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Original Article

Erianin induces apoptosis of colorectal cancer cells via activation of JNK signaling pathways

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Abstract: The current study aimed to investigate the anti-cancer activity of erianin on human colorectal carcinoma cell lines, exploring the underlying molecular mechanisms. After administration with erianin, inhibition of proliferation was quantitatively measured with colorimetric MTT assays. Morphologic changes were observed with DAPI staining. Flow cytometry analysis was conducted with Annexin-V-FITC/PI double staining, aiming to determine the effects of erianin on cell cycle and apoptosis levels. Western blotting was used to detect expression of cell cycle and apoptosis-related proteins after erianin treatment. Sp600125, a JNK signaling inhibitor, was used to confirm the regulatory effects of the anti-neoplastic activity of erianin. Results suggest that the proliferation of human colorectal cells could be significantly inhibited after 24 hours of treatment with erianin, in a dose-dependent manner. The IC_{50} value was 70.96 nM for SW620 and 106.52 nM for HCT116. DAPI staining showed typical morphologic changes of apoptosis after erianin treatment. With increased concentrations of erianin, the cell number at the G2/M phase was increased after 24 hours. Significant increases in the number of apoptotic cells were observed in SW620 cells via Annexin-V-FITC/PI double staining. Furthermore, erianin activated expression of p21 and p27 and suppressed expression of CDK1 and Cyclin B1, inducing cell cycle G2/M phase arrest. Apoptosis-related proteins (p53, Bax) were upregulated by erianin, while PARP and Bcl-2 proteins were downregulated. Phosphorylation levels of JNK increased, in a dose-dependent manner, after erianin treatment. A selective inhibitor of JNK, sp600125, weakened apoptosis levels of SW620 cells induced by erianin. Results suggest that erianin may play a role in proliferation and apoptosis of colorectal cancer cells by affecting JNK pathways, paving the road for further application of erianin in colorectal cancer therapy.

Keywords: Erianin, colorectal cancer, cell cycle and apoptosis, JNK signaling pathway

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both men and women worldwide [1]. It is more common in developed countries, with more than 65% of cases found [2]. CRC has become a life-threatening disease in China. Current treatment methods of colorectal cancer include surgery, radiation therapy, chemotherapy, targeted therapy, and comprehensive treatment. However, patients will still be in the shadow of recurrence or metastatic lesions, leading to poor prognosis. Over 50% of patients suffer from liver metastases, a leading cause of death. Therefore, seeking effective anti-neoplastic drugs with high efficacy and low toxicity is necessary, an urgent topic for CRC treatment [3, 4].

Anti-cancer compounds could be explored from natural plants. Nearly 74% of bioactive natural products or their derivatives have been discovered for cancer treatment, such as taxol, podophyllotoxin, and camptothecin. The Golden-bow Dendrobium or Fried-egg Orchid (*Dendrobium chrysotoxum*) belongs to one of the Traditional Chinese Medicine (TCM) herbs. *Dendrobium* or its extracts have been reported to possess multiple functions, including antioxidant [5, 6], anti-platelet aggregation [7], antimicrobial [8], and anti-tumor activities [9]. Erianin is the major bioactive compound of *Dendrobium chrysotoxum*. Previous studies have identified the multiple anti-neoplastic activity of erianin [10]. Wang et al. showed that erianin induced cell cycle G2/M-phase arrest, apoptosis, and autophagy via ROS/JNK signaling pathways in human

osteosarcoma cells [11]. Sun et al. showed that erianin induced apoptosis in T47D cells through reducing Bcl-2 expression and activating caspase signaling [12]. Previous studies have identified the multiple anti-neoplastic activity of erianin [10], suggesting that the activity may involve JNK pathways, a member of the MAPK superfamily. This pathway is an important signaling pathway involved in inflammation development [11]. JNK is activated and phosphorylated in response to numerous stimuli, including oxidant stress and cytokines [13, 14]. CUI et al. [15] demonstrated that erianin significantly suppressed proliferation of SW480 cancer cells, associated with induced apoptosis and cell cycle G2/M arrest by downregulation of XIAP and Bcl-xL protein expression and activation of Caspase-9, Caspase-7, Caspase-3, and PARP activity. However, the effects of erianin on proliferation of SW620 and HCT116 cells have not been reported. The current study provides evidence concerning the effects of erianin on human colorectal cancer cells through inducing apoptosis and cell cycle arrest via activation of JNK kinase signaling pathways.

Materials and methods

Cell lines and reagents

Human colorectal cancer cell lines HCT116 and SW480 were obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China). Fetal bovine serum and RPMI1640 medium were purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA. Cell culture plasticware was purchased from Corning. Antibodies for PARP, including Caspase-3, Caspase-9, Cyto-C, Bcl-2, Bax, JNK, CDK1, Cyclin B1, Cyclin A, p21, p27, and GAPD, as well as secondary peroxidase-conjugated antibodies, were obtained from Cell Signaling Technology, with dilutions of 1:1000 (for primary antibodies) and 1:3000 (for secondary antibodies). Kits for flow cytometry and apoptosis were purchased from BD biosciences, US. Specific c-Jun amino-terminal-kinase (JNK) inhibitor sp-600125, MTT, DAPI, and poly-L-lysine (PLL) were obtained from Sigma.

MTT assays

Cells grown in the logarithmic phase were trypsinized and seeded in 96-well plates at 10,000 cells/well. After incubation for 24 hours, the medium with different dosages (0, 10, 20, 40, 80, 160, 320 nM) of erianin was administrated. Each concentration had triplicated wells and was incubated for another 48 hours in a CO_2 incubator. Next, the MTT solution was added with a final concentration of 0.5 mg/mL. They were incubated for another 4 hours at 37°C. The supernatant was discarded and 150 μl DMSO was added to dissolve the formazan. Plates were read at 570 nm with 630 nm as reference wavelength using a micro-plate reader (cMax plus, Molecular device). IC_{50} was calculated by GraphPad Prism 6 (Version 6.01).

DAPI staining

Cells were plated into six-well plates with PLL-treated round cover glass. They were cultured to 70% confluence. Erianin was added into the growth medium with varying concentrations (0, 40, 80, 160 nM) for 24 hours. After treatment, the cells were washed with PBS 3 times, fixed in 4% (w/v) paraformaldehyde for 30 minutes, and washed with PBS again. DAPI solution (1 mg/mL in methanol, Sigma) was then added for 15 seconds to stain the nuclei. The slides were removed with fine forceps and washed with PBS, coated on the glass slide. Images of cell morphology and apoptosis were captured via inverted fluorescence microscopes (Olympus, Japan).

Cell cycle analysis

Cells were seeded into six-well plates and treated with varying concentrations of erianin (0, 40, 80, 160 nM) for 24 hours. After treatment, they were collected by EDTA-free trypsin, washed twice with PBS, and fixed at 4°C for 1 hour using 70% ethanol overnight. Obtained cells were stained with a propidium iodide (PI) solution containing 100 μg/mL RNase, at room temperature, in the dark for 30 minutes the next day. They were analyzed with flow cytometry, measuring the intensity of PI staining.

Apoptosis staining

Cells were seeded into six-well plates and treated with varying dosages of erianin (0, 40, 80, 160 nM). After 24 hours of treatment, the cells were collected with 0.25% EDTA-free trypsin, washed twice with PBS, and resuspended in 300 μl Annexin-V binding buffer. They were then incubated with 5 μl FITC-conjugated Annexin-V antibody for 15 minutes in the dark at room temperature. This was followed by incubation with 10 μl PI for 15 minutes in the dark at room temperature. Finally, 200 μl Annexin-V binding

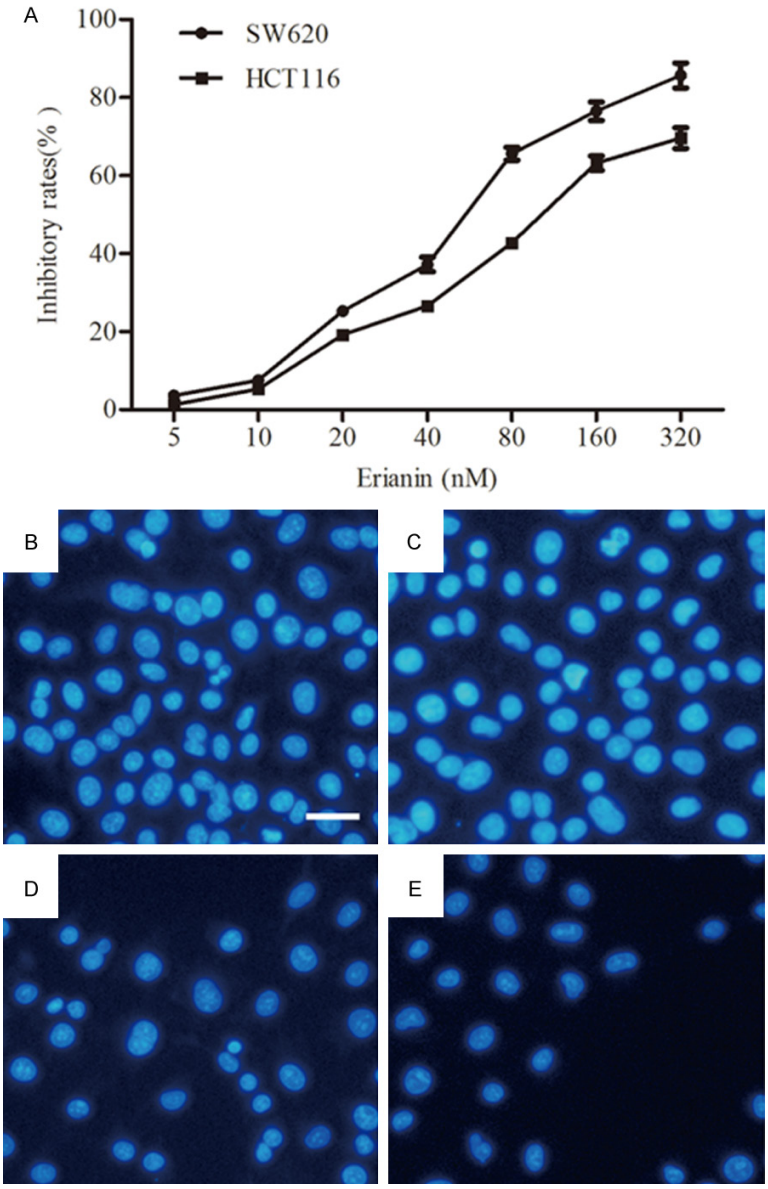

Figure 1. Erianin inhibited the proliferation of SW620 cells, in a dose-dependent manner. (A) SW620 cells and HCT116 cells were treated with graded concentrations of erianin (0, 10, 20, 40, 80, 160, 320 nM) for 24 hours. Cell proliferation potential was evaluated by MTT assays. The inhibition rate of proliferation of untreated control cells is represented as 0%; (B-E) Morphological changes of SW620 cells treated with different dosages at 40 nM (C), 80 nM (D), and 160 nM (E) of erianin for 24 hours with 0 nM (B) as control. Scale bar indicates 20 μm.

buffer was mixed with the cells and filtered with nylon mesh (pore size at 50 μm). Flowcytometry analysis was performed using the BD Accuri™ C6 flow cytometer within 1 hour.

Western blotting analysis

Cells were cultured in 6 cm dishes at a density of 5×10^6/mL. They were then treated with different concentrations of erianin (0, 40, 80, and 160 nM) for 24 hours. The cells were collected, washed with cold PBS, and lysed in ice-cold RIPA buffer containing a protease inhibitor. Protein concentrations were determined with the BCA kit (Pierce). The proteins were separated on 10% SDS polyacrylamide gel and electro-transferred to nitrocellulose (NC) membranes. The membranes were blocked with 5% non-fat milk solution (dissolved in TBS-T buffer) for 1 hour at room temperature. They were incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. A secondary antibody was added with a dilution of 1:3000 after 3 washings with TBS-T. They were then washed with TBS-T 3 times at room temperature. Positive bands on the membranes were identified after treatment with reagents in enhanced chemiluminescence (ECL) kits, following manufacturer recommendations.

Statistical analysis

Inhibitation rates of the group treated with both sp600125 and erianin and the group only treated with erianin were compared via statistical analysis. Analysis was performed using Student's t-tests with Graph Pad Prism 6 software. *P*-values less than 0.05 indicate that inhibitation rates of the sp600625-erianin treating group and erianin treating group were significantly different.

Results

Erianin inhibits the proliferation of SW620 cells

To investigate the effects of erianin on human colorectal cancer cells, MTT assays were conducted, evaluating the viability of SW620 and HCT116 cells treated with different dosages of erianin. Results indicated that erianin could

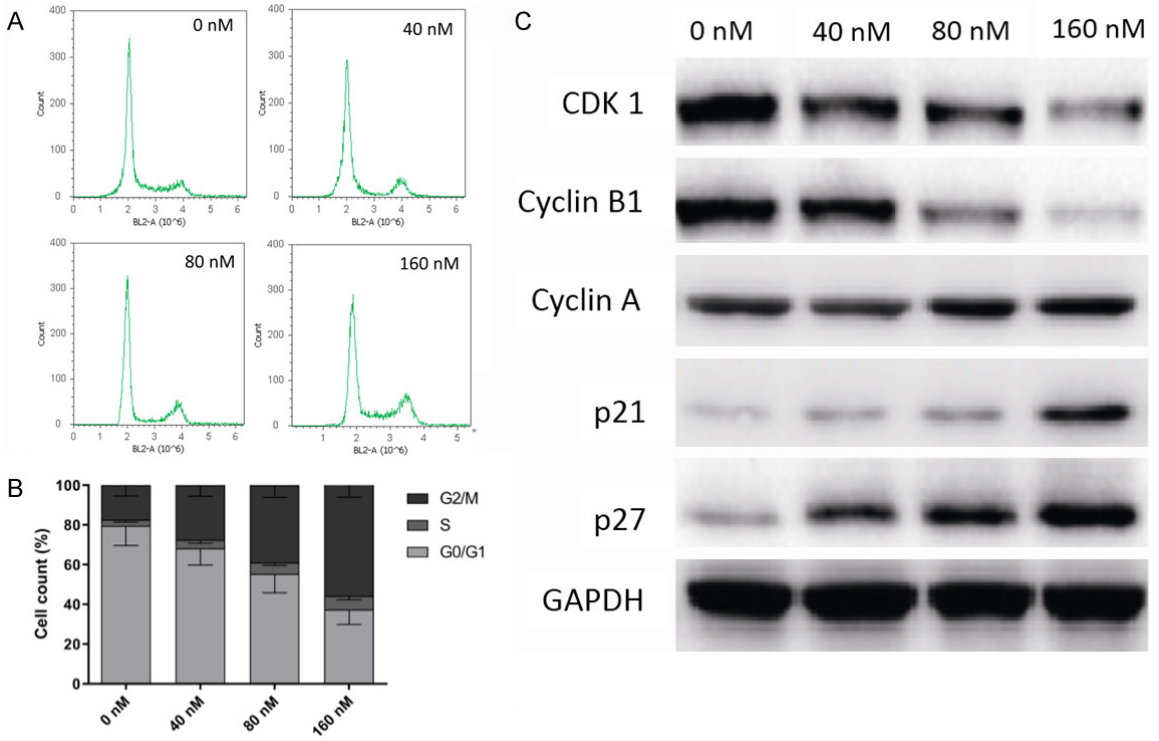

Figure 2. Erianin-induced SW620 cells cycle arrest at G2/M phase. A. Erianin induced SW620 cells cycle arrest. SW620 cells were treated with different concentrations of erianin (0, 40, 80, 160 nM) for 24 hours. Propidium iodide (PI) staining was done to determine the proportion of cell cycle; B. The percentage of cells in different phases of cell cycle after flow cytometry analysis; C. Western blotting showed the effects of erianin on expression of cell-cycle related proteins in SW620 cells, with GAPDH as the internal control.

inhibit the proliferation of SW620 cells relying on different doses. Erianin significantly decreased the viability of SW620 cells at 80, 160, and 320 nM after 24 hours of treatment (**Figure 1A**). The IC_{50} value of erianin was calculated according to the cell inhibition rate curve. After 24 hours of exposure with erianin, the IC_{50} value of erianin was 70.96±0.35 nM for SW620 cells and 106.52±0.56 nM for HCT116. Types of cell morphology of SW620 cells treated with different concentrations of erianin were visualized with a fluorescence microscope (**Figure 1B-E**). Live and dead cells were stained with normal chromatin, while apoptotic cells showed condensed and fragmented chromatin. After staining with DAPI, morphological changes of SW620 cells treated with different dosages at 40, 80, and 160 nM indicated the occurrence of apoptosis under erianin treatment.

Erianin causes cell cycle arrest of SW620 cells at G2/M phase

This study further investigated whether erianin could inhibit cancer cell proliferation through inducing cell cycle arrest. Indeed, erianin-treated SW620 cells showed significantly increased amounts of cells at the G2/M phase, especially at the concentration of 80 and 160 nM (**Figure 2A**). In addition, flow cytometry analysis illustrated that the proportion of cells at the G2/M phase was also obviously increased and cells at the G0/G1 phase were greatly reduced in erianin-treated SW620 cells (**Figure 2B**). Furthermore, expression levels of proteins which are relative to cell cycle were detected by Western blotting. Erianin induced cell cycle arrest via upregulation of p21 and p27, combined with downregulation of CDK1 and Cyclin B1, in a dose-dependent manner (**Figure 2C**). Accumulation of Cyclin A and the downregulation of Cyclin B-CDK1 might have contributed to erianin-induced G2/M phase arrest in SW620 cells.

Erianin induces apoptosis of SW620 cells

To further evaluate erianin-induced apoptosis in SW620 cells, Annexin V-FITC/PI double staining assays were performed, as shown in **Figure**

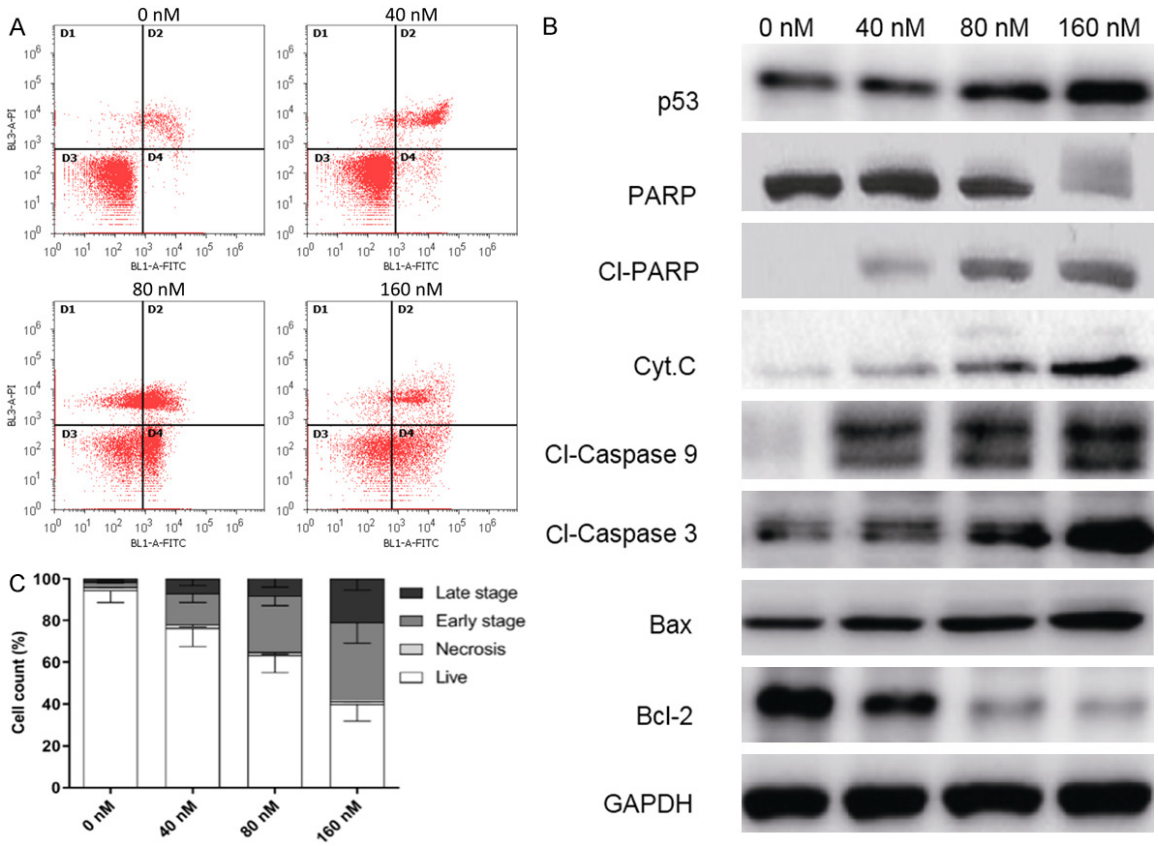

Figure 3. Erianin induced apoptosis of SW620 cells. A. Visualization of apoptotic status by Annexin V-FITC/PI staining assays on erianin-treated SW620 cells by flow cytometry analysis; B. Percentages of negative (live) cells, Annexin V-FITC-positive (early-stage apoptotic) cells, PI-positive (necrotic) cells, or Annexin V-FITC and PI double-positive (late-stage apoptotic) cells; C. Western blot analysis showed the effects of erianin on protein levels of apoptotic-related genes in SW620 cells. GAPDH served as the internal control.

3A. It was found that the number of apoptotic cells was gradually increased, in a dose-dependent manner of erianin. Both the proportion of early- and late-apoptotic cells gradually increased (**Figure 3B**). Underlying mechanisms of erianin-induced apoptosis were then investigated via Western blot analysis. Decreased expression of Bcl-2, a gene responsible for the suppression of apoptosis, and increased expression levels of proteins that were involved in the promotion of apoptosis, including p53, PARP, and BAX, were observed in SW620 cells after treatment with different concentrations of erianin (**Figure 3C**). In addition, cleaved form of Caspase 3 and Caspase 9 were upregulated, indicating the activation of both proteins. Results indicate that erianin-induced apoptosis involved the activation of caspase signaling pathways.

Erianin induces apoptosis of SW620 cells via activation of JNK signaling pathways

JNK has been verified to play an important role in cell cycle progression and apoptosis regulation. Its specific antagonist has been reported to inhibit tumor cell invasion and metastasis in various tumor models [16-18]. Revealing the underlying mechanisms of erianin-induced apoptosis on a molecular level, the current study detected the status of c-Jun N-terminal kinase (JNK) proteins in erianin-treated cells. While total protein levels of JNK remained unchanged, phosphorylation levels of JNK increased, in a dose-dependent manner, after erianin treatment (**Figure 4A**). Furthermore, this study utilized a selective inhibitor of JNK, sp600125 (concentration 5 μM), with erianin to treat SW620 cells for 24 hours. MTT assays indicated that sp600125 could compensate

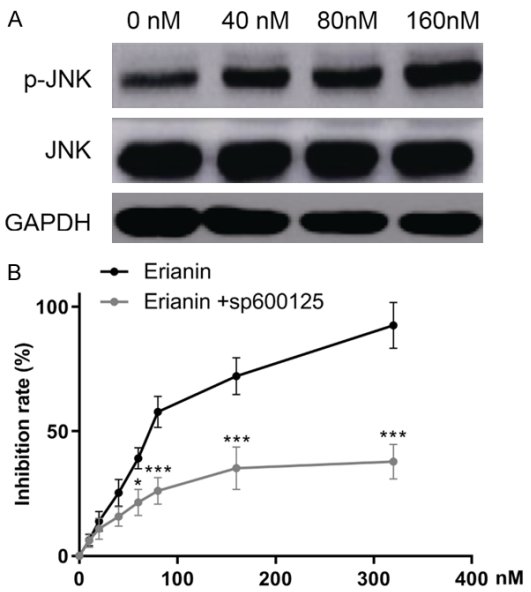

Figure 4. Erianin induced apoptosis of SW620 cells via activation of JNK signaling pathways. A. Western blot analysis showed the effects of erianin on JNK protein and phosphorylation levels in SW620 cells. GAPDH was the internal control; B. A selective inhibitor of JNK, sp600125 (concentration 5 μM), compensated the inhibitory effects of erianin on proliferation of SW620 cells. *p<0.05, ***p<0.001.

the inhibitory effects of erianin to the proliferation of SW620 cells (**Figure 4B**). In general, activation of JNK signaling pathways contributed to erianin-induced anti-proliferation of SW620 cells.

Discussion

Dendrobium chrysotoxum, an herb from Tranditional Chinese Medicine, has been reported to inhibit the proliferation of many cancer cells [19-22]. Erianin, the major bioactive compound of *Dendrobium chrysotoxum*, has shown potential anti-neoplastic activity. The current study evaluated the effects of erianin on colorectal cancer cell lines, exploring underlying molecular mechanisms.

Results demonstrated that erianin treatment could significantly decrease cell viability, in a dose-dependent manner, at 24 hours. More importantly, treatment with erianin induced apoptosis in SW620 cells and exhibited the classical apoptotic cell morphology of condensed and fragmented chromatin. This study also found that erianin induced cell cycle arrest at the G2/M phase, in a dosage-dependent manner. The regulatory system of cell cycle is quite precise and complex. CDKs play a key role in the controlling of cell proliferation by maintaining the cell cycle [11]. Current results demonstrated that treatment with erianin significantly downregulated expression levels of multiple CDKs and upregulated expression of p21, p27, and Cyclin A. This suggests that erianin could target multiple CDKs, suppressing cancer cells effectively. Further investigations are required to elucidate how erianin affects the function of CDKs *in vivo* and *in vitro*.

Furthermore, flow cytometric analysis via Annexin V-FITC/PI double staining assays was performed. Results showed that erianin increased the amounts of early- and late-stage apoptotic cells, in a dose-dependent manner. Apoptosis consists of two activating mechanisms, intrinsic and extrinsic pathways. Intrinsic pathways start from the stimulus to the cells, then mitochondria releases cytochrome c due to constitution of channels in the outer membrane of the mitochondria. Released cytochrome c combines with apoptotic protease that can activate factor-1 (Apaf-1). This is followed by binding to pro-caspase-9, generating a caspase cascade. Results suggest that apoptosis of SW620 cells and activation of the caspase cascade were greatly enhanced by treatment with erianin. However, detailed mechanisms concerning how erianin participates in the process of apoptosis should be further investigated.

Lastly, the current study showed that erianin treatment promoted phosphorylating JNK without changing expression levels of JNK proteins in SW620 cells. Combined with sp600125, a specific JNK inhibitor, erianin could no longer possess inhibitory activity towards cell proliferation. Non-cytotoxic doses of sp600125 were used in all the treatments due to preliminary experiments. In summary, suppression of JNK activity and JNK signaling pathways further results in the loss of erianin-induced cell apoptosis. Present data deepens the knowledge and understanding of erianin anti-neoplastic function.

In conclusion, erianin restrains the proliferation of SW620 cells through inducing cell arrest and apoptosis via activation of JNK signaling pathways. The present study suggests the poten-

tial application of erianin for colorectal cancer treatment, providing potential underlying mechanisms.

Disclosure of conflict of interest

None.

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