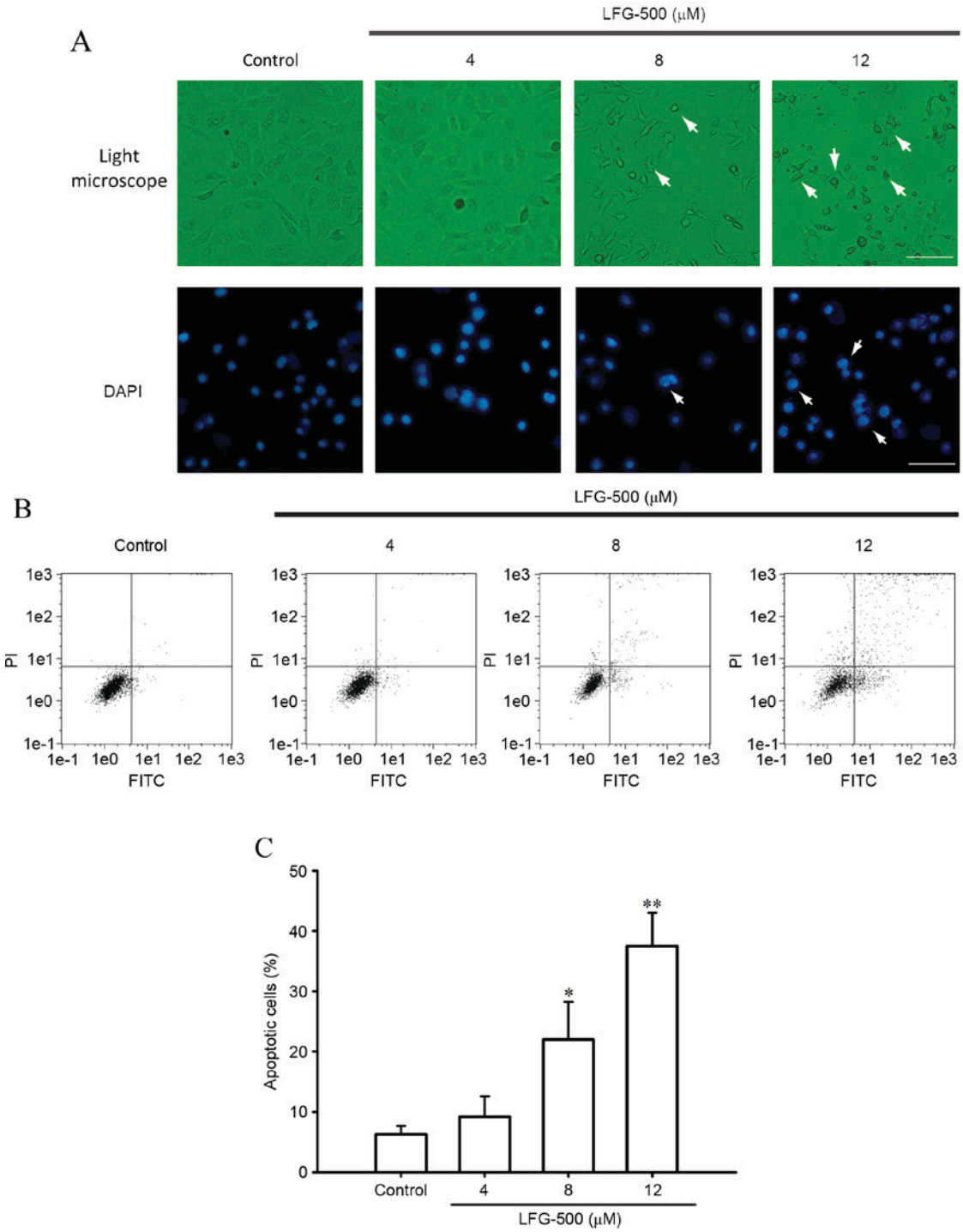

Figure 2. LFG-500 induces apoptosis in SKOV3 cells. (A) Morphological changes of SKOV3 cells treated with LFG-500 compared with untreated SKOV3 control cells. Top: Control cells and cells treated with LFG-500 were incubated for 24 h before observation with an inverted light microscope. Arrows indicate the round and shrunken cells. Bottom: Fluorescent DAPI staining of nuclei in LFG-500 treated cells and control cells. Arrows indicate the apoptotic bodies (magnification, x200; scale bars, 100 μm). (B) Flow cytometry analysis of apoptotic cells. Cells were treated with 4, 8 or 12 μM LFG-500 for 24 h, and then stained by Annexin V and PI. Early apoptotic cells were identified by Annexin V-positive and PI-negative staining (top right); late apoptotic cells were identified by Annexin V-positive and PI-positive staining (lower right). (C) Percentages of apoptotic cells. *P<0.05, **P<0.01 vs. control cells. LFG-500, synthetic flavonoid compound; SKOV3, human ovarian carcinoma cell line.

LFG-500 regulates expression of apoptosis-related proteins. To determine the mechanism of apoptosis induction by LFG-500, western blot analysis was performed to investigate the effects of LFG-500 on the expression of apoptosis-related proteins. Following treatment with LFG-500, the expression of caspase-3 was reduced relative to untreated control cells (Fig. 3A and B). This reduction in caspase-3 expression was significant for the 8 and 12 μM LFG-500 treatments (P<0.01). Moreover, the expression and ratio of the anti-apoptotic Bcl-2 protein and the pro-apoptotic Bax protein are the critical factors for initiating apoptosis via the mitochondria (24,25). Therefore, the expression of Bcl-2 and Bax in SKOV3 cells

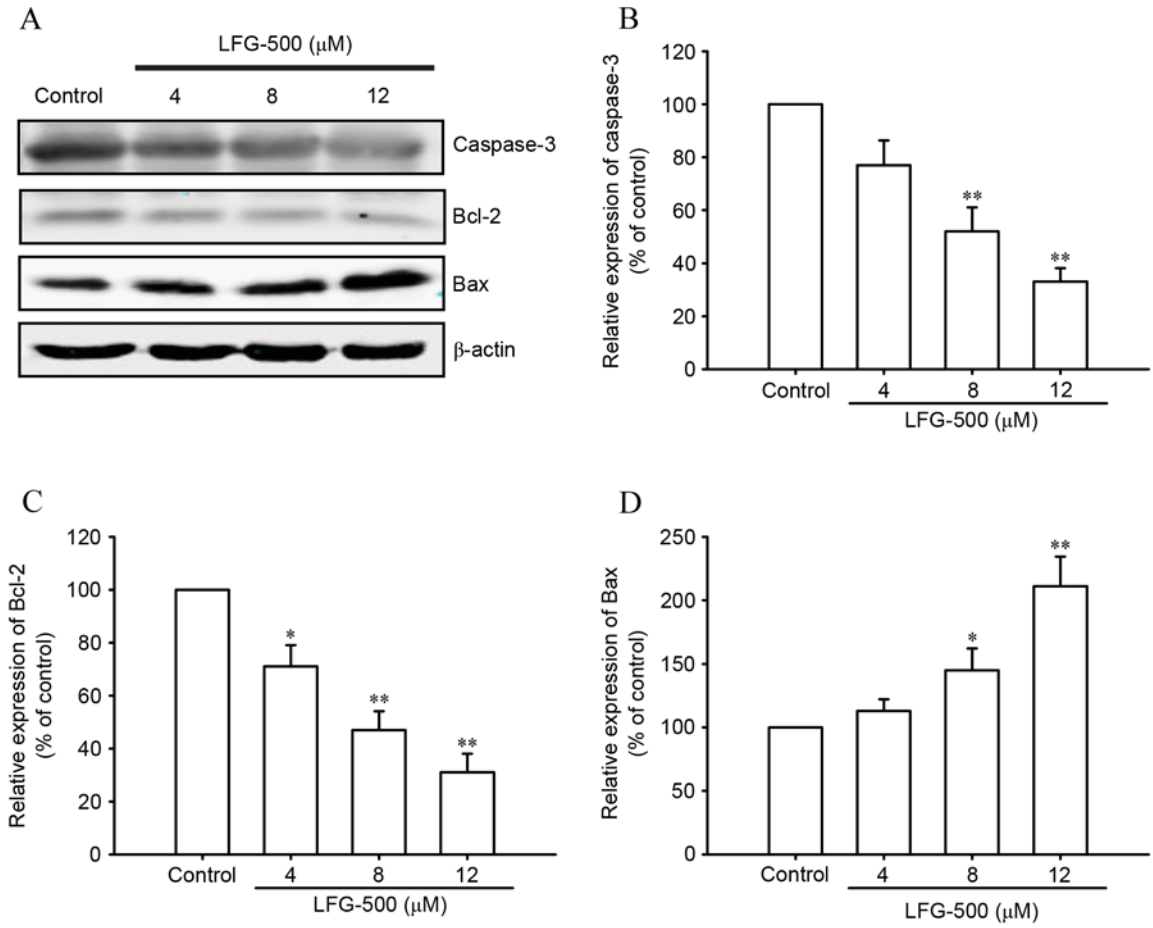

Figure 3. LFG-500 regulates the expression of apoptosis-related proteins. (A) Expression of caspase-3, Bcl-2 and Bax in SKOV3 cells. The cells were treated with various concentrations (4, 8 and 12 μM) of LFG-500 for 24 h. The protein expression levels of caspase-3, Bcl-2 and Bax were analyzed by western blotting. β-actin was used as a loading control. Following western blotting, densitometric analysis of the proteins was performed for (B) caspase-3, (C) Bcl-2 and (D) Bax. *P<0.05, **P<0.01 vs. control cells. LFG-500, synthetic flavonoid compound; Bcl-2, B-cell lymphoma 2 protein; Bax, Bcl-2-associated X protein; SKOV3, human ovarian carcinoma cell line.

was also examined by western blotting. The data in Fig. 3A demonstrates that LFG-500 decreased Bcl-2 expression while simultaneously upregulating Bax expression, indicating that LFG-500 increased the Bax/Bcl-2 ratio in SKOV3 cells. In particular, for Bcl-2 the decrease in expression was significant for 4 (P<0.05), 8 and 12 μM (P<0.01) LFG-500 (Fig. 3C), while for Bax the increase in expression was significant at 8 (P<0.05) and 12 μM (P<0.01) LFG-500 (Fig. 3D). These results indicate that LFG-500 may trigger the mitochondrial apoptotic pathway in SKOV3 cells.

LFG-500 induces apoptosis in SKOV3 cells through ROS accumulation and loss of ΔΨm. It has been demonstrated that an elevated quantity of intracellular ROS is a common mediator of apoptosis (26). Therefore, to further clarify the mechanism by which LFG-500 induces apoptosis, the levels of ROS were detected. As shown in Fig. 4A, exposure to LFG-500 increased ROS levels in SKOV3 cells relative to the control. This increase was significant for LFG-500 concentrations of 8 (P<0.05) and 12 μM (P<0.01; Fig. 4B), with 12 μM LFG-500 increasing ROS levels by 90%. In addition to ROS levels, the loss of ΔΨm is considered to be a critical stage in mitochondrial apoptosis (27). Thus, the ΔΨm level was examined in SKOV3 cells exposed to different concentrations of LFG-500. The data in Fig. 4C demonstrates that LFG-500 reduced the ΔΨm. The percentage of ΔΨm-collapsed cells over a 24 h period increased to 7.5, 22.6 and 35.7% with increasing LFG-500 concentrations of 4, 8 and 12 μM, respectively (Fig. 4C). Collectively, these results suggest that LFG-500 may induce apoptosis in SKOV3 cells by promoting ROS accumulation and the mitochondrial apoptotic pathway.

Discussion

Ovarian cancer accounts for approximately 3% of cancers among women in the USA, though it has a higher mortality than all other cancers of the female reproductive system (28). Numerous questions surrounding ovarian cancer remain unknown, particularly those regarding the optimal therapeutic approaches for primary and recurrent disease. The development of novel therapies and potent chemotherapeutic drugs for the treatment of ovarian cancer is required (29). In recent years, it has been suggested that novel, naturally occurring anticancer agents may be an effective treatment (30).

Flavonoids are a diverse and historically important family of natural products. Various plants and spices containing flavonoid

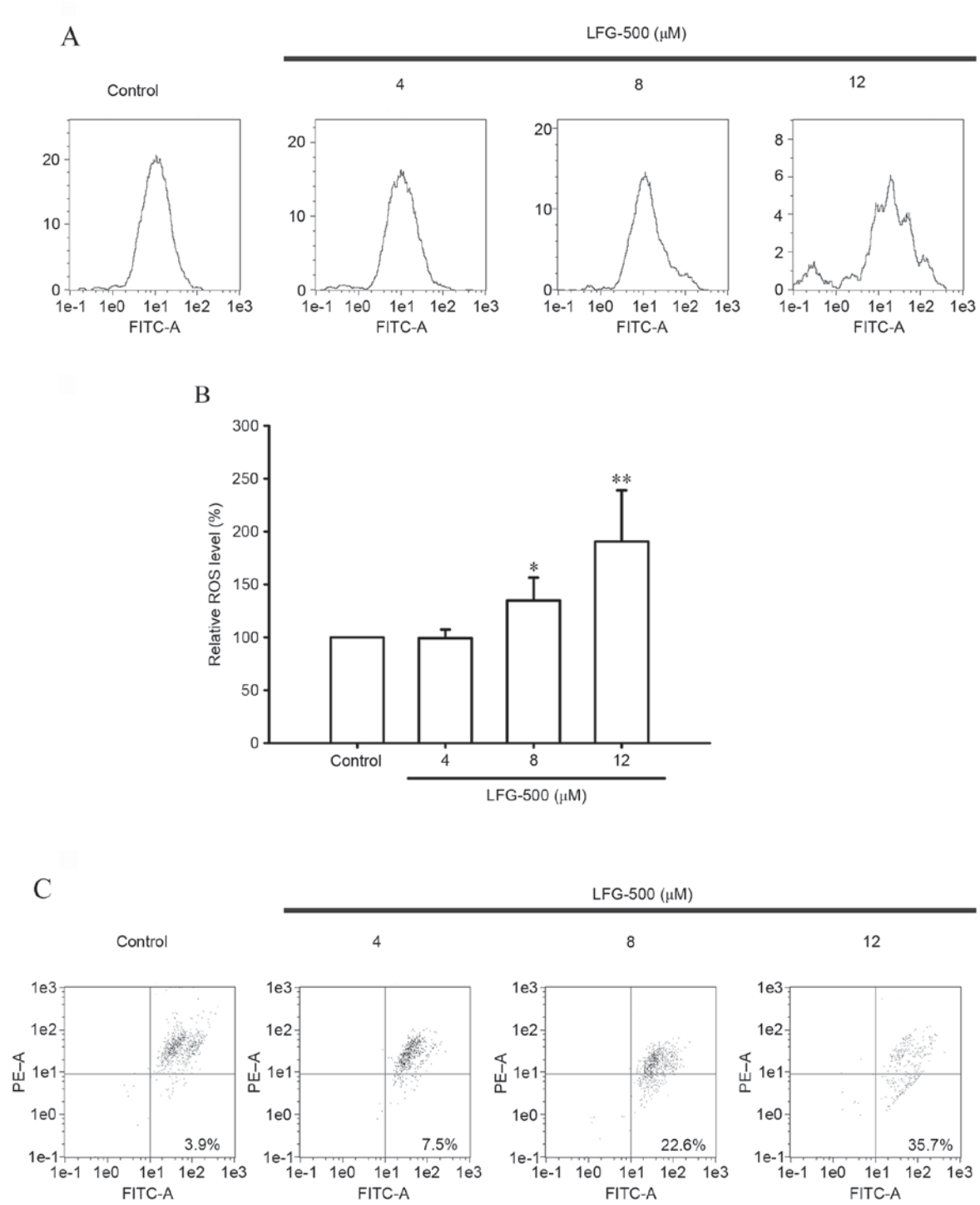

Figure 4. LFG-500 induces ROS accumulation and loss of the ΔΨm in SKOV3 cells. (A) LFG-500 treatment increases the ROS levels in SKOV3 cells. (B) Flow cytometry analysis was performed to determine ROS levels in LFG-500-treated cells and the results were quantified relative to untreated control cells. (C) LFG-500 treatment decreases the ΔΨm in SKOV3 cells. Analysis of JC-1 fluorescence, as a marker of low ΔΨm, was performed by flow cytometry. The percentage in the right lower section of the fluorocytogram indicates the quantity of ΔΨm collapsed cells relative to the cell total of each group. *P<0.05, **P<0.01 vs. control cells. LFG-500, synthetic flavonoid compound; ROS, reactive oxygen species; ΔΨm, mitochondrial transmembrane potential; SKOV3, human ovarian carcinoma cell line; JC-1, J-aggregate-forming lipophilic cation-1.

derivatives have demonstrated their potency as preventive and therapeutic agents (31). Among the range of pharmacological activities of flavonoids, their antitumor activity is of particular interest (32). The anticarcinogenic effects of flavonoids have been observed *in vitro* and *in vivo,* in which the compounds have been found to inhibit and protect against various stages of cancer processes, including proliferation, inflammation, angiogenesis, invasion and metastasis (33). These actions are attributable to their poly-phenolic structure (34). However, the resulting bioavailability of orally administered flavonoids is poor (35), due to extensive first-pass metabolism of the compounds, likely occurring at their hydroxyl groups (36). Therefore, LFG-500 was designed as a flavonoid with improved oral bioavailability. In LFG-500, a piperazine group

and a benzyl group at the hydroxyl positions were introduced to prevent metabolism of the flavonoid. These substitutions also give LFG-500 greater lipid solubility, enabling it to enter the intracellular space more efficiently (18). Based on the anticancer activities of flavonoids and the optimized structure of LFG-500, it was hypothesized that the novel flavonoid may be a promising anticancer agent (17). In previous studies (17,18), LFG-500 was found to exert growth-inhibitory effects on several cancer cells, such as HepG2 and MDA-MB-231 cells. The present study demonstrated that LFG-500 had potential antitumor activity in human ovarian cancer SKOV3 cells by inducing apoptosis. This effect was associated with ROS accumulation and the mitochondrial apoptotic pathway.

In the present study, the MTT assay showed that LFG-500 exerted a growth inhibitory effect on SKOV3 cells. Subsequent data indicated the inhibitory effect of LFG-500 was via the induction of apoptosis. Apoptosis refers to an active form of cell death, with stereotypic morphological changes occurring during the process (37). It is characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation and chromosomal DNA fragmentation. The results of the present study were consistent with these characteristics. In addition, to evaluate the mechanism by which LFG-500 induced apoptosis, Annexin V/PI-double staining was performed. During apoptosis, the asymmetric distribution of phospholipids in the plasma membrane is lost, and phosphatidylserine (PS) is translocate to the outer leaflet of the membrane (38). Annexin V has a high affinity for PS and binds to cells with exposed PS on their outer surface. The combination of Annexin V and PI allows for the distinction between early apoptotic cells, late apoptotic cells and viable cells (39). The results of the Annexin V/PI assay in this study verified that LFG-500 inhibits the growth of SKOV3 cells by induction of apoptosis.

Apoptosis is thought to play key roles in developmental processes, maintenance of homeostasis and elimination of damaged cells (40). The caspase family is an executioner of apoptosis, in which caspase-3 is the main factor (41). Caspase-3 is activated in apoptotic cell by death receptor and mitochondrial pathways, and subsequently cleaves poly (ADP-ribose) polymerase to induce cell apoptosis (42,43). As an executioner of caspase, the caspase-3 zymogen has virtually no activity until it is cleaved after apoptotic signaling events have occurred (44). The present study found that LFG-500 inhibited the expression of caspase-3, indicating that levels of the cleaved form increased, which in turn induced apoptosis.

Furthermore, apoptosis is a balanced system under tight regulation by anti-apoptotic and pro-apoptotic effectors, particularly by proteins of the Bcl-2 family. The anti-apoptotic protein Bcl-2 promotes cell survival, whereas the pro-apoptotic protein Bax induces the apoptotic program of cell death. Thus, the ratio of Bax/Bcl-2 plays a critical role in determining cell fate (45). Numerous anticancer agents induce apoptosis by influencing proteins of the Bcl-2 family (46). In the present study, LFG-500 increased the expression ratio of Bax to Bcl-2 in the ovarian cancer cell line, therefore promoting apoptosis.

The signaling involved in apoptosis occurs via the death receptor (extrinsic) pathway or the mitochondrial (intrinsic) pathway. The death receptor pathway is activated by ligand-bound death receptors of the tumor necrosis factor superfamily, resulting in the activation of initiator caspases (caspase-8) (47). The mitochondria pathway involves the mitochondria and Bcl-2 family of proteins (48). In this system, the Bax/Bcl-2 ratio determines whether cells undergo mitochondria-mediated apoptosis. In the present study, the finding that LFG-500 treatment increased the ratio of Bax/Bcl-2 suggests that the flavonoid may induce apoptosis by triggering the mitochondrial apoptotic pathway.

Mitochondrial damage is key in cell death, and in certain models of apoptosis, it is one of the early events in the process (49). Oxidative stress, a signaling messenger that may promote apoptosis (50,51), causes alterations in various biological structures in mitochondria (52). Excessive levels of ROS cause oxidative damage to the mitochondrial membranes and impair membrane integrity. This leads to the release of cytochrome c and apoptosis inducing factor from the mitochondria, along with disruption of the $\Delta\Psi m$, resulting in caspase activation and apoptosis (53). It is well established that several chemotherapeutic agents cause cell apoptosis through ROS accumulation (54). The results of the present study revealed that treatment with LFG-500 caused the levels of ROS to increase. This was accompanied by loss of the $\Delta\Psi m$, indicating that LFG-500 induced mitochondrial apoptosis by increasing the levels of ROS.

In this study, it was found that LFG-500 triggered the mitochondrial apoptotic pathway; however, the effects of LFG-500 on upstream signaling pathways also require investigation. Upstream of the mitochondria, the activation of c-Jun N-terminal kinases (JNK) and p38 mitogen activated protein kinase is associated with apoptosis induction, whereas extracellular signal-regulated kinases is cytoprotective (55). In particular, it is suggested that elevated ROS levels mediate JNK activation, resulting in cytochrome c release and cell death (56). Thus, further mechanistic studies are required to determine whether LFG-500 triggers mitochondrial apoptosis via regulation of these signaling molecules. More importantly, the ability of LFG-500 to inhibit ovarian cancer growth and to induce apoptosis *in vivo* requires further study.

Collectively, present results indicate that LFG-500 effectively induced apoptosis in human ovarian cancer SKOV3 cells *in vitro,* by increasing the Bax/Bcl-2 ratio and reducing caspase-3 protein expression. In turn, these effects may be potentially associated with activation of the mitochondrial apoptotic pathway by promoting ROS accumulation. These results are potential evidence for the anticancer effects of LFG-500, suggesting that LFG-500 may be a promising therapeutic agent for the treatment of ovarian cancer.

Acknowledgements

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