

Baicalein inhibits the invasion of gastric cancer cells by suppressing the activity of the p38 signaling pathway

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Abstract. Baicalein, one of the major flavonoids in *Scutellaria baicalensis*, has been used in anti-inflammatory and anticancer therapies for a long time. However, the antimetastatic effects and related mechanism(s) in gastric cancer remain unclear. In the present study, we tested the hypothesis that administration of baicalein may inhibit the proliferation, motility and invasion of human gastric cancer cell lines by regulating the p38 signaling pathway. In the present study, we found that baicalein could inhibit migration and invasion of gastric cancer cells. Additionally, after treating with baicalein for 24 h, the expression levels of matrix metalloproteinase (MMP)-2 and -9 as well as proteinase activity in gastric cancer cells were reduced in a dose-dependent manner. Moreover, baicalein clearly reduced the phosphorylated levels of p38. Combined treatment with p38 activator partially blocked the antimetastatic effects of baicalein, while p38 inhibitor (SB203580) and baicalein resulted in a synergistic reduction in MMP-2 and -9 expression; the invasive ability of gastric cancer cells was also inhibited. In conclusion, baicalein inhibits gastric cancer cell invasion and metastasis by reducing cell motility and migration via suppression of the p38 signaling pathway, suggesting that baicalein is a potential therapeutic agent for gastric cancer.

Introduction

Gastric cancer is the fourth most commonly diagnosed cancer and the second leading cause of cancer-related mortality worldwide (1), leading to thousands of deaths per year. The potent invasion and metastatic ability of gastric cancer is one of the key factors that affect poor prognosis (2). Therefore, it is critical to detect the underlying mechanisms of the invasion and metastasis of gastric cancer, and to develop therapeutic treatment for gastric cancer.

The extracellular matrix (ECM) plays an important role in tumor development and metastasis (3). Matrix metalloproteinases (MMPs), a family of Zn^{2+} containing Ca^{2+} -dependent proteolytic enzymes, play important roles in degrading basement membranes and are intricately involved in cancer invasion and metastasis (4). MAPKs play important roles in intracellular signaling during proliferation, differentiation, cellular stress responses and apoptosis (5). Increased activation of p38 signaling pathway is observed in gastric cancer, and is correlated with the invasion by regulating the expression of MMPs (6,7).

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one), as one of the major flavonoids with a defined chemical structure in *Scutellaria baicalensis*, has long been used in oriental medicine. Baicalein has been found to have anticancer effects (4,8,9). However, the antimetastatic effects and related mechanism(s) in gastric cancer cells have not previously been determined. In the present study, we tested the hypothesis that administration of baicalein may inhibit the proliferation, motility and invasion of human gastric cancer cells via the p38 signaling pathway *in vitro*.

Materials and methods

Reagents. Fetal bovine serum (FBS), streptomycin and penicillin were obtained from HyClone. Baicalein, SB203580 and anisomycin were from Sigma. Anti-p38 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-MMP-2 and anti-MMP-9 antibodies were purchased from Cell Signaling. Anti- β -actin was purchased from Santa Cruz.

Cell culture. The human gastric cancer cell lines SGC7901 and MGC803 were purchased from Type Culture Collection, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. All cells were incubated at 37°C with 5% CO_2 .

Cell viability assays. Cell survival was detected using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions. First, cells were seeded in 96-well culture plates (5×10^4 cells/well). Then, cells were treated with different concentrations of baicalein. After incubating for 24 h, the cells were washed twice with PBS and incubated with 5 mg/ml MTT

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Table I. Primers for RT-PCR.

Gene	Forward	Reverse
β -actin	CCATCGTCCACCGCAAAT	CATGCCAATCTCATCTTGT
MMP-2	CTCATCGCAGATGCCTGGAA	TTCAGGTAATAGGCACCCTTGAAGA
MMP-9	GTCCACCCTTGTGCTCTTCC	GCCACCCGAGTGTAACCAT

MMP, matrix metalloproteinase.

(Sigma) for 4 h. Finally, the optical density was measured in a microplate reader at 562 nm formazan absorbance.

In vitro invasion and migration assays. The *in vitro* invasion and migration activity was measured according to previously described methods with minor changes (10,11). Cells were pretreated with 0, 10, 20 and 40 μ M baicalein or SB203580 (20 μ M) or anisomycin (25 μ g/ml) for 24 h, and surviving cells were harvested and seeded to Transwell chamber (Corning) then incubated for 24 h at 37°C. Then, the cells on the upper side of inserts were completely removed by swabbing, while the cells on the bottom side of the filter were fixed, stained and measured. For invasion assay, 10 ml Matrigel (25 mg/50 ml; BD Biosciences, Bedford, MA, USA) was applied to 8-mm pore size polycarbonate membrane filters and the bottom chamber contained standard medium.

Real-time quantitative PCR. Total RNAs were prepared by using the RNeasy Mini kit (Invitrogen). cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR (RT-PCR) was performed using SYBR-Green II in accordance with the PrimeScript RT-PCR kit protocol (Takara). Gene-specific primer pairs used for amplification are shown in Table I. β -actin was used as an endogenous control. The analysis of the relative gene copy number data for MMP-2 and -9 was performed using the $2^{-\Delta\Delta C_t}$ method.

Gelatin zymography. Following treatment with baicalein for 24 h, samples of conditioned media were collected. Then, gelatin zymography was performed as previously described (4). Briefly, appropriate volumes of the unboiled samples (adjusted by vital cell number) were separated by 0.1% gelatin-8% SDS-PAGE electrophoresis. Then, the gels were soaked in 2.5% Triton X-100 for three times at room temperature, once for 30 min, and incubated in reaction buffer (10 mM CaCl_2 , 40 mM Tris-HCl and 0.01% NaN_3 , pH 8.0) at 37°C for 12 h. Gels were rinsed with distilled water, stained with Coomassie brilliant blue R-250. The gelatinolytic activities were quantified and analyzed by an image analysis system (Bio-Rad Laboratories, Richmond, CA, USA).

Western blot analysis. After treating with baicalein or SB203580 or anisomycin, proteins were extracted with lysis buffer (40 mmol/l Tris-HCl, 1 mmol/l EDTA, 150 mmol/l KCl, 100 mmol/l NaVO_3 , 1% Triton X-100, 1 mmol/l PMSF, pH 7.5). Then the proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. To block non-specific binding, membranes were blocked

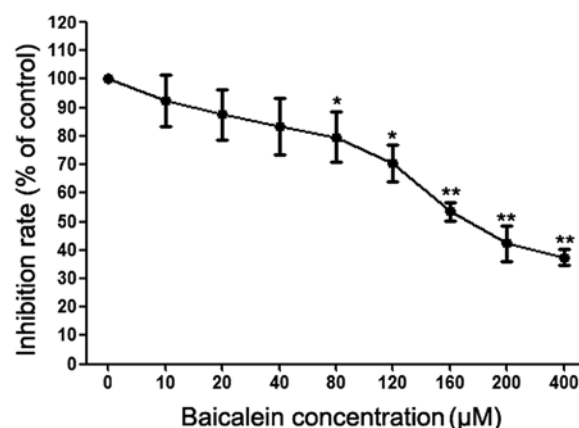


Figure 1. Cellular viability of SGC7901 cells treated with baicalein. SGC7901 cells were treated with baicalein, and after 24 h their viability was measured using an MTT assay. Values represent means \pm SD of three independent experiments, each performed in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared with the control group.

in defatted milk [5% in Tris-buffered saline with Tween-20 (TBST) buffer] at room temperature for 1 h and were then incubated with antibodies against p38 MAPK, p-p38 MAPK, MMP-2, -9 or β -actin overnight at 4°C. The membranes were then incubated with appropriate second antibodies for 1 h at room temperature. The bands were detected with an enhanced chemiluminescence kit (ECL Plus; Amersham, Freiburg, Germany) and exposed by autoradiography. The densitometric analysis was performed using ImageJ software (GE Healthcare, Buckinghamshire, UK).

Statistical analysis. Experiments were repeated three times, and the results of the studies are expressed as the means \pm SD. All statistical tests and corresponding p-values were two sided. $p < 0.05$ was considered to indicate a statistically significant difference. We performed correlation analysis by the Z-test.

Results

Baicalein inhibits the proliferation of gastric cancer cells. The antiproliferation effects of baicalein at various concentrations (0–400 μ M) on SGC7901 cells are shown in Fig. 1. At 80 μ M, baicalein clearly inhibited the proliferation of SGC7901 cells, therefore we chose a concentration range of baicalein lower than this for all subsequent experiments.

Baicalein inhibits the migration and invasion of gastric cancer cells. Fig. 2 shows the effect of baicalein on cell migration

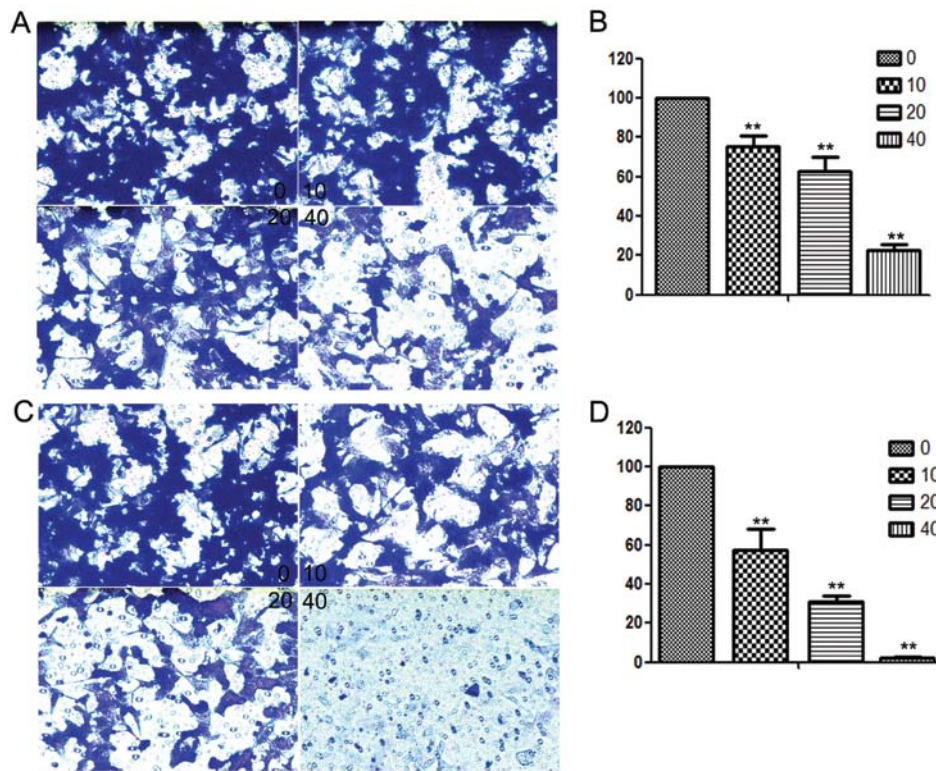


Figure 2. Effects of baicalein on the migration and invasion of SGC7901 cells. (A) For the migration assay, cells pre-incubated with various concentrations of baicalein (0, 10, 20 and 40 μ M) were plated onto the upper wells of the chamber. FBS (10%) was added to the lower wells for 24 h to induce cell migration. After 16 h, cells on the bottom side of the filter were fixed, stained and measured. Migration was assessed by measuring area of migrated cells in five microscopic fields/well at x200 magnification. Cell migration spontaneous migration in DMSO was designated as control. (B) The percent migration rate was expressed as a percentage of the control (0 μ M). (C) For the invasion assay, SGC7901 cells were assayed. Cells pre-incubated with various concentrations of baicalein (0, 10, 20 and 40 μ M) were plated onto the upper wells of the chamber. FBS (10%) was added to the lower wells for 24 h to induce cell invasion. After 24 h, cells on the bottom side of the filter were fixed, stained and measured. Invasion was assessed by measuring the area of migrated cells in five microscopic fields/well at x200 magnification. Cell migration spontaneous migration in DMSO was designated as control. (D) The percent invasion rate was expressed as a percentage of the control (0 μ M). Values represent the means \pm SD of three independent experiments performed in triplicate. ** p <0.01 compared with the control group.

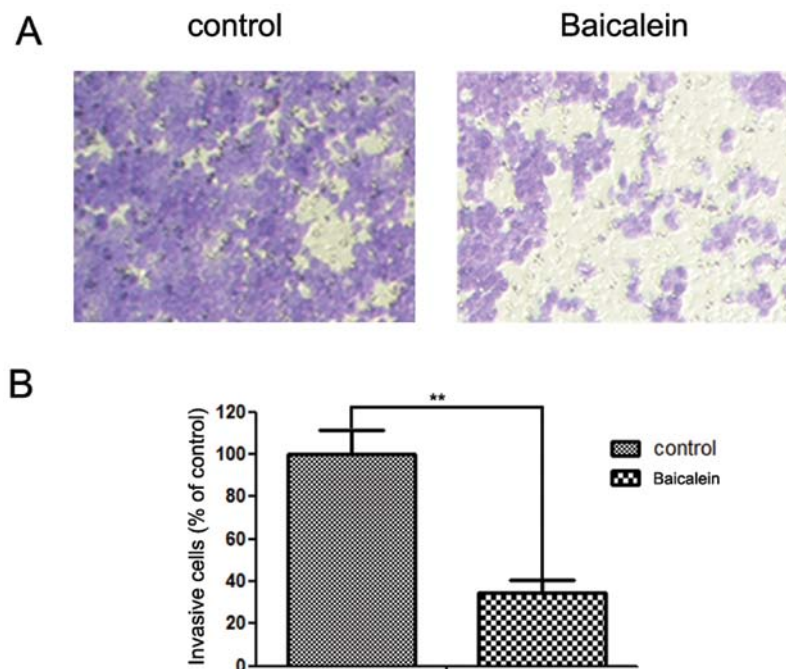


Figure 3. Baicalein inhibits the invasion of MGC803 cells. (A) For the invasion assay, cells pre-incubated with various concentrations of baicalein (0 and 40 μ M) were plated onto the upper wells of the chamber. FBS (10%) was added to the lower wells for 24 h to induce cell invasion. After 24 h, cells on the bottom side of the filter were fixed, stained and measured. Invasion was assessed by measuring the area of migrated cells in five microscopic fields/well at x200 magnification. Cell spontaneous invasion in DMSO was designated as control. (B) The percent invasion rate was expressed as a percentage of the control (0 μ M). Values represent the means \pm SD of three independent experiments performed in triplicate. ** p <0.01 compared with the control group.

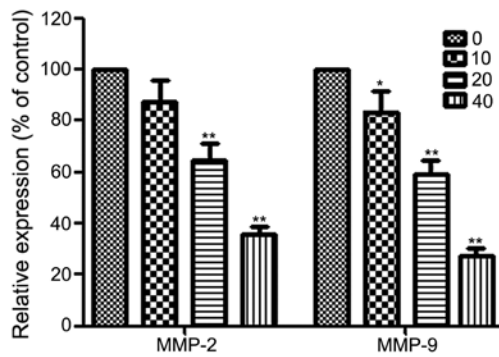


Figure 4. Inhibitory effect of baicalein on the transcriptional levels of MMP-2 and -9 in SGC7901 cells. We used RT-PCR to investigate the inhibitory effect of baicalein on MMP-2 and -9 in SGC7901 cells. SGC7901 cells were treated with 0 and 40 μ M baicalein for 12 h and then subjected to RT-PCR. We found that baicalein significantly reduced the transcriptional levels of MMP-2 and -9 in a concentration-dependent manner. Values represent the means \pm SD of three independent experiments performed in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared with the control group.

and invasion in SGC7901 cells that were treated with 0, 10, 20 and 40 μ M of baicalein for 16 h (cell migration) and 24 h (cell invasion), respectively. We showed that baicalein reduced the invasion and migration of SGC7901 cells substantially in a concentration-dependent manner. Quantification analysis indicated that the inhibition rate of migration and invasion were ~24.27, 37.13, 77.48 and 42.43, 68.81, 97.52%, respectively. Similar antimetastatic effect of baicalein was observed in MGC803 cells (data shown in Fig. 3).

Inhibitory effect of baicalein on the transcriptional levels of MMP-2 and -9. SGC7901 cells were treated with 0, 10, 20 and 40 μ M baicalein for 24 h and then subjected to RT-PCR. We found that baicalein significantly reduced the transcriptional levels of MMP-2 and -9 in a concentration-dependent manner (Fig. 4).

Baicalein suppresses the expression and activity of MMP-2 and -9. The expression and activity of MMP-2 and -9 in SGC7901 cells that were exposed to different concentrations of baicalein were examined. Cells were treated with 0, 10, 20 and 40 μ M baicalein for 24 h and then subjected to western blotting. Fig. 5A and B show that baicalein significantly reduced the protein levels of MMP-2 and -9 in a concentration-dependent manner compared with the control group. Gelatin zymography was performed to assess the activity of MMP-2 and -9 in cells treated with various concentrations of baicalein. As shown by gelatinolytic activity data, baicalein inhibited the activity of MMP-2 and -9 in a concentration-dependent manner (Fig. 5C and D). The effect of baicalein on the expression of MMP-2 and -9 in MGC803 is shown in Fig. 6.

The p38 signaling pathway is involved in the antimetastatic mechanism of baicalein. In human gastric cancer cells, activation of p38 is required for the invasion process and the mechanism is correlated with proteinases (7,12,13); thus, we investigated the effect of baicalein on the p38 signaling pathway in SGC7901 cells. Western blotting showed that baicalein reduced the phosphorylation of p38 in a concentration-dependent manner (Fig. 7A and B). Fig. 8 shows the effect of baicalein on the phosphorylation of p38 in MGC803 cells.

In order to examine whether the inhibitory effect of baicalein on cell invasion and MMP-2 and -9 expression was correlated with inhibition of the p38 signaling pathway, SGC7901 cells were pretreated with a p38 inhibitor (SB203580, 20 μ M) for 30 min and then incubated in the presence or absence of baicalein for 24 h. The results showed that treatment with SB203580 and baicalein significantly reduced both cell invasion (Fig. 7C and D) as well as MMP-2 and -9 protein expression (Fig. 7E and F).

Furthermore, chemical anisomycin, a p38 activator, was employed to confirm the role of the p38 signaling pathway. Fig. 9A shows the effects of anisomycin and baicalein on the invasion of SGC7901 cells. Quantification analysis indicated

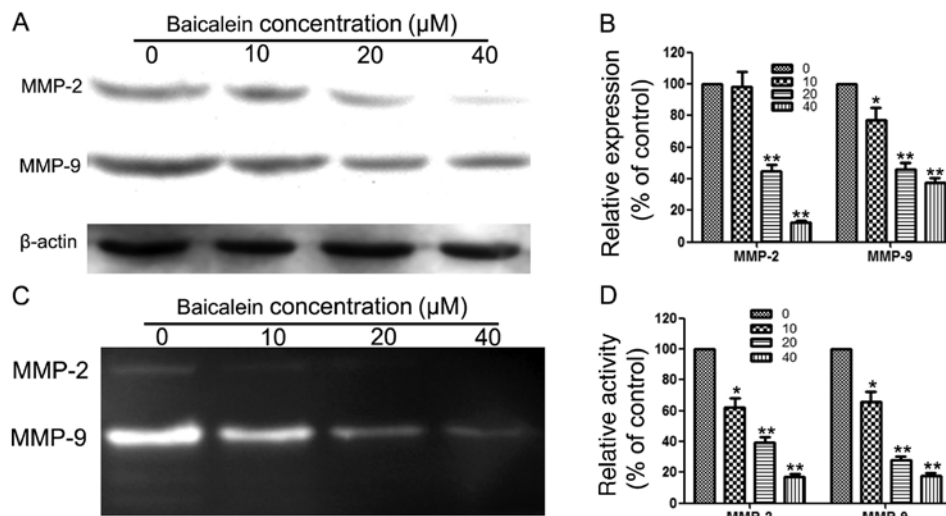


Figure 5. Baicalein suppresses the expression and activity of MMP-2 and -9 in SGC7901 cells. (A) SGC7901 cells were treated with baicalein (0, 10, 20 and 40 μ M) for 24 h and then subjected to western blotting to analyze the protein levels of MMP-2 and -9. (B) Quantification of the protein levels of MMP-2 and -9 in SGC7901 cells. (C) SGC7901 cells were treated with baicalein (0, 10, 20 and 40 μ M) for 24 h and then the activity of MMP-2 and -9 was analyzed. (D) Quantification of the activity of MMP-2 and -9 in SGC7901 cells. Values represent the means \pm SD of three independent experiments performed in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared with the control group.

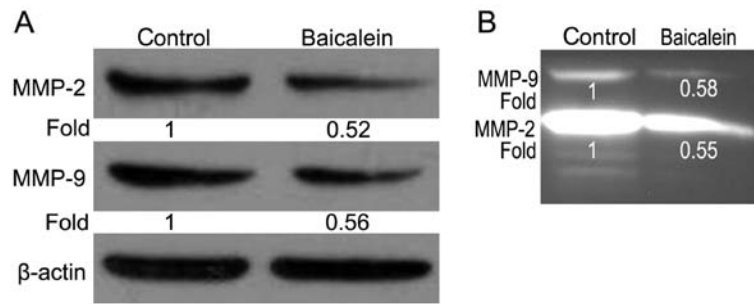


Figure 6. Baicalein suppresses the expression and activity of MMP-2 and -9 in MGC803 cells. MGC803 cells were treated with baicalein (0 and 40 μ M) for 24 h and then subjected to western blotting to analyze the protein (A) and activity (B) levels of MMP-2 and -9.

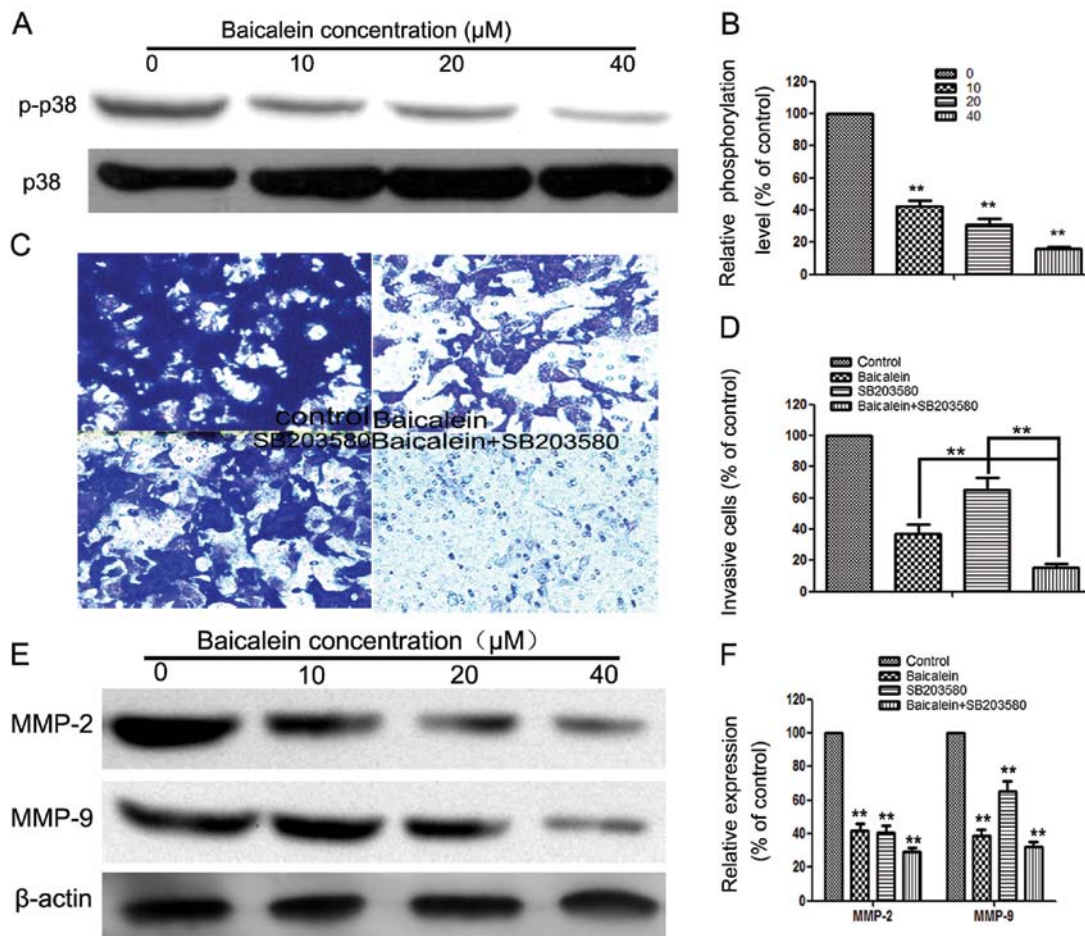


Figure 7. Effect of baicalein on the p38 signaling pathway, and effects of the p38 inhibitor (SB203580) and baicalein on cell invasion and MMP-2 and -9 expression in SGC7901 cells. (A) The protein levels of p38 and p-p38. (B) Phosphorylation densities of p38 were digitally scanned. Values represent the means \pm SD of three independent experiments performed in triplicate. ** p <0.01 compared with the control group. (C) Cells were pretreated with SB203580 (10 μ M) for 30 min and then incubated in the presence or absence of baicalein (40 μ M) for 24 h. Cellular invasiveness was measured using the Transwell chamber invasion assay. (D) The percent invasion rate was expressed as a percentage of control. (E and F) SGC7901 cells were treated and then subjected to western blotting to analyze the protein levels of MMP-2 and -9. Values represent the means \pm SD of three independent experiments performed in triplicate. ** p <0.01 compared with the control group.

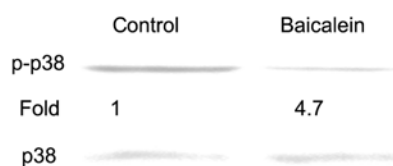


Figure 8. Baicalein reduces the activity of the p38 signaling pathway in MGC803 cells. After treating with baicalein (40 μ M) for 24 h, the expression of p38 and p-p38 was detected.

that anisomycin was able to enhance the invasion ability of SGC7901 cells, and anisomycin was able to reverse the inhibitory effect of baicalein on the invasion of SGC7901 cells (Fig. 9B and C) and MMP-2 and -9 protein expression (Fig. 9D and E). These results reveal that the inhibition of both cell invasion and MMP-2 and -9 expression by baicalein occurs through the suppression of the p38 signaling pathway.

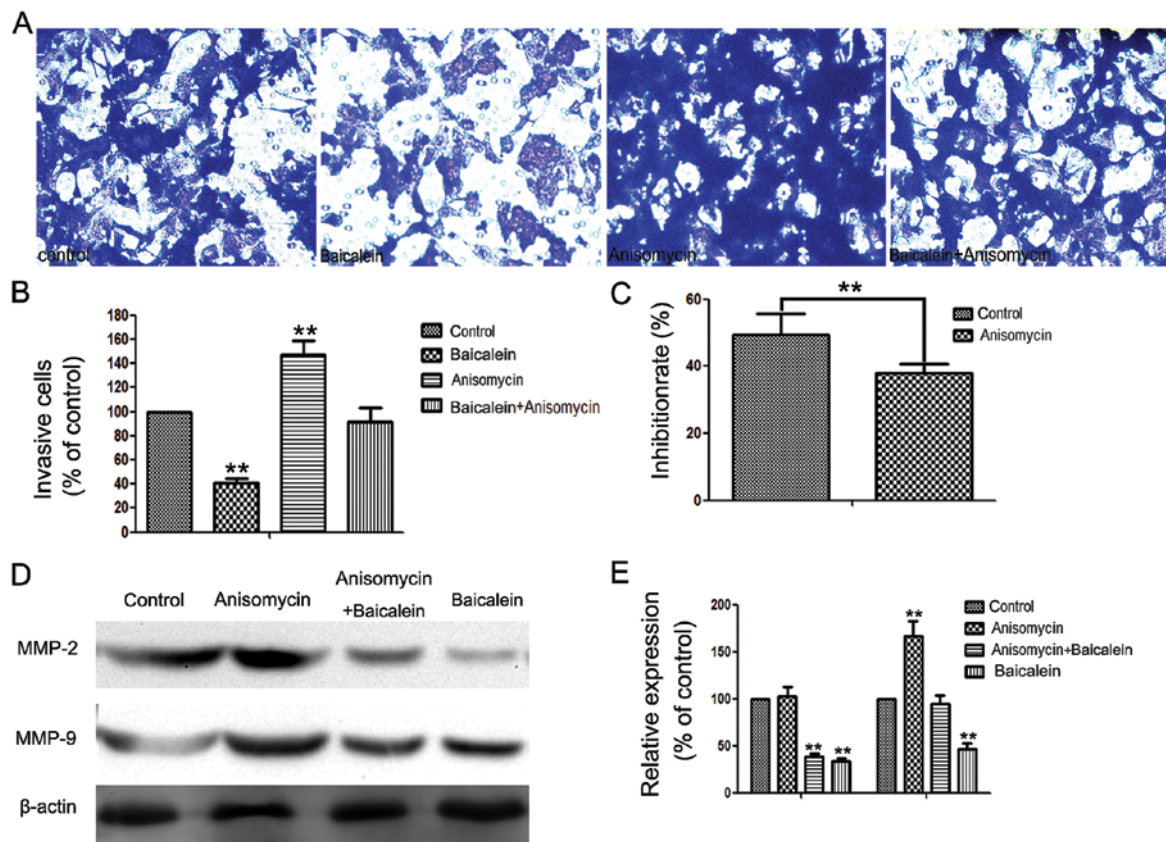


Figure 9. Effects of the p38 activator (anisomycin) and baicalein on cell invasion. (A) Cells were pretreated with anisomycin (25 μ g/ml) for 30 min and then incubated in the presence or absence of baicalein (40 μ M) for 24 h. Cellular invasiveness was measured using the Transwell chamber invasion assay. (B) The percent invasion rate was expressed as a percentage of control. (C) The inhibition rates of baicalein on two groups of cells. (D and E) SGC7901 cells were treated and then subjected to western blotting to analyze the protein levels of MMP-2 and -9. Values represent the means \pm SD of three independent experiments performed in triplicate. ** $p < 0.01$ compared with the control group.

Discussion

Gastric cancer is a serious public health problem worldwide (1), and administration of the antitumor natural product baicalein has been confirmed in many types of cancers (14-17). However, the antimetastatic effect and related mechanism(s) in gastric cancer cells remain unclear. In the present study, we investigated whether baicalein has anti-invasive and antimetastatic abilities in gastric cancer cells *in vitro* by regulating the expression of MMPs via inhibition of the p38 signaling pathway. According to our literature search, this is the first scientific report of the antimetastatic effect of baicalein on gastric cancer.

In order to elucidate the effect of baicalein on cell migration and invasion, Transwell chamber assay was performed. As it has been shown previously that baicalein inhibits the growth of various human cancer cell types (18), the results showed that baicalein can significantly inhibit the migration and invasion of gastric cancer cells at non-toxic doses ($<80 \mu$ M). These results showed that the inhibition of invasion by baicalein in SGC7901 cells was not due to cytotoxicity.

Metastasis is one of the leading causes of cancer-related mortality among gastric cancer patients. Degradation of the extracellular matrix (ECM) of blood or lymph vessels is critical to metastasis, since loss of the ECM allows cancer cells to invade the blood or lymphatic system and spread to other tissues

and organs (4). MMPs, particularly MMP-2/-9, are responsible for breaking down the ECM (19,20). Previous studies found that baicalein could inhibit the expression of MMP-2/-9 in various types of tumors (21-23). To clarify the mechanism of action of baicalein on gastric cancer, we investigated whether the inhibitory effect of baicalein on cell invasion is through regulation of the expression of MMPs. In the present study, we found that baicalein could significantly inhibit the expression and activity of MMP-2/-9 in gastric cancer cells. These results indicate that the antimetastatic effect of baicalein on SGC7901 cells was correlated with modulation of MMPs.

The synthesis of proteinases is regulated by multiple signaling cascades, including the p38 signaling pathway (22,24,25). The p38 signaling pathway is widely expressed in various tissues and has much broader functions physiologically (26). The p38 signaling pathway induces the expression of MMPs and thereby promotes the degradation of ECM proteins, which leads to cell invasion (27). A previous study found that baicalein could inhibit the invasion of cancer cells by reducing the expression of MMP-2/-9 via regulating the p38 signaling pathway (22). To further explore the possible mechanism(s) of baicalein in the inhibition of gastric cancer invasion, we determined the levels of phosphorylation of p38 in SGC7901 cells. The results showed that the phosphorylation of p38 in cells treated with baicalein was significantly reduced relative to that in control cells. Baicalein combined

with a p38 inhibitor (SB203580) significantly reduced gastric cancer cell invasion and was accompanied by downregulation of MMP-2/-9. However, p38 chemical activator (anisomycin) could block these effects induced by baicalein, suggesting baicalein directly downregulates the p38 signaling pathway.

In conclusion, the present study demonstrated the inhibitory effect of baicalein on the invasion and metastatic abilities of gastric cancer cells. Furthermore, the decrease in the expression of MMP-2/-9-induced by baicalein is attributed to an inhibition of the p38 signaling pathway. This mechanism may contribute to the inhibition of invasion and metastasis in SGC7901 cells by baicalein. These findings present a new potential therapeutic application of baicalein in antimetastatic therapy for gastric cancer.

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