

Baicalein inhibits the migration and invasion of colorectal cancer cells via suppression of the AKT signaling pathway

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Abstract. The anticancer effect of baicalein has been known for a number of years. However, its anti-metastatic effect and associated mechanisms in colorectal cancer (CRC) remain unclear. The present study investigated the hypothesis that treatment with baicalein may inhibit the proliferation, motility and invasion of human CRC cell lines via regulation of the protein kinase B (AKT) signaling pathway. Baicalein was demonstrated to significantly inhibit the migration and invasion of CRC cells ($P=0.01$). Additionally, after treatment with baicalein for 24 h, the protein expression levels of matrix metalloproteinase-2 (MMP-2) and MMP-9 in CRC cells were significantly reduced in a dose-dependent manner ($P=0.01$). Furthermore, treatment with baicalein significantly reduced the expression levels of phosphorylated AKT ($P=0.01$). In conclusion, baicalein appears to inhibit CRC cell migration and invasion by reducing the expression of MMP-2 and MMP-9 via suppression of the AKT signaling pathway. Thus, baicalein is a potential novel therapeutic agent for patients with CRC.

Introduction

Colorectal cancer (CRC) is a growing threat to public health. It is the second most fatal type of cancer and affects >1 million individuals annually worldwide (1,2). Although the removal of primary CRC tumors by surgical resection is typically straightforward, $\leq 50\%$ of patients with CRC suffer a relapse and eventually succumb to metastatic disease (3), showing that cancer metastasis is the most frequent cause of CRC-associated mortality. Therefore, the development of novel therapeutic agents is required to combat CRC.

Metastasis involves multiple processes, of which degradation of the extracellular matrix (ECM) is an important component (4). Through degradation of the ECM, cancer

cells escape the primary tumor, invade the blood or lymph vessels and migrate to new locations in the body. MMP-2 and MMP-9 are crucial molecules in the degradation of ECM, as well as affecting the metastasis of cancer cells in other ways, such as by inducing angiogenesis (5). In addition, the protein kinase B (AKT) pathway is key in the invasion of cancer cells via the regulation MMPs-2 and -9 (6-8).

Baicalein, which predominantly occurs as an active compound in the root of *Scutellaria baicalensis* Georgi, is a purified flavonoid with a defined chemical structure (4). Baicalein has been demonstrated to exert antitumor activity in various types of cancer, including CRC (9-11). However, to the best of our knowledge, to date, no studies have been conducted with regard to the anti-metastatic effect of baicalein in CRC. Therefore, the aim of the present study was to explore the effect of baicalein on the invasive ability of CRC, as well as to investigate the underlying mechanisms of this process.

Materials and methods

Cell culture. The DLD-1 colorectal carcinoma cell line (American Type Culture Collection, Rockville, Maryland, USA) was maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 $\mu\text{g/ml}$ streptomycin and 100 units/ml penicillin (all from Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO_2 at a temperature of 37°C.

Cell viability assays. Cell survival was assessed by performing a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (4). Briefly, cells were plated in 96-well culture plates at a density of 5×10^3 cells per well and treated with various concentrations of baicalein (0-120 μM). After a 24-h incubation, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with 5 mg/ml MTT (Sigma-Aldrich) for 4 h. The cells were subsequently washed with PBS, solubilized in dimethyl sulfoxide and the optical density of each well was measured using an enzyme-linked immunosorbent assay plate reader (680; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 490 nm.

Migration and invasion assays. The *in vitro* migration and invasion assays were conducted in a Transwell® chamber with a Costar® 8- μm pore size polycarbonate membrane (Corning Life Sciences, Cambridge, MA, USA) coated with Matrigel™

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or not, as described previously (11). Briefly, cells were trypsinized and suspended to a final concentration of 1×10^6 cells/ml in serum-free medium. The suspended cells were treated with varying concentrations of baicalein (0, 10, 20 and 30 μM) for 24 h prior to seeding and then added into each upper chamber of the Transwell. The bottom chamber contained medium supplemented with 10% FBS to serve as a chemoattractant. Following incubation for 24 h at 37°C in a humidified atmosphere of 5% CO_2 , all of the non-invaded cells were removed from the upper face of the Transwell membrane using a cotton swab, and the invaded cells were fixed, stained and counted.

Western blot analysis. Western blot analysis was performed as previously described (4). Following treatment with various concentrations of baicalein (0, 10, 20 and 30 μM), 1×10^6 cells were suspended in 100 μl lysis buffer (1 mmol/l EDTA, 40 mmol/l Tris-HCl, 150 mmol/l KCl, 100 mmol/l NaVO_3 , 1% Triton X-100 and 1 mmol/l phenylmethylsulphonyl fluoride; pH 7.5). The proteins (60 μg) were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were subsequently blocked with non-fat milk [5% in Tris-buffered saline and Tween-20 (TBST) buffer] at 37°C for 1 h to prevent non-specific binding. After 1 h, the membranes were incubated overnight with antibodies against polyclonal AKT (cat no. 9272), monoclonal phosphorylated-AKT (p-AKT; cat no. 4060), polyclonal MMP-2 (cat no. 4022), polyclonal MMP-9 (cat no. 3852) or monoclonal β -actin (cat no. 3700; Cell Signaling Technology, Beverly, MA, USA) in TBST containing 5% non-fat milk at a temperature of 4°C (12,13). The membranes were subsequently incubated with a horseradish peroxidase goat anti-mouse (cat no. 7076) or anti-rabbit IgG (cat no. 7077) antibodies (Cell Signaling Technology) for 1 h at room temperature. The bands were detected using an enhanced chemiluminescence (ECL) kit (Amersham ECL Plus; GE Healthcare, Freiburg, Germany) and exposed to autoradiography film. Densitometric analysis was performed using Image J software (National Institutes of Health, Bethesda, MD, USA). The results are expressed as relative expression (%) compared with the control

Statistical analysis. All statistical analyses were performed using SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA). Experiments were repeated three times and data were analyzed using Student's t-test. All statistical tests and corresponding P-values were two sided. $P < 0.05$ was considered to indicate a statistically significant difference. Correlation analysis was performed using a Z-test (13,14).

Results

Baicalein inhibits the proliferation of DLD1 cells. The anti-proliferation effects of baicalein at various concentrations (0–120 μM) on DLD1 cells are shown in Fig. 1. At a concentration of 50 μM , baicalein significantly inhibited the proliferation of the DLD1 cells ($P < 0.05$). However, at concentrations $< 50 \mu\text{M}$, the inhibitory effect of baicalein was not significant; thus, a concentration range of baicalein $< 50 \mu\text{M}$ was selected for all subsequent experiments.

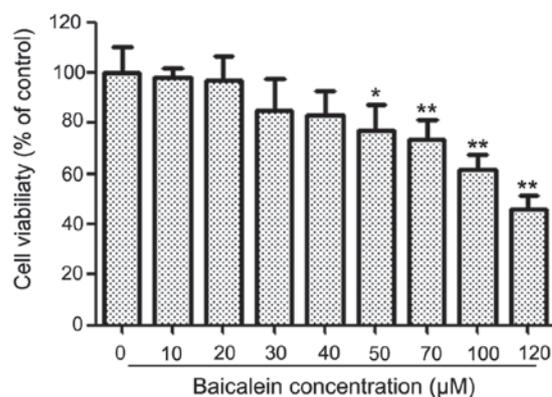


Figure 1. Cellular viability of DLD1 cells. DLD1 cells were treated with increasing concentrations of baicalein and after 24 h their viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values represent the mean \pm standard deviation of three independent experiments performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ vs. control group.

Baicalein inhibits the migration and invasion of DLD1 cells.

Fig. 2 shows the effect of baicalein on cell migration and invasion in DLD1 cells that were treated with 0, 10, 20 and 30 μM baicalein for 24 h, respectively. Treatment with baicalein significantly reduced the invasion and migration of DLD1 cells in a concentration-dependent fashion ($P < 0.01$).

Baicalein suppresses the expression of MMP-2 and MMP-9.

The protein expression levels of MMP-2 and MMP-9 in DLD1 cells that were exposed to different concentrations of baicalein was examined. Cells were treated with 0, 10, 20 and 30 μM baicalein for 24 h and then subjected to western blotting. Fig. 3A and B shows that baicalein significantly reduced the protein expression levels of MMP-2 and MMP-9 in a concentration-dependent manner compared with the control group ($P < 0.01$).

Involvement of the AKT signaling pathway in the anti-metastatic mechanism of baicalein.

The present study also investigated effect of baicalein on the AKT signaling pathway in DLD1 cells. Western blotting indicated that treatment with baicalein significantly reduced the relative expression levels of p-AKT compared with AKT in a concentration-dependent manner ($P < 0.01$; Fig. 4A and B).

Discussion

Baicalein has been demonstrated to exhibit antitumor effects in various types of cancer (9,15,16). However, the antimetastatic effect and the associated mechanisms in CRC cells remains unclear. The present study revealed that baicalein significantly inhibits the invasive and metastatic ability of CRC cells by regulating the expression levels of MMP-2 and -9 via inhibition of the AKT signaling pathway. To the best of our knowledge, the present study is the first to investigate the antimetastatic effect of baicalein on CRC cells.

A Transwell chamber assay was employed to detect the antimetastatic effect of baicalein on CRC cells. The results demonstrated that treatment with baicalein significantly reduced the migration and invasion of CRC cells. These

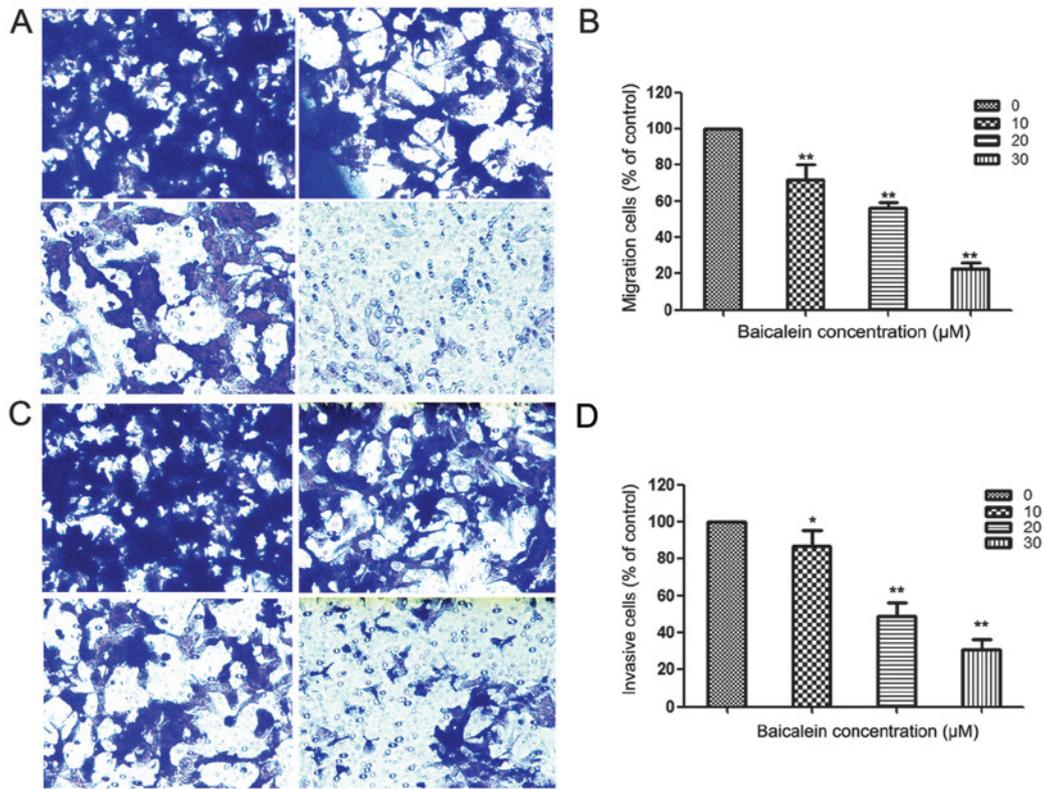


Figure 2. Effect of baicalein treatment on the invasive ability of DLD1 cells. (A) For the migration assay, cells pre-incubated with baicalein (0, 10, 20 and 30 μM) were plated onto the upper wells of the Transwell® chamber. The spontaneous migration in cells treated with dimethyl sulfoxide (DMSO) was designated as the control. (B) The migration rate (%) was expressed as a percentage of the control (0 μM baicalein). (C) For the invasion assay, DLD1 cells pre-incubated with baicalein (0, 10, 20 and 40 μM) were plated onto the upper wells of the chamber and fetal bovine serum (10%) was added to the lower wells for 24 h to induce cell invasion. After 24 h, invaded cells on the bottom side of the filter were fixed, stained and measured. The spontaneous migration in cells treated with DMSO was designated as the control. (D) The invasion rate (%) was expressed as a percentage of the control (0 μM baicalein). Values represent the mean ± standard deviation of three independent experiments performed in triplicate. *P<0.05 and **P<0.01 vs. control group.

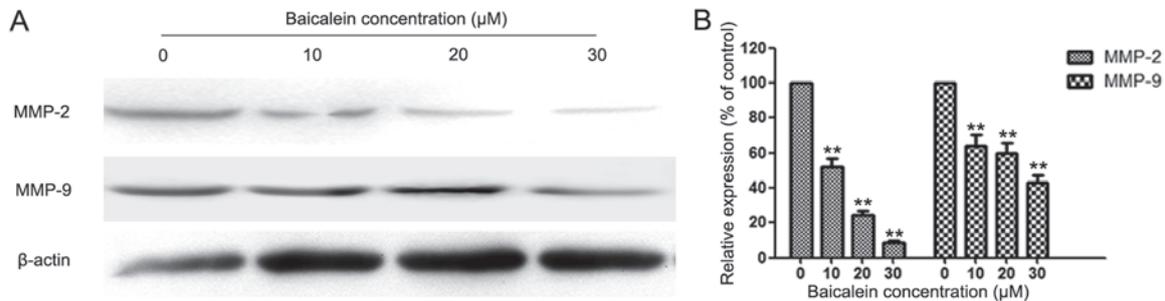


Figure 3. Baicalein treatment suppresses the protein expression levels of MMP-2 and -9 in DLD1 cells. (A) DLD1 cells were treated with baicalein (0, 10, 20 and 30 μM) for 24 h and then subjected to western blotting to analyze the expression of MMP-2 and -9. (B) Quantification of MMP-2 and -9 expression. Values represent the mean ± standard deviation of three independent experiments performed in triplicate. *P<0.05 and **P<0.01 vs. control group. MMP, matrix metalloproteinase.

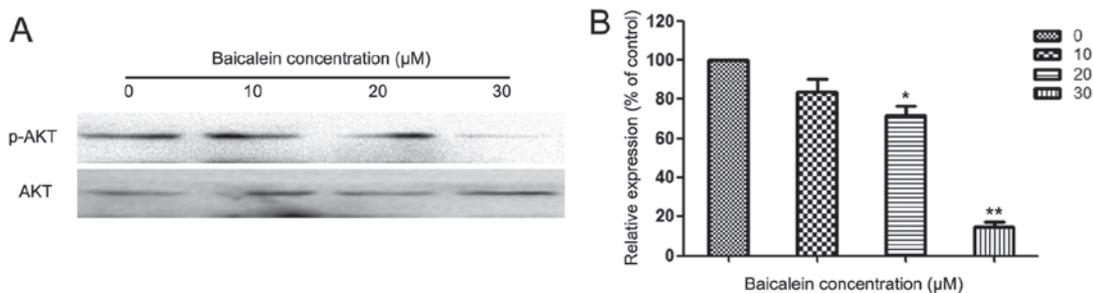


Figure 4. Effect of treatment with increasing concentration of baicalein (0, 10, 20 and 30 μM) on the AKT signaling pathway. (A) Western blot of the protein expression levels of AKT and p-AKT. (B) Quantified phosphorylation densities of AKT by digital scanning. Values represent the mean ± standard deviation of three independent experiments performed in triplicate. *P<0.05 and **P<0.01 vs. control group. AKT, protein kinase B; p-AKT, phosphorylated.

results are in agreement with previously conducted studies, in which baicalein inhibited the migration and invasion of hepatocellular carcinoma, glioma, breast cancer and tongue cancer cells (4,11,17,18).

Metastasis of cancer cells is a complex multistep process involving cell adhesion, invasion and motility. One of the key steps in metastasis, which involves MMP-2 and -9, is degradation of the ECM of blood or lymph vessels (19). In CRC patients, plasma MMP-2 levels are correlated with disease stage and survival (19), while MMP-9 expression is correlated with the progression of CRC and hence may be a potential target for the treatment of CRC (20,21). Furthermore, MMP-2 and MMP-9 expression in normal mucosa from patients with CRC are independently associated with the outcome of patients with CRC (22). Therefore, to elucidate the mechanism of the antimetastatic effect of baicalein, the present study determined the expression levels of MMP-2/-9 in CRC cells treated with baicalein, and baicalein was demonstrated to significantly inhibit the protein expression levels of MMP-2/-9 in CRC cells.

Numerous cell signaling pathways are critical in the regulation of MMPs (4,17,23,24). For example, the AKT signaling pathway is associated with the expression of MMP-2/-9 in healthy and cancerous tissues (24,25). Furthermore, the antitumor effects of baicalein treatment are associated with the AKT signaling pathway (26). Hence, interruption of this signaling pathway is one possible approach for the development of novel antimetastatic therapeutic agents. Therefore, the present study investigated the effect of baicalein treatment on the activity of the AKT signaling pathway in CRC cells, and baicalein was shown to significantly reduce the activity of the AKT signaling pathway by inhibiting AKT phosphorylation, subsequently leading to the suppression of CRC cell migration and invasion. However, further investigation is required to determine at what stage the pathway is inhibited.

In conclusion, the present study demonstrated the effect of baicalein treatment on the invasion and metastatic capabilities of CRC cells. Baicalein treatment was demonstrated to significantly correlate with decreased protein expression levels of MMP-2 and MMP-9. Furthermore, the reduced expression of MMP-2 and MMP-9 induced by baicalein was attributed to inhibition of the AKT signaling pathway. This mechanism may contribute to the inhibition of CRC cell invasion and metastasis caused by treatment with baicalein. Thus, the findings of the present study reveal a novel potential therapeutic application of baicalein in antimetastatic CRC therapy. Future studies should focus on determining the anti-metastatic effect of baicalein *in vivo*.

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