Luteolin induces apoptosis *in vitro* through suppressing the MAPK and PI3K signaling pathways in gastric cancer

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Abstract. Luteolin, an active component of traditional Chinese medicine, exhibits potential for anti-tumor proliferation; however, the molecular events occurring in such process and the signal transduction pathways involved are currently unknown. Our group previously reported that luteolin inhibited proliferation and induced apoptosis in the gastric cancer cell line BGC-823. The aim of the present study was to investigate the mechanism by which the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathways regulate the apoptosis *in vitro* of BGC-823 cells following treatment with luteolin. It was observed that luteolin induced apoptosis through the intrinsic pathway by increasing the levels of caspase-3, caspase-9 and cytochrome *c*, and the ratio of B-cell lymphoma (Bcl)-2 associated X protein (Bax) to Bcl-2. Luteolin suppressed the phosphorylation of extracellular signal-regulated kinase in the MAPK signaling pathway, as well as suppressing the phosphorylation of AKT, PI3K and mechanistic target of rapamycin in the PI3K signaling pathway. In addition, luteolin combined with LY294002 markedly increased the Bax/Bcl-2 ratio, while when combined with U0126, luteolin had less effects on the Bax/Bcl-2 ratio compared with luteolin treatment alone, suggesting that both the MAPK and PI3K signaling pathways are involved in the apoptosis induced by luteolin. Furthermore, luteolin attenuated the MAPK and PI3K signaling pathways by increasing the expression of specific dual-specificity phosphatases and decreasing the expression of chemokine (C-X-C motif) ligand 16 at the messenger RNA level, respectively. Taken together, the present results demonstrate that luteolin is a potential chemotherapeutic agent against gastric cancer by exerting a dual inhibition on the MAPK and PI3K signaling pathways.

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Introduction

Gastric carcinoma (GC) is the third most common cause of cancer mortality worldwide, and >50% of GC cases occur in Eastern Asia (1,2). Therapy for GC includes surgical resection, radiation and chemotherapy (3). Surgical resection is the curative treatment for patients with early stages of disease. However, ~20% of patients survive 5 years after surgery, and the majority of patients with advanced GC, which is characterized by poor prognosis and metastasis, eventually relapse (4). At present, the use of chemotherapy and combination treatments are alternative therapeutic strategies for controlling advanced GC (5). Therefore, it is an urgent requirement to identify new chemotherapeutic agents for preventing gastric cancer metastasis and improving the 5-year survival rates of gastric cancer patients (6,7).

A malignant tumor could be developed from a normal cell in various mechanisms, including self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (8). Regarding growth signals, the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathways serve crucial roles in controlling fundamental cellular processes, including growth, proliferation, differentiation, migration and apoptosis (9). Emerging evidence has suggested that sustained activation of the MAPK and PI3K signaling pathways is responsible for anti-apoptosis and carcinogenesis (10). Data from several groups have suggested that constitutively activated extracellular signal-regulated kinase (ERK) is involved in the progression of certain types of human cancer, including carcinomas of the breast (11), colon (12) and prostate (13). Activation of the PI3K signaling pathway was significantly associated with the tumor development and

progression of human gastric cancer, based on 56 gastric cancer specimens (14). In addition, An *et al* (15) investigated a total of 290 patients with pT2b gastric cancer and elucidated that phosphorylated (p-) mammalian target of rapamycin (mTOR) was expressed in patient-derived gastric cancer samples, and that mTOR activation was associated with the extent of lymph node metastasis and poor survival in patients with gastric cancer. Therefore, inhibition of the PI3K and MAPK signal transduction pathways may represent a promising strategy in the treatment of the initiation and progression of gastric cancer.

A body of studies suggest that luteolin (3',4',5,7-tetrahydroxyflavone), a natural flavonoid compound highly enriched in a number of medicinal herbals, including *Lonicera japonica*, *Scutellaria barbata* and *Ajuga decumbus* (16), possesses diverse biological activities, including anti-inflammatory (17), antioxidant (18) and antiproliferative effects (19). Furthermore, it has been documented that luteolin could arrest the cell cycle and induce apoptosis in a wide variety of cancer cells *in vitro*, including prostate cancer cells (20), AGS human gastric cancer cells (21,22), SMMC7721 liver cancer cells (23), COLO205 human colorectal cancer cells and HeLa human cervical cancer cells (24). It has also been reported that luteolin was able to significantly decrease colon cancer incidence and the number of tumors per rat when administered at the initiation and post-initiation stages of carcinogenesis (25). To date, various well-controlled clinical trials have been carried out to evaluate the chemopreventive potential of luteolin in human subjects (26-30). However, the molecular events and signal transduction pathways involved in the mechanism of action of luteolin in gastric cancer remain to be elucidated.

Therefore, the purpose of the present study was to investigate the role of the MAPK and PI3K signaling pathways in regulating luteolin-induced apoptosis *in vitro*. The present findings highlight the potential of luteolin as an anti-cancer therapeutic agent that targets MAPK and PI3K signaling in gastric cancer cells.

Materials and methods

Reagents and antibodies. Luteolin, MTT and dimethyl sulfoxide were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RPMI-1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Annexin V-FITC apoptosis detection kit and anti-cytochrome *c* antibody (catalogue no. 556433) were purchased from BD Biosciences (San Jose, CA, USA). Primary antibodies, including anti-p-PI3K (Y607; catalogue no. YP0765), anti-p-mTOR (S2448; catalogue no. YP0176) and anti-β-actin (catalogue no. YT0099) antibodies, were from ImmunoWay Biotechnology Company (Plano, TX, USA), while anti-p-AKT (Ser473; catalogue no. 4051), anti-p-p38 (Thr180/Tyr182; catalogue no. 9216), anti-p-ERK1/2 (Thr202/Tyr204; catalogue no. 9106), anti-p-c-Jun N-terminal kinase (JNK) (Thr183/Tyr185; catalogue no. 4668), anti-B-cell lymphoma (Bcl)-2 (catalogue no. 15071), anti-Bcl-2 associated X protein (Bax) (catalogue no. 2772), anti-caspase-3 (catalogue no. 9668) and anti-caspase-9 (catalogue no. 9508) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Caspase-3 (3G2, catalogue no. 9668) is mouse monoclonal antibody that detects endogenous levels of full length (35 kDa) and the large fragment (17/19 kDa) of caspase-3 resulting from cleavage at aspartic acid 175. Caspase-9 (C9, catalogue no. 9508) is mouse monoclonal antibody that detects endogenous levels of the pro form and cleaved fragments of caspase-9 (47,37 and 35 kDa). The horseradish peroxidase-conjugated secondary antibodies anti-rabbit immunoglobulin (Ig)G (catalogue no. sc-2357) and anti-mouse IgG (catalogue no. sc-516102) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). U0126 (catalogue no. S1102) and LY294002 (catalogue no. S1105) were purchased from Selleck Chemicals (Houston, TX, USA). All other chemicals were purchased from Sangon Biology Engineering Technology Service, Ltd. (Shanghai, China) and were of analytical grade.

Cancer cell culture. The human gastric cancer cell line BGC-823 was obtained from the Cell Center at Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a 5% CO_2 incubator. The cells were sub-cultured every 2 or 3 days and routinely checked visually under an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) for potential contamination. No contamination was identified.

Western blot analysis. Cells (1.0×10^6) were seeded in 10-cm dishes. When cells were in logarithmic growth phase, they were treated with luteolin at the 0, 20, 40 and 60 μM for 48 h. Subsequently, BGC-823 cells were washed twice with PBS (pH 7.4) and lysed in 100 μl radioimmunoprecipitation assay buffer (AR0102) that purchased from Boster Biotech (Wuhan, China). The lysed cells were removed from the culture dish by gentle scraping with a cell scraper (#3010; Corning Incorporated, USA) and transferred to a microcentrifuge tube. The samples were centrifuged at 13,000 rpm for 5 min at 4˚C, and the supernatant was then transferred to a new tube. Total protein concentration was determined using the Pierce BCA Protein assay kit (Thermo Fisher Scientific, Inc.). Proteins were separated by 10% SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane (0.2 μm; Merck KGaA, Darmstadt, Germany) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Blocking was carried out for 2 h in TBST [Tris-buffered saline (TBS) containing 1‰ Tween-20, v/v] with 5% non-fat milk at room temperature. The primary antibodies (1:1,000) were incubated with the membrane overnight at 4˚C. Following three washes in TBST, secondary antibodies (1:2,000) were added and incubated at room temperature for 2 h. The blots were washed with TBST three times, and detection was then performed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (qPCR) assay. After BGC-823 cells were exposed to 0, 20, 40 and 60 μM luteolin for 48 h, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA concentration and purity were determined based on the measurement of

absorbance at 260 and 280 nm. RNase-free DNase I (Takara Bio, Inc., Japan) was used to remove the DNA contamination. M-MLV Reverse Transcriptase (Fermentas, Thermo Fisher Scientific, Inc.; Pittsburgh, PA, USA) was used according to the manufacturer's protocol to treat 2 μg total RNA for synthesizing first-strand complementary DNA (cDNA). The cDNA was then subjected to qPCR for evaluation of the relative messenger RNA (mRNA) levels. Gene-specific amplification was performed using an StepOne™ (96 wells) Real-Time PCR system (Applied Biosystems® ABI; Thermo Fisher Scientific, Inc.) with a 20 μl PCR reaction mixture containing 1 μl cDNA (synthesized as described above), 10 μl 2X Fast SYBR-Green Master Mix (Applied Biosystems® ABI, USA), forward and reverse primers with a final concentration of 0.25 μM. The amplification conditions were 95˚C for 15 min, followed by 40 cycles of 95˚C for 15 sec, 56˚C for 20 sec and 72˚C for 30 sec. The relative expression levels of the target genes were normalized to the geometric mean of the internal control gene, *GAPDH*. Each gene was performed in a set of three replicates. No template control was included in all batches. The Cq (threshold cycles) values were used to calculate the mRNA levels by the formula $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ treatment - \Delta Ct\ control]}$ (31). The primers used in qPCR analysis were obtained from Sangon Biology Engineering Technology Service, Ltd. (Shanghai, China), and their sequences are reported in Table I.

Statistical analysis. The data were expressed as the mean ± standard deviation (n=3). All calculations were performed with SPSS version 16 (SPSS, Inc., Chicago, IL, USA). Statistical significance was analyzed by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Luteolin induces activation of caspases. Our group previously reported that luteolin inhibited BGC-823 cell proliferation and induced apoptosis in a dose-dependent manner (32). In the present study, to further examine whether luteolin-induced apoptosis resulted from activating caspase enzymes, the activities of initiator caspase (caspase-9) and effector caspase (caspase-3) (33,34) were measured by western blotting in BGC-823 cells. The results revealed that luteolin treatment increased the expression of cleaved caspase-9 and caspase-3 in a dose-dependent manner (Fig. 1). Next, the level of cytochrome *c* in the cytoplasm was detected (35-38), as this is a key step in the process of caspase activation during apoptosis. As shown in Fig. 1, luteolin induced an increase of cytoplasmatic cytochrome *c* in a dose-dependent manner. These results implied that luteolin-induced apoptosis in BGC-823 cells may occur through the intrinsic pathway.

Effects of luteolin on the expression of Bcl-2 family proteins. The involvement of the intrinsic pathway in apoptosis is regulated by proteins of the Bcl-2 family, which comprises anti-apoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax) proteins (39). The ratio of Bax/Bcl-2 could determine whether the cell undergoes apoptosis (40,41). In the present study, luteolin treatment of BGC-823 cells resulted in decreased of Bcl-2 in a dose-dependent manner (Fig. 2A). Compared with that in control cells, the ratio of Bax/Bcl-2 significantly increased at the highest concentrations of luteolin in treated cells (Fig. 2B). These data further suggested that luteolin induced apoptosis via the intrinsic pathway in BGC-823 cells.

Table I. Primers used in quantitative polymerase chain reaction analysis.

Gene name	Primer sequence (5'-3')
DUSP1	F: TTTGAGGGTCACTACCAG
	R: GAGATGATGCTTCGCC
DUSP2	F: AGTCACTCGTCAGACC
	R: TGTTCTTCACCCAGTCAAT
DUSP4	F: CAAAGGCGGCTATGAG
	R: GGTTATCTTCCACTGGG
DUSP5	F: CTGAGTGTTGCGTGGA
	R: AGTCTATTGCTTCTTGAAAGT
DUSP6	F: CGAGACCCCAATAGTGC
	R: AATGGCCTCAGGGAAA
DUSP7	F: TCATTGACGAAGCCCG
	R: GCGTATTGAGTGGGAACA
DUSP9	F: ATCCGCTACATCCTCAA
	R: AGGTCATAGGCATCGTT
DUSP10	F: CTGAACATCGGCTACG
	R: GGTGTAAGGATTCTCGGT
CXCL16	F: CAGCAAGCCAAGAGGA
	R: TGACAAAGGCATAGAGCA
GAPDH	F: AAGGTCGGAGTCAACGGATT
	R: CTCCTGGAAGATGGTGATGG

DUSP, dual-specificity phosphatase; CXCL16, chemokine (C-X-C motif) ligand 16; F, forward; R, reverse.

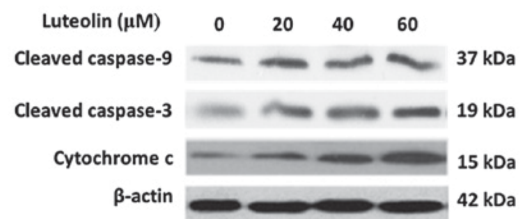

Figure 1. Luteolin induces the activation of caspases in BGC-823 cells. Cells were seeded in 60-mm plates and cultured to 80-90% confluence. The cells were then treated with various doses of luteolin (20, 40 and 60 μM) for 48 h. Cells treated with dimethyl sulfoxide alone were used as the control. Whole-cell extracts were subjected to immunoblot analysis using antibodies specific for caspase-9, caspase-3, cytochrome *c* and β-actin.

Luteolin suppresses the PI3K and MAPK signaling pathways. The key elements of the PI3K and MAPK signaling pathways were evaluated by western blotting in BGC-823 cells exposed to luteolin at 0, 20, 40 and 60 μM for 48 h. As shown in Fig. 3A, luteolin reduced the expression level of p-PI3K, p-AKT and p-mTOR in a dose-dependent manner. These results suggested that luteolin treatment suppressed the PI3K signaling pathway.

With regards to the MAPK signaling pathway, after BGC-823 cells were treated with luteolin at 0, 20, 40 and

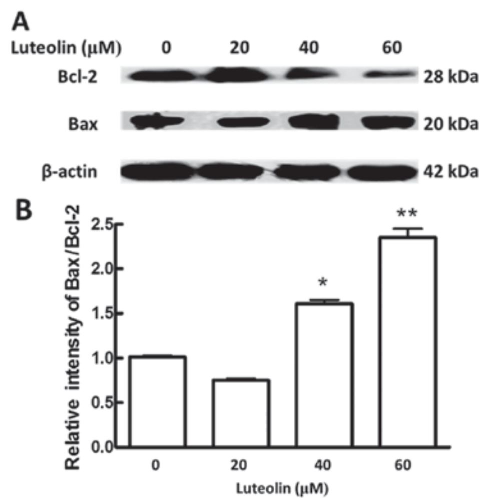

Figure 2. Effects of luteolin on the expression levels of Bcl-2 family proteins in BGC-823 cells. (A) Cells were treated as described above, and the cell extracts were subjected to immunoblotting using specific antibodies against Bcl-2, Bax and β-actin. (B) The intensity of Bax/Bcl-2 protein signals was calculated. Values are the mean ± standard deviation. The data were analyzed by one-way analysis of variance. *P<0.05 and **P<0.01 compared with the control group. Bcl, B-cell lymphoma; Bax, Bcl-2 associated X protein.

60 μM for 48 h, the results of western blotting indicated that the levels of p-ERK1/2 were reduced markedly in a dose-dependent manner, while the levels of p-p38 and p-JNK exhibited no significant change (P>0.05). These results suggested that luteolin treatment suppressed the ERK1/2 signaling pathway, but not the JNK or p38 signaling pathways, in BGC-823 cells (Fig. 3B).

To confirm the roles of the ERK1/2 or PI3K signaling pathways in luteolin-induced apoptosis in BGC-823 cells, the ERK inhibitor U0126 (42) and the AKT inhibitor LY294002 (43) were used for treating the cells in the absence or presence of 60 μM luteolin. The effects were examined according to the ratio of Bax to Bcl-2. The results indicated that exposure of BGC-823 cells to U0126 or LY294002 alone did not significantly alter the Bax/Bcl-2 ratio compared with that of control cells (Fig. 3C and D; P>0.05). The Bax/Bcl-2 ratio in the combination U0126 plus luteolin group increased compared with that in the control group, while such ratio was lower than that observed upon luteolin treatment alone (P<0.05). The Bax/Bcl-2 ratio in the combination LY294002 plus luteolin group increased compared with that in the control group, being much higher than that observed following luteolin treatment alone (P<0.05). Taken together, these results indicated that the ERK and PI3K signaling pathways were involved in the apoptosis induced by luteolin in BGC-823 cells. Notably, the Bax/Bcl-2 ratio in the LY294002 plus luteolin group was markedly higher than that in the U0126 plus luteolin group (~3.7 vs. 1.6), respectively, indicating that the PI3K signaling pathway has much stronger effects on luteolin-induced apoptosis than the MAPK signaling pathway.

Effects of luteolin on the expression of dual-specificity phosphatase (DUSP) and chemokine (C-X-C motif) ligand 16 (CXCL16) genes in BGC-823 cells. Dual-specificity phosphatases (DUSPs) are a heterogeneous group of protein phosphatases that have ability to dephosphorylate both tyrosine and serine/threonine residues (44) and serve a critical role in the inactivation of different isoforms of MAPK (45) DUSPs share common features, including a cluster of basic amino acids as part of the kinase interactive motif (KIM) on their amino terminus. The KIM confers substrate specificity and is the least homologous region demonstrating individual substrate preferences (46,47). Therefore, the transcription level of DUSP1-DUSP10 was examined. The mRNA levels of *DUSP1*, *2*, *4* and *5* were upregulated in luteolin-treated BGC-823 cells compared with those in controls cells, while the mRNA levels of *DUSP6*, *7*, *9* and *10* did not change during the treatment (Fig. 4). *DUSP1*, *2*, *4* and *5* prefer to use the ERK as their substrates. This result was consistent with the decrease of p-ERK protein in the western blotting results of the present study, indicating that luteolin exhibits the potential to regulate the expression of specific *DUSP* genes, resulting in an attenuation of the MAPK signaling pathway.

Chalabi-Dchar *et al* (48) reported that blocking *CXCL16* activity abrogated activation of the PI3K/AKT pathway, implying the *CXCL16* axis may regulate the activity of PI3K pathway. Therefore, the expression of *CXCL16* was monitored at an mRNA level. The results showed that the *CXCL16* mRNA level was greatly downregulated in a dose manner after treatment with luteolin, suggesting that luteolin efficiently suppressed the mRNA level of *CXCL16*. Hence, the attenuation of PI3K pathway may be ascribed to the suppression of *CXCL16* in BGC-823 cell lines following luteolin treatment.

Discussion

In the present study, the apoptotic process induced by luteolin in BGC-823 cells was associated with the activities of caspases and the expression of Bcl-2 family proteins, which is in agreement with previous reports regarding the pro-apoptotic effects of luteolin on other cancer cells, including the gastric cancer cell line AGS (21,22), human prostate cancer cells (49) and the human colon cancer cell line HT-29 (50). However, the underlying mechanism of luteolin-induced apoptosis is not well understood yet.

MAPKs have been linked to diverse cellular events, including proliferation, senescence, differentiation, migration and apoptosis (51). A well-characterized apoptotic signaling cascade is regulated by MAPKs, including JNK, ERK and p38 MAPK (51). The ERK1/2 signaling pathway primarily responds to growth and differentiation factors, and the p38 and JNK signaling pathways primarily responds to stress conditions (52,53). The present study noticed that the phosphorylation of ERK1/2 decreased significantly following luteolin treatment, while that of p38 or JNK did not change in the course of luteolin treatment, suggesting that p38 and JNK are not involved in the regulation of apoptosis in the BGC-823 cell line. It has been reported that ERK1/2 participates in apoptotic signaling via post-translational regulation of the Bcl-2 family members, including Bcl-2 interacting mediator of cell death (Bim) (54). Phosphorylation of Bim by ERK1/2 results in a change in the Bax/Bcl-2 ratio, which determines whether cell apoptosis occurs (55). In the present study, although U0126 treatment alone did not induce apoptosis in BGC-823 cells, when combined with luteolin, it increased the

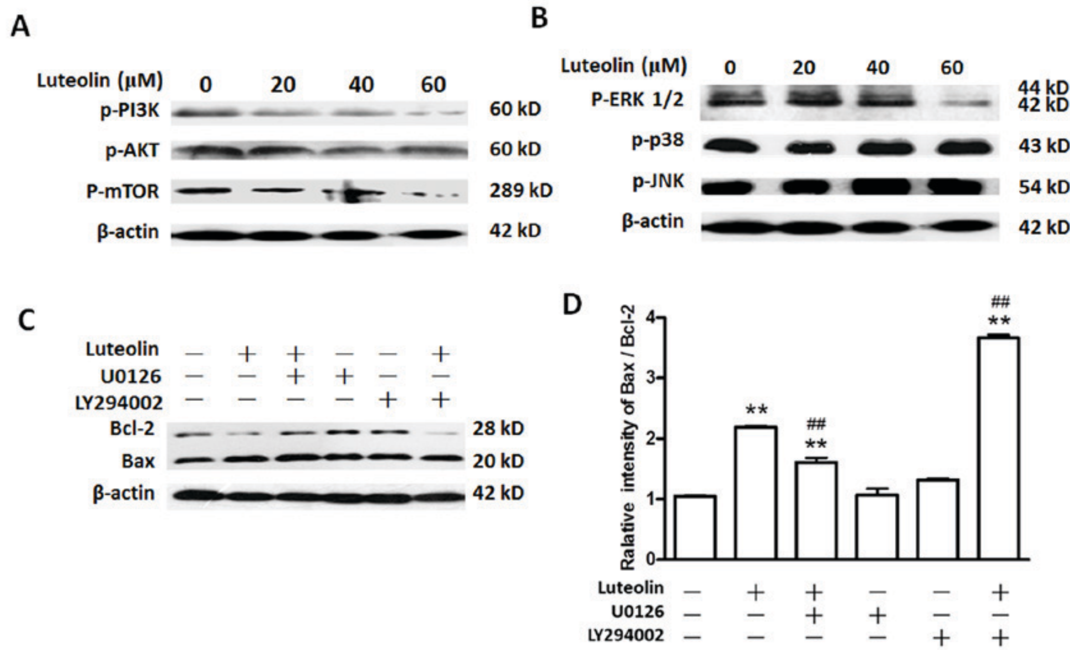

Figure 3. Luteolin inhibits the activation of the PI3K and MAPK signaling pathways in BGC-823 cells. Cells were seeded in 60-mm plates and cultured to 80-90% confluence. The cells were then treated with various doses of luteolin (20, 40 and 60 μM) for 48 h. Cells treated with dimethyl sulfoxide alone were used as the control. (A) Luteolin inhibited the PI3K signaling pathway in BGC-823 cells. Cells were treated as described above, and the cell extracts were subjected to immunoblot analysis using anti-p-PI3K, anti-p-AKT, anti-p-mTOR and anti-β-actin antibodies. (B) Effect of luteolin on ERK1/2, p38 and JNK pathways. Cells were treated as described above, and the cell extracts were subjected to immunoblotting using anti-p-ERK, anti-p-p38, anti-p-JNK and anti-β-actin antibodies. (C) Effects of luteolin on the Bcl-2 and Bax in BGC-823 cells. Cells were treated as described above, and the cell extracts were subjected to immunoblotting using anti-p-ERK, anti-Bcl-2, anti-Bax and anti-β-actin antibodies. (D) The relative intensity of Bax/Bcl-2. Values are the mean ± standard deviation. The data were analyzed by one-way analysis of variance. **The control group vs. all the other groups (P<0.001); ##luteolin treatment groups vs. luteolin+U0126 and luteolin+LY294002 treatment groups (P<0.001). PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; mTOR, mammalian target of rapamycin; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; Bcl, B-cell lymphoma; Bax, Bcl-2 associated X protein.

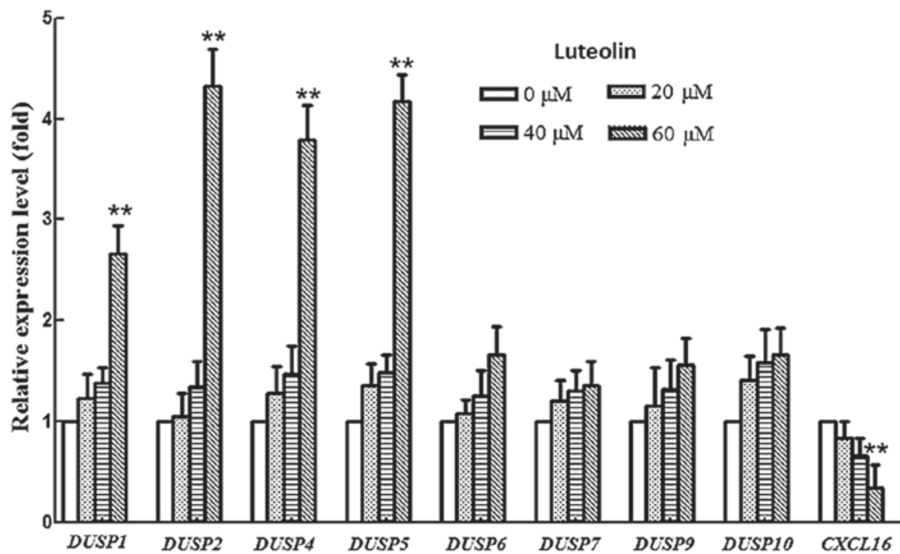

Figure 4. Effects of luteolin on the expression of *DUSP* and *CXCL16* genes in BGC-823 cells. Values are the mean ± standard deviation of three independent experiments. The data were analyzed by one-way analysis of variance. **P<0.01 compared with the control group. DUSP, dual-specificity phosphatase; CXCL16, chemokine (C-X-C motif) ligand 16.

Bax/Bcl-2 ratio, suggesting that luteolin-induced apoptosis was mediated by the ERK1/2 signaling pathway.

A major determinant of the biological outcome of MAPK signaling is the duration and magnitude of kinase activation, which can be achieved by serine/threonine phosphatases, tyrosine-specific phosphatases or dual-specificity phosphatases (DUSPs) (56,57). DUSPs, whose family consists of 25 members, can specifically dephosphorylate ≥1 MAPKs, and their substrate specificity is dependent on the cell type and context (44,46). DUSP1, 2, 4 and 5 are mitogen- and

stress-inducible nuclear DUSPs, which prefer using ERK as a substrate compared with JNK or p38. DUSP6, 7 and 9 are cytoplasmic ERK-specific DUSPs, while DUSP8 and 10 are JNK/p38-specific phosphatases present in both the nucleus and cytoplasm (58). The present study revealed that *DUSP1*, *2*, *4* and *5* mRNA expression was upregulated, while the expression of other *DUSPs* was unchanged, upon luteolin treatment. These results indicated that highly expressed *DUSP1*, *2*, *4* and *5* specifically dephosphorylated ERK1/2 as a substrate, which may explain the decrease in phosphorylation of ERK1/2. No difference in p-JNK or p-p38 was observed between cells treated with or without luteolin, which may be due to the unchanged expression of other *DUSPs*. To date, no study has reported that luteolin has the potential to regulate the expression of *DUSP* genes on GC. Taken together, increased mRNA levels of specific *DUSPs* in luteolin-treated BGC-823 cell lines resulted in decreased p-ERK1/2 in the present study, and the suppression of p-ERK1/2 could increase the ratio of Bax to Bcl-2, eventually triggering apoptosis.

The PI3K pathway relies on an array of intracellular events that have been intensively studied in previous years (59). The PI3K signaling pathway may influence apoptosis regulation, including the regulation of the Bcl-2 family proteins (10,60). One of the important members of the Bcl-2 family of proteins is Bax, which can be phosphorylated at the inhibition site Ser184 near the C-terminus by AKT, leading to suppression of the apoptotic activity mediated by Bax (61). Thus, the activity of PI3K in cancer cells protects them from undergoing apoptosis; conversely, inhibiting the activity of PI3K can induce apoptosis in cancer cells (62,63). In the present study, it was demonstrated that luteolin may inhibit p-PI3K, p-AKT and p-mTOR in the gastric cancer cell line BGC-823, suggesting that the PI3K signaling pathway may be involved in luteolin-induced apoptosis. Further experiments verified that combined treatment with LY294002 and luteolin had an enhanced effect on the induction of apoptosis, as evidenced by a significant increase in the Bax/Bcl-2 ratio, indicating that luteolin-induced apoptosis mainly occurred through the PI3K signaling pathway.

The activation of PI3K family members is a universal event in response to cytokines, growth factors and hormones (59,64). Chemokines, a superfamily of chemotactic cytokines consisting of nearly 50 cytokine members and 20 chemokine receptors (65,66), are classified into four major families based on the relative position of their cysteine residues near the NH_2 terminus: CC, CXC, C and CX3C (67). Xing *et al* reported that aberrant expression of CXCL16 and CXCR6 may be involved in gastric carcinogenesis, and that the expression and serum concentration of CXCL16 could indicate the aggressiveness and prognosis of GCs (68). Furthermore, a previous study elucidated that PI3K/AKT/mTOR signaling may be involved in the CXCL16/CXCR6 biological axis (69). In that study, the phosphorylation of AKT was reduced with decreased CXCR6 expression, and mTOR was activated by CXCL16's stimulation of CXCR6 (69). Since the PI3K signaling pathway is involved in the activation of the CXCR6/CXCL16 axis (70,71), a number of therapy options may target blocking this axis or exploit other antibodies against this signaling pathway in order to prevent metastasis. Based on such strategy, various antibodies have been designed to specifically block the CXCL16/CXCR6 axis; however, the positive outcomes are poor (72-74). The present study demonstrated that luteolin efficiently inhibited *CXCL16* mRNA expression. In the present study, based on the suppression of PI3K/AKT/mTOR signaling, it was hypothesized that luteolin may inhibit the expression of *CXCL16*, resulting in the suppression of the signaling PI3K pathway. To the best of our knowledge, the present study is the first to report that chemicals have the potential to regulate the CXCL16/CXCR6 axis, which may be beneficial in the development of a more effective anti-metastasis therapeutic strategy for gastric cancer. The present results also provide the first evidence suggesting that luteolin treatment may also alter the tumor microenvironment.

In summary, the present study has demonstrated that luteolin could induce apoptosis in the BGC-823 cell line through the intrinsic pathway. Luteolin upregulated the mRNA levels of specific *DUSPs*, which suppressed the protein phosphorylation of ERK1/2. In addition, luteolin attenuated the mRNA levels of *CXCL16*, leading to the suppression of the PI3K signaling pathway, and both the ERK1/2 and the PI3K signaling pathways were closely associated with the regulation of apoptosis in the gastric cancer cell line BGC-823. The present results provide useful information for considering luteolin as an attractive chemotherapeutic agent against gastric cancer.

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