Alantolactone induces gastric cancer BGC-823 cell apoptosis by regulating reactive oxygen species generation and the AKT signaling pathway

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Abstract. Alantolactone (ALT), a natural sesquiterpene lactone, has been suggested to exert anti-cancer activities in various cancer cell lines. However, the effects and mechanisms of action of ALT in human gastric cancer remains to be elucidated. In the present study, the effects of ALT on BGC-823 cells were examined and the underlying molecular mechanisms associated with these effects were investigated. Cell viability was detected by using an MTT assay. Cell cycle, cell apoptosis and the level of reactive oxygen species (ROS) were assessed by flow cytometry, and the expression levels of proteins of interest were analyzed by western blot assay. The results demonstrated that ALT triggered apoptosis and induced G0/G1 phase arrest in a dose-dependent manner. Furthermore, the expression level of the anti-apoptosis protein Bcl-2 was downregulated, and expression of the pro-apoptosis proteins Bax and cleaved PARP were significantly upregulated. The cell cycle-associated proteins cyclin-dependent kinase inhibitor 1 and cyclin-dependent kinase inhibitor 1B were also increased, while cyclin D1 was deceased. In addition, ALT induced apoptosis via the inhibition of RAC-alpha serine/threonine-protein kinase (AKT) signaling and ROS generation, which was effectively inhibited by the ROS scavenger, N-acetyl cysteine. Therefore, the results from the present study indicated that the ROS-mediated inhibition of the AKT signaling pathway serves an important role in ALT-induced apoptosis in BGC-823 cells. In conclusion, the results demonstrated that ALT exerted significant anti-cancer effects against gastric cancer cells in vitro.

Introduction

Gastric carcinoma is currently the third most common cause of cancer mortality worldwide; 50% of all gastric carcinomas occur in Eastern Asia, and it is particularly common in China (1,2). At present, the effective treatment strategies for gastric carcinoma include surgery, radiotherapy, chemotherapy and targeted therapy (3,4). Surgical resection followed by adjuvant chemotherapy remains the most effective therapeutic option. Unfortunately, relapse and metastasis of gastric tumors (5), and resistance to chemotherapy are common. Therefore, novel therapeutic agents for gastric carcinoma therapy are urgently required.

Increasing attention has been paid to the application of natural products for chemopreventive cancer therapy. Terpenoids are phytochemicals traditionally used for medicinal purposes. Preclinical studies have demonstrated that terpenoids present in plant foods or isolated from medicinal plants, including germacrone from Rhizoma Curcuma or lactucopicrin from Cichorium intybus L, exhibit significant anti-cancer effects against various types of cancer cell in vivo and in vitro (6-8). Alantolactone (ALT), a sesquiterpene lactone compound isolated from Inula helenium, has exhibited multiple biological properties, including anti-bacterial, anti-inflammatory and anti-cancer activities (9). Notably, ALT was suggested to exhibit potential anti-cancer activity against various types of cancer, including human colorectal cancer (10), liver cancer (11,12), leukemia (13), breast cancer (14), lung cancer (15,16) and cervical cancer (17,18). ALT may inhibit breast cancer growth via anti-angiogenic activity by inhibiting vascular endothelial growth factor receptor 2 and RAC-alpha serine/threonine-protein kinase B (AKT) signaling (19). In human cervical cancer cells, ALT induces apoptosis via generation of reactive oxygen species (ROS) and inhibition of the B-cell lymphoma 2 (Bcl-2)/Bcl-2 associated X apoptosis regulator (Bax) signaling pathway (17). In SK-MES-1 lung squamous cancer SK-MES-1 cells, ALT may trigger apoptosis and induce cell cycle G1/G0 phase arrest. Furthermore, ALT may enhance the chemosensitivity of A549 cells to doxorubicin via ROS-mediated apoptosis (20). However, the exact mechanism underlying the anti-cancer
activity of ALT in human gastric cancer cells remains to be elucidated.

The present study aimed to elucidate the anti-cancer effects and associated molecular mechanisms of ALT in BGC-823 cells, and to evaluate the potential of ALT for its application as a novel naturally-derived agent for the treatment of gastric cancer.

Materials and methods

Reagents. ALT, N-acetyl cysteine (NAC) and MTT were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Primary antibodies to detect cyclin D1 (cat. no., 2978), cyclin-dependent kinase inhibitor 1 (p21; cat. no., 2947), cyclin-dependent kinase inhibitor 1B (p27; cat. no., 3686), Bax (cat. no., 5023), Bcl-2 (cat. no., 15071), poly (adenosine 5'diphosphate-ribose) polymerase (PARP; cat. no., 5625), phosphorylated (p)-AKT (cat. no., 4060), AKT (cat. no., 9272) and GAPDH (cat. no., 5174) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit and cell cycle detection kit were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). The ROS assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. The BGC-823 human gastric cancer cell line was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO2.

MTT cytotoxicity assay. Cell viability was measured using an MTT assay as described previously (21). The BGC-823 cells were seeded into 96-well plates at a density of 1x10^3 cells/well and treated following culture for 24 h. The BGC-823 cells were treated with ALT (0, 10, 20, 40, 60 or 100 µM), and incubated for an additional 4 h at 37°C. In total, 0.5 mg/ml MTT solution was then added to the medium, and the cells were incubated for an additional 4 h at 37°C. The culture was then removed, and dimethyl sulfoxide (150 µl/well) was added to dissolve the solid residue. The absorbance at 490 nm (A490) was determined using an ELISA microplate reader, and all experiments were performed at least 3 times. Percentage of cell viability was calculated as follows: Cell viability (%)=(A490 sample/A490 blank)/(A490 control-A490 blank) x100.

Cell cycle assay. Cell cycle arrest was detected using a cell cycle detection kit according to manufacturer's protocol (Nanjing KeyGen Biotech Co., Ltd.). Briefly, the BGC-823 cells were seeded at 1x10^3 cells/well in 6-well plates and treated with different ALT concentrations (0, 10, 20, 40 or 60 µM) for 24 h. The BGC-823 cells were fixed with 75% ethanol at 4°C overnight. PI was then used to stain the DNA of the samples for 15 min at 25°C in the dark, and flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine the cell cycle and analyzed by Modfit LT 3.0 software (Verity Software House, Topsham, ME, USA). All experiments were performed at least three times.

Assessment of apoptosis. Cell apoptosis was assessed using an Annexin V-FITC/PI kit according to manufacturer's protocol (Nanjing KeyGen Biotech Co., Ltd.) and the apoptotic rate was analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences). BGC-823 cells were seeded at 1x10^6 cells/well in 6-well plates and treated different ALT concentrations (0, 10, 20, 40 or 60 µM) for 24 h. Subsequently, cells were stained with Annexin V-FITC and PI in binding buffer for 15 min at 25°C in the dark. According to manufacturer's protocol, 5 µl Annexin V-FITC and 5 µl PI were added per sample. The apoptotic cells were then detected on a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA). A total of three independent experiments were performed.

ROS detection. ROS generation was measured using a ROS assay kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute) and the ROS levels were analyzed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences). Briefly, BGC-823 cells were treated with the indicated concentrations (0, 10, 20, 40 and 60 µM) of ALT for 6 h. In addition, BGC-823 cells were pre-treated with 10 mM NAC for 1 h, and with 40 µM ALT for 6 h. The cells were then incubated with 30 µM 2',7'-dichlorodihydrofluorescein diacetate at 37°C for 30 min. The cells were analyzed by FACSCalibur flow cytometer (BD Biosciences). The results were analyzed with FlowJo 7.6 software (FlowJo LLC) and all experiments were performed at least three times.

Western blot analysis. Cells were lysed using radioimmunoprecipitation assay (Beyotime Institute of Biotechnology, Haimen, China) on ice for 30 min. The proteins were quantified with Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins (40 µg) were separated by 6-12% SDS-PAGE and then transferred onto nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA) and blocked with 5% non-fat milk in TBS-containing 0.05% Tween 20 for 2 h at room temperature. The membranes were incubated with anti-cyclin D1 (1:1,000 dilution), anti-p21 (1:1,000 dilution), anti-p27 (1:1,000 dilution), anti-Bax (1:1,000 dilution), anti-Bcl-2 (1:1,000 dilution), anti-p-AKT (1:1,000 dilution), anti-AKT (1:1,000 dilution), anti-cleaved PARP and anti-GAPDH (1:3,000 dilution) antibodies at 4°C overnight. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig)G (cat. no. 7076; 1:25,000) and HRP-conjugated anti-rabbit IgG (cat. no. 7074; 1:20,000; both Cell Signaling Technology, Inc.) secondary antibodies for 2 h at 25°C. Visualization was performed using a SuperSignal West Pico chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation. Differences between groups were determined by
one-way analysis of variance followed by Dunnett's or Tukey's post-hoc tests. The analyses were performed using SPSS v.19 statistical software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

ALT inhibits cell proliferation in BGC-823 cells. The effect of ALT on cell proliferation was determined using an MTT assay. The cytotoxicity of ALT was evaluated by treating BGC-823 cells with different concentrations (0, 10, 20, 40, 60, 80 and 100 µM) for 24 h. As demonstrated in Fig. 1, ALT significantly inhibited the viability of BGC-823 cells in a concentration-dependent manner, exhibiting significant differences compared with the control group. These results indicated that ALT significantly suppressed the growth of BGC-823 human gastric cancer cells.

ALT induces cell cycle arrest at the G0/G1 phase in BGC-823 cells. Inhibition of cell proliferation via anti-tumor drugs is often accompanied by changes in cell cycle progression (22,23). In the present study, flow cytometry was used to analyze the distribution of the cell cycle in BGC-823 cells following treatment with ALT (20-60 µM) for 24 h. G0/G1 phase arrest was observed in BGC-823 cells exposed to ALT compared with the control group. As demonstrated in Fig. 2A, ALT induced G0/G1 phase arrest of BGC-823 cells in a dose-dependent manner. The percentage of cells in the G0/G1 phase was 57.50±0.10, 66.57±0.05, 71.24±0.88, 72.31±0.17 and 81.97±0.53% in the 0, 10, 20, 40 and 60 µM groups, respectively (Fig. 2A). To gain insight into the mechanism of G0/G1 phase arrest, the expression levels of cyclinD1, p21 and p27, which regulate G1 to S phase progression, were detected. As presented in Fig. 2B, the level of cyclin D1 was decreased, and levels of p21 and p27 were significantly increased following stimulation with ALT. These results indicated that ALT induces G0/G1 phase arrest via downregulation of cyclin D1 expression and upregulation of p21 and p27 expression in BGC-823 cells.

ALT induces apoptosis in BGC-823 cells. Anti-tumor drugs often execute cytotoxic effects via the induction of apoptosis (24,25). The effect of ALT on BGC-823 cell apoptosis was evaluated using Annexin V-FITC/PI double staining and flow cytometry. The percentage of apoptotic cells was 1.15±0.01, 22.69±0.13, 25.10±0.14, 39.53±0.37 and 50.93±0.29% in the 0, 10, 20, 40 and 60 µM groups, respectively, after 24 h (Fig. 3A). The data suggested that ALT significantly induced apoptosis in a dose-dependent manner. To reveal the molecular mechanism involved in ALT-induced apoptosis, the levels of anti-apoptosis protein Bcl-2 and pro-apoptosis proteins Bax and cleaved PARP were detected by western blot analysis. When BGC-823 cells were treated with 40 µM ALT, Bax and cleaved PARP protein levels were increased in a time-dependent manner, whereas Bcl-2 levels were decreased in a time-dependent manner (Fig. 3B). These results suggested that ALT may trigger apoptosis in BGC-823 cells partially via a mitochondrial-dependent pathway.

ROS accumulation is associated with ALT-induced apoptosis. Several studies have revealed that anti-tumor drug-induced cell death is associated with intracellular ROS accumulation in multiple cancer types (26-28). Therefore, whether ROS were involved in ALT-induced apoptosis was also investigated. Intracellular ROS generation was detected following treatment with different ALT concentrations (0, 10, 20, 40 or 60 µM) for 6 h. ALT significantly increased intracellular ROS generation in a concentration-dependent manner in BGC-823 cells (data not shown). Subsequently, to determine whether the increased ROS generation served an important role in ALT-induced cell apoptosis, the cells were pretreated with NAC 1 h prior to treatment with ALT for 24 h. Pretreatment with NAC effectively prevented ALT-induced ROS accumulation (Fig. 4A). Additionally, NAC also abolished ALT-inhibited cell proliferation and ALT-induced cell apoptosis (Fig. 4B and C). Furthermore, western blot analysis revealed that NAC decreased the expression of Bax and the cleavage of PARP, and increased Bcl-2 expression (Fig. 4D). Collectively, the results indicated that ALT-induced apoptosis was associated with ROS accumulation.

ALT induces BGC-823 cell apoptosis via inhibition of AKT signaling. Accumulating evidence has revealed that the AKT pathway is one of the major signaling pathways closely associated with cancer progression (29,30). To determine whether the AKT signaling pathway is involved in ALT-induced apoptosis, western blot analysis was used to assay the effects of ALT on p-AKT and AKT protein expression. As presented in Fig. 5A, BGC-823 cells were treated with 40 µM ALT for 3, 6, 12 and 24 h. The phosphorylation of AKT was significantly decreased in a time-dependent manner. ROS have been demonstrated to be associated with the apoptosis induced by anti-cancer drugs via regulation of AKT pathways (31). To additionally investigate the association between ROS and the AKT pathway, cells were treated with or without NAC for 1 h, and then western blot analysis was used to examine p-AKT and AKT protein expression levels in BGC-823 cells. As presented in Fig. 5B, NAC significantly increased AKT phosphorylation.
Together, these results suggest that ALT induced apoptosis through ROS-mediated inactivation of the AKT signaling pathway in BGC-823 cells.

Discussion

The emergence of drug resistance remains a major barrier for successful cancer treatments. Therefore, exploring and developing novel and effective anti-cancer drugs is essential. ALT, a sesquiterpene lactone, is a potential anti-cancer therapeutic agent. In a previous study, the anti-cancer effects of ALT were demonstrated in various tumor cells, including breast (14), lung (20,32), cervical (17,18), liver (12), colorectal (10) and colon cancer (33). However, to the best of our knowledge, there is a lack of thorough experimental data describing the anti-tumor effects of ALT in BGC-823 human gastric cancer cells. The present study demonstrated that ALT exhibited significant anti-cancer effects in BGC-823 cells: ALT inhibited cell proliferation, and induced G0/G1 cell cycle arrest and apoptosis in BGC-823 cells. Furthermore, ALT promoted BGC-823 cell apoptosis via ROS-induced AKT signaling.

Cell cycle arrest is an important mechanism associated with anti-cancer drug-induced proliferation inhibition (34,35). Previous studies have indicated that ALT may inhibit cell cycle progression in lung and colorectal cancer cells (10,32). Similarly, flow cytometric analysis demonstrated that ALT induced G0/G1 cell cycle arrest of BGC-823 cells in a concentration-dependent manner. Furthermore, previous studies have demonstrated that anti-cancer drugs promote G0/G1/S transition and inhibit cell proliferation by regulating the expression of cyclin D1 (36,37). Additionally, p27 and p21 serve suppressive roles in G0/G1/S transition by inhibiting the activity of cyclin/cyclin-dependent kinase complexes (37,38). To additionally investigate the molecular basis by which ALT inhibited cell cycle-associated proteins was determined by
western blot analysis. ALT markedly decreased the expression levels of cyclin D1, whereas the expression of p21 and p27 were increased. These data provide evidence that ALT inhibited the proliferation of BGC-823 cells via ALT-induced G0/G1 phase arrest.

Apoptosis is a critical homeostatic mechanism involved in anti-cancer drug-induced proliferation inhibition (35). The mitochondrial apoptotic pathway has a critical role in drug-mediated apoptosis and is often regarded as a potential anti-cancer target (39-41). Bcl-2 family protein members, including Bax and Bcl-2, serve important roles in the mitochondrial apoptotic pathway. Previous data has indicated that ALT induces apoptosis in a variety of human cancer cells: Cui et al (14) suggested that ALT induced the mitochondrial-mediated apoptotic pathway by increasing the Bax/Bcl-2 ratio and PARP cleavage in MDA-MB-231 cells; Jiang et al (17) also demonstrated that the Bcl-2/Bax signaling pathway was associated with ALT-induced HeLa cell apoptosis. In the present study, treatment with ATL significantly induced apoptosis of BGC-823 cells by increasing the Bax/Bcl-2 ratio and PARP cleavage in BGC-823 cells. These data suggest that the mitochondrial apoptotic pathway serves a key role in ALT-mediated BGC-823 cell apoptosis.

The AKT signaling pathway is an important anti-apoptosis pathway that promotes cell survival and resistance to cell apoptosis induced by chemotherapeutic agents in various cancer types (42). Inactivation of the AKT signaling pathway may inhibit cell growth and induce cell apoptosis in various cancer cells. Therefore, whether the AKT pathway was associated with ALT-induced apoptosis of BGC-823 cells was examined in the present study. The data demonstrated that ALT decreased the phosphorylation of AKT in a time-dependent manner. This indicated that AKT signaling may be involved in ALT-mediated apoptosis of BGC-823 cells.

ROS have been suggested to be involved in the initiation and the promotion of tumor development at different stages of carcinogenesis (43,44). In fact, numerous studies have
demonstrated that various anti-cancer drugs exert their effects via ROS-dependent pathways (45-47). ALT was demonstrated to induce apoptosis of MDA-MB-231 cells via ROS-mediated mitochondrial dysfunction (14). Jiang et al. (17) also revealed that ROS may mediate apoptosis in human cervical cancer cells by increasing the Bax/Bcl-2 ratio. The results of the present study demonstrated that ALT treatment increased ROS generation in a concentration-dependent manner in BGC-823 cells (data not shown). Furthermore, pretreatment with NAC for 1 h reversed the ALT-induced production of ROS and cell apoptosis, and NAC significantly decreased the Bax/Bcl-2 ratio and PARP cleavage. The results suggested that ALT induced ROS-dependent apoptosis in BGC-823 cells. In addition, ROS generation was demonstrated to be involved in chemotherapeutic agent-mediated apoptosis and may be an upstream regulator of AKT-mediated signaling pathways (48,49). In the present study, pretreatment with NAC for 1 h reversed the AKT inhibition induced by ALT. These results suggested that the apoptosis of BGC-823 cells was induced by ALT via ROS generation, which was then modulated the AKT signaling.

In conclusion, the results demonstrated that ALT induced apoptosis and G0/G1 phase arrest in BGC-823 cells in a

Figure 4. ROS are involved in ALT-induced apoptosis. (A) BGC-823 cells were pre-treated with NAC (10 mM) for 1 h, and then ALT (40 µM) was added to the cells for 6 h. Flow cytometry was used to detect ROS levels. (B) BGC-823 cells were pre-treated with NAC (10 mM) for 1 h, and then ALT (40 µM) was added to the cells for 24 h. MTT was used to measure cell viability. (C) BGC-823 cells were incubated with ALT and NAC as aforementioned. Flow cytometry was used to detect apoptosis in BGC-823 cells. (D) BGC-823 cells were pre-treated with NAC (10 mM) for 1 h, and then ALT (40 µM) was added to the cells for 12 h. Western blot analysis was used to detect the expression levels of Bax, Bcl-2 and cleaved PARP. *P<0.05 and **P<0.01 vs. the control group. ROS, reactive oxygen species; ALT, alantolactone; NAC, N-acetyl cysteine; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated protein; PARP, poly (adenosine 5'diphosphate-ribose) polymerase; PI, propidium iodide; ctrl, control.

Figure 5. ALT induces BGC-823 cell apoptosis via reactive oxygen species-mediated AKT signaling. (A) BGC-823 cells were treated with ALT (40 µM) for 3, 6, 12 and 24 h. The levels of p-AKT and AKT were analyzed by western blot analysis. (B) BGC-823 cells were pre-incubated with NAC (10 mM) for 1 h, and then ALT (40 µM) was added to the cells for 12 h. The expression levels of p-AKT and AKT were measured by western blot analysis. ALT, alantolactone; NAC, N-acetyl cysteine; AKT, RAC-alpha serine/threonine-protein kinase; p, phosphorylated; ctrl, control.
concentration-dependent manner. In addition, ALT induced the apoptosis of BGC-823 cells via ROS-mediated inactivation of the AKT signaling pathway; therefore, ALT may be a promising candidate drug for the treatment of gastric cancer. However, additional studies are required to validate the anti-cancer activity of ALT in xenograft mouse models in vivo.

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Competing interests

Not applicable.

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Availability of data and materials

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

Authors' contributions

XZ conducted the experiments, analyzed the data, contributed to the design of the study and prepared the manuscript. XZ and HMZ performed the western blotting and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

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