Alpinetin promotes Bax translocation, induces apoptosis through the mitochondrial pathway and arrests human gastric cancer cells at the G_2/M phase

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Abstract. Alpinetin is a natural flavonoid widely distributed in *Zingiberaceae*. Previous studies have demonstrated that alpinetin markedly inhibits tumour growth. However, the molecular mechanisms underlying the antitumour effects of alpinetin are unclear. Bcl-2-associated X protein (Bax) translocation is known to activate the mitochondrial-dependent endogenous apoptosis pathway. The aim of the current study was to investigate the roles of Bax and the mitochondrial pathway during alpinetin-induced gastric cancer cell apoptosis and the effects of alpinetin on the cell cycle. Human gastric cancer cells were treated with various doses of alpinetin and an MTT assay was performed to measure cell viability, flow cytometry to measure the apoptotic rate, changes in the cell cycle and mitochondrial membrane potential and western blot analysis to detect the expression levels of relevant proteins. Results demonstrate that alpinetin induces apoptosis in human gastric cancer cells in a dose- and time-dependent manner. During the early stages of apoptosis, alpinetin may alter mitochondrial membrane potential leading to release of cytochrome c from mitochondria, activation of caspase family members and ultimately apoptosis of human gastric cancer cells. Results of the present study indicate that alpinetin-induced human gastric cancer cell apoptosis is associated with the mitochondrial pathway.

Introduction

Gastric cancer is one of the most common malignancies in the world, and ~800,000 people are diagnosed with this disease each year (1). Gastric cancer has a high mortality rate and is the second leading cause of cancer mortality (2). The average five-year survival rate for gastric cancer is <10% (3). Neoadjuvant therapy, radiotherapy and additional drug therapies coupled with surgery have greatly improved the survival rate of gastric cancer survival rate (4). In Western countries, preoperative and postoperative adjuvant chemotherapies have become the standard regimen for gastric cancer treatment and in Asia, postoperative conventional chemotherapy is also performed for gastric cancer treatment, indicating the importance of postoperative drug treatment for gastric cancer (5). Conventional chemotherapeutic treatments include 5-fluorouracil-based combination regimens containing mitomycin C or anthracycline antibiotics. Although this treatment regimen has been demonstrated to significantly reduce the tumour recurrence rate, a number of disadvantages, including expensive treatment costs and serious side effects, have prevented many patients from benefiting from this chemotherapy. Therefore, the identification of a more economical drug associated with fewer toxic side effects for gastric cancer treatment has become the focus of research in recent years.

Flavonoids have long been utilised in traditional medicine and are associated with low toxicity. In addition, previous studies have identified these compounts to exhibit significant antitumour effects (6-8). Alpinetin is a natural flavonoid, primarily found in *Zingiberaceae,* including turmeric, cardamom and *radix curcumae* (9). A number of studies have demonstrated that alpinetin has marked antitumour and inhibitory effects on tumour cell proliferation and inhibits the growth of numerous types of tumour cells, including breast, colon, lung, cervical and liver cancer cells (10-12). However, the inhibitory effects of alpinetin on human gastric cancer cell growth has not been investigated. In addition, the mechanisms by which alpinetin mediates its antitumourigenic effects remain poorly understood.

In the present study, the cytotoxic and pro-apoptotic effects of alpinetin in human gastric cancer cells were investigated. Furthermore, the relationship between alpinetin-induced apoptosis in human gastric cancer cells and the mitochondrial apoptosis pathway was investigated.

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Materials and methods

Materials and chemicals. Alpinetin (≥98% purity) was obtained from the National Institute for Food and Drug control (Beijing, China). Propidium iodide (PI), 5,5',6,6'-tetrachloro-1, 1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). The Annexin V-FITC/PI kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. Human gastric cell lines AGS (gastric adenocarcinoma) and N87 (gastric cancer) were purchased from American Type Culture Collection (Manassas, VA, USA). Following passaging, AGS cells were cultured in F-12K medium containing 10% foetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan UT, USA), 100 U/ml penicillin and streptomycin (Gibco-BRL, Carlsbad, CA, USA) and N87 cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and streptomycin. Cells were cultured in incubators containing 5% CO_2 and 95% O_2 at 37˚C.

Cell viability. Cells ($1x10^5$ cells/ml) were seeded in 96-well plates. Following adherence, cells were treated with various doses of alpinetin with six replicates for each concentration. A negative control group without drugs was also established. Cells were placed in an incubator with a 5% CO_2 atmosphere and incubated for 24, 48 and 72 h prior to the colorimetric reaction. MTT solution (20 μl; 5 mg/ml) was added to each well and the plate was incubated for 4 h in a CO_2 atmosphere incubator. Following incubation, culture medium was removed and 150 μl dimethylsulfoxide (DMSO) was added to each well and mixed by agitation at room temperature for 10 min. The optical density (OD; A_{570} nm) of each well was measured using a microplate reader.

Detection of apoptosis by Annexin V-FITC/PI double staining. During the early stages of apoptosis, the cell membrane loses its symmetry, and phosphatidylserine, which is normally located in the inner leaflet of the plasma membrane, becomes exposed on the outer leaflet of the plasma membrane. Cells were collected using the trypsin digestion method and cell density was adjusted to $1x10^6$ cells/ml. Annexin V-FITC (5 μl) and PI (5 μl) were then added. Cells were stained in the dark at 4˚C for 30 min, followed by flow cytometry analysis.

Cell cycle analysis. Cells in each experimental group were collected using the trypsin digestion method, washed with phosphate-buffered saline (PBS) and fixed overnight in 70% cold ethanol at 4˚C. The ethanol was then discarded followed by a PBS wash. Cell density was adjusted to $1x10^6$ cells/ml and the final volume was 100 μl. DNAStain comprehensive dye solution (500 μl) containing 50 mg/l RNase, 100 mg/l PI and 1 ml/l Triton X-100 was added to the cells which were then placed them in the dark at room temperature for 30 min prior to flow cytometry.

Mitochondrial membrane potential detection. Cell density was adjusted to $1x10^6$ cells/ml. JC-1 dye (10 μg/ml), which was dissolved in DMSO, was added to the cells, mixed thoroughly and the cells were incubated in the dark for 30 min in an incubator at 37˚C with 5% CO_2 atmosphere followed by three PBS washes. A flow cytometer (BD Biosciences) was used for analysis. FL1-H and FL2-H represented the green and red fluorescence intensity, respectively. CellQuest analysis software was used for the quantitative analysis.

Detection of caspase activity. Detection of caspase-3 and -9 activities was performed as described previously (13). The Perkin-Elmer LS-50B fluorescence spectrophotometer (Waltham, MA, USA) was used to measure changes in fluorescence intensity at excitation and emission wavelengths of 380 and 460 nm, respectively. Alterations in caspase activity were determined by comparing caspase expression levels in the alpinetin-treated and control groups.

Mitochondria isolation, protein extraction and western blot analysis. Mitochondria separation, purification and protein extraction was performed as described previously (14). Following quantification using the bicinchoninic acid method, samples were loaded into an SDS-PAGE gel and separated. Proteins were transferred to a polyvinylidene fluoride membrane using the semi-dry method and the membrane was blocked overnight in 5% non-fat dry milk at 4˚C. Following this, the membrane was washed in Tris-buffered saline with Tween (TBST), primary antibodies were added followed by 1 h hybridisation at 37˚C and TBST washes. Secondary antibodies were then added followed by a 1 h hybridisation at 37˚C, a TBST wash, 5 min of the chromogenic reaction and autoradiography. OD values were analysed and determined using Quantity One software and the results were expressed as the ratio of the sample OD value to the OD value of the internal reference.

Statistical analysis. SPSS 16.0 software was used for statistical analysis. Values are presented as the mean ± SD. Statistical analysis was performed using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Alpinetin inhibits human gastric cancer cell proliferation. Various doses of alpinetin (0, 40, 80, 120 and 160 μM) were used to treat human gastric cancer cell lines, AGS and N87, for 24, 48 and 72 h. Cell viability was measured by MTT assay. Results demonstrate that alpinetin inhibits human gastric cancer cell proliferation in a time- and dose-dependent manner (Fig. 1). As the alpinetin concentration was increased from 0 to 160 μM, the A_{570} nm value measured for the human gastric cancer cells gradually decreased and this decrease was found to be most significant at 120 μM (IC_{50}).

Alpinetin induces apoptosis in human gastric cancer cells. To investigate whether alpinetin induces apoptosis in human gastric cancer cells, AGS cells were treated with various doses of alpinetin for 48 h and flow cytometry was used to detect apoptosis (Fig. 2). Compared with the control group, as the alpinetin dose increased, the number of apoptotic gastric cancer cells significantly increased. These results indicate that

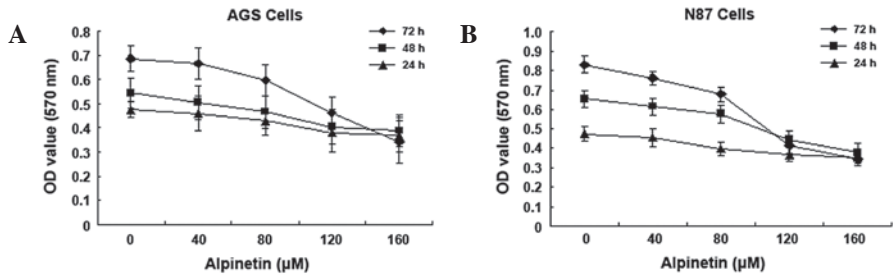

Figure 1. Alpinetin inhibited the growth of human gastric cancer cells. Following treatment of (A) AGS and (B) N87 cells for 24, 48 and 72 h with various alpinetin concentrations (0, 40, 80, 120, 160 μM), an MTT assay was used to determine cell viability. Results are representative of six independent experiments.

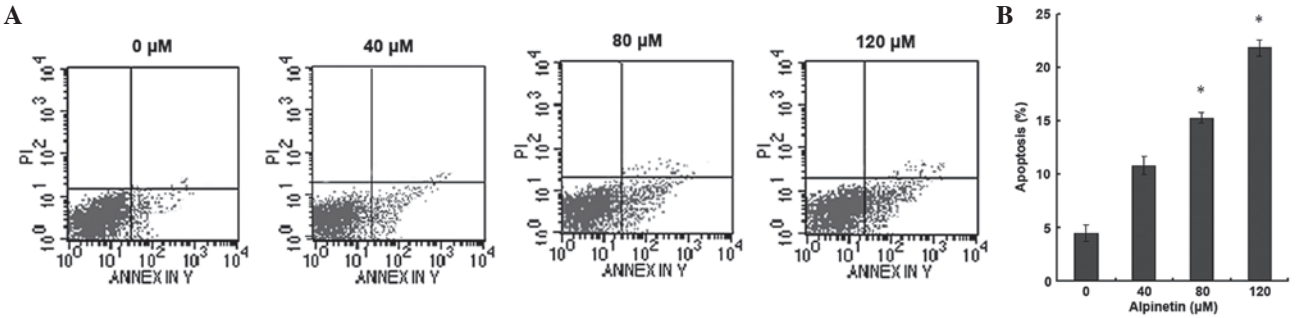

Figure 2. Alpinetin induced apoptosis in AGS cells. Cells were treated with various alpinetin doses for 48 h. (A) Flow cytometry detection of Annexin V/PI double staining. (B) Histogram demonstrating AGS cell apoptosis rate. *P<0.05, vs. control group. Data are representative of three independent experiments. PI, propidium iodide.

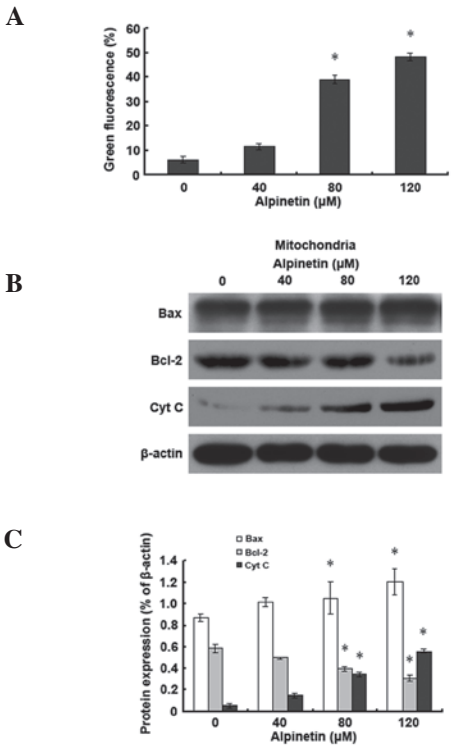

Figure 3. Alpinetin induced apoptosis in AGS cells via the mitochondrial pathway. Cells were treated with various alpinetin doses for 48 h. (A) Detection of mitochondrial membrane potential using flow cytometry staining for JC-1. (B) Western blot analysis of Bax, Bcl-2 and Cyt C protein expression levels. (C) Histogram demonstrating Bax, Bcl-2 and Cyt C protein levels. *P<0.05, vs. control group. Data are representative of three independent experiments. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Cyt C, cytochrome C.

alpinetin induces apoptosis in a dose-dependent manner and inhibits proliferation of gastric cancer cells.

Alpinetin induces apoptosis in human gastric cancer cells via the mitochondrial pathway. To further understand the molecular mechanisms of alpinetin-induced apoptosis in human gastric cancer cells, AGS cells were treated with various doses of alpinetin for 48 h. Western blot analysis was used to detect changes in levels of Bcl-2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2) and cytochrome C (Cyt C) and JC-1 staining was applied to detect changes in mitochondrial membrane potential (Fig. 3). Results demonstrated that, compared with the control group, as the alpinetin dose increased, mitochondrial Bax levels increased and Bcl-2 levels decreased in the alpinetin treatment group. Mitochondrial membrane potential was subsequently decreased and cytosolic Cyt C levels increased gradually. Observations indicate that alpinetin promotes Bax and Bcl-2 translocation, leading to reduced mitochondrial membrane potential and release of Cyt C into the cytoplasm, ultimately leading to apoptosis of human gastric cancer cells.

Alpinetin activated caspase in human gastric cancer cells. To detect the effect of alpinetin on the caspase family of apoptotic proteins, AGS cells were treated with various doses of alpinetin for 48 h and changes in caspase-3 and -9 activity were detected using a fluorescence spectrophotometer (Fig. 4). Data demonstrate that, following treatment of AGS cells with various doses of alpinetin, caspase-3 and -9 activities were significantly increased and peaked at 120 μM, indicating that

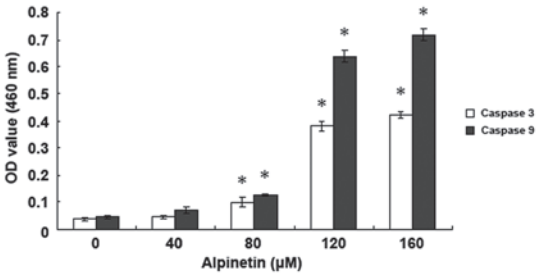

Figure 4. Alpinetin induced activation of caspase family member proteins in AGS human gastric cancer cells. Following treatment of AGS cells with various alpinetin doses for 48 h, a fluorescence spectrophotometer was used to measure changes in caspase-3 and -9 activities. *P<0.05, vs. control group. Data are representative of three independent experiments.

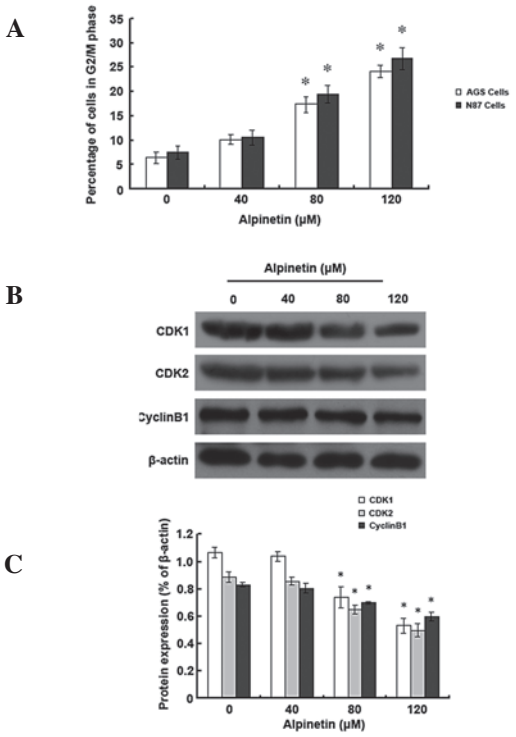

Figure 5. Alpinetin arrested human gastric cancer cells at the G_2/M phase. AGS and N87 cells were treated with various alpinetin doses for 48 h. (A) Flow cytometry analysis of cell cycle changes. (B) Western blot analysis of protein expression levels of CDK1 and 2 and cyclin B1. (C) Histogram presenting CDK1 and 2 and cyclin B1 protein levels. *P<0.05, vs. control group. Data are representative of three independent experiments. CDK, cyclin-dependent kinase.

alpinetin induces activation of caspase family members and caspase-dependent apoptosis in AGS cells.

Alpinetin induces cell cycle arrest at the G_2/M phase in human gastric cancer cells. To investigate whether alpinetin regulates cell cycle distribution of human gastric cancer cells, AGS and N87 cells were treated with various alpinetin doses for 48 h and flow cytometry was performed to detect cell cycle progression (Fig. 5). Results revealed that, as the alpinetin dose increased, the number of AGS and N87 cells entering the G_2/M phase increased and the majority of the cells were blocked at the G_2/M phase in the alpinetin-treatment group compared with the control group. Furthermore, we found that protein expression levels of cyclin-dependent kinase (CDK) 1 and 2 and cyclin B1 were significantly reduced. Our data indicate that the alpinetin-induced anti-proliferative effect may also be mediated by G_2/M phase arrest.

Discussion

Gastric cancer is a common malignancy. Each year, ~800,000 individuals are diagnosed with gastric malignancies worldwide, accounting for 9% of newly diagnosed malignancies (15). The gastric cancer mortality rate ranks second among all malignant tumours. Advances in research over the past 10 years have led to a gradual improvement in diagnosis of gastric cancer; however, the efficacy of gastric cancer treatments remains poor (16). Although surgical resection is the primary gastric cancer treatment method, it has long been recognised that malignant tumours are systemic diseases. Surgery only removes the tumour and the post-operative recurrence rate remains high. Therefore, drug therapies specifically targeting gastric cancer which aim to decrease the gastric cancer recurrence rate have become an important area of research. Although chemotherapeutic drugs have been demonstrated to reduce the recurrence rate of gastric cancer, adverse reactions in patients caused by chemotherapeutic drugs, including bone marrow suppression, cannot be ignored.

Previous studies have identified a number of features in plant-derived antitumour drugs, including diversity, fewer side effects and adverse reactions. Flavonoids have been demonstrated to exhibit anti-inflammatory, -allergic, -oxidative, -damage and -tumour effects (17,18). In recent years, studies on flavonoids have entered a new phase with these compounds revealed to inhibit proliferation of cancer cells, including breast, colon, lung, cervical and liver cancers and lymphoma cells (19-23). In addition, alpinetin has also been identified to inhibit proliferation of leukemic cells (24). However, few studies have reported the effects of alpinetin on gastric cancer cells and its underlying mechanism. In the present study, we found that alpinetin inhibits proliferation and induces apoptosis of gastric cancer cells.

At present, the occurrence of malignant tumours is considered to be associated with abnormal proliferation and decreased apoptosis of tumour cells. Therefore, it has been proposed that the growth of tumour cells may be reduced or inhibited by promoting apoptosis using a variety of methods (25,26). Previously, we found that alpinetin regulates expression of Bcl-2 family members and X-linked inhibitor of apoptosis protein, which promotes release of Cyt C and further activates apoptotic proteases that eventually affect proliferation of pancreatic cancer cells and induce apoptosis. In this study, alpinetin was identified to have significant inhibitory effects on human gastric cancer cell proliferation. Using flow cytometry, we demonstrated that alpinetin induces apoptosis in human gastric cancer cells. Apoptosis is often closely linked with the cell cycle. Previous studies have revealed that blocking tumour cells at a certain cell cycle phase using various methods induces apoptosis of tumour cells or terminates tumour cell growth (27,28). In the current study alpinetin was identified to significantly inhibit cell cycle alterations in human gastric cancer cells, arresting the cell cycle at the G_2/M phase, thereby inhibiting cell proliferation.

The induction of apoptosis is considered to be an effective antitumour method. There are two major signal transduction pathways that trigger apoptosis, the endogenous mitochondrial and the exogenous death receptor pathway. This study found that human gastric cancer cells treated for 48 h with various alpinetin doses caused Bax and Bcl-2 translocation followed by Cyt C release. These results indicate that alpinetin-induced apoptosis in human gastric cancer cells may be mediated by the mitochondrial pathway. Previous studies have demonstrated that high expression levels of Bax in patients with gastric cancer improve chemotherapy efficacy (29). Bcl-2/Bax family members are key regulatory factors in the endogenous mitochondrial apoptotic pathway (30,31). Upon stimulation with pro-apoptotic factors, Bax translocates from the cytoplasm to the mitochondrial membrane, which alters the permeability of the mitochondrial membrane and promotes release of Cyt C from the mitochondria into the cytoplasm (32). The apoptotic cascade pathway is subsequently initiated, eventually leading to apoptosis.

Due to its ability to activate apoptosis-related proteases during the apoptotic process, the activation of caspase family members is an important prerequisite for apoptosis (33). Previous studies have found that Bax translocation leads to alterations in mitochondrial membrane potential, triggering release of Cyt C and further activating caspase-9 to promote apoptosis (34). The present study demonstrates that following alpinetin treatment of human gastric cancer cells, activated caspase-3 and -9 were enhanced in a dose-dependent manner and release of Cyt C from the mitochondria to the cytoplasm was correspondingly increased. Release of Cyt C from the mitochondria into the cytoplasm activates caspase-3 and -9 and therefore is key to the apoptosis pathway (35).

In summary, results of the present study demonstrate that alpinetin inhibits human gastric cancer cell proliferation and induces apoptosis in these cells via the mitochondrial pathway in a dose-dependent manner. In addition, alpinetin arrests the cell cycle at the G_2/M phase. Therefore, alpinetin may be a potential compound for the treatment of gastric cancer in the future.

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