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Baicalin Suppresses Migration, Invasion and Metastasis of Breast Cancer *via* p38MAPK Signaling Pathway

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Abstract: Metastasis is the major cause of death in breast cancer patients. In this study, we investigated the effects of baicalin, a natural compound, on cell migration, invasion and metastasis using human breast cancer MDA-MB-231 cell line as model system. Baicalin not only dose-dependently inhibited MDA-MB-231 cells migration and *in vitro* invasion, but also suppressed the tumor outgrowth and the pulmonary metastasis of MDA-MB-231 cells in xenograft model. Importantly, treatment of baicalin caused little change in body weight, liver and kidney function of recipient animals. Tumorigenesis-inhibitory effect is likely linked to the capability of baicalin to downregulate metalloproteinase (MMP)-2, MMP-9, urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR) expression in MDA-MB-231 cells. As baicalin blocked p38 mitogen-activated protein kinase (MAPK) activity and treatment of p38MAPK inhibitor SB203580 led to the reduction of MMP-2, MMP-9, uPA and uPAR expressions, we concluded that baicalin suppresses the tumorigenecity of MDA-MB-231 cells by down-regulating MMP-2, MMP-9, uPA and uPAR expressions through the interruption of p38MAPK signaling pathway.

Keywords: Baicalin, breast cancer, MDA-MB-231 cell, metastasis, side effects.

INTRODUCTION

Breast cancer is one of the most common malignancies and its incidence and mortality vary considerably worldwide [1]. Currently, the incidence and the mortality of breast cancer in China are increasing rapidly and the increasing of mortality is associated with the metastasis of breast cancer and medical care [2, 3]. To date, chemotherapy is the most frequently-used treatment for metastatic breast cancer. However, adjacent normal cells are also destroyed during chemotherapy [4]. Because of the limitation and the toxicity of chemotherapy, it is recommended to look for safer and more effective agents from the natural products to prevent and suppress metastasis of breast cancer.

Baicalin (Fig. **1A**) is a natural compound isolated from *Baikal Skullcap Root* that has been used as a herbal medicine in China for thousand years. Early studies demonstrate that baicalin possesses a potent anti-tumor capability in human pancreatic [5], lung [6, 7], breast [8, 9], prostate [10], liver cancer cells [11] and malignant melanoma cells [12]. We have previously reported that baicalin was able to induce apoptosis in human breast cancer MCF-7 cells [13]. Others have shown that baicalin can inhibit migration and metastasis of lung cancer cells [7] and melanoma cells [12]. However, whether baicalin can inhibit the metastasis of human breast cancer cells is unknown.

Degradation of extracellular matrix (ECM) by proteolytic enzymes and subsequent cancer invasion are the essential early steps of metastasis [14]. Metalloproteinase (MMP) and urokinase-type plasminogen activator (uPA) are two important proteolytic enzymes that play important roles in the degradation of ECM and basement membrane (BM). It has been reported that up-regulated MMP-2, MMP-9, uPA and uPAR expressions are often associated with the increased breast cancer cell invasion and metastasis [15, 16].

Mitogen-activated protein kinase (MAPK) signaling pathways are actively involved in cell proliferation, differentiation, apoptosis, angiogenesis and tumor metastasis [17]. In recent years, MAPK signaling pathways have been shown to regulate degradation of ECM, cells migration and invasion of cancer cells [18]. For example, p38MAPK signaling pathway can facilitate breast cancer invasion and metastasis by up-regulating uPA expression [19, 20].

In this study, we investigated the effects of baicalin on various tumorigenic properties of highly metastatic human breast MDA-MB-231 cells. We showed that baicalin inhibited migration, invasion and metastasis of MDA-MB-231 cells. The tumorigenesis-suppressing role of baicalin is due to its ability to reduce MMP-2, MMP-9, uPA, and uPAR expression. We further demonstrated that baicalin's inhibitory effect on p38 MAPK activity is likely responsible for baicalin-caused reduction in MMP-2, MMP-9, uPA, and uPAR expression.

MATERIALS AND METHODS

Materials

Baicalin was obtained from Shanghai Standardization for the Traditional Research Center and dissolved in dimethyl sulfoxide (DMSO). Matrigel, 3-(4, 5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide (MTT) were purchased from Sigma (St Louis, MO, USA). SB203580 was obtained from Biomol (Philadelphia, PA, USA). Antibodies used in this study were MMP-9, MMP-2, TIMP-1, TIMP-2, uPA, uPAR, p-NF-κB (p50) and p-NF-κB (p65) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p38MAPK, p-p38MAPK, p-ERK, p-SAPK/JNK from Cell Signaling Technology (Boston, MA, USA). IRDye^{TM} fluorescence antibodies were obtained from Li-Cor Bioscience (Lincoln, NE, USA).

Cell Culture

Human breast cancer MDA-MB-231 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 0.01 mg/mL insulin, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5% CO_2 in a humidified atmosphere.

Cell Viability Assay

Cell viabilities were determined by MTT assay. Briefly, MDA-MB-231 cells (5×10^4 cells/well) were seeded in 96-well culture plates. After overnight incubation, various concentrations of baicalin were added to cells for varying times followed by the

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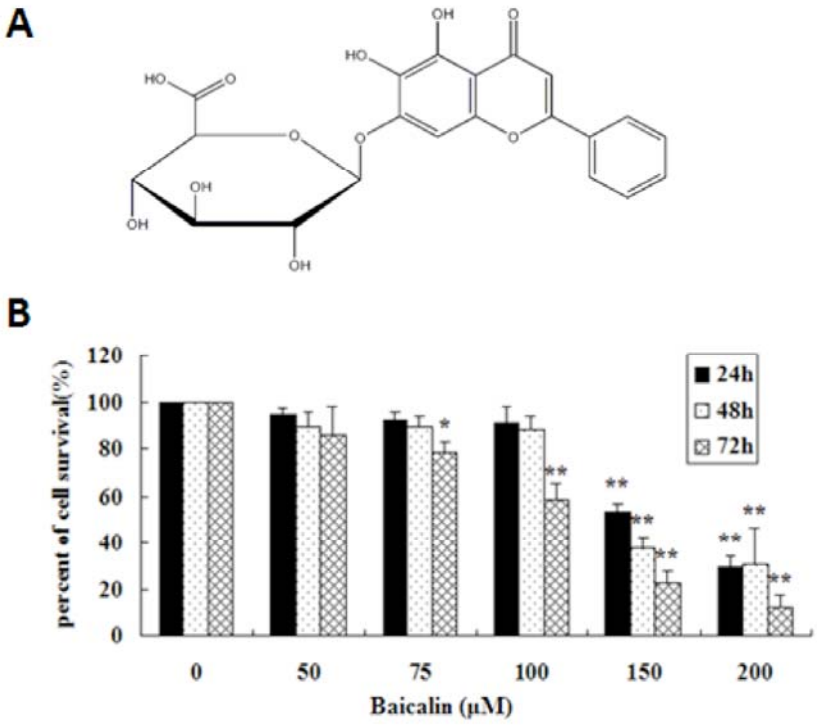

Fig. (1). A, The chemical structure of baicalin. B, Effect of baicalin on breast cancer cell viability. The effects of baicalin on MDA-MB-231 cell viabilities were observed by MTT assay. Cells were treated with Baicalin50, 75, 100, 150, 200 μM for 24, 48, and 72 h, respectively. Results were presented as means ± SD of three independent experiments; SD denoted by error bars. * $p < 0.05$, ** $p < 0.01$ *vs.* untreated cells.

addition of 20 μL MTT at 37 °C for 4 h. Formed formazan crystals were dissolved in 150 μL DMSO and optical density (OD) was measured at 490 nm using an ELISA plate reader (BioTek, Winooski, Vermont, USA).

Wound Healing Assay

MDA-MB-231 cells were seeded at a density of 1~5 × 10^5cells/well in 12-well culture plates and allowed to form a confluent monolayer. The layer of cells was scraped with a 20-200 μL micropipette tip to create a wound of ~1 mm width. Cells were washed twice with PBS and replaced with serum-free medium containing various concentrations of baicalin. At 0, 24, 48 and 72 h, cells were washed with PBS and then fixed with 4% paraformaldehyde followed by staining with 0.5% Coomassie Brilliant Blue. Images of the wounds were monitored under a phase-contrast microscope at 100-fold magnification.

Migration and Invasion Assays

Cell migration was analyzed with the aid of Transwells (Corning, USA). To analyze cell invasion, the upper surface of filter membrane in the upper compartment of Transwell was coated with 30 μg Matrigel. Cells ($1x10^5$) suspended in 200 μL of serum-free medium were seeded onto the upper compartment of the Transwell and the lower chambers were filled with medium containing 10% FCS and various concentrations of baicalin. After 24 h, cells remained in the upper chamber were removed and cells on the undersurface of the filters were fixed with 70% ethanol followed by staining with 0.5% Coomassie Brilliant Blue for 10 min. The migrated or invaded cells were visualized and counted from six randomly selected fields (× 100 magnifications) under a phase-contrast microscope.

Effects of Baicalin on Breast Cancer MDA-MB-231 Xenograft

Six-week-old female athymic nude mice were obtained from Laboratory Animal Center at Shanghai University of Traditional Chinese Medicine and housed in pathogen-free condition throughout the experimental duration. Briefly, mice were injected with $3x10^6$ MDA-MB-231 cells (suspended in matrigel). One day after tumor cell inoculation, mice received either 1% DMSO/10% Tween-80 in PBS (sham-treated group, n=8) or 100 mg⁄kg baicalin (baicalin-treated group, n=8) every two days through intraperitoneal injection. Mice were closely monitored, their bodies were weighed and tumor weights were measured weekly. Eight weeks after tumor cell inoculation, mice were sacrificed and tumors were excised. Lungs were also collected from sacrificed animals, sectioned and stained with hematoxylin and eosin (H&E). Representative fields for each group were photographed and the metastatic nodules were counted.

Function Tests of Liver and Kidney

At the time of necropsy, 1mL blood was collected through eye-bleeding and centrifugated at 3,000 rpm for 10 minutes to obtain sera. Sera were analyzed for the levels of glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), creatinine (Cr) and urea nitrogen (BUN) using the respective colorimeter testing kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA)

To detect the effect of baicalin on the secretion of MMP-2 and MMP-9 from MDA-MB-231 cells, the cells were treated with 25, 50 and 100 μM baicalin for 48 h, supernatants were then collected and analyzed by ELISA using Human MMP-9 (R&D Systems, Minneapolis, MN, USA) and Human MMP-2 ELISA Kits (RayBiotech, Norcross, GA, USA) according to the manufacturers' instruction.

Western Blot Analysis

Whole cell lysatse were electrophoresed on 8% or 10% SDS-PAGE. The levels of MMP-2, MMP-9, TIMP-1, TIMP-2, uPA,

uPAR, p38MAPK, p-p38MAPK, p-ERK, p-SAPK/JNK, p-NF-κB (p50), p-NF-κB (p65) and GAPDH were detected by first incubating with the respective primary antibodies (1:1,000~5,000) and visualized by IRDye^{TM}700DX (red) or IRDye^{TM}800DX (green) conjugated secondary antibodies (1:10,000~20,000). Images were generated using Odyssey Infrared Imaging System (Li-Cor Biosciences, NE, USA). Quantitative analyses of Western blots were performed using Alpha Ease FC (FluorChem FC2) software. Relative protein expression was standardized to the level of GAPDH.

Statistical Analyses

All data are the means ± SD. Comparisons between the groups were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA). $p < 0.05$ is considered significant.

RESULTS

Baicalin Inhibits Breast Cancer Cell Migration

Baicalin has previously been shown to induce apoptosis of various cancer cell types [5-13]. To determine the general effect of baicalin on breast cancer cell survival, varying concentration of baicalin was added to the culture of MDA-MB-231 cells for up to 3 days. MTT assay showed that significant baicalin-induced cell death only occurred at the concentration of 150 μM after 72 h or 200 μM after 48 h (*vs.* untreated cells, $p < 0.05$) (Fig. **1B**). We subsequently examined the effect of baicalin on cell migration by performing wound healing assay with non-lethal concentrations and times (≤ 100 μM, ≤ 48 h). As shown in Fig. (**2A**), the gaps in 25, 50, 100 μM baicalin-treated groups were significantly wider than those of the untreated group at 24, 48, and 72 h. We further analyzed cell migration by Transwell assay. Similar to what was seen with wound healing assay, baicalin effectively inhibited cell migration and over 50% and 65% reduction could be seen in MDA-MB-231 cells treated with 50 μM and 100 μM respectively (Fig. **2B** and **2C**). Since baicalin at these concentrations affected little cell viability, these results indicate that baicalin can effectively inhibit the motility of MDA-MB-231 cells.

Baicalin Inhibits Breast Cancer Cell Invasion

To determine the effect of baicalin on invasion, MDA-MB-231 cells were treated with 25, 50 and 100 μM baicalin followed by allowing cells to invade in Matrigel-coated transwells for 24 h. The number of cells invaded was reduced by baicalin in a dose-dependent manner (Fig. **3**). Compared with untreated group, baicalin at 50 and 100 μM blocked approximately 60 and 70% of invasion respectively ($p < 0.01$). These data clearly show that baicalin is a strong suppressor of breast cancer cell invasion.

Effects of Baicalin on Breast Cancer MDA-MB-231 Xenograft

The ability of baicalin to potently block *in vitro* invasion prompted us to investigate its effectiveness to inhibit *in vivo* metastasis. Athymic nude mice were injected with MDA-MB-231 cells for 1 day followed by (every two days) intraperitoneal injection of 100 mg⁄kg baicalin or vehicle (sham-treated) for 2 months. We prepared H&E-stained section from the lungs excised from sacrificed animals and performed histological examination to determine the number of metastatic nodules in lungs. The average number of tumor nodules in sham-treated group was 21.60 ± 3.92 while was 8.6 ± 1.51 in baicalin-treated group ($p < 0.01$) (Fig. **4B**), suggesting that baicalin effectively decreases tumor cell colonization to the lung. Another noticeable difference between two groups was that the sizes of these nodules were significantly larger in sham-treated group than those in baicalin-treated group (Fig. **4A**). Moreover, tumors weighed 0.46 ± 0.07g in average in sham-treated group while average weight was 0.10 ± 0.05g in baicalin-treated group (Fig. **4C**), representing over 78% of inhibition in tumor outgrowth. Together, these results suggested that baicalin can block breast tumor outgrowth and metastasis.

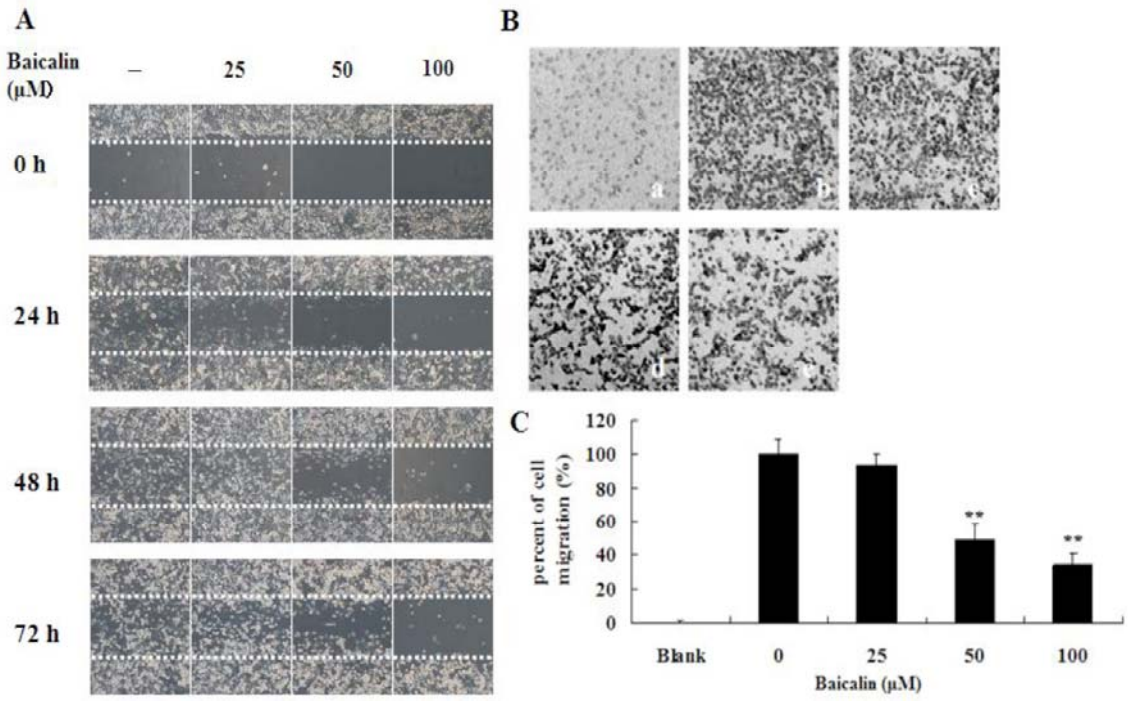

Fig. (2). Effect of baicalin on MDA-MB-231 cell migration. A. Images of wound healing assays (×100 magnifications). Cells were seeded into 12 well cell culture plates and cultured to near confluence. The wounded monolayer was incubated in free-FCS RPMI-1640 containing 0, 25, 50, 100 μM of baicalin for 24, 48, and 72 hours. **B.** Transwell chamber was used for the migration assay (× 100 magnifications). MDA-MB-231 cells were treated with 0(b), 25(c), 50(d) or 100(e) μM of baicalin for 24 h. (a) Blank (not adds cell). **C.** Percent of cell migration. Stand error bars represent three independent experiments and each experiment was triplicate. ** $p < 0.01$ *vs.* untreated cells.

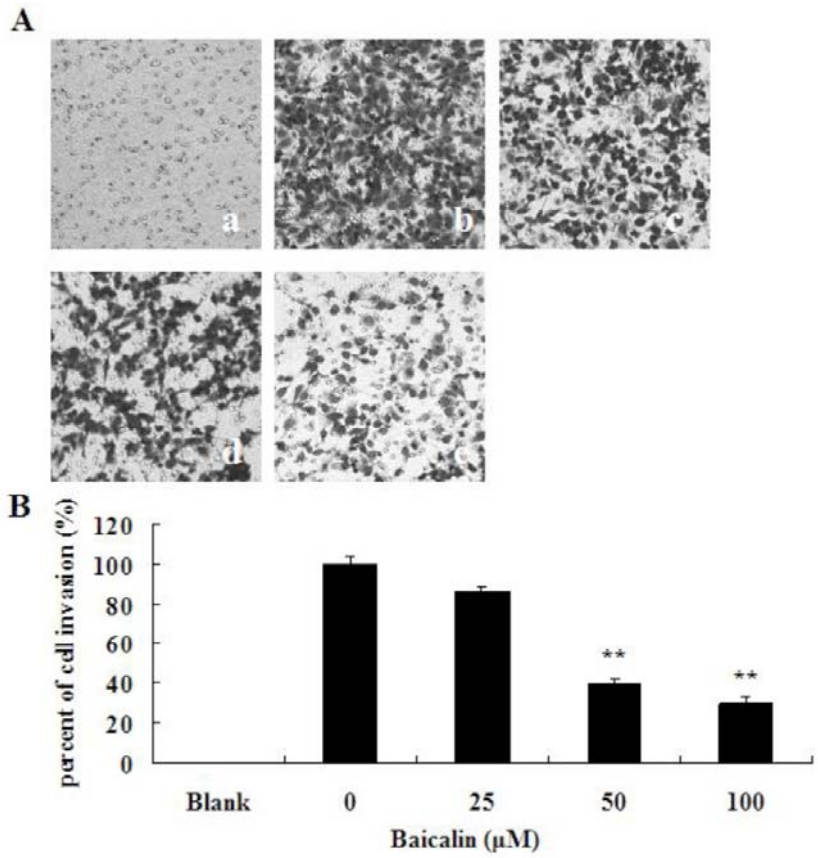

Fig. (3). Effect of baicalin on MDA-MB231 cell invasion. A. Transwell chamber invasion assay (× 100 magnifications). The filter membranes were coated with matrigel. MDA-MB-231 cells were treated with 0 (b), 25 (c), 50 (d) or 100 (e) μM baicalin for 24 h. (a) Blank (not adds cell). **B.** Percent of cell invasion. Stand error bars represent three independent experiments and each experiment was triplicate. ** $p < 0.01$ *vs.* untreated cells.

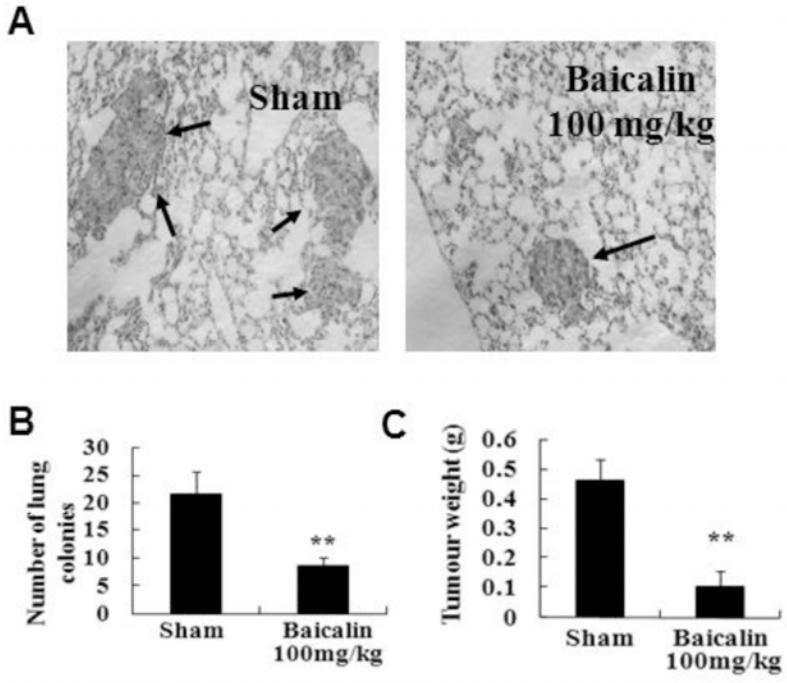

Fig. (4). Baicalin inhibits the pulmonary growth and metastasis in MDA-MB-231 breast cancer xenograft. Mice were given 100mg/kg baicalin (i.p.) for 8 weeks (n=8). Lung tissues were removed and fixed with Bouin's solution for 24 h. Metastatic nodules on the lungs were counted under a dissecting microscope (× 100 magnifications). **A.** Histological appearance of representative lungs from sham- and baicalin-treated mice. **B.** The quantization of metastatic lung nodules. **C.** Tumor weights by sham- and baicalin-treated. ** $p < 0.01$ *vs.* sham-treated group.

Baicalin Treatment does not elicit Side Effects in Mice

To determine whether baicalin treatment caused side effects, the body weights of mice were measured every week. Among normal, sham- and baicalin-treated groups, there were no significant differences in their body weights (Fig. **5A**). When liver and kidney functions were further analyzed, we found that there was no significant difference in the levels of serum GPT (Fig. **5B**), GOT (Fig. **5C**), Cr (Fig. **5D**) and BUN (Fig. **5E**) between normal, sham- and baicalin-treated groups. Together, these results implicate that baicalin can be safely used *in vivo* for suppressing breast tumorigenecity.

Baicalin Reduces MMP-2 and MMP-9 Secretion from MDA-MB-231 Cells

MMP-2 and MMP-9 are known to play essential role in cancer cell invasion and metastasis by facilitating the degradation of ECM and BM. We thus measured the amount of MMP-2 and MMP-9 secreted from MDA-MB-231 cells with or without baicalin treatment. As shown in Fig. (**6**), baicalin greatly decreased the secretion of MMP-2 and MMP-9 from MDA-MB-231 cells ($p < 0.01$).

Effect of Baicalin on Proteolytic Enzymes, MAPK and NF-κB

To determine whether the expression of proteolytic enzymes was affected by baicalin, we compared the levels of MMP-2, MMP-9, uPA and uPAR in MDA-MB-231 cells treated with or without baicalin. Western blotting with the respective antibodies showed that the levels of uPA, uPAR, MMP-2 and MMP-9 expressions were all decreased by 100 μM baicalin (Fig.**7A**). In contrast, the levels of TIMP-1 and TIMP-2 were not altered by baicalin (Fig. **7A**). These results suggested that baicalin is most likely to inhibit MDA-MB-231 cell migration, invasion and metastasis by downregulating the levels of proteolytic enzymes. Moreover, baicalin also reduced the levels of both p38 MAPK and phospho-p38MAPK without altering the levels of p-ERK1/2, p-SAPK/JNK, p-NF-κB (p50) and p-NF-κB (p65) (Fig.**7B**).

Regulation of Cell Invasion and MMP-2, MMP-9, uPA and uPAR Expressions by Baicalin and SB203580

To investigate the potential functional link between the expressions of proteolytic enzymes and p38 MAPK, we treated MDA-MB-231 cells with p38 MAPK-specific inhibitor SB203580 in the presence or absence of baicalin followed by analyzing cell invasion and proteolytic proteins expressions. Both the ability of MDA-MB231 cells to invade and the levels of MMP-2, MMP-9, uPA and uPAR expressions were suppressed by 15 μM SB203580. However, combined treatment of SB203580 and baicalin did not exhibit significantly greater inhibitory effect (Fig. **8**). These results suggest that baicalin is most likely to decrease MMP-2, MMP-9, uPA, uPAR expressions and *in vitro* invasion by blocking p38 MAPK activity.

DISCUSSION

Most of late stage breast cancer patients do not die from local complications of their primary tumors, but rather from metastasis. Therefore, finding way to prevent and suppress tumor invasion and metastasis can be very beneficial to decrease the mortality of breast cancer patients.

There are achievements in the development of drugs targeting primary tumors; however, agents that can effectively suppress cancer metastasis are still lacking [21]. Here, we show that baicalin, a component of *Baikal Skullcap Root,* is potent agent to inhibit breast cancer cell migration and invasion (Figs. **1**, **2** and **3**). Importantly, we demonstrate that baicalin can effectively suppress both tumor outgrowth and spontaneous metastasis at a dose without eliciting severe toxicity to the recipients (Fig. **4** and **5**). A previous study showed that baicalin suppressed lung cancer metastasis at the dosage of 100 mg/kg *in vivo* [22]. We found that baicalin at this dosage also block breast cancer metastasis. Together, these findings indicate that baicalin may represent a promising agent to suppress cancer metastasis.

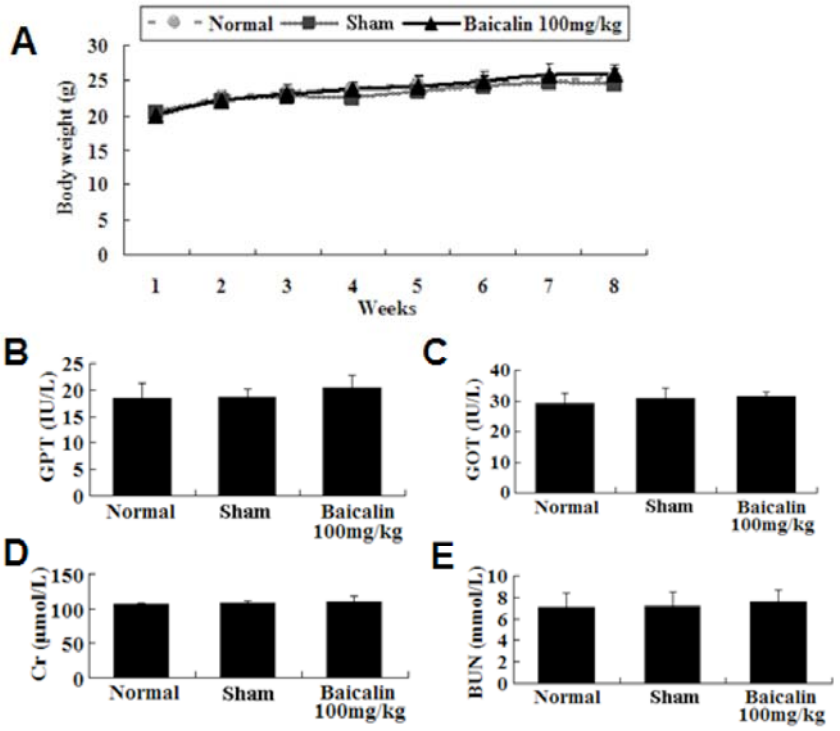

Fig. (5). Effects of baicalin on body weight, liver and kidney functions in mice. Mice were treated with saline or 100 mg/kg baicalin for 8 weeks. Body weights **(A)** were measured every week. GPT **(B)**, GOT **(C)**, Cr **(D)** and BUN **(E)** were measured at 8 weeks. Results were presented as means ± SD, n=8.

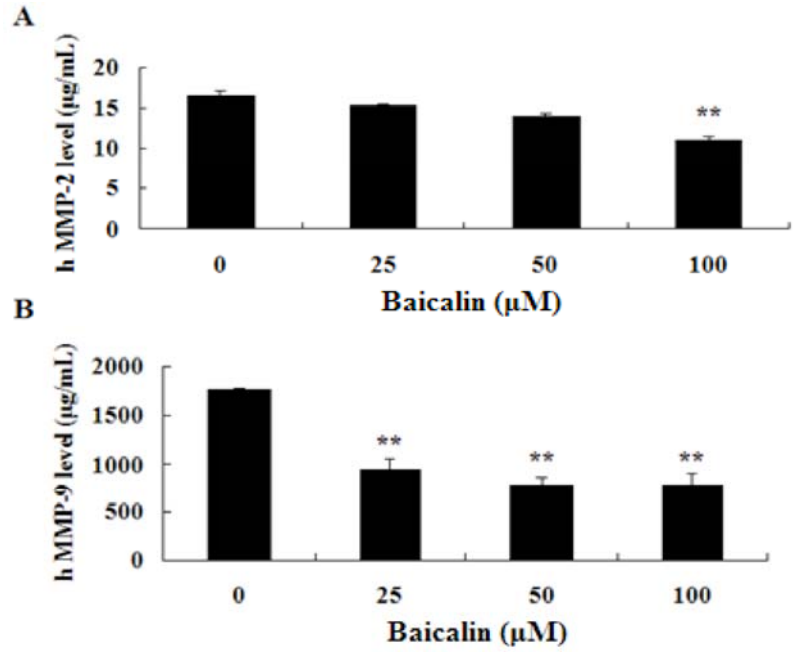

Fig. (6). Effects of baicalin on the secretion MMP-2 and MMP-9 from MDA-MB-231 cells. Cells were treated for 48 h with or without 25, 50 and 100 μM baicalin, respectively. Then each supernatant of the cell culture was collected and analyzed by ELISA. **A.** Level of MMP-2 secretion. **B.** Level of MMP-9 secretion. Each experiment was repeated three times. ** $p < 0.01$ *vs.* untreated cells.

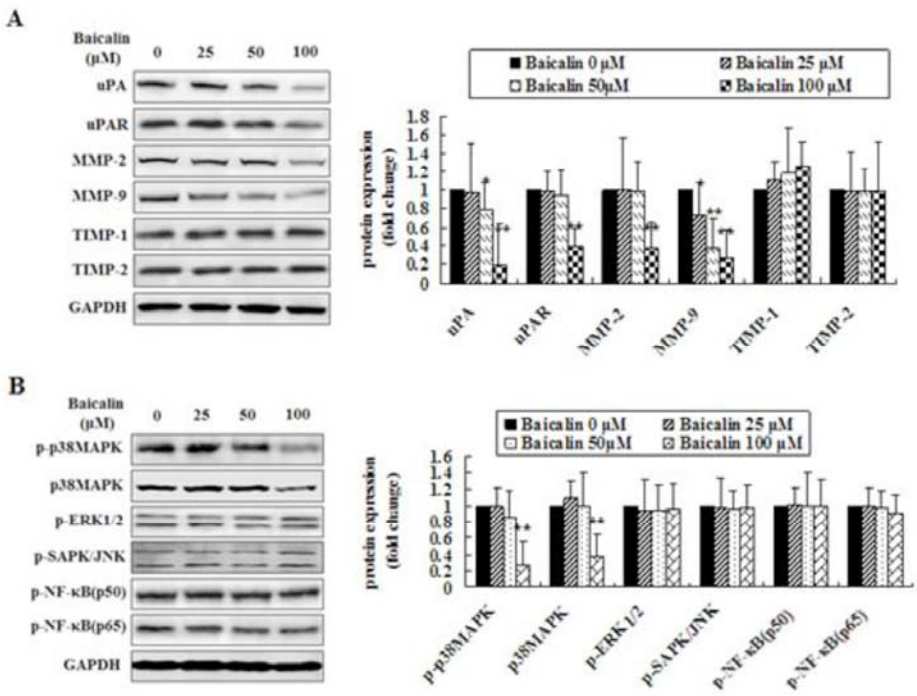

Fig. (7). Effect of baicalin on proteolytic enzymes, MAPK and NF-κB in MDA-MB-231 cells. Cells were treated with or without 25, 50, 100 μM baicalin for 24 h. The proteins were expressed by Western blot. The density ratio of proteins to GAPDH was shown as relative expression. **A.** Proteolytic enzyme expressions. **B.** MAPK and NF-κB expressions. Values are expressed mean ± SD. Experiments were repeated three times. ** $p < 0.01$ *vs.* untreated cells.

Cancer metastasis is a complex multi-step process involving cancer cells migration and invasion [23]. ECM and BM are the barriers for cancer cells to travel to the distant sites. Extensive studies have convincingly demonstrated the critical role of MMP-2, MMP-9 and uPA in degradation of ECM [24, 25]. Over expression of these enzymes is frequent in advanced stage of breast cancers and are the indicators of poor prognosis [26, 28]. With MDA-MB-231 cell line as the model system, we showed that baicalin significantly inhibited the expression of MMP-2, MMP-9, uPA and uPAR. Therefore, we provided evidence that baicalin inhibits invasion, migration and metastasis of MDA-MB-231 cells through its ability to downregulating MMP-2, MMP-9, uPA and uPAR expression.

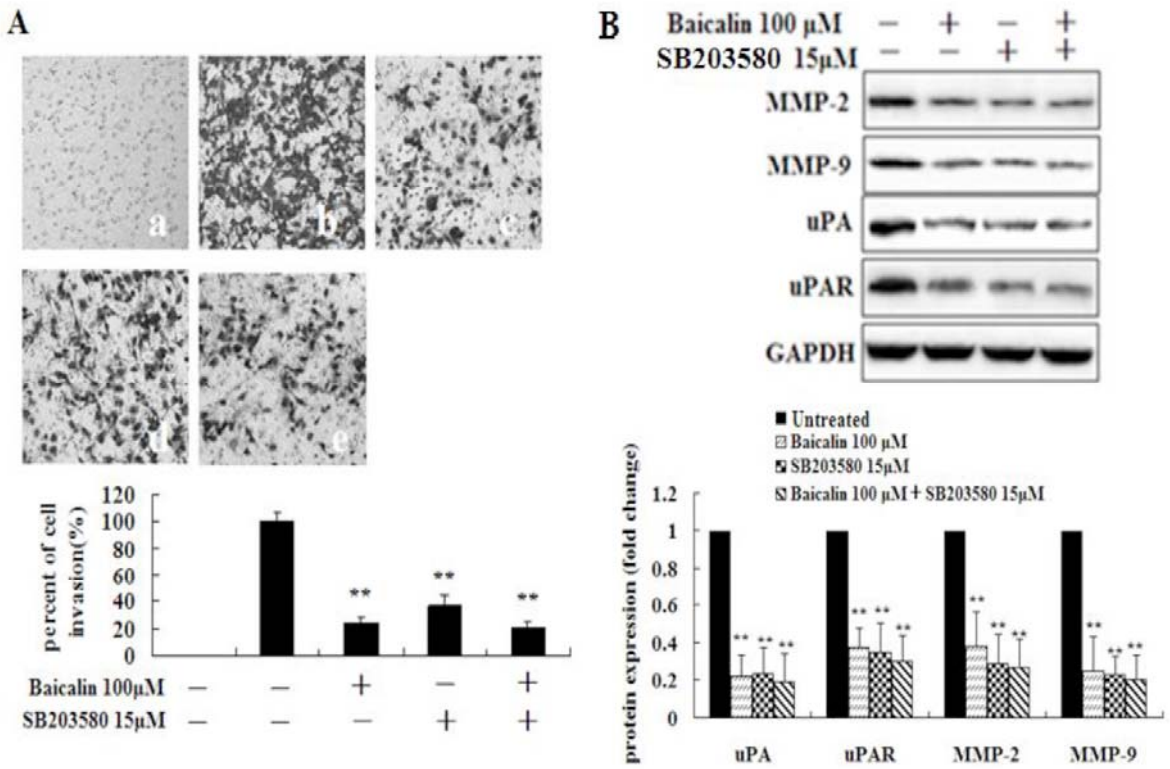

Fig. (8). Effect of baicalin, SB203580 and their combination on MDA-MB-231 cell invasion and MMP-2, MMP-9, uPA and uPAR expressions. A. Transwell chamber was used for the invasion assay (× 100 magnifications). The filter membranes were coated with matrigel. Cells were untreated (b) or treated with 100 μM baicalin (c), 15 μM SB203580 (d) and 100 μM baicalin plus 15 μM SB203580 (e) for 24 h. (a) Blank (not adds cell). Results were presented as means ± SD of three independent experiments. ** $p < 0.01$ *vs.* untreated cells. B. MDA-MB-231 cells were treated with or without 100 μM baicalin, 15 μM SB203580 and 100 μM baicalin plus 15 μM SB203580 for 48 h. The proteins expressed by Western blot. The density ratio of proteins to GAPDH was shown as relative expression. Values are expressed mean ± SD. Experiments were repeated three times. ** $p < 0.01$ *vs.* untreated cells.

MAPK and NF-κB are the two important signaling pathways in MAPK signaling pathways including ERK, JNK, p38MAPK are critically involved in the process of cancer progression and metastasis by regulating cell proliferation, differentiation and apoptosis, angiogenesis, invasion and tumor metastasis [19, 29, 30]. ERK1/2 [31], JNK [32], p38MAPK [21, 32, 33] were the important three MAPK pathways for cancer invasion and metastasis. In this study, we showed that baicalin decreased the level of activation-required p38 MAPK phosphorylation without altering ERK, JNK or NF-κB activities in MDA-MB-231 cells (Fig.**7B**). As p38 MAPK-specific inhibitor SB203580 inhibited invasion and combined treatment of baicalin and SB203580 did not further increase the inhibitory effect over the use of baicalin or SB203580 alone (Fig. **8**). Our findings indicate that baicalin is most likely to suppress breast tumor cell invasion/metastasis by intercepting p38 MAPK signaling pathway.

P38 MAPK signaling pathway has been shown to play an important role in maintaining high levels of MMP-2 and MMP-9, two critical proteinases in proteolytic process of breast cancer cells [34-36]. We showed that p38 MAPK-specific inhibitor SB203580 was able to decrease MMP-2/9 expression to a degree that baicalin was able to do and that combined treatment of baicalin and SB203580 reduced the level of these proteolytic enzymes in a similar degree as either one used alone (Fig. **8**), indicating that a similar signaling pathway mediates baicalin and SB203580-caused reduction in MMP-2 and MMP-9 expression. Together, our results suggest a model (Fig. **9**) in which baicalin intercepts p38MAPK signaling pathway, which in turn reduces MMP-2/9, uPA and uPAR expressions, leading to the blockage of breast cancer cell invasion, migration and metastasis.

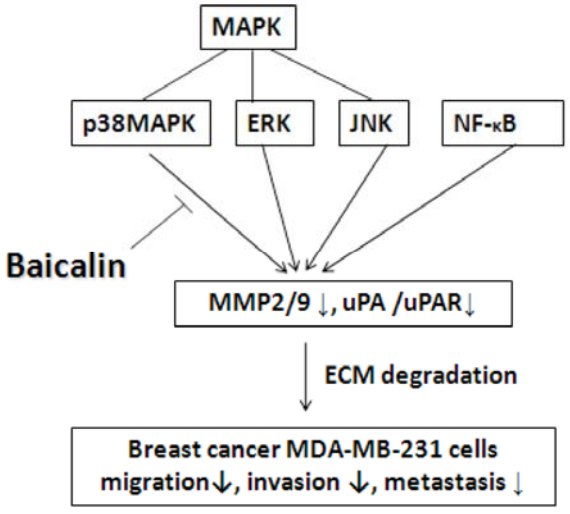

Fig. (9). A schematic diagram illustrating of the molecular mechanism of baicalin inhibited MDA-MB-231 breast cancer cell migration, invasion and metastasis. The mechanisms of baicalin effects might be through inhibiting the phosphorylation of p38MAPK to reduce MMP2/9, uPA/uPAR expressions and activities, leading to the decrease of cells invasion, migration and me tastasis.

In conclusion, we demonstrate a strong inhibitory capability of baicalin on breast cancer cell invasion, migration and metastasis. To our knowledge, our study is the first to reveal that baicalin possess such capability. Since the use of baicalin does not elicit significant toxicity to the recipient, baicalin is expected to be well tolerated by cancer patients and may thus be safely employed as a potential metastasis-suppressing agent.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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PATIENT CONSENT

Declared none.

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