Amentoflavone triggers cell cycle G2/M arrest by interfering with microtubule dynamics and inducing DNA damage in SKOV3 cells

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Abstract. Ovarian cancer is the seventh most common cancer and the second most common cause of cancer-associated mortality among gynecological malignancies worldwide. The combination of antimitotic agents, such as taxanes, and the DNA-damaging agents, such as platinum compounds, is the standard treatment for ovarian cancer. However, due to chemoresistance, development of novel therapeutic strategies for the treatment of ovarian cancer remains critical. Amentoflavone (AMF) is a biflavonoid derived from the extracts of Selaginella tamariscina, which has been used as a Chinese herb for thousands of years. A previous study demonstrated that AMF inhibits angiogenesis of endothelial cells and induces apoptosis in hypertrophic scar fibroblasts. In order to check the influence of AMF on cell proliferation, the effects of AMF on cell cycle and DNA damage were measured by cell viability, flow cytometry, immunofluorescence and western blotting assays in SKOV3 cells, an ovarian cell line. In the present study, treatment with AMF inhibited ovarian cell proliferation, increased P21 expression, decreased CDK1/2 expression, interrupted the balance of microtubule dynamics and arrested cells at the G2 phase. Furthermore, treatment with AMF increased the expression levels of phospho-Histone H2AX (γ-H2AX; a variant of histone 2A, that belongs to the histone 2A family member X) and the DNA repair protein RAD51 homolog 1 (Rad51), indicating the occurrence of DNA damage since γ-H2AX and Rad51 are both key markers of DNA damage. Consistent with previous findings, the results of the present study suggest that AMF is a potential therapeutic agent for the treatment of ovarian cancer. In addition, the effects of AMF on cell cycle arrest and DNA damage induction may be the molecular mechanisms by which AMF might exert its potential therapeutic benefits in ovarian cancer.

Introduction

According to statistics, the incidence rate of ovarian cancer in 2018 was 3.4%, worldwide (1). Ovarian cancer is the eighth most common cancer in female and the second most common cause of cancer-associated mortality among gynecological malignancies worldwide (1). A combination of antimitotic agents, such as taxanes, and DNA-damaging agents, such as platinum compounds remains the principle treatment for ovarian cancer (2), whereby 60-85% of patients with high-grade ovarian cancer initially respond to this regimen; however, the majority of these patients eventually relapse due to chemoresistance (3,4). Furthermore, most patients with high-grade ovarian cancer are resistant to paclitaxel and associated microtubule inhibitors (3,4). Thus, development of novel therapeutic strategies for the treatment of ovarian cancer remains critical.

Several anticancer drugs exert their effects through the cell cycle. For example, methotrexate, vinca alkaloids and bleomycin play function by arresting cells in S phase or G2/M phase. The cell cycle is a complex multi-step process that is regulated by different mechanisms, including cyclin-dependent kinase (CDK) pathways, metabolic adaptations and redox-dependent signaling. CDK complexes play key regulatory roles in cell cycle progression (5). In CDK-dependent pathways, the catalytic activities of CDKs are modulated by the interactions between cyclins and CDK inhibitors (CKIs) (6). In this progression, cyclins and CKIs serve as brakes to halt cell cycle progression under unfavorable conditions, such as when DNA damage is present (7). P21, a member of the cyclin-dependent kinase inhibition protein/kinase inhibition protein family of CKIs, is activated following DNA damage and metabolic stress, which arrests cell cycle progression in the G1/S and G2/M phases by inhibiting Cyclin D/CDK4 and CDK6, and Cyclin E/CDK2 activities, respectively (8).

In addition to cyclin-CDK complexes, several other cell cycle-associated targets exist for antitumor therapies. For
example, taxanes and colchicine can also induce cell cycle arrest by influencing microtubule (MT) stability (9,10). MTs are hollow cylindrical tubes consisting of 13 aligned protofilaments, formed from repeating α-tubulin and β-tubulin heterodimers (11). MTs undergo polymerization and depolymerization, while the dynamic balance between them plays a central role in cell meiosis. Disruption of this balance caused by factors, such as low temperature and drugs halts meiosis. Taxanes are MT regulators that block cell meiosis in G2/M by binding to tubulin, thus promoting MT polymerization and eventually inducing apoptosis (12). In addition to directly affecting tubulin, MT regulators can also influence the expression of MT-associated proteins. For example, stathmin is a MT de-polymerizing protein that regulates MT dynamics and spindle assembly through binding to α/β-tubulin heterodimers (13). The high expression level of stathmin decreased the sensitivity of ovarian cancer to paclitaxel (14). However, taxanes and anti-stathmin therapy produced a synergistic anti-cancer effect, and stathmin knockdown, by transfecting the expression construct containing full-length stathmin cDNA in the antisense orientation, increased taxanes sensitivity (15). A previous study has demonstrated that p53 induces cell arrest at the G2/M checkpoint by downregulating stathmin, while its expression is activated following DNA damage (16).

Plant-derived flavones, such as morelloflavone and ginkgo, have also been reported to play an important role in preventing cancer progression including prostate and lung cancer cells (17,18). Amentoflavone (AMF) is a biflavonoid extracted from the Chinese herb Selaginella tamariscina, which displays several pharmacological properties, including antitumor, anti-inflammatory and antiviral effects (19-22). A previous study demonstrated that AMF inhibits angiogenesis of endothelial cells and induces apoptosis in hypertrophic scar fibroblasts (23). Although it has been demonstrated that AMF inhibits the development of different types of cancer, its underlying molecular mechanisms in ovarian cancer remain unclear.

Thus, the present study aimed to investigate the effect of AMF on ovarian cancer progression and the underlying mechanisms involved in the observed effects. The results demonstrated that AMF decreased ovarian cancer cell viability and induced cell cycle arrest, by disrupting the balance of MT dynamics and increasing the levels of DNA damage. Taken together, the results of the present study suggest that AMF may act as a therapeutic agent in the treatment of ovarian cancer.

Materials and methods

Cell culture, cell line and reagents. The SKOV3 human ovarian cancer cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine (all from Thermo Fisher Scientific, Inc.), 100 units of penicillin/ml and 100 µg of streptomycin/ml (both from Corning Life Sciences) at 37°C in a humidified atmosphere of 5% CO2 and subcultured every 2-3 days. AMF was purchased from Shanghai Winherb Medical Science Co. Ltd., with a purity of 99%. A total of 100 mmol/l stock solution of AMF was prepared in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) and stored at -20°C until further experimentation.

Cell viability assay. SKOV3 cells were seeded in 96-well plates at a density of 5,000 cells/well (100 µl). After 24 h, cells were treated with different concentrations of AMF (0, 50, 75, 100, 150 and 200 µmol/l) for 48 h at 37°C. Cell viability was determined via the CellTiter 96 Aqueous One Solution Proliferation assay (Promega Corporation) at a wavelength of 490 nm, using a multi-well spectrophotometer (Agilent Technologies, Inc.). All experiments were performed in triplicate.

Flow cytometric analysis. A total of 1x105 SKOV3 cells/well were seeded in 6-well overnight and treated with different concentrations of AMF (0, 100 and 150 µmol/l) for 48 h at 37°C. Cells were fixed in 70% ethanol overnight at 4°C, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA), digested with RNaseA (Thermo Fisher Scientific, Inc.) and subsequently stained with propidium iodide (BD Biosciences) in the dark for 30 min at 37°C, prior to cell cycle analysis using a FACS Calibur flow cytometer (BD Biosciences) and analyzed using ModFit LT Windows 3.2 (Verity Software House, Inc.).

Immunofluorescence. A total of 1x105 SKOV3 cells/well were seeded onto coverslips in a six-well plate. Following incubation for 24 h at 37°C, cells were treated with different concentrations of AMF (0, 100 and 150 µmol/l) for 48 h at 37°C. Subsequently, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.25% Triton X-100 for 10 min and blocked with 1% BSA for 30 min at room temperature. Cells were incubated with primary antibodies against phospho-Histone H2AX (γ-H2AX; 1:200 v/v; cat. no. 9718), α-tubulin (1:200 v/v; cat. no. 2144) or β-tubulin (1:200 v/v; cat. no. 2146), from Cell Signaling Technology Inc., overnight at 4°C. Subsequently, cells were incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:500 v/v; cat. no. 4416; Cell Signaling Technology, Inc.) for 1 h at room temperature. Nuclei were stained with 0.1 µg/ml DAPI (Santa Cruz Biotechnology Inc.) for 5 min at room temperature. Cell images were observed under a Nikon Eclipse E600 fluorescence microscope (magnification, x400; Nikon Corporation) and analyzed using NIS-Elements D 4.50 software (Nikon Corporation).

Western blotting. A total of 1x105 SKOV3 cells/well were seeded into 100-mm cell culture dishes and treated with different concentrations of AMF (0, 100 and 150 µmol/l) for 48 h at 37°C. Total protein was extracted using RIPA lysis buffer (150 mM NaCl, 50 mM Tris with pH 7.4, 1% NP40, 0.1% SDS and 0.5% sodium deoxycholate; Beyotime Institute of Biotechnology) supplemented with 10 mM phenylmethanesulphonyl fluoride (Amresco, Inc.) and 10X phosphatase inhibitor (Roche Applied Science). Total protein concentration was determined using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.), 20 µg protein samples per lane were loaded and separated via 10% SDS-PAGE and electroblotted. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes (Merck KGaA) and blocked with 5% (w/v) non-fat milk powder in TBST [10 mM Tris, pH 7.5, 150 mM NaCl and 0.1% (v/v) Tween 20] for 2 h at room temperature. Membranes were incubated with primary antibodies against GAPDH (1:1,000 v/v; cat. no. 2118;
Cell Signaling Technology Inc.), β-tubulin (1:1,000 v/v; cat. no. 2146; Cell Signaling Technology Inc.), Cyclin-B1 (1:1,000 v/v; cat. no. 12231; Cell Signaling Technology Inc.), CDK2 (1:1,000 v/v; cat. no. ab32147; Abcam), p-CDK1 (1:1,000 v/v; cat. no. 4539; Cell Signaling Technology Inc.), P21 (1:1,000 v/v; cat. no. 2947; Cell Signaling Technology Inc.), γ-H2AX (1:1,000 v/v; cat. no. 9718; Cell Signaling Technology Inc.), stathmin (1:1,000 v/v; cat. no. ab52630; Abcam), Rad51 (1:1,000 v/v; cat. no. ab133534; Abcam) or CDK1 (1:1,000 v/v; cat. no. ab18; Abcam) overnight at 4˚C. The membranes were washed three times with TBST and subsequently incubated with horse radish peroxidase-conjugated secondary antibodies (1:2,000 v/v; cat. no. 7074 or 7076; Cell Signaling Technology Inc.) diluted in TBST for 1 h at room temperature. Membranes were re-washed three times with TBST, and protein bands were visualized using the electrochemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc.) and imaged using the ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories, Inc.). Image Lab 3.0 software (Bio-Rad Laboratories, Inc.) was used for semi-quantitative analysis of band signals.

Statistical analysis. Statistical analysis was performed using SPSS software v17.0 (IBM Corp.) and data are presented as the mean ± standard deviation (SD) of at least three independent experiments. One-way analysis of variance and Student-Newman-Keuls post-hoc test were used to compare difference between multiple groups. Unpaired Student's t-test was used to test statistical significance between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

AMF inhibits SKOV3 cell viability. The effect of AMF on SKOV3 cell viability was assessed via the CellTiter 96 Aqueous One Solution Proliferation assay. Cells were treated with different concentrations of AMF (50-200 µmol/l) for 48 h. The results demonstrated that AMF significantly inhibited SKOV3 cell viability in a dose-dependent manner, from 75 µmol/l onwards compared with the control cells (Fig. 1A).

AMF induces S phase and G2 cell cycle arrest of SKOV3 cells. In order to determine the molecular mechanism underlying the inhibitory effect of AMF on SKOV3 cell viability, the effect of AMF on the cell cycle was assessed. SKOV3 cells were treated with 100 or 150 µmol/l AMF for 48 h and flow cytometric analysis was performed to determine cell cycle distribution. The results demonstrated that treatment with AMF slightly increased the percentage of SKOV3 cells in the S or G2 phases, and decreased the percentage of SKOV3 cells in the G1 phase (Fig. 1B and C). Comparing with the percentage of SKOV3 G1 or G2 phase cells in the control group, the percentage of G1 phase cells in 150 µmol/l AMF group was significantly decreased, while the percentages of G2 phase cells in 100 and 150 µmol/l AMF groups were significantly increased. Taken together, these results suggest that AMF arrests cell cycle at the G2 phase and interferes with cell meiosis and cell proliferation.

AMF regulates the expression of cell cycle-associated proteins in SKOV3 cells. In order to further investigate the molecular mechanisms by which AMF arrests the cell cycle, the effect of AMF on the expression levels of proteins associated with cell cycle progression was assessed via western blot analysis. The expression levels of p-CDK1 and CDK2 decreased in SKOV3 cells treated with 100 or 150 µmol/l AMF for 48 h (Fig. 1D and E). Furthermore, CDK1 expression decreased in SKOV3 cells treated with 100 and 150 µmol/l AMF; however, a significant decrease was only observed in the cells treated with 150 µmol/l of AMF (Fig. 1D and E). The p-CDK1/CDK1 ratio decreased in cells treated with AMF; however, no significant differences were observed compared with the control cells (Fig. 1D and E). Notably, cyclin B1 expression was significantly downregulated in SKOV3 cells treated with 100 µmol/l AMF and upregulated in cells treated with 150 µmol/l AMF. P21, a well-known inhibitor of the cell cycle, significantly increased in AMF-treated cells compared with AMF-untreated ovarian cells (Fig. 1E).

AMF interferes with tubulin expression and spindle assembly. MTs are made from tubulin heterodimers and are vital for several cellular processes, such as spindle assembly for cell meiosis (24). MTs have complex polymerization characteristics and are stable and long lasting during interphase (25). Conversely, MTs become short and dynamic during mitosis. Stathmin regulates cell cycle progression by influencing the dynamics of MTs (13).

The present study assessed the influence of AMF on MT structure via immunofluorescent staining of α/β-tubulin in SKOV3 cells, and determined the expression levels of stathmin and β-tubulin via western blot analysis. Immunofluorescence staining demonstrated that α/β-tubulin were long and condensed in AMF-treated cells compared with the control cells (Fig. 2A and B). Furthermore, the expression levels of β-tubulin and stathmin were significantly downregulated following treatment with AMF (Fig. 2C and D). Taken together, these results indicate that spindle assembly and cell meiosis are altered in AMF-treated SKOV3 cells.

AMF induces DNA damage in SKOV3 cells. In order to determine whether AMF induces DNA damage in SKOV3 cells, the expression levels of γ-H2AX, a specific marker of DNA double-strand breaks (DSBs) (26), were assessed via immunofluorescence and western blot analyses. Immunofluorescence staining demonstrated that the percentage of γ-H2AX-positive cells significantly increased in AMF-treated SKOV3 cells compared with the control cells (Fig. 3A and B). Furthermore, the number of DNA-damaged cells and fluorescence intensity increased with AMF in a dose-dependent manner. Compared with the control cells, western blot analysis indicated that the protein expressions were significantly higher in 100 and 150 µmol/l AMF-treated cells for γ-H2AX and in 150 µmol/l AMF-treated cells for Rad51 (Fig. 3C and D). Taken together, these results suggest that AMF induces extensive DNA damage. Rad51 formation is a hallmark of homologous recombination repair (HRR), which is often induced by DNA damage (27). The results of the present study indicated that DSBs were induced by AMF and the cells attempted to repair DNA damage through the HRR signaling pathway.
Discussion

Previous studies have reported the anticancer effects of AMF in different types of tumor (28-32). For example, AMF has been demonstrated to be associated with apoptosis and the inhibition of metastasis and angiogenesis of tumors. Furthermore, AMF has exhibited its anticancer effect in SiHa and CaSki cervical cancer cells by the suppressing expression levels of the human papilloma virus protein, E7 (33). The present study investigated the antitumor effect of AMF on ovarian cancer, and the results demonstrated that AMF decreased SKOV3 cell viability and induced cell cycle arrest in the S and G2 phases, in a dose-dependent manner. Furthermore, AMF induced DNA damage and interfered with MT function and meiosis.
Several antitumor drugs, such as paclitaxel and cisplatin, decrease cell proliferation by either inducing cell death or arresting cell cycle progression. Previous studies have reported that AMF induces cell apoptosis (23,32,33). The results of the present study demonstrated that treatment with AMF significantly increased the proportion of G2 phase SKOV3 cells. The proportion of S phase cells was also upregulated, however, no significant difference was observed compared with the control cells. The results of the present study demonstrated that AMF was able to induce cell cycle arrest of ovarian cancer cells in S and G2 phases, which is in partly inconsistent with previous findings that have demonstrated that AMF is able to arrest cells either in S or G1 phases (34‑36). These results suggest that the effect of AMF on cell cycle is influenced by cell type, AMF concentration or/and treatment time. There are some AMF‑like bioflavonoids, such as bilobetin, isoginkgetin and morelloflavone, which can also arrest cell cycle at G2/M phase by inducing cell apoptosis or inhibiting the activation of Raf/MEK/ERK kinases (18). It is well known that CDKs, CKIs and cyclins also play key roles in cell cycle progression (6,37,38). CDK1, in combination with cyclin A and B, regulates the transition from G2 to M phase (39). P21, a member of the CKIs, can promote cell cycle arrest as a response to several stimuli such as DNA damage and oxidative stress by regulating G1/S or G2/M transitions, respectively (8). P21 is predominantly induced by p53, which is activated by several stressors, including DNA damage. P21 is also known to inhibit the activity of cyclinA/CDK1/2, which results in cell cycle arrest in the S phase (40,41). In the present study, the expression levels of p‑CDK1 and CDK2 significantly decreased, while P21 expression increased in AMF‑treated SKOV3 cells, suggesting that AMF may induce G2/M cell cycle arrest by upregulating P21 and downregulating CDK1 and CDK2. A previous study demonstrated that paclitaxel or eribulin can arrest cell meiosis and lead to the accumulation of mitotic marker proteins, such as cyclin B1 (42). In the present study, cyclin B1 was significantly downregulated following treatment with 100 µmol/l AMF, but upregulated following treatment with 150 µmol/l AMF. A reason for this increase in cyclin B1 expression may be due to the protein accumulation induced by meiosis arrest.

MT acts as a key drug target in tumor cells due to its roles in determining and supporting cell shape, cell division, transport and signal transduction (43). Previous studies have demonstrated that paclitaxel or eribulin can arrest cell meiosis by regulating MT polymerization (12,44,45). Under normal conditions, there is a balance between polymerization and de-polymerization of MTs, whereby
disruption of this balance destroys the normal MT structures observed (46,47). Stathmin is a MT destabilizing protein that inhibits tubulin dimer polymerization and contributes to the formation of cell spindles (48). Previous studies have reported that stathmin is upregulated in the highly malignant types of breast and ovarian cancers (49,50). Furthermore, downregulation of stathmin inhibits cell viability and induced apoptosis in several types of cancer cells (51,52). Paclitaxel is an efficacious MT-stabilizing antitumor drug, particularly used in the treatments of ovarian, breast and non-small cell lung cancers (53). Paclitaxel is considered to shift the assembly equilibrium of MTs towards the depolymeric state, thus blocking cell entry into meiosis by suppressing MT dynamics (53). The results of the present study demonstrated that treatment with AMF decreased the expression levels of stathmin and β-tubulin in SKOV3 cells. Immunofluorescence staining was performed to assess MT structures, using antibodies against α- and β-tubulin. The results indicated that MT structures were distorted, and the cell spindle-like structures were disturbed following treatment with AMF. Furthermore, AMF blocked cell cycle at the G2/M phase, interfered with MT dynamics and downregulated the expression levels of proteins associated with MT structures. Taken together, these results indicate that the effects of AMF on ovarian cancer cells are like those of paclitaxel.

Following cell DNA damage, DNA repair mechanisms are activated and DNA replication and meiosis are interrupted. Mammalian cells have evolved a series of DNA repair systems, including non-homologous end-joining and homologous recombination (HR) (54). H2AX, a variant of H2A, is rapidly phosphorylated at Ser 139 and accumulates at DSB sites with other proteins including Rad51 and BRCA1/2 (55,56). Among these, Rad51 is a key factor involved in HR (57,58). Rad51 is recruited to γ-H2AX sites and polymerizes at the resection-generated single-stranded DNA ends, which leads to invasion and exchange between homologous DNA sequences (58). The results of the present study demonstrated that Rad51 expression markedly increased in AMF-treated SKOV3 cells. Furthermore, the intensity and expression of γ-H2AX were significantly upregulated in AMF-treated SKOV3 cells. Considering the key roles γ-H2AX and Rad51 play in DNA damage repair, the results of the present study suggest that AMF induces DNA damage and activates the HR
repair system in AMF-treated SKOV3 cells. The present study demonstrated that AMF triggered cell cycle G2/M arrest and induced DNA damage in ovarian cancer cells, even though it may be better to use several ovarian cancer cell lines than just to use SKOV3 cell line. We will be aimed at confirming these findings and prove the antitumor effect of AMF in ovarian cancer cell line derived xenograft mouse models.

In conclusion, the results of the present study demonstrated that AMF inhibited human ovarian cancer cell proliferation by triggering cell cycle arrest at the G2/M phase. Furthermore, AMF was demonstrated to interfere with MT dynamics and induce DNA damage. Thus, AMF may act as an antitumor drug by exerting its effects on MT dynamics and inducing DNA damage.

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Availability of data and materials

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

Authors' contributions

ZL, JZ, AL and XL contributed to the initial conception and design of the experiments. HS, XX, SQ, PW and LD performed the experiments. ZL and JZ wrote the paper. ZZ revised the experimental design and analyzed the 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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