Identification of an antitumor effect of demethylzeylasteral on human gastric cancer cells

YANG YANG, MENGLIN ZHAO, TING HU, FANG SU, FENG QIAN and ZISHU WANG

Department of Medical Oncology, The First Affiliated Hospital of Bengbu Medical College, Anhui Province Key Laboratory of Translational Cancer Research, Bengbu Medical College, Bengbu, Anhui 233004, P.R. China

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Abstract. Gastric cancer is a common malignancy in China, with the second highest mortality rate worldwide. Advanced gastric cancer usually exhibits a poor prognosis with a low 5-year survival rate. Therefore, developing novel drugs for the treatment of this cancer will be beneficial for patients. Demethylzeylasteral, an extract of tripterygium wilfordii, has shown positive anticancer activities. However, the possible antitumor effect of demethylzeylasteral on gastric cancer cells and its underlying molecular mechanism remain to be determined. In the present study, the Cell Counting Kit-8 and colony formation assays revealed that demethylzeylasteral impeded the proliferation of human gastric cancer cells in a dose-dependent manner. Furthermore, the Transwell assay identified an inhibitory effect of demethylzeylasteral on the migration of MKN-45 cells, while flow cytometry found that treatment with demethylzeylasteral induced apoptosis and decreased the mitochondrial membrane potential in the cancer cells. Further investigation revealed that demethylzeylasteral downregulated the phosphorylation of ERK1/2, AKT, and GSK-3β in MKN-45 cells. Notably, decreased expression of Bcl-2 and increased expression of Bax, cleaved caspase-3, cleaved caspase-9 and cleaved PARP were detected in the cancer cells treated with demethylzeylasteral. The present study demonstrated that demethylzeylasteral exhibits therapeutic potential for gastric cancer.

Correspondence to: Professor Zishu Wang or Professor Feng Qian, Department of Medical Oncology, The First Affiliated Hospital of Bengbu Medical College, Anhui Province Key Laboratory of Translational Cancer Research, Bengbu Medical College, 287 Changhuai Road, Bengbu, Anhui 233004, P.R. China
E-mail: wzshahbb@163.com
E-mail: fengqian@sjtu.edu.cn

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Introduction

The global burden of cancer continues to increase due to a variety of factors (1). Gastric cancer is the fourth most common cancer worldwide and is the second most common cause of cancer-associated mortality (2). The treatment of gastric cancer includes surgery, chemotherapy, radiotherapy and molecular targeted therapy, among which surgery combined with chemo-radiotherapy is the most effective treatment regimen (3). However, the treatment has entered a bottleneck period due to congenital or acquired drug resistance and postoperative recurrence (4). Therefore, identification of new targets and signaling pathways related to the progression of gastric cancer may be beneficial for the treatment of gastric cancer.

Tumor occurrence and development are closely associated with uncontrolled cell proliferation, while malignant cells often escape apoptosis to obtain unlimited proliferation capacity (5). In this case, three types of evasion mechanisms of apoptosis exist, including the weakening of caspase function, the damage of the death receptor pathway, and the destruction of the balance between anti-apoptotic and pro-apoptotic proteins (6). Therefore, targeting cancer cell apoptosis by modulating key proteins or enzymes in the apoptosis-related signaling pathway has become an area of focus in cancer research (7). Notably, a number of natural products have been shown to act in apoptosis signaling pathways involved in cancer cell death. Camptothecin, a quinoline alkaloid, induced apoptosis of human myeloid leukemia cells by upregulating proapoptotic proteins and downregulating cyclins (8). Matrine, an alkaloid derived from the Sophora flavescensait, promoted apoptosis of gastric cancer cells by boosting the pro-apoptotic proteins, altering the ratio of Fas/FasL and activating caspase-3 (9,10). These findings have led researchers to focus on developing new potential anticancer agents.

Demethylzeylasteral is a triterpene monomer extracted from tripterygium wilfordii, which has been widely used in the study of anti-inflammatory immune regulation, antifertility and estrogen metabolism regulation (11-14). In recent years, the anticancer properties of demethylzeylasteral have been continuously studied. Li *et al* (15) reported that demethylzeylasteral significantly impeded the invasion of triple-negative breast cancer by blocking the TGF-β signaling pathway (15). Meanwhile, this compound may suppress glioma growth by mediating the miR-30e-5p/MYBL2 axis (16). Notably,

demethylzeylasteral was found to evoke the apoptosis of melanoma cells by downregulating the level of MCL1 (17). However, the anticancer activity of demethylzeylasteral on gastric cancer cells and its underlying mechanism have not been investigated.

The present study aimed to determine and characterize the anticancer properties of demethylzeylasteral on human gastric cancer cells. The present study demonstrated that demethylzeylasteral impeded the viability and migration of gastric cancer cells, while inducing cancer cell apoptosis. Furthermore, treatment with demethylzeylasteral attenuated the ERK1/2 pathway, as well as a decreasing the levels of phosphorylated Akt (p-Akt) and phosphorylated GSK3β (p-GSK3β) in the cancer cells. The present study demonstrated that demethylzeylasteral has a therapeutic potential for gastric cancer.

Materials and methods

Reagents. The reagents used in this study were purchased as follows: Demethylzeylasteral (CAS:107316-88-1) from Target Molecule Corp.; fetal bovine serum (FBS) from Shanghai Nuova Pharmaceutical Technology Co., Ltd.; 0.25% Trypsin-EDTA, RPMI-1640 medium and penicillin/streptomycin from Gibco; Thermo Fisher Scientific, Inc.; dimethyl sulfoxide (DMSO) and enhanced chemiluminescent (ECL) substrate from Thermo Fisher Scientific, Inc.; Wright-Giemsa Stain it from Jian Cheng Technology Company; crystal violet from Sangon Biotechnology; Annexin V-FITC/PI apoptosis detection kit from Multi Sciences (Lianke) Biotech, Co., Ltd.; phosphate-buffered solution (PBS), mitochondrial membrane potential (MMP) assay kit with JC-1, and Cell Counting Kit-8 (CCK-8) from Beyotime Institute of Biotechnology; methanol and ethanol from Macklin Reagent Co., Ltd.; primary antibodies against Bax, cleaved PARP, caspase-9, cleaved caspase-3, Bax, c-Myc, GSK-3β, p-GSK-3β (Ser9), ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), Akt, p-Akt and β-actin from Cell Signaling Technology, Inc.; horseradish peroxidase-conjugated secondary antibodies against rabbit and mice from Jackson ImmunoResearch Inc.

Demethylzeylasteral liquid preparation. A total of 10 mg demethylzeylasteral was dissolved in DMSO for preparation of 100 mmol/ml solution, and the solution was kept at -20˚C for a long-term storage. For the experiments, the solution was diluted with DMSO to the indicated concentrations.

Cell culture. Human gastric cancer MKN-45 cells were purchased from American Type Culture Collection. Human normal gastric mucosal GES-1 cells were obtained from the Cell Bank of the Chinese Academy of Sciences. The cells were grown in a 37˚C incubator with 5% CO_2 and cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin for 24 h.

Cytotoxicity assay. CCK-8 was used to assess the cytotoxicity of the compound. In brief, MKN-45 cells were seeded onto 96-well plates (5,000 cells/well) and incubated with 0.2 μl DMSO or demethylzeylasteral at various concentrations (50 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, 30 μM, 50 μM or 100 μM) for 24 h. Next, 10 μl CCK-8 solution was applied to each well, followed by incubation for 2 h at 37˚C. Finally, the absorbance at a wavelength of 450 nm was measured using a microplate reader. Similarly, the effects of different concentrations of demethylzeylasteral on the activity of human normal gastric mucosa GES-1 cells were also investigated using the same method.

Colony formation assay. A colony formation assay was utilized to assess cell proliferation. MKN-45 cells in a single cell suspension state were seeded onto a 6-well plate at a density of 500 cells per well, and then treated with 2 μl DMSO or demethylzeylasteral at different concentrations (100 nM, 500 nM, 1 μM or 10 μM). The cells were grown in RPMI-1640 medium containing 10% FBS for 1 week until each colony reached 100-200 cells. Colonies were fixed in 100% methanol at room temperature for 20 min and stained using Giemsa staining at room temperature for 20 min. Finally, the plate was washed moderately with running tap water, and the colony with at least 100 cells was counted. Digital photography of colonies was conducted, and the data was analyzed using Prism software 7.0 (GraphPad Software, Inc.).

Transwell assay. The Transwell assay was used to assess the effect of demethylzeylasteral on gastric cancer cell migration. A total of 200 μl serum-free cell suspension containing $2x10^5$ MKN-45 cells and 600 μl RPMI-1640 medium with 5% FBS were added to the upper chamber of the transwell chambers (8-μm pore size, Corning Inc.) and lower compartment of the Transwell chamber, respectively. Next, 2 μl DMSO or demethylzeylasteral at different concentrations (1, 3 or 10 μM) were applied to the upper chamber. After growth for 24 h, the cells migrating to the submembrane surface were fixed with methanol for 20 min at room temperature and stained with 0.5% crystal violet for 20 min at room temperature. Finally, the stained cells were photographed under a Leica optical microscope at x100 magnification and ImageJ computer software 1.6 (National Institutes of Health) was used to calculate the area of cell migration.

Measurement of MMP. Alteration in MMP in apoptosis was investigated using an MMP-specific fluorescent probe, 5, 5', 6, 6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazole-carbocyanide iodine (JC-1; Beyotime Institute of Biotechnology). In brief, MKN-45 cells were treated with 2 μl DMSO or demethylzeylasteral at different concentrations (1, 3 or 10 μM) for 24 h and centrifuged for 5 min at -4˚C and 500 x g. The cells were stained with JC-1 staining solution for 20 min in the dark at 37˚C. After being washed twice with JC-1 buffer, the MKN-45 cells were re-suspended in JC-1 buffer and analyzed on a flow cytometer (BD FACSVerse™).

Apoptosis assay. Annexin V-FITC/PI apoptosis detection kit was used to assay apoptosis in MKN-45 cells treated with demethylzeylasteral, according to the manufacturer's protocol. MKN-45 cells were seeded onto 12-well plates ($5x10^5$ cells/well) and grown at 37˚C overnight. Next, the cells were incubated with 2 μl DMSO or demethylzeylasteral at different concentrations (1, 3 or 10 μM) for 24 h. Following treatment, the cells were harvested by centrifugation (5 min at -4˚C and 500 x g), rinsed twice with PBS and re-suspended in 500 μl Annexin-binding buffer. Finally, the cells were stained with 5 μl Annexin-V-FITC and 10 μl PI for 15 min at 4˚C, and the fluorescence was determined using a flow cytometer

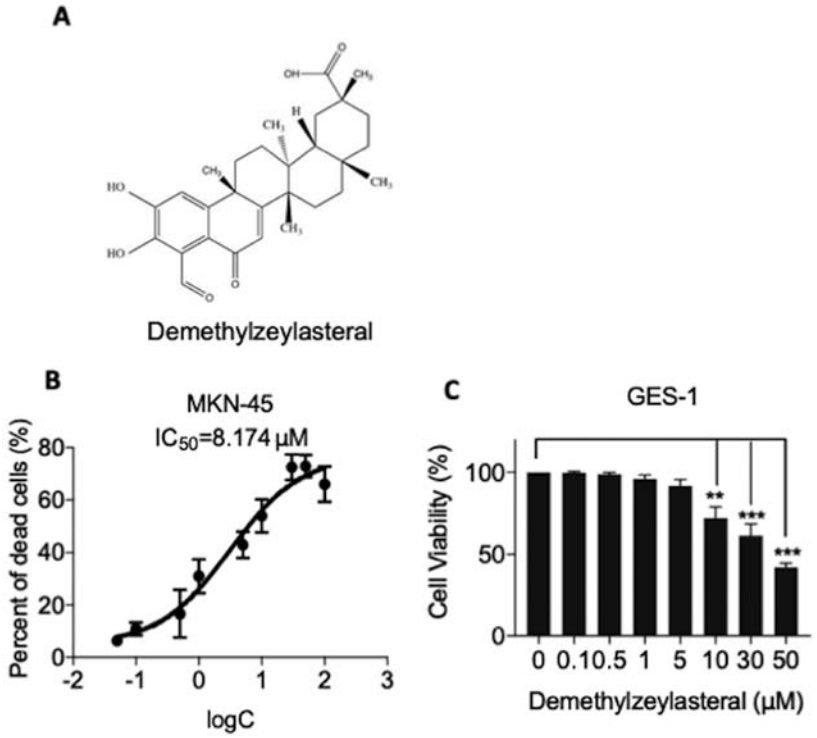

Figure 1. Chemical structure and cytotoxic effect of demethylzeylasteral. (A) Chemical structure of demethylzeylasteral (molecular weight: 480.59 g/mol). (B) CCK-8 assay was used to quantitatively analyze the cell death rate of MKN-45 cells treated with various indicated concentrations of demethylzeylasteral. The X-title was logarithm of the concentrations. (C) GES-1 cells were treated with demethylzeylasteral (DMSO: 0.1, 0.5, 1, 5, 10, 30 and 50 μM), and cell viability was detected using a CCK-8 assay. **P<0.01 and ***P<0.001, compared with the DMSO group. CCK-8, Cell Counting Kit-8; DMSO, dimethyl sulfoxide.

(BD FACSVerse^{TM}; BD Biosciences). FlowJo 7.6 computer software (BD Biosciences) was used to analyze the data.

Western blot analysis. Protein expression was detected using western blot analysis. MKN-45 cells were seeded onto 6-well plates (1x10^6 cells/well) and treated with 2 μl DMSO or demethylzeylasteral at different concentrations for 24 h at 37˚C. Following treatment, MKN-45 cells were washed twice with cold PBS, mixed with the loading buffer in each well, and then heated at 99˚C for 10 min. Protein lysates were separated by electrophoresis on 12 or 15% SDS-PAGE, and then transferred onto nitrocellulose membranes. After being blocked with fresh 5% skimmed milk at room temperature for 2 h, the membranes were incubated with primary antibodies (Cell Signaling Technology, Inc.; 1:1,000) against Bax (cat. no. 5023), cleaved PARP (cat. no. 5625), cleaved caspase-9 (cat. no. 52873), cleaved caspase-3 (cat. no. 9669), Bcl-2 (cat. no. 15071), c-Myc (cat. no. 13987), GSK-3β (cat. no. 9315), p-GSK-3β (Ser9; cat. no. 5558), ERK1/2 (cat. no. 4695), p-ERK1/2 (cat. no. 4376), Akt (cat. no. 4685), p-Akt (cat. no. 9563) and β-actin (cat. no. 8457) at 4˚C overnight. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies against rabbit and mice at room temperature for 2 h. The target proteins in the membrane were detected and visualized using the Chemiluminescence Luminol kit. ImageJ software (National Institutes of Health) was used to measure the intensity of the bands.

Statistical analysis. Demethylzeylasteral structure was drawn using ChemDraw Professional 16.0 software (PerkinElmer, Inc.). All statistical data are expressed as the mean ± standard error of the mean, and all experiments were repeated at least three times. GraphPad Prism 7.0 was used to perform Student's t-tests or one-way analysis of variance with a Dunnett's post hoc test to analyze the significance of the results. P<0.05 was considered to indicate a statistically significant difference.

Results

Demethylzeylasteral has cytotoxic effects on human gastric cancer cells. The chemical structure of demethylzeylasteral was depicted in Fig. 1A. CCK-8 assay was applied to test whether demethylzeylasteral has a cytotoxic effect on human gastric cancer MKN-45 cells. As shown in Fig. 1B, demethylzeylasteral exhibited a concentration-dependent cytotoxic effect on MKN-45 cells. Additionally, the 50% inhibitory concentration (IC_{50}) values were found to be 8.174 μM for the MKN-45 cells. In the experiments, human gastric mucosa GES-1 cells were included as a control. As shown in Fig. 1C, the CCK-8 assay revealed that demethylzeylasteral at concentrations below 10 μM (0.1, 0.5, 1 or 5 μM) had no significant influence on the survival rate of GES-1 cells, while cytotoxicity was evident in the control cells treated with 10 μM or higher concentrations of demethylzeylasteral (10, 30 or 50 μM).

Demethylzeylasteral inhibits gastric cancer cell proliferation. In the present study, MKN-45 cells were treated with various concentrations of demethylzeylasteral for 1 week. As shown in Fig. 2A, the treatment decreased colony formation in all tested gastric cancer cells. Furthermore, quantitative analysis of the colonies indicated that demethylzeylasteral decreased the proliferation of gastric cancer cells in a dose-dependent manner.

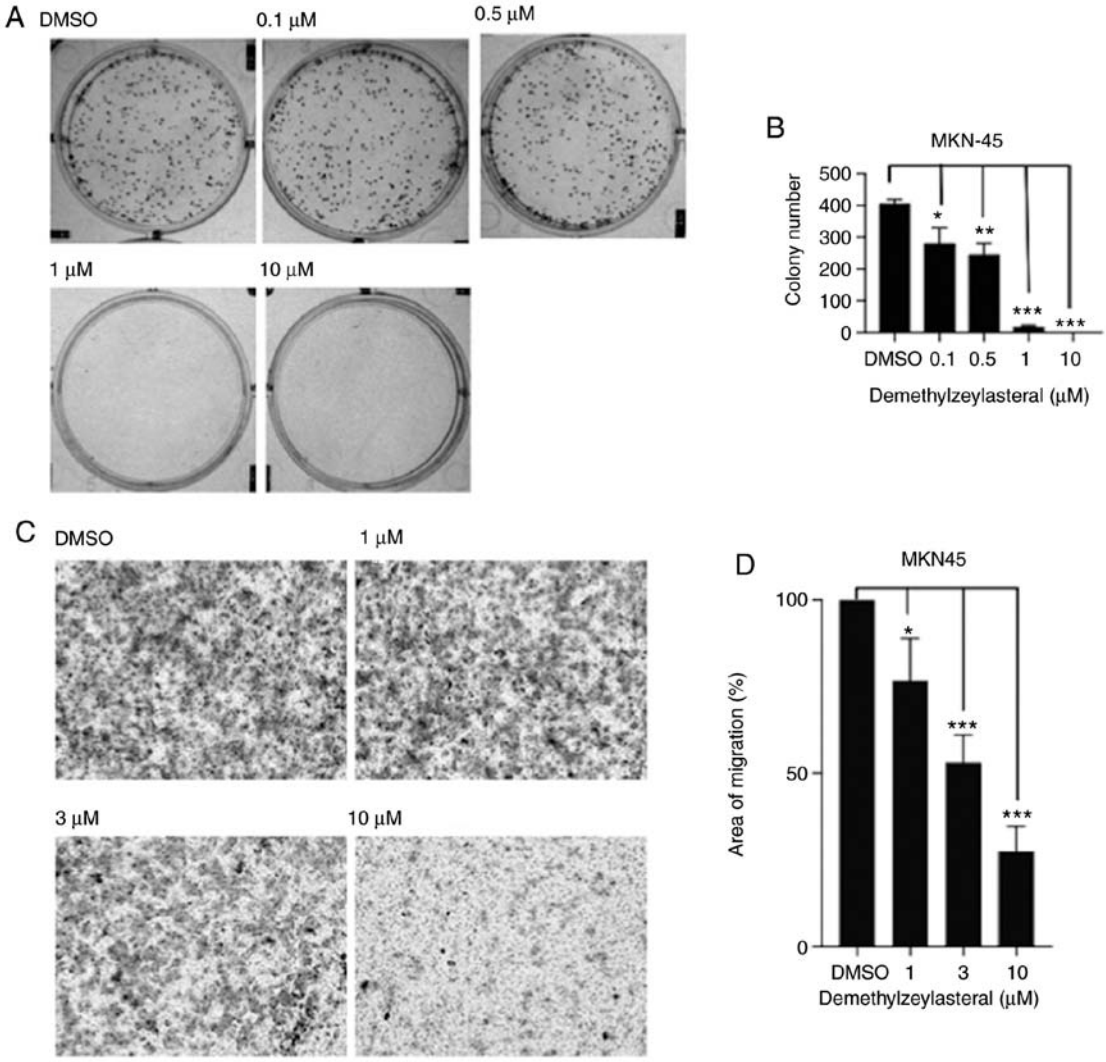

Figure 2. Anti-proliferative and migration inhibition effects of demethylzeylasteral on MKN-45 cells. (A) Representative images of colonies on MKN-45 cells. (B) Quantification of the colony formation on the cancer cells was performed. Note that histogram data on the cells treated with 10 μM demethylzeylasteral were minimal due to the formation of few colonies. (C) Images of MKN-45 cell migration in the Transwell assay. MKN-45 cells were treated with DMSO or various concentrations of demethylzeylasteral for 24 h. (D) Quantitative analysis of cell migration. P<0.05 was considered to indicate a statistically significant difference. *P<0.05, **P<0.01 and ***P<0.001, compared with the DMSO group. DMSO, dimethyl sulfoxide.

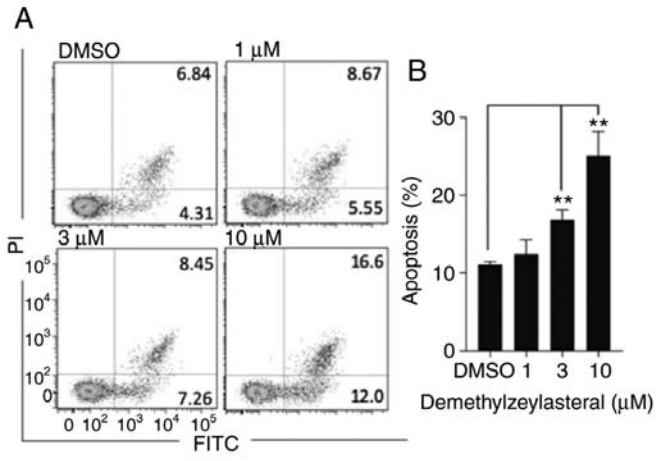

Figure 3. Demethylzeylasteral induces the apoptosis of MKN-45 cells. (A) Flow cytometric analysis of the cancer cells treated with DMSO or different concentrations of demethylzeylasteral for 24 h. (B) Quantitative analysis of the apoptotic cells. P<0.05 was considered to indicate a statistically significant difference. **P<0.01, compared with the DMSO group. DMSO, dimethyl sulfoxide.

Demethylzeylasteral suppresses cancer cell migration. The effects of demethylzeylasteral on the migration of human gastric cancer cells were investigated using an *in vitro* Transwell assay. As shown in Fig. 2C, a significant decrease in the number of cells migrating to the lower surface of the filter was observed in the group of MKN-45 cells treated with demethylzeylasteral, compared with that in the DMSO-treated group. Further statistical analysis revealed that treatment with demethylzeylasteral at various concentrations suppressed the migration of MKN-45 cells in a dose-dependent manner (Fig. 2D).

Demethylzeylasteral induces apoptosis of MKN-45 cells. It has been reported that numerous compounds inhibit tumor cell growth by inducing apoptosis (18). To determine whether the anti-proliferative effect of demethylzeylasteral is associated with apoptosis, Annexin V-FITC/PI double staining and flow cytometry were used to detect the number of apoptotic cells. As shown in Fig. 3A and B, compared with the control group,

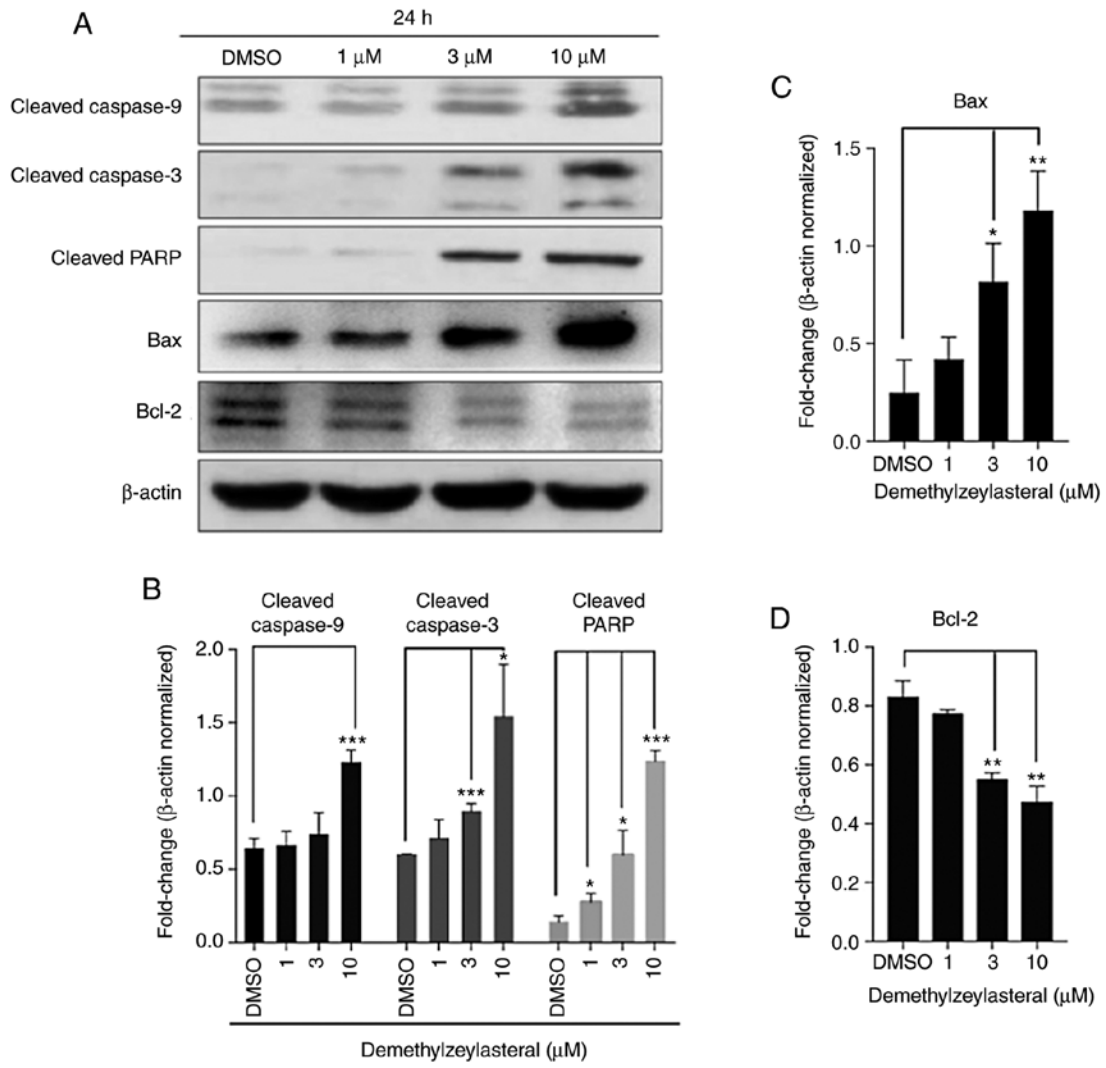

Figure 4. Demethylzeylasteral increases the expression of Bax, Bcl-2, cleaved Caspase-9, cleaved Caspase-3 and cleaved PARP. (A) The expression of Bax, Bcl-2, cleaved Caspase-9, cleaved Caspase-3, and cleaved PARP in MKN-45 cells treated with DMSO or various concentrations of demethylzeylasteral. β-actin was used as a loading control. (B-D) ImageJ software-based quantitative analysis of cleaved PARP, cleaved Caspase-9, cleaved Caspase-3, Bcl-2 and Bax. P<0.05 was considered to indicate a statistically significant difference. *P<0.05, **P<0.01, and ***P<0.001 compared with the DMSO group. DMSO, dimethyl sulfoxide.

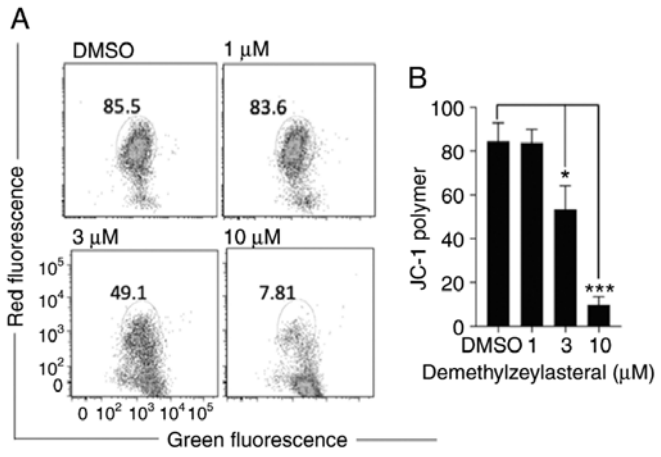

Figure 5. Demethylzeylasteral decreased the MMP in MKN-45 cells. (A) Flow cytometry of MMP changes in MKN-45 cells incubated with DMSO or different concentrations of demethylzeylasteral for 24 h. (B) The percentage of polymer was calculated for determining the quantitative change of MMP. *P<0.05 and ***P<0.001, compared with the DMSO group. MMP, mitochondrial membrane potential; DMSO, dimethyl sulfoxide.

treatment with demethylzeylasteral at various concentrations for 24 h resulted in a significant increase in the rate of apoptosis from 11.15% (DMSO) to 28.6% (10 μM). Notably, the number of apoptotic cells was gradually increased along with increasing concentrations of demethylzeylasteral (Fig. 3B). Furthermore, western blot analysis revealed that markedly upregulated expression of pro-apoptotic proteins, including Bax, cleaved caspase-9, cleaved caspase-3 and cleaved PARP, as well as downregulated expression of an anti-apoptotic protein, Bcl-2, were detected in MKN-45 cells treated with demethylzeylasteral (Fig. 4). These observations indicated that demethylzeylasteral may induce apoptosis of MKN-45 cells.

Demethylzeylasteral affects the MMP in MKN-45 cells. It has been shown that the cleavage of caspase-3, and caspase-9 and PARP is increased during the mitochondria-dependent apoptosis (19). JC-1 staining was used to investigate whether demethylzeylasteral promotes apoptosis via the mitochondrial-dependent pathway. As shown in Fig. 5A and B, the

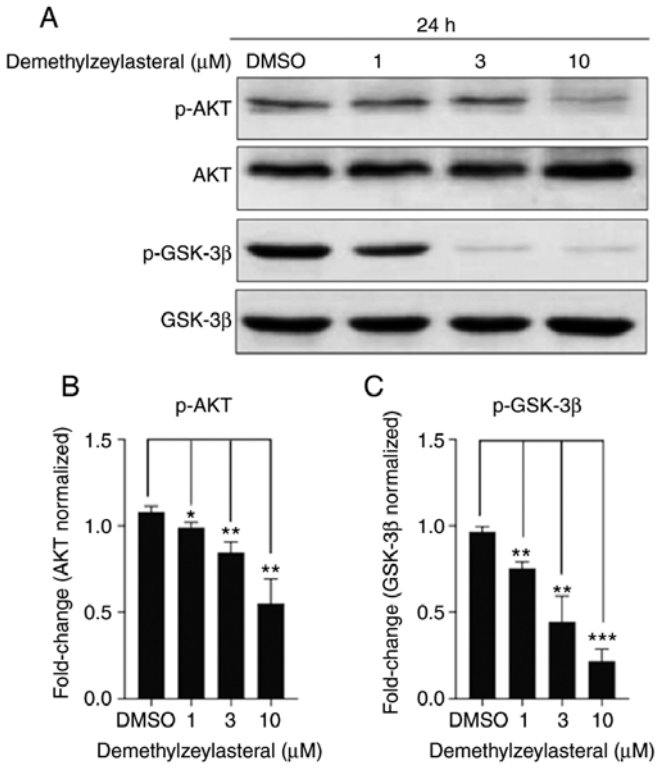

Figure 6. Demethylzeylasteral downregulates the Akt/GSK-3β pathway. (A) The expression of Akt, p-Akt, GSK-3β and p-GSK3β (ser9) in MKN-45 cells incubated with DMSO or demethylzeylasteral at different concentrations for 24 h was detected by Western blot analysis. (B and C) ImageJ software-based quantitative analysis of phosphorylation of Akt and GSK-3β. P<0.05 was considered to indicate a statistically significant difference. *P<0.05, **P<0.01 and ***P<0.001, compared with the DMSO group. DMSO, dimethyl sulfoxide; p-, phosphorylated.

JC-1 polymer was significantly decreased in MKN-45 cells treated with demethylzeylasteral at various indicated concentrations for 24 h. Furthermore, the change in JC-1 from red to green fluorescence indicated a loss of the MMP (Fig. 5A). Taken together, these results suggested that demethylzeylasteral-induced apoptosis in the cancer cells may involve the mitochondria-dependent pathway.

Demethylzeylasteral treatment downregulates Akt/GSK3β pathway. Akt is a canonical oncogenic kinase serving a prominent role in tumor progression (20). To improve understanding of the molecular mechanism underlying the antitumor effect of demethylzeylasteral on the cancer cells, the levels of total Akt, total GSK-3β, p-Akt and p-GSK-3β (ser9) in MKN-45 cells treated with demethylzeylasteral were investigated. As depicted in Fig. 6A, treatment with increasing concentrations of demethylzeylasteral led to a stepwise decrease in the levels of p-Akt and p-GSK-3β, while the levels of total Akt and GSK-3β remained unchanged. Quantitative analysis revealed a dose-dependent decrease in the levels of p-Akt and p-GSK-3β (Fig. 6B and C).

Demethylzeylasteral attenuates the ERK1/2 pathway. It has been documented that the activation of the MAPK pathway contributes toward the development of gastric cancer. To clarify whether the anticancer properties of demethylzeylasteral are associated with MAPK signaling pathways in gastric cancer cells, the expression of JNK, ERK1/2 and p38 protein in demethylzeylasteral-treated cells was measured using western blot analysis. The analysis revealed that, compared with the DMSO-treated cells, the level of p-ERK1/2 was decreased in the cancer cells treated with demethylzeylasteral, while no significant change in the levels of phosphorylated-JNK and phosphorylated-p38 was detected in the cells treated with various concentrations of demethylzeylasteral (Fig. 7). ERK1/2 serves a pro-tumorigenic role in numerous cancer types and has hundreds of substrates (21), some of which control cell growth, differentiation, survival and death by regulating the phosphorylation and activation of transcription factors. Given that c-Myc is a downstream target of ERK1/2 (22,23), the present study aimed to analyze the expression of c-Myc protein in MKN-45 cells incubated with demethylzeylasteral. As shown in Fig. 7A, treatment with demethylzeylasteral decreased the expression of c-Myc, while downregulating p-ERK1/2 in the cancer cells. Taken together, these data suggested that the ERK pathway may be involved in demethylzeylasteral-mediated cell proliferation and apoptosis in MKN-45 cells.

Discussion

Gastric cancer is the second leading cause of cancer-associated mortality after lung cancer. Although the rate of early diagnosis has been improved, the therapeutic outcomes of gastric cancer requires improvement due to postoperative recurrence, acquired drug resistance and late metastasis (4,24). Therefore, developing novel drugs for the treatment of gastric cancer may contribute toward promoting the therapeutic outcomes. Demethylzeylasteral is a natural compound from tripterygium wilfordii, which possesses multiple pharmacological effects. Although this compound has been shown to be associated with breast cancer, glioma and malignant melanoma, determining whether it has a broad-spectrum antitumor effect requires further investigation. The present study aimed to investigate the potential anticancer effect of demethylzeylasteral on gastric cancer cells.

The present study investigated the anti-proliferative activity of demethylzeylasteral by using gastric cancer MKN-45 cell line. The CCK-8 assay demonstrated that demethylzeylasteral had a dose-dependent cytotoxic effect on cancer MKN-45 cells. Demethylzeylasteral was found to downregulate the Akt/GSK-3β pathway, as well as the ERK1/2 pathway. Furthermore, it was found that treatment with demethylzeylasteral decreased MMP, while downregulating Bcl-2 expression and upregulating Bax, cleaved caspase-9, cleaved caspase-3 and cleaved PARP expression.

Apoptosis or programmed cell death is mediated by the endogenous mitochondrial pathway and the exogenous death receptor pathway (25). The complex mechanism involves numerous signaling pathways (6); among them, the intrinsic pathway of mitochondria-induced apoptosis is dependent on the expression of a series of proteins, including the Bcl-2 family, which contains anti-apoptotic and pro-apoptotic proteins. Bcl-2 family proteins mediate the activation of cysteine aspartic acid specific proteases (caspases) for inducing apoptosis (26-28). Caspases are the core of apoptosis mechanism, as they act as both the promoters and the executors of

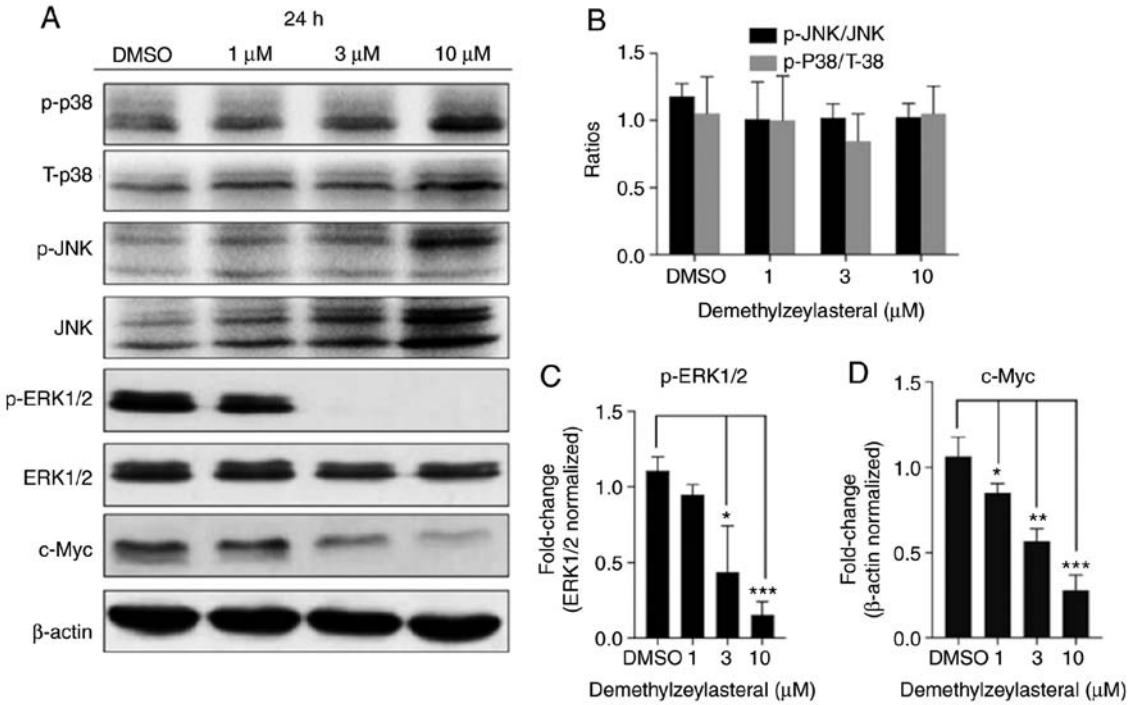

Figure 7. Demethylzeylasteral attenuates the ERK1/2 pathway. (A) Western blot analysis of phosphorylation of p38, JNK and ERK1/2, as well as c-Myc expression in MKN-45 cells treated with DMSO or demethylzeylasteral at various concentrations. β-actin was applied as a loading control. (B-D) ImageJ software-based quantitative analysis of p-p38, p-JNK, p-ERK1/2 and c-Myc expression. $P<0.05$ was considered to indicate a statistically significant difference. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with the DMSO group. DMSO, dimethyl sulfoxide; p-, phosphorylated.

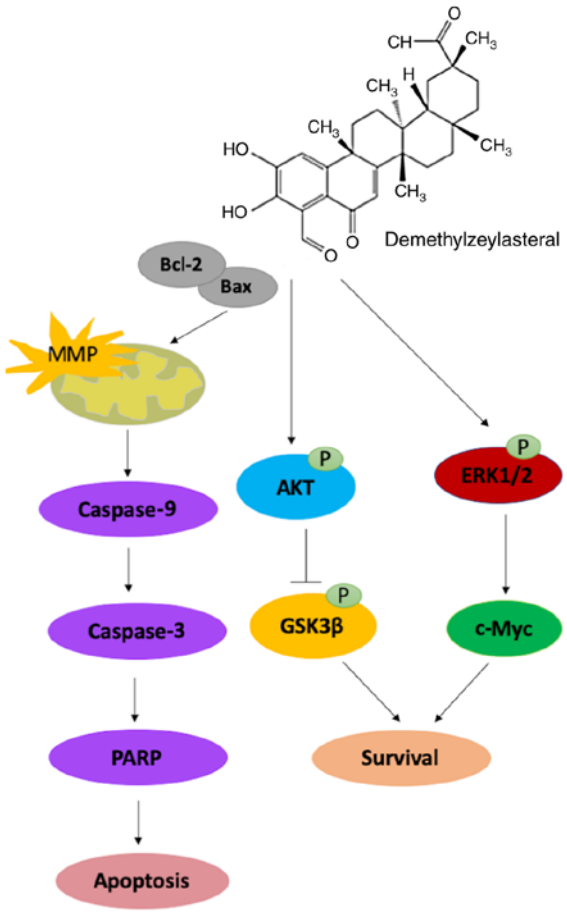

Figure 8. Schematic representation of the proposed mechanism underlying the antitumor effect of demethylzeylasteral on human gastric cancer cells. MMP, mitochondrial membrane potential.

cell death (29). Once activated, the initiator caspases will cleave and activate executioner caspases, which subsequently perform critical cleavage on specific cell substrates, ultimately leading to apoptosis (30). Activated Caspase-9 in the endogenous mitochondria-dependent pathway in turn results in the activation of Caspase-3 (31). Caspase-3 is a key component in the caspase-dependent apoptosis pathway, which triggers the cleavage of downstream substrate PARP (32), leading to chromatin lysis and apoptosis (18). In the present study, treatment of MKN-45 cells with demethylzeylasteral caused an upregulation in the levels of cleaved Caspase-9, cleaved Caspase-3 and cleaved PARP; a decreased expression of anti-apoptotic protein Bcl-2; increased expression of pro-apoptotic protein Bax; and disruption of MMP. All these data suggested that demethylzeylasteral-elicited apoptosis in MKN-45 cells may involve the activation of the caspase cascade in the endogenous death pathway (mitochondrial pathway).

Akt, a serine/threonine kinase, is highly amplified in gastric cancer and regulates numerous biological and pathological processes, including apoptosis, cell proliferation and glucose usage (33,34). Apatinib is known to be significant for the treatment of advanced gastric cancer (35). Compound Astragalus polysaccharide (AsPs) works with apatinib against gastric cancer by inhibiting the Akt pathway in AGS cells (36). In addition, *helicobacter pylori* (HP) has been highlighted due to its association with and involvement in the occurrence of gastric cancer associated with the Akt/GSK-3β signaling pathway (37). Additionally, clinical studies have demonstrated that Akt phosphorylation is involved in tumor invasion, and p-Akt status may be associated with early recurrence and poor prognosis (38). Akt promotes survival and cell cycle

by phosphorylating cellular proteins, including GSK-3α and GSK-3β (39); GSK-3α and GSK-3β constitute two main isoforms of GSK-3, a multifunctional serine/threonine protein kinase. It has been reported that GSK-3 is regulated by activated Akt, and activated GSK-3 (non-phosphorylated state) regulates cell cycle and apoptosis (40). It is worth noting that GSK-3β is inactivated once phosphorylated on serine 9. Multiple studies have demonstrated that Dioscin, β-sitosterol and Isobavachalcone, respectively, induced apoptosis of osteosarcoma, pancreatic cancer and colon cancer cells by downregulating Akt/GSK-3β pathway (41-43). The present study demonstrated that the treatment of MKN-45 cells with demethylzeylasteral in induced cell growth inhibition and apoptosis, while suppressing the Akt/GSK-3β pathway. These findings suggested that the anti-proliferative and apoptosis-inducing effects of demethylzeylasteral on gastric cancer cells are associated with the Akt/GSK-3β signaling pathway.

The MAPK signaling pathway includes JNK, ERK and p38, and serves a considerable role in cell proliferation, survival and apoptosis (44). The present study demonstrated that significantly decreased p-ERK1/2, but minor changes in the phosphorylation of JNK or p38, were detected in the cancer cells treated with demethylzeylasteral. ERK 1/2 is a key modulator of cell proliferation, and inhibitors of the ERK pathway are currently used as potential anticancer agents in clinical trials (45). Furthermore, ERK1/2 phosphorylation-targeting compounds were found to serve a vital role in the survival of gastric cancer. Wang *et al* (46) reported that 1,4-naphthoquinone derivatives evoked apoptosis of gastric cancer cells by downregulating the ERK pathway (46). An extract from Triptolide has been identified to have antitumor effects on gastric cancer cells (47). Furthermore, recent studies have shown that Triptolide may prevent the proliferation and metastasis of esophageal cancer cells via the ERK1/2 pathway (48). The aforementioned two compounds differ from demethylzeylasteral in chemical properties and are greatly limited in clinical application due to their undesirable side effects. Once translocated to the nucleus, p-ERK1/2 activates multiple transcription factors, including CREB, NF-κB and c-Myc, thereby regulating various cellular processes, including cell survival and proliferation (22,23). The present study observed a dose-dependent inhibitory effect of demethylzeylasteral on the expression of p-ERK 1/2 in cancer cells. Meanwhile, treatment with demethylzeylasteral significantly decreased the expression level of c-Myc. We hypothesize that demethylzeylasteral inhibits gastric cancer cell proliferation and induces apoptosis of cancer cells by modulating the ERK1/2 pathway and downregulating c-Myc expression.

Toxicity has long been a key factor limiting the clinical application of novel research drugs. The growing advancement in technology suggests that the toxicity of demethylzeylasteral may be decreased through either increasing its solubility via modification of the chemical structure or administration combined with other chemotherapy drugs. More importantly, structural and chemical modification of demethylzeylasteral will enable more *in vivo* experiments to be conducted in future studies. Therefore, decreased toxicity may make it possible for demethylzeylasteral to become a novel drug for the treatment of gastric cancer.

In conclusion, the results of the present study suggested that demethylzeylasteral inhibited the proliferation and migration of gastric cancer cells, while promoting the apoptosis of cancer cells via the mitochondria-dependent pathway. Further investigation suggested that the Akt/GSK-3β and ERK1/2 signaling pathways may be involved in the demethylzeylasteral-induced antitumor effects on the cancer cells (Fig. 8). These results suggested that demethylzeylasteral has therapeutic potential for the treatment of gastric cancer.

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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

YY, ZW and FQ designed the study. YY, MZ and TH collected experimental materials and carried out the experiments. FS performed the statistical analyses. YY wrote the manuscript. FQ and ZW made critical revisions to the manuscript. ZW given final approval of the version to be published. All authors read and approved the final version.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
2. Ang TL and Fock KM: Clinical epidemiology of gastric cancer. Singapore Med J 55: 621-628, 2014.
3. Valastyan S and Weinberg RA: Tumor metastasis: Molecular insights and evolving paradigms. Cell 147: 275-292, 2011.
4. Park JY, von Karsa L and Herrero R: Prevention strategies for gastric cancer: A global perspective. Clin Endosc 47: 478-489, 2014.
5. Klener P Jr, Andera L, Klener P, Necas E and Zivny J: Cell death signalling pathways in the pathogenesis and therapy of haematologic malignancies: Overview of therapeutic approaches. Folia Biol (Praha) 52: 119-136, 2006.
6. Wong RS: Apoptosis in cancer: From pathogenesis to treatment. J Exp Clin Cancer Res 30: 87, 2011.
7. Bianco R, Melisi D, Ciardiello F and Tortora G: Key cancer cell signal transduction pathways as therapeutic targets. Eur J Cancer 42: 290-294, 2006.

8. Ullmannova V and Haskovec C: Gene expression during camptothecin-induced apoptosis in human myeloid leukemia cell line ML-2. Neoplasma 51: 175-180, 2004.
9. Luo C, Zhu Y, Jiang T, Lu X, Zhang W, Jing Q, Li J, Pang L, Chen K, Qiu F, *et al*: Matrine induced gastric cancer MKN45 cells apoptosis via increasing pro-apoptotic molecules of Bcl-2 family. Toxicology 229: 245-252, 2007.
10. Dai ZJ, Gao J, Ji ZZ, Wang XJ, Ren HT, Liu XX, Wu WY, Kang HF and Guan HT: Matrine induces apoptosis in gastric carcinoma cells via alteration of Fas/FasL and activation of caspase-3. J Ethnopharmacol 123: 91-96, 2009.
11. Wang Q, Xiao Y, Liu T, Yuan H and Li C: Demethylzeylasteral ameliorates inflammation in a rat model of unilateral ureteral obstruction through inhibiting activation of the NF-κB pathway. Mol Med Rep 16: 373-379, 2017.
12. Hu Q, Yang C, Wang Q, Zeng H and Qin W: Demethylzeylasteral (T-96) treatment ameliorates mice lupus nephritis accompanied by inhibiting activation of NF-κB pathway. PLoS One 10: e133724, 2015.
13. Zhao JW, Wang GH, Chen M, Cheng LH and Ji XQ: Demethylzeylasteral exhibits strong inhibition towards UDP-glucuronosyltransferase (UGT) 1A6 and 2B7. Molecules 17: 9469-9475, 2012.
14. Bai JP, Shi YL, Fang X and Shi QX: Effects of demethylzeylasteral and celastrol on spermatogenic cell Ca^{2+} channels and progesterone-induced sperm acrosome reaction. Eur J Pharmacol 464: 9-15, 2003.
15. Li L, Ji Y, Fan J, Li F, Li Y, Wu M, Cheng H and Xu C: Demethylzeylasteral (T-96) inhibits triple-negative breast cancer invasion by blocking the canonical and non-canonical TGF-β signaling pathways. Naunyn Schmiedebergs Arch Pharmacol 392: 593-603, 2019.
16. Zhang K, Fu G, Pan G, Li C, Shen L, Hu R, Zhu S, Chen Y and Cui H: Demethylzeylasteral inhibits glioma growth by regulating the miR-30e-5p/MYBL2 axis. Cell Death Dis 9: 1035, 2018.
17. Zhao Y, He J, Li J, Peng X, Wang X, Dong Z, Zhao E, Liu Y, Wu Z and Cui H: Demethylzeylasteral inhibits cell proliferation and induces apoptosis through suppressing MCL1 in melanoma cells. Cell Death Dis 8: e3133, 2017.
18. Wang Y, An R, Umanah GK, Park H, Nambiar K, Eacker SM, Kim B, Bao L, Harraz MM, Chang C, *et al*: A nuclease that mediates cell death induced by DNA damage and poly(ADP-ribose) polymerase-1. Science 354: aad6872, 2016.
19. Lee HJ, Lee HJ, Lee EO, Ko SG, Bae HS, Kim CH, Ahn KS, Lu J and Kim SH: Mitochondria-cytochrome C-caspase-9 cascade mediates isorhamnetin-induced apoptosis. Cancer Lett 270: 342-353, 2008.
20. Gao C, Yuan X, Jiang Z, Gan D, Ding L, Sun Y, Zhou J, Xu L, Liu Y and Wang G: Regulation of AKT phosphorylation by GSK3β and PTEN to control chemoresistance in breast cancer. Breast Cancer Res Treat 176: 291-301, 2019.
21. Sammons RM, Perry NA, Li Y, Cho EJ, Piserchio A, Zamora-Olivares DP, Ghose R, Kaoud TS, Debevec G, Bartholomeusz C, *et al*: A novel class of common docking domain inhibitors that prevent ERK2 activation and substrate phosphorylation. ACS Chem Biol 14: 1183-1194, 2019.
22. Chambard JC, Lefloch R, Pouyssegur J and Lenormand P: ERK implication in cell cycle regulation. Biochim Biophys Acta 1773: 1299-1310, 2007.
23. Liu F, Yang X, Geng M and Huang M: Targeting ERK, an Achilles' Heel of the MAPK pathway, in cancer therapy. Acta Pharm Sin B 8: 552-562, 2018.
24. den Hoed CM and Kuipers EJ: Gastric cancer: How can we reduce the incidence of this disease? Curr Gastroenterol Rep 18: 34, 2016.
25. Heo SK, Yun HJ, Park WH and Park SD: Emodin inhibits TNF-alpha-induced human aortic smooth-muscle cell proliferation via caspase- and mitochondrial-dependent apoptosis. J Cell Biochem 105: 70-80, 2008.
26. Guerra F, Arbini AA and Moro L: Mitochondria and cancer chemoresistance. Biochim Biophys Acta Bioenerg 1858: 686-699, 2017.
27. Akl H, Vervloessem T, Kiviluoto S, Bittremieux M, Parys JB, De Smedt H and Bultynck G: A dual role for the anti-apoptotic Bcl-2 protein in cancer: Mitochondria versus endoplasmic reticulum. Biochim Biophys Acta 1843: 2240-2252, 2014.
28. Birkinshaw RW and Czabotar PE: The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation. Semin Cell Dev Biol 72: 152-162, 2017.
29. Van Opdenbosch N and Lamkanfi M: Caspases in cell death, inflammation, and disease. Immunity 50: 1352-1364, 2019.
30. Stennicke HR and Salvesen GS: Caspases-controlling intracellular signals by protease zymogen activation. Biochim Biophys Acta 1477: 299-306, 2000.
31. Hill MM, Adrain C and Martin SJ: Portrait of a killer: The mitochondrial apoptosome emerges from the shadows. Mol Interv 3: 19-26, 2003.
32. Bhola PD and Letai A: Mitochondria-Judges and executioners of cell death sentences. Mol Cell 61: 695-704, 2016.
33. Revathidevi S and Munirajan AK: Akt in cancer: Mediator and more. Semin Cancer Biol 59: 80-91, 2019.
34. Staal PS: Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: Amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci USA 84: 5034-5037, 1987.
35. Roviello G, Ravelli A, Fiaschi AI, Cappelletti MR, Gobbi A, Senti C, Zanotti L, Polom K, Reynolds AR, Fox SB and Generali D: Apatinib for the treatment of gastric cancer. Expert Rev Gastroenterol Hepatol 10: 887-892, 2016.
36. Wu J, Yu J, Wang J, Zhang C, Shang K, Yao X and Cao B: Astragalus polysaccharide enhanced antitumor effects of Apatinib in gastric cancer AGS cells by inhibiting AKT signalling pathway. Biomed Pharmacother 100: 176-183, 2018.
37. Geng W and Zhang HY: Research on the mechanism of HP mediated PI3K/AKT/GSK3β pathways in gastric cancer. Eur Rev Med Pharmacol Sci 21 (3 Suppl): S33-S37, 2017.
38. Nakanishi K, Sakamoto M, Yamasaki S, Todo S and Hirohashi S: Akt phosphorylation is a risk factor for early disease recurrence and poor prognosis in hepatocellular carcinoma. Cancer 103: 307-312, 2005.
39. Manning BD and Cantley LC: AKT/PKB signaling: Navigating downstream. Cell 129: 1261-1274, 2007.
40. Namba T, Kodama R, Moritomo S, Hoshino T and Mizushima T: Zidovudine, an anti-viral drug, resensitizes gemcitabine-resistant pancreatic cancer cells to gemcitabine by inhibition of the Akt-GSK3β-Snail pathway. Cell Death Dis 6: e1795, 2015.
41. Cao ZQ, Wang XX, Lu L, Xu JW, Li XB, Zhang GR, Ma ZJ, Shi AC, Wang Y and Song YJ: β-sitosterol and gemcitabine exhibit synergistic anti-pancreatic cancer activity by modulating apoptosis and inhibiting epithelial-mesenchymal transition by deactivating Akt/GSK-3β signaling. Front Pharmacol 9: 1525, 2019.
42. Li Y, Qin X, Li P, Zhang H, Lin T, Miao Z and Ma S: Isobavachalcone isolated from Psoralea corylifolia inhibits cell proliferation and induces apoptosis via inhibiting the AKT/GSK-3β/β-catenin pathway in colorectal cancer cells. Drug Des Devel Ther 13: 1449-1460, 2019.
43. Liu W, Zhao Z, Wang Y, Li W, Su Q, Jia Q, Zhang J, Zhang X, Shen J and Yin J: Dioscin inhibits stem-cell-like properties and tumor growth of osteosarcoma through Akt/GSK3/β-catenin signaling pathway. Cell Death Dis 9: 343, 2018.
44. Roux PP and Blenis J: ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68: 320-344, 2004.
45. Kohno M and Pouyssegur J: Pharmacological inhibitors of the ERK signaling pathway: Application as anticancer drugs. Prog Cell Cycle Res 5: 219-224, 2003.
46. Wang H, Luo YH, Shen GN, Piao XJ, Xu WT, Zhang Y, Wang JR, Feng YC, Li JQ, Zhang Y, *et al*: Two novel 1,4-naphthoquinone derivatives induce human gastric cancer cell apoptosis and cell cycle arrest by regulating reactive oxygen species-mediated MAPK/Akt/STAT3 signaling pathways. Mol Med Rep 20: 2571-2582, 2019.
47. Chang HJ, Kim MH, Baek MK, Park JS, Chung IJ, Shin BA, Ahn BW and Jung YD: Triptolide inhibits tumor promoter-induced uPAR expression via blocking NF-kappaB signaling in human gastric AGS cells. Anticancer Res 27: 3411-3417, 2007.
48. Yanchun M, Yi W, Lu W, Yu Q, Jian Y, Pengzhou K, Ting Y, Hongyi L, Fang W, Xiaolong C, *et al*: Triptolide prevents proliferation and migration of esophageal squamous cell cancer via MAPK/ERK signaling pathway. Eur J Pharmacol 851: 43-51, 2019.