Butein suppresses cervical cancer growth through the PI3K/AKT/mTOR pathway

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Abstract. Cervical cancer is the second most common women carcinoma worldwide and the fourth leading cause of cancer-associated mortality in women. Butein, a bioactive flavonoid isolated from numerous native plants, has been shown to induce apoptosis and inhibits migration and invasion in numerous human cancer cells. However, to the best of our knowledge, the effect of butein on human cervical cancer cells has not been reported. The present study aimed to determine the effect of butein on cell growth, apoptosis, migration and invasion and identify the associated molecular mechanism involved using HeLa human cervical cancer cells *in vitro*, and on tumor growth in a nude mouse model. It was found that butein notably inhibited cell viability, colony formation, migration and invasion, induced cell cycle at the G2/M stage and cell apoptosis, as well as enhanced caspase-3, -8 and -9 activity in HeLa cells in a dose-dependent manner. When administered intraperitoneally, butein inhibited the tumor growth of human cervical cancer xenograft tumors in the nude mouse model. Additionally, treatment with butein significantly increased reactive oxygen species (ROS) generation and reduced the phosphorylation of PI3K, AKT and mTOR expression, which contributes to the inhibition of the tumor growth of cervical cancer and reduction of oxidative stress. These findings suggested that butein serves as a potential therapeutic agent for the treatment of cervical cancer.

Introduction

Cervical cancer is the second most common cancer among women worldwide and the fourth leading cause of cancer-associated mortality in women, accounting for 9% of total new cases of cancer and 8% of all cancer deaths. Over 85% of these mortalities occur in developing countries with approximately 250,000 women succumbing to the disease annually (1,2). Although the mortality rate has declined due to improvement in screening, diagnostic, prognostic and treatment modalities in recent years, treatments currently available including surgery, radiotherapy, and chemotherapy are often unsatisfactory, particularly for patients with advanced stage of cervical cancer (3,4). Chemotherapy drugs have been widely used for patients with advanced cervical cancer. However, many patients acquire resistance to chemotherapeutic agents, leading to treatment failures (4,5). Additionally, chemotherapy drugs exhibit high toxicity in normal tissues (4-6). Therefore, the identification of new drugs with few side-effects are required to improve the survival and quality of life of cervical cancer patients.

Natural products are suitable alternatives that can be used instead of platinum-based drugs since they are much more effective and have minimal side-effect consequences compared to synthetic drugs (7,8). Numerous natural products have been identified as promising sources of drugs for cancer prevention and treatment, based on their ability to attack multiple molecular targets (9-13). Butein (2',3,4,4'-2',4',3,4- or 3,4,2',4'-tetrahydroxychalcone, chemical structure shown in Fig. 1A), an important natural products, can be isolated in various plants including *Toxicodendron vernicifluum* (*Rhus verniciflua*), *Semecarpus anacardium* and *Dalbergia odorifera*, and has exhibited different pharmacological effects, including antioxidant, anti-inflammatory, anti-allergic and anti-angiogenic activities (14-18). Accumulating evidence has demonstrated that butein suppressed tumor growth by inhibiting cell proliferation and inducing cell apoptosis in various human cancer cells, such as colorectal carcinoma (19), lung cancer (20), melanoma (21), prostate cancer (22), leukemia (23), breast carcinoma (24), osteosarcoma cells (25), hepatocellular carcinoma (26) and neuroblastoma cells (27). Butein has recently received wide attention as a useful chemopreventive and chemotherapeutic agent due to exhibiting only minimal toxicity in normal cells (28,29). A preliminary clinical trial on the effects of flavonoids containing butein in gastric cancer patients showed that this drug was safe with good tolerability, exhibiting a marked decrease in tumor size (30). Nonetheless, to the best of our knowledge, its potential role in apoptosis and tumor inhibition, as well as the underlying anticancer mechanism in cervical cancer remains to be elucidated.

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The aim of the present study was to evaluate the potency of butein in inhibiting cervical cancer cell proliferation, colony formation, migration and invasion, inducing cell apoptosis and cell-cycle arrest *in vitro*, as well as to identify the underlying molecular mechanisms associated with anticancer activity. In addition, tumor growth ability in nude mice was detected to define the butein treatment effect in the tumorigenesis of cervical cancer *in vivo*.

Materials and methods

Reagents and antibodies. Butein was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Butein was dissolved in dimethylsulfoxide (DMSO) and stored at -20˚C until use. The concentration of DMSO used was <1% in the control and the butein-containing medium.

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Propidium iodide (PI) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA). Stock solutions of PI and MTT were prepared by dissolving 1 mg of each compound in 1 ml of phosphate-buffered saline (PBS). The solution was stored at 4˚C in the dark and used within 1 month. Caspase-3, -8 and -9 Colorimetric Activity Assay kits were purchased from R&D Systems (Minneapolis, MN, USA). Apoptotic Cells Detection kit (Annexin V-FITC/ethidium homodimer III staining) was purchased from PromoKine Systems (Heidelberg, Germany).

For western blot analysis, the antibodies used were: a mouse monoclonal anti-human Bcl2, mouse monoclonal anti-human Bax, mouse monoclonal anti-human MMP-2, and mouse monoclonal anti-human MMP-9, all purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-human AKT, mouse monoclonal anti-human phosphorylated (p)-AKT, mouse monoclonal anti-human PI3K, mouse monoclonal anti-human (p)-PI3K, mouse monoclonal anti-human mTOR, mouse monoclonal anti-human (p)-mTOR, were obtained from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-human β-actin and anti-mouse immunoglobulin G-horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines and culture. The HeLa human cervical cancer cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China). Cultured cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37˚C in a humidified atmosphere containing 5% CO_2.

Cell viability. Cell viability was determined by MTT assay as previously described (31). Briefly, HeLa cells were cultured in 96-well plates and treated with different concentrations of butein (0-100 μM) for 72 h. Cell viability was determined via an MTT assay. Absorbance at 570 nm was measured in an ELISA plate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

Colony formation assay. HeLa cells were seeded in 6-well plates at a density of $1x10^3$ cells/well. After being cultured for 24 h, the cells were treated with the indicated concentrations of butein (0, 15, 25 and 50 μM). The cells were then washed with drugs-free medium and allowed to grow for 14 days in drugs-free conditions. After 14 days, the cells were fixed in paraformaldehyde and stained with Giemsa for 15 min, and images of the colonies were captured by a digital camera (Olympus, Tokyo, Japan). The percentage of colony formation was calculated by adjusting control (untreated cells) to 100%.

Cell cycle analysis. The cells were seeded at a density of $1x10^6$ cells/well in 6-well plates in complete medium for 16 h. At the end of treatment with the indicated concentration of butein, the cells were collected and fixed with ice-cold 70% ethanol overnight at -20˚C. After centrifugation at 3,000 x g for 5 min, the cell pellets were added into 4 μg/ml PI solution containing 1% Triton X-100 and 100 μg/ml RNase at 37˚C for 30 min. The samples were analyzed by a flow cytometer (FACSCalibur), and the percentage of cell-cycle phases was quantified using ModFit LT software 2.0 (BD Biosciences, San Jose, CA, USA).

Cell apoptosis assays. The cells were plated at a density of $5x10^5$ cells/60-mm Petri dish in complete medium for 16 h, and then treated with the indicated concentrations of butein for 24 h. Apoptotic cells were assessed using an Apoptotic Cells Detection kit according to the manufacturer's instructions. The cells were collected and resuspended in 500 μl of binding buffer, and 5 μl of Annexin V-FITC and 5 μl of PI were added. The cells were cultured for 10 min at room temperature. The samples were analyzed by flow cytometer using CellQuest software (BD Biosciences).

In addition, the expression of Bax and Bcl-2 were determined by western blotting 24 h after treatment with the different concentrations of butein.

Caspase activity assay. Activities of caspase-3, -8 and -9 were detected as previously described (21). Briefly, HeLa cells were seeded in 6-well plates at a density of $4x10^5$ cells/well and cultured for 24 h, then treated with indicated concentration of butein for 2 h. The cells were washed with cold PBS and collected by centrifugation at 1,500 rpm for 5 min at 4˚C. The cells were lysed using a cell extraction buffer (BioSource International, Camarillo, CA, USA) with protease inhibitor cocktail (Sigma) and PMSF (BioSource), incubated on ice for 30 min and vortexed for 30 sec. The samples were centrifuged at 10,000 rpm for 10 min at 4˚C to remove the supernatant. The caspase-3, -8 and -9 levels in cell lysates were measured using the caspase-3, -8 and -9 colorimetric kit according to the manufacturer's instructions. The optical density was read using a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 405 nm. The relative caspase activity of the untreated group (0 μM butein treatment) was taken as 100.

Cell invasion and migration assays. The cell invasion and migration assays were carried out using Transwell inserts (Corning, Corning, NY, USA) according to the manufacturer's instructions. For the invasion assay, filters were precoated with Matrigel (BD Biosciences, Bedford, MA, USA) for 30 min. Approximately $5x10^4$ cells were placed in the upper chamber in serum-free DMEM, and 500 μl of DMEM containing 20% FBS and the indicated concentrations of butein (0, 15, 25 and

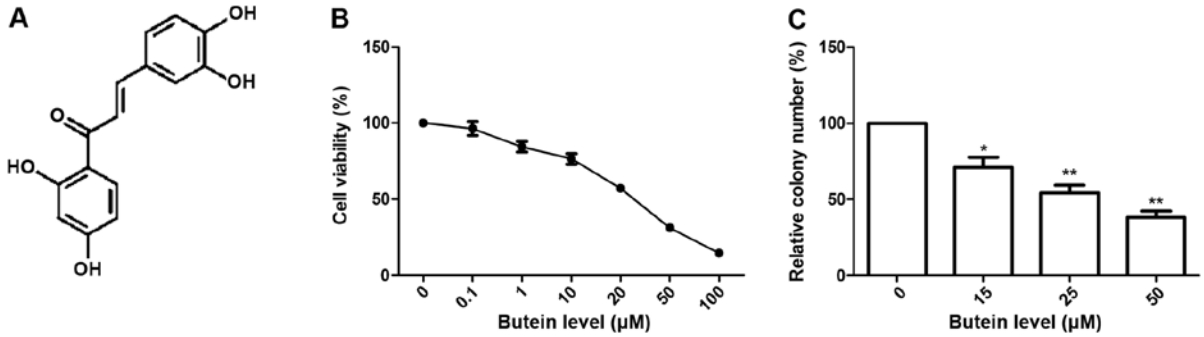

Figure 1. Butein inhibits cell viability and colony formation of HeLa cells in a dose-dependent manner. (A) Butein structure. (B) HeLa cells were incubated with different concentrations of butein (0-100 μM) for 72 h. Cell viability was examined by MTT assay. (C) Cell colony formation of HeLa cells was determined following treatment with the indicated concentrations of butein (0, 15, 25 and 50 μM). *P<0.05 and **P<0.01, vs. control (0 μM).

50 μM) were placed in the lower chamber in the invasion and migration assays. After 24 h of treatment, the cells on the upper side of the filters were mechanically removed by wiping with a cotton swab. Cells migrating to the lower surface of filter were fixed in 70% ethanol for 15 min and stained with 0.2% crystal violet for 15 min. The invaded or migrated cells were photographed and quantified by counting the number of stained cells in five randomly selected fields under a light microscope (Olympus).

Reactive oxygen species (ROS) measurement. Generation of intracellular ROS was examined by flow cytometry as previously described (32). Briefly, 5x10^4 HeLa cells were seeded in 60-mm dishes overnight, and exposed to different concentrations of butein (0, 15, 25 and 50 μM) for 1 h. The cells then were stained with 2 μM hematoxylin and eosin (H&E) or 5 μM H_2DCFDA at 37˚C for 30 min. The cells were collected and fluorescence was analyzed using a flow cytometer. The cells were also pretreated with 10 mM NAC prior to butein exposure and analysis of ROS generation.

Western blot assay. At the end of treatment, the cells were lysed in the ice-cold whole cell extract buffer containing protease inhibitors (Sigma) for 30 min, and centrifuged at 10,000 rpm for 10 min at 4˚C. The protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equal amounts of total proteins (20 μg) were subjected to electrophoresis using 8-12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% dry milk in TBS-T overnight at 4˚C and then incubated with specific primary antibody at room temperature for 2 h. After washing with TBS-T twice, the membranes were incubated with anti-mouse immunoglobulin G-horseradish peroxidase-conjugated secondary antibodies, followed by washing three times with TBS-T. The protein bands were then visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA, USA). Blots were stripped and reprobed with anti-β-actin for the loading variations. Quantity One software (Bio-Rad) was used to analyze the intensity of bands on X-ray film by semi-quantification.

Xenograft mouse model. Twenty female BALB/c nude mice (6 weeks old) were obtained from the Experiments Animal Center of Changchun Biological Institute (Changchun, China), and maintained under specific pathogen-free conditions based on the guidelines established by the Institutional Animal Care and Use Committee of Jilin University. HeLa cells (2x10^6) mixed with 10% Matrigel (R&D Biosystems) were subcutaneously injected into the right flank of each mouse. Tumor-bearing mice were randomly divided into two groups with 10 animals each. Animals received either intraperitoneal (i.p.) injection of 1% polysorbate resuspended in deionized water (control group) or butein (5 mg/kg in 100 μl) on alternate days for 4 weeks. The mice were weighed weekly to determine any toxicity associated with butein treatments, and the tumors were measured using digital vernier calipers. The volume was calculated as: (π/6 x length x width x height). There was no mortality during the treatment regimen. At the end of the study, the animals were euthanized by CO_2 asphyxiation, the tumor tissues were harvested and weighed. Sections of each tumor tissue were wax-embedded and H&E stained to study cell apoptosis *in vivo* by TUNEL using In Situ Cell Death Detection kit (Roche Diagnostics, Branchburg, NJ, USA) according to the manufacturer's instructions.

Statistical analysis. For the *in vitro* and *in vivo* studies, the statistical significance was evaluated by the two-tailed Student's t-test and ANOVA, and data are presented as the mean ± SD (standard deviation). *In vitro* assessments were performed in three independent experiments to confirm the results. Statistical analyses were performed using the GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant result.

Results

Butein decreases cell viability and colony formation of HeLa cells. To examine the proliferation effects of butein, HeLa cells were treated with different dose of butein (0-100 μM) for 72 h, and the percentage of viable cells was determined. Results shown in Fig. 1B indicated that butein decreased cell viability of HeLa cells in a dose-dependent manner. The IC_{30} values (the effective dose that inhibits 30% growth) and IC_{50} values

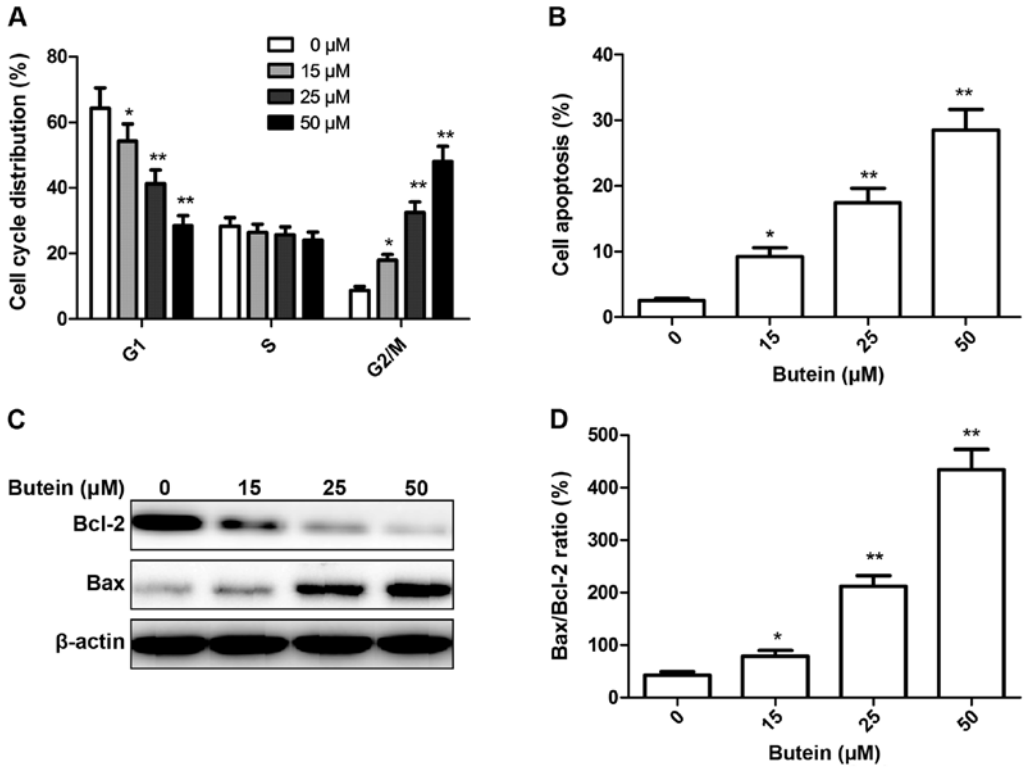

Figure 2. Butein-induced G2/M phase arrest and cell apoptosis of HeLa cells in a dose-dependent manner. HeLa cells were treated with the indicated concentrations of butein (0, 15, 25 and 50 μM) for 48 h, and (A) cell cycle distribution and (B) apoptosis were analyzed. (C) Western blot analysis of Bax-2 and Bcl-2 protein expression following treatment with the indicated concentrations of butein. β-actin was used as an internal control. (D) Bax/Bcl-2 ratio was calculated after treatment with the indicated concentrations of butein (0, 15, 25 and 50 μM). *P<0.05 and **P<0.01, vs. control (0 μM).

(the effective dose that inhibits 50% growth) for treatment of HeLa cells by butein were 14.89 and 24.76 μM, respectively. Based on the results we selected the concentrations of 15 μM (IC_{30}), 25 μM (IC_{50}) and 50 μM (2xIC_{50}) salidroside for subsequent experiments.

The effects of butein on the cell colony formation of HeLa cells were also investigated. It was found that butein significantly inhibited the colony formation of HeLa cells in a dose-dependent manner (P<0.05, Fig. 1C).

Butein-induced G2/M phase arrest and apoptosis of HeLa cells. To determine whether the growth inhibitory effect of butein in HeLa cells was associated with the induction of the cell cycle, we monitored cell-cycle progression using flow cytometry following treatment with the indicated concentrations of butein (0, 15, 25 and 50 μM). Exposure to butein resulted in an increase of G2/M phase cells, accompanied by a decrease in G1 phase cells (Fig. 2A). The effect observed at 50 μM butein was the greatest, with ~48% of cells being in the G2/M phase, compared to ~9% in the control condition.

The effects of butein on the cell apoptosis of HeLa cells were also examined. HeLa cells were treated with butein at different concentrations of 0, 15, 25 and 50 μM for 48 h, and cell apoptosis was analyzed by flow cytometry (Fig. 2B). It was found that butein significantly increased total apoptotic rate of HeLa cells in a dose-dependent manner (P<0.05). Consequently, our results demonstrated that treatment with butein inhibits HeLa cell growth by inducing G2/M transition and cell apoptosis.

To determine the potential mechanism of butein, the effects of cell apoptosis, apoptosis-related protein, Bax and Bcl-2 expression were examined by western blot analysis. The results revealed a significant upregulation of Bax expression and downregulation of Bcl-2 expression in HeLa cells treated with butein in a dose-dependent manner (Fig. 2C). Butein upregulated the Bax/Bcl-2 ratio in HeLa cells in a dose-dependent manner (Fig. 2D), which triggered the release of cytochrome *c* from the mitochondria and stimulated pro-caspase-3, thereby promoting cell apoptosis.

Butein increased caspase-3, -8 and -9 activities of HeLa cells. To examine the contribution of caspases in the butein-induced apoptosis, the role of caspase-3, -8 and -9 was investigated after treatment with different concentration butein (0, 15, 25 and 50 μM). It was found that treatment with butein resulted in a significant increase in the activities of caspase-3, -8 and -9 of HeLa cells in a dose-dependent manner (P<0.05, Fig. 3A-C).

Butein decreases cell migration and invasion of HeLa cells. To investigate the effect of butein on migration and invasion of HeLa cells, the cells were seeded on Transwell with uncoated (for migration) or Matrigel-coated (for invasion) filters. After 48 h of treatment, we examined the migration activity and invasive potential of HeLa cells, and found that butein at 15, 25 or 50 μM significantly inhibited the migration and invasion of HeLa cells (Fig. 4A and B). These results suggested that butein suppressed the migration and invasion of HeLa cells in a dose-dependent manner.

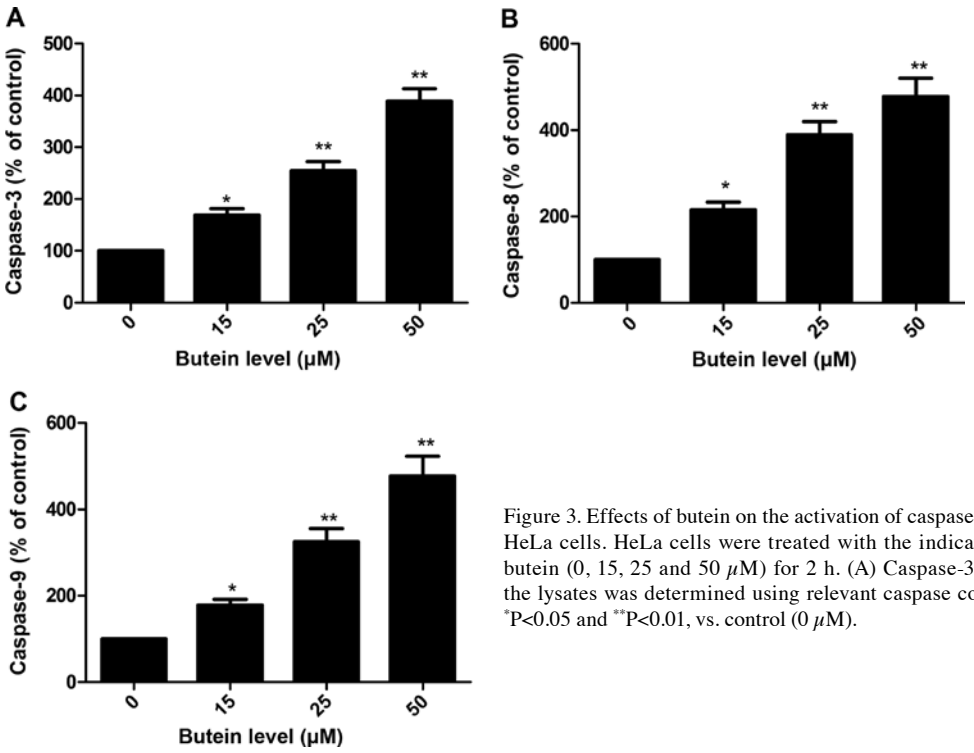

Figure 3. Effects of butein on the activation of caspase-3, -8 and -9 in human HeLa cells. HeLa cells were treated with the indicated concentrations of butein (0, 15, 25 and 50 μM) for 2 h. (A) Caspase-3, (B) -8 and (C) -9 in the lysates was determined using relevant caspase colorimetric assay kits. *P<0.05 and **P<0.01, vs. control (0 μM).

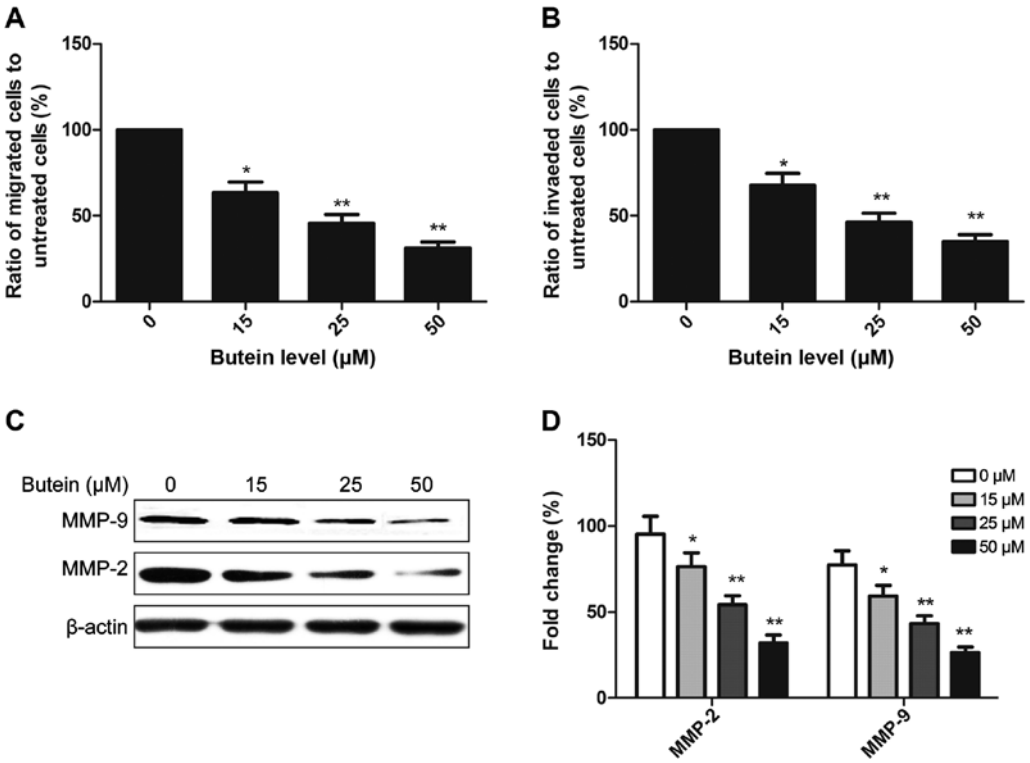

Figure 4. Butein suppresses the migration and invasion of HeLa cells. (A) The number of migrated cells was determined using Transwell assay (without Matrigel) following treatment with the indicated concentrations of butein (0, 15, 25 and 50 μM). (B) The number of invaded cells was determined using the Transwell matrix penetration assay (with Matrigel) following treatment with the indicated concentrations of butein (0, 15, 25 and 50 μM). (C) Western blot analysis of MMP-2 and MMP-9 protein expression following treatment with the indicated concentrations of butein (0, 15, 25 and 50 μM). β-actin was used as an internal control. (D) Relative quantification of MMP-2 and MMP-9 protein expression by densitometric analysis following treatment with the indicated concentrations of butein (0, 15, 25 and 50 μM). *P<0.05; **P<0.01, vs. control (0 μM).

To determine the potential mechanism of butein effect on cell migration and invasion, MMP-2 and MMP-9 expression was determined by western blotting following treatment with butein. It was found that butein significantly reduced MMP-2 and MMP-9 expression of HeLa cells in a dose-dependent manner (Fig. 4C and D).

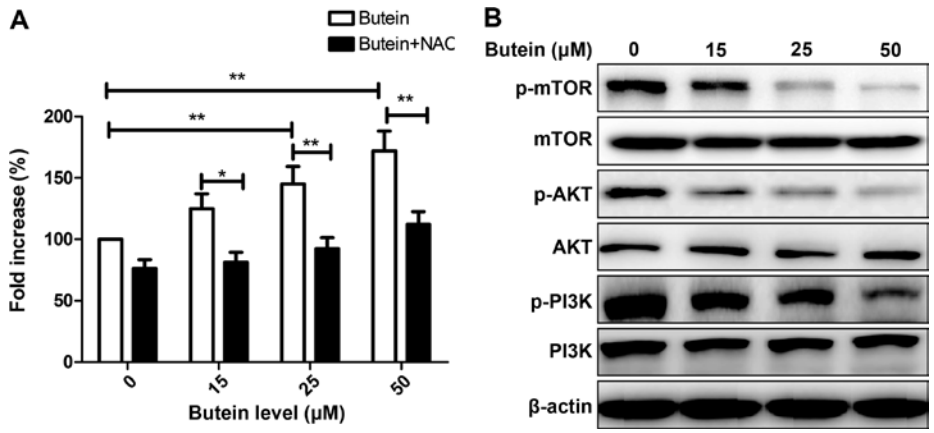

Figure 5. Effects of butein on intracellular ROS formation and the PI3K/AKT/mTOR signaling pathway in HeLa cells. (A) HeLa cells were treated with butein or pretreated with NAC, then treated with butein, and the intracellular accumulation of ROS was assessed. *P<0.05 and **P<0.01. (B) HeLa cells were treated with butein at 0, 15, 25 and 50 μM for 24 h. The phosphorylation of PI3K, AKT and mTOR was determined by western blotting. β-actin was used as an internal control. ROS, reactive oxygen species.

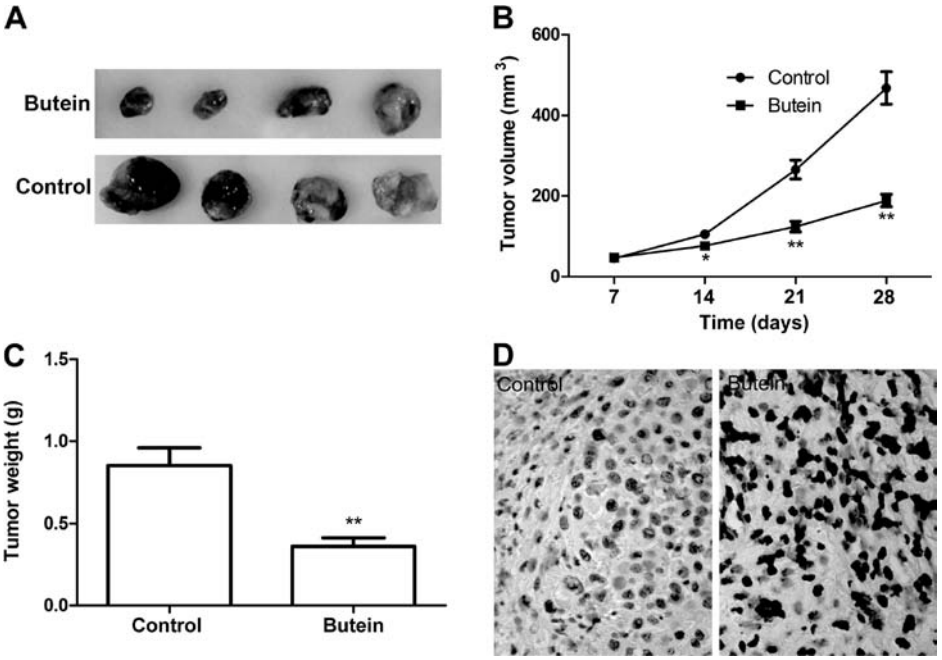

Figure 6. Antitumor activity of butein on human cervical tumor xenografts in nude mice. (A) The visible cervical tumors were harvested from sacrificed mice. (B) Tumor volume of tumor tissue at different time points. (C) Weight of tumor tissue from the butein and control groups. (D) Cell apoptosis of tumor tissues were determined by TUNEL. (D) *P<0.05 and **P<0.01, vs. control.

Effect of butein on ROS generation and the PI3K/AKT/mTOR pathway of HeLa cells. Previous findings have shown that generation of ROS plays an important role in regulating cell apoptosis and cell cycle (27,32). To determine whether butein affected the ROS level, ROS generation was determined using the DCFDA staining method following treatment with the indicated concentrations of butein. As shown in Fig. 5A, treatment with butein significantly induced ROS generation in HeLa cells in a dose-dependent manner. ROS generation was reduced by pretreatment with the antioxidant agent NAC (10 mM) (Fig. 5A).

It is well known that the PI3K/AKT/mTOR signaling pathways play keys role in cell proliferation, survival, motility and angiogenesis. We evaluated the effect of butein on this signaling pathway. Measurements of the phosphorylation/activation pattern of PI3K, AKT and mTOR were carried out by western blotting 24 h after treatment with the indicated concentration of butein. Treatment of HeLa cells with butein caused a dose-dependent inhibition in the expression of phosphorylated PI3K, AKT and mTOR, while the total protein levels of PI3K, AKT and mTOR in different concentrations of butein group were not altered (Fig. 5B).

Butein inhibits cervical tumor growth in a xenograft mouse model. We performed a HeLa xenograft mouse study to test the effectiveness of butein in preventing tumor growth. Results showed that treatment with butein (5 mg/kg) on alternate days for 4 weeks caused a significant inhibition of HeLa tumor xenograft growth in the butein treatment group compared to the control group (untreated group) (Fig. 6A-C). We did

not observe any gross signs of toxicity and/or possible side-effects/mortality during the butein treatment relative to the control group, suggesting butein is a safe anticancer agent.

In addition, we determined cell apoptosis of tumor tissue by TUNEL. The results of TUNEL showed that butein obviously induced the cell apoptosis of tumor tissue compared to the control group (Fig. 6D). These data suggested that butein treatment suppressed the tumor growth of cervical cancer *in vivo*.

Discussion

Cervical cancer is the fourth leading cause of cancer-associated mortality in women worldwide (1,2). The development of novel strategies and anticancer drugs with fewer side effects for cervical cancer therapy is crucial. Butein is a polyphenol and belongs to one of the chalcone flavonoid subgroups. Chalcones including butein have been approved for clinical trials for the treatment of cancer and viral and cardiovascular diseases, and findings suggest that these compounds reached reasonable plasma concentrations without causing toxicity (28,33). Butein is one of the compound of chalcones that possess anticancer abilities against varying human cancer cells (19-27). In the present study, the anticancer activities of butein were investigated in a cervical cancer model *in vitro* and *in vivo*. The *in vitro* studies demonstrate that butein provides an effective therapeutic approach for cervical cancer since butein significantly inhibits proliferation, migration and invasion, and induced tumor apoptosis in HeLa cells in dose-dependent manners. The *in vivo* mouse models also confirmed that butein suppresses tumor growth with minor side effects in the nude mouse model. These findings suggest that butein serves as a potential therapeutic agent for the treatment of cervical cancer.

The balance of expression of pro- and anti-apoptotic members of the Bcl-2 family of proteins has been shown to play an important role in apoptosis (34). An increased the ratio of pro-apoptotic (Bax) to anti-apoptotic (Bcl-2) proteins altered the membrane potential of the mitochondria, released apoptogenic factors to the cytoplasma, activated the caspase cascade and eventually lead to cell apoptosis (34,35). It has been reported that butein induced cell apoptosis via the upregulation of Bax expression and downregulation of Bcl-2 expression in neuroblastoma cells, and activation of caspase-3 and cleavage of PARP (27). Caspases are known for being activated during apoptosis in a self-amplifying cascade, playing a crucial role in the execution of apoptosis (23). In the present study, we found that butein significantly induced cell apoptosis of HeLa cells, and increased caspase-3, -8 and -9 activity, and upregulated the Bax/Bcl-2 ratio suggesting butein induces cell apoptosis by regulating the Bax/Bcl-2 ratio and caspase activity.

Alteration of intracellular reactive oxygen species (ROS) production modulates several physiological functions including stimulating mitotic cell division, regulating cell apoptosis and inducing cell senescence (36). It has been demonstrated that elevated ROS binds to lipids, proteins or DNA to produce oxidative stress and eventually cause cell death (37). Several studies have shown that butein induced apoptosis in cancer cells via the elevation of intracellular ROS levels. For example, Moon *et al* (32) showed that butein induces ROS generation, modulates JNK, ATM and Chk activity, and causes G2/M arrest in hepatoma cells. Yang *et al* (38) reported that butein induced apoptosis through a generation of ROS and deregulation of ERK1/2 and p38MAPK in triple-negative [triple-negative breast cancer (TNBC)] MDA-MB-231 cells. Chen *et al* (27) demonstrated that butein-triggered neuroblastoma cells undergo apoptosis via the generation of ROS, alteration of the Bcl-2/Bax ratio, and cleavage of pro-caspase-3 and PARP. Consistent with these studies, the present study showed that butein inhibited cervical cancer cell growth and induced cell apoptosis through the generation of ROS.

PI3K/Akt is major signal transduction pathway regulating cell apoptosis, proliferation, differentiation and metastasis (39). The constitutive activation of the PI3K/Akt signaling pathway has been shown to function as a major determinant of tumor cell growth and survival in a number of solid tumors (40). Downstream in the PI3K/Akt pathway there is a distal component known as mTOR (39). Treatment of human PCa cells with butein resulted in a decrease in the expression of phosphorylation PI3K (p-PI3K) and phosphorylation of Akt (p-AKT) (22). Liu *et al* found that butein inhibited the Akt/mTOR/p70S6K translational machinery, leading to the downregulation of MMP-9 and uPA, and subsequently exerting an antimetastatic effect (41). In the present study, our results showed that butein inhibited p-PI3K, p-AKT and p-mTOR, suggesting that butein suppressed cervical cancer growth, at least partially by regulating the PI3K/AKT/mTOR signaling pathway.

In conclusion, to the best of our knowledge, this is the first study to show that butein inhibits cell viability, colony formation, migration and invasion, and induced G2/M phase arrest in HeLa cells. Our results also show that butein significantly induced-HeLa cell apoptosis by regulating the Bcl-2/Bax ratio, and increasing caspase-3, -8 and -9 activity. The *in vivo* results showed that butein suppressed tumor growth in a xenograft mouse model. In addition, butein increased the generation of ROS, and inhibited PI3K/AKT/mTOR pathway activation, which may contribute to a decrease in oxidative stress and suppression of tumor growth. Collectively, our findings suggest that butein serves as a chemotherapeutic agent for the future treatment of cervical cancer.

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