Abstract. Flavonoids are structurally similar to steroid hormones, particularly estrogens, and therefore have been studied for their potential effects on hormone-dependent cancers. Baicalein is the primary flavonoid derived from the root of *Scutellaria baicalensis* Georgi. In the present study, we investigated the effects of baicalein on 17β-estradiol (E2)-induced migration, adhesion and invasion of MCF-7 and SK-BR-3 breast cancer cells. The results demonstrated that baicalein suppressed E2-stimulated wound-healing migration and cell-Matrigel adhesion, and ameliorated E2-promoted invasion across a Matrigel-coated Transwell membrane. Furthermore, baicalein interfered with E2-induced novel G protein-coupled estrogen receptor (GPR30)-related signaling, including a decrease in tyrosine phosphorylation of epidermal growth factor receptor (EGFR) as well as phosphorylation of extracellular signal-regulated kinase (ERK) and serine/threonine kinase Akt, without affecting GPR30 expression. The results also showed that baicalein suppressed the expression of GPR30 target genes, cysteine-rich 61 (CYR61) and connective tissue growth factor (CTGF) induced by E2. Furthermore, baicalein prevented GPR30-related signaling activation and upregulation of *CYR61* and *CTGF* mRNA levels induced by G1, a specific GPR 30 agonist. The results suggest that baicalein inhibits E2-induced migration, adhesion and invasion through interfering with GPR30 signaling pathway activation, which indicates that it may act as a therapeutic candidate for the treatment of GPR30-positive breast cancer metastasis.

Introduction

Breast cancer is the most common malignancy affecting women and is the leading cause of cancer-related mortality among females worldwide (1). Despite advances in the diagnosis and treatment of breast cancer, approximately a third of patients develop metastatic disease, with a median survival time of less than 3 years (2). Preventing metastasis is important for effectively overcoming breast cancer. Estrogen plays an important role, not only in the initiation and proliferation of breast cancer, but also in cancer metastasis (3). Most of the biological effects of estrogen are mediated by binding and activating classic estrogen receptors (ERs), ERα and ERβ, to stimulate the transcription of target genes via genomic and non-genomic mechanisms (4). Based on this knowledge, hormonal therapies that interfere with ERα function are currently applied in patients with ERα-positive breast cancer, and beneficial effects have been achieved. Although there is general success with ERα expression and responsiveness to ERα-targeted agents, some patients do not respond to endocrine therapy, and most patients appear to have acquired resistance (5). In addition, some types of ERα-negative breast cancer cells remain estrogen responsive, indicating the existence of an alternative receptor for estrogen.

G protein-coupled estrogen receptor (GPR30), as a member of the 7-transmembrane GPR family, mediates both rapid signaling and transcriptional events in response to estrogen. It mediates estrogenic-rapid non-genomic actions, through the Gβγ subunit downstream, release of heparin-bound EGF (HB-EGF) and metalloproteinase (MMP)-dependent transactivation of epidermal growth factor receptors (EGFRs), as well as activation of mitogen-activated protein kinases (MAPKs), adenylyl cyclase, and phosphoinositide 3-kinase (PI3K) (6). Subsequently, it also triggers transcriptional responses by activating various transcription factors, such as *FOS*, *JUN*, *EGRI*
and ATF3 following the protein kinase signaling cascade (7). GPR30 expression is positively associated with the features of breast tumor progression, including tumor size, HER2/neu status, and metastases. Yet, ERα is inversely associated with HER2/neu and tumor size (8). ERα in breast cancer generally indicates good prognosis and treatment responsiveness with anti-estrogen agents (9); yet, GPR30 is overexpressed in invasive breast cancer and is positively associated with a metastatic phenotype (10). GPR30 is implicated in breast cancer metastasis and it may provide a new potential target for endocrine therapy.

The incidence of breast cancer is much higher in Western countries than in Asian ones. Epidemiological studies contribute these differences to dietary factors, particularly soy consumption. Flavonoids are considered to have a significant potential in breast cancer chemoprevention (11). Their polyphenolic ring is structurally similar to the steroid nucleus of 17β-estradiol (Fig. 1A) and may have estrogenic or anti-estrogenic activity (12). Baicalein is the primary flavonoid derived from the root of Scutellaria baicalensis Georgi and is found in soybeans and fruit, which bears the three-ring structure of the flavone backbone with phenolic hydroxyl at the 5', 6', and 7' position (Fig. 1A). It possesses several health benefits including anti-inflammatory, antioxidant, antiviral, neuroprotective, and anticancer effects (13). It has also been shown to have antitumor effects in various hormone-dependent cancers such as breast, colon, and prostate cancer. Previous studies have found that baicalein suppresses 17β-estradiol (E2)-induced ER transactivation in MCF-7 cells (14) and displaces >85% of estradiol binding in mouse uterine cytosol (15). Additionally, baicalein exhibits effective inhibitory activity against E2/insulin-like growth factor 1 (IGF-1)-induced proliferative events in breast cancer cells (16). These investigations propose that baicalein may exert anti-estrogenic activity.

The present study investigated the inhibitory activity of baicalein on E2-induced migration, adhesion, and invasion of MCF-7 and SK-BR-3 breast cancer cells. Furthermore, we investigated its effect on GPR30-related signaling, including phosphorylation of EGFR as well as extracellular regulated kinase (ERK), serine/threonine kinase Akt activation, and GPR30-mediated gene expression.

Materials and methods

Reagents and antibodies. Baicalein (purity >98%) was provided by Professor Zhiyu Li (China Pharmaceutical University, Jiangsu, China). It was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 0.1 M and stored at -20°C. E2 and G1 from Sigma-Aldrich (St. Louis, MO, USA) were dissolved in DMSO as a stock solution at 10⁻¹ M and stored at 4°C. Matrigel was obtained from BD Biosciences (Bedford, MA, USA). Bovine serum albumin (BSA) was purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). 3-(4,5-Dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) and cytosine β-D-arabinofuranoside hydrochloride were purchased from Sigma-Aldrich. The GPR30 (N-15)-R antibody (sc-48525-R, polyclonal antibody, rabbit anti-human, 1:800) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Primary antibodies against p-ERK (T202/Y204) (AP0484, polyclonal antibody, rabbit anti-human, 1:1,000), extracellular regulated protein kinase 1/2 (ERK1/2) (L352) (BSI112, polyclonal antibody, rabbit anti-human, 1:1,000), and GAPDH (1A6) (MB001, monoclonal antibody, mouse anti-human, 1:1,000) were purchased from Bio-World (Dublin, OH, USA). Primary antibodies for p-Akt (Ser473) (193H12) (#4058, monoclonal antibody, rabbit anti-human, 1:1,000), Akt (11E7) (#4685, monoclonal antibody, rabbit anti-human, 1:1,000), p-EGFR (Tyr1173) (53A5) (#4407, monoclonal antibody, rabbit anti-human, 1:1,000) and EGFR (D38B1) (#4267, monoclonal antibody, rabbit anti-human, 1:1,000) were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (1:3,000) were purchased from Santa Cruz Biotechnology, Inc.

Cell lines and culture. Human breast cancer cell lines MCF-7 and SK-BR-3 were obtained from the Kunming Cell Bank of the Chinese Academy of Sciences (Yunnan, China). MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, and SK-BR-3 cells were maintained in RPMI-1640 (all from Hyclone, Logan, UT, USA) with 10% FBS (Gibco-BRL, Carlsbad, CA, USA), both supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (both from Sigma-Aldrich). These cells were incubated at 37°C in humidified air with 5% CO₂.

Cell treatment. Before the indicated treatments, cells were cultured in phenol red (PR)-free medium without serum for 24 h. Cells were then treated with or without 10 nM of E2 (or 1 µM G1) and different concentrations of baicalein (5, 10, and 15 µM) in PR-free medium without serum for a specified period of time. Control cells were incubated in DMSO (0.01%, v/v).

MTT assay. The 96-well microculture plates were individually seeded with 100 µl of the respective cell suspension and 100 µl of treatment medium. After incubation at 37°C for 24 h, 20 µl of MTT (5 mg/ml) was added, and the plates were incubated for an additional 4 h at 37°C. The medium was then removed, and 100 µl DMSO was added and mixed thoroughly. Spectrophotometric absorbance values at 570 nm were measured using a microplate reader (Bio-Rad, Lincoln, NE, USA). The results are expressed as inhibitory rates with respect to the controls, calculated using the following formula:

\[
\text{Inhibitory rate (\%) = \left(\frac{A_\text{control} - A_\text{treated}}{A_\text{control}}\right) \times 100.}
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Wound-healing assay. Cells were seeded into 12-well plates at 90% confluency. Monolayers were wounded with a micropipette tip and rinsed with phosphate-buffered saline (PBS) 3 times to remove any floating cells. Then the cells were incubated in the absence or presence of E2 (10 nM) with or without various concentrations of baicalein (5, 10, and 15 µM) for 24 h. Cytosine β-D-arabinofuranoside hydrochloride (10 µM) was added 1 h before the test treatment to inhibit cell proliferation (17). Images were captured at 0 and 24 h after wound application. The level of cell migration into the wound was quantified as the migration rate compared against the cells at 0 h for each group.
Cell adhesion assay. The cell adhesion assay was performed as described (18). Briefly, 96-well plates were coated with Matrigel (50 µg/well) at 37˚C for 1 h and blocked with 0.5% BSA (v/v) at 4˚C for 4 h. After treatment with E2 (10 nM) or E2 (10 nM) plus baicalein (5, 10, and 15 µM) for 24 h, the cells were harvested and suspended in serum-free medium. Cells at a density of 2x10⁵ cells/ml (100 µl) were added into the plates and then incubated at 37˚C for 1 h. After incubating for adhesion, the medium was carefully suctioned out. The wells were then washed with PBS to remove non-adherent cells. The colorimetric MTT assay was employed to analyze the number of adherent cells. Spectrometric absorbance was measured at 570 nm by using a microplate reader (Bio-Rad). The results are expressed as adhesive cells with respect to the control as a percentage.

Invasion assay. Invasive ability of the cells was measured by assay using a Transwell chamber (Millipore, Billerica, MA, USA) containing membranes with an 8-µm pore size and, coated with Matrigel as previously described (19). The cells were exposed to E2 (10 nM) with or without different concentrations of baicalein (5, 10, and 15 µM) for 24 h. The cells were then trypsinized and suspended at a final concentration of 5x10⁵ cells/ml in serum-free medium. The cell suspension was added into each 10-mm upper chamber, and medium with 10% FBS was added into the bottom chamber as a chemoattractant. After incubating for 24 h, the upper surfaces of the membranes were swabbed to remove non-invaded cells, and the cells that attached to the lower surface were fixed in 100% methanol, stained with hematoxylin and eosin (H&E) (Beyotime Institute of Biotechnology, Jiangsu, China), and counted under a microscope. Five randomly chosen fields were analyzed for each group.

Western blot analysis. Cells were collected after E2 (10 nM) or G1 (1 µM) with or without baicalein (5, 10, and 15 µM) treatment for 1 h, and lysed in lysis buffer (Beyotime Institute of Biotechnology). The lysates were clarified by centrifugation at 4°C for 15 min at 13,000 x g. The concentration of protein in the supernatants was measured using bicinchoninic acid (BCA) assay kit (Beyotime Institute of Biotechnology) with a Varioskan Multimode microplate spectrophotometer (Thermo Waltham, MA, USA). Total proteins (30 µg/lane) were separated on a 12 or 8% SDS-PAGE gel, and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk and subsequently incubated with the indicated antibodies. Blots were visualized by using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). Digital images of the blots were produced by a Syngene Gel Imaging System and quantified with GeneSnap (Syngene, Frederick, MD, USA) software.

Real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted after a 1-h treatment with 10 nM E2 or G1 (1 µM) in the presence or absence of baicalein (5, 10 and 15 µM) by using an RNA extraction kit (code no. 9767; Takara, Dalian, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a Strand Complementary DNA Synthesis kit (code no. 6210A; Takara). Real time PCR was performed on an iCycle iQ (Bio-Rad) using 20 ng of cDNA template and 10 µM primers. Cysteine-rich 61 (CYR61) primers (forward, 5'-ACT...
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Figure 2. Effects of baicalein on the E2-induced migration of MCF-7 and SK-BR-3 breast cancer cells. (A) Confluent monolayers were scratched and incubated in the absence or presence of E2 (10 nM) with or without various concentrations of baicalein (5, 10, and 15 µM) for 24 h. The images were captured at 0 and 24 h after wounding (magnification, ×100). These figures are representative of 3 separate experiments. (B) The inhibition of E2-stimulated migration by baicalein. Values are mean ± SEM. *P<0.05 compared with the E2-treated group. E2, 17β-estradiol.

TCA TGG TCC CAG TGC TC-3' and reverse, 5'-AAA TCC GGG TTT CTT TCA CA-3') (20), connective tissue growth factor (CTGF) primers (forward, 5'-ACC TGT GGG ATG GGC ATC T-3' and reverse, 5'-CAG GCG GCT CTG CTT CTC TA-3') (21) and β-actin primers (forward, 5'-AGT TGC GTT ACA CCC TTT C-3' and reverse, 5'-CCT TCA CCG TTC CAG TTT-3') (22) were synthesized by Sangon Biotech (Shanghai, China). The mRNA levels were measured using SYBR Premix Ex Taq™ (code no. RR037A; Takara). The reactions were performed for 30 sec at 95°C, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The melting curve was analyzed at 55 to 95°C to detect a single gene-specific peak and to verify the absence of primer dimer peaks. The CYR61 or CTGF mRNA level was quantified in relation to β-actin mRNA level by using the 2^−ΔΔ Ct method.

Statistical analysis. Statistically significant differences were calculated by one-way ANOVA followed by the Bonferroni post-hoc test for multiple-group comparisons. A P<0.05 was considered to indicate a significant difference, and data are expressed as mean ± standard error of the mean (SEM). The data shown in this test were obtained from 3 independent experiments.

Results

Cytotoxic effect of baicalein on MCF-7 and SK-BR-3 cells. To investigate the effect of baicalein on cell viability, MCF-7 and SK-BR-3 cells were treated with various concentrations of baicalein for 24 h, and viability was detected by MTT assay. The IC_{50} values of baicalein in the MCF-7 and SK-BR-3 cells after 24 h were 66.3±5.9 and 103.5±9.4 µM, respectively (Fig. 1B). According to the results, baicalein at 5, 10 and 15 µM had no obvious cytotoxic effect on the MCF-7 or SK-BR-3 cells in the presence or absence of E2 (Fig. 1C), and were used in following study.
Baicalein inhibits E2-induced cell migration. After treatment with E2, MCF-7 and SK-BR-3 cells were found to have significantly enhanced migration into the wound, while cells treated with baicalein had less migratory ability (Fig. 2A). E2 stimulation for 24 h significantly increased the migration rate from 13±2 to 52±3% in the MCF-7 cells, and from 10±4 to 40±5% in the SK-BR-3 cells, respectively. After treatment with 15 µM baicalein for 24 h, the migration rate decreased to 17±2% in the MCF-7 cells and 13±4% in the SK-BR-3 cells, respectively (Fig. 2B). These results demonstrated that baicalein suppressed E2-induced cell migration.

Baicalein suppresses E2-promoted cell adhesion to Matrigel. Adhesion of tumor cells to extracellular matrix (ECM) is an essential step for tumor invasion and metastasis. We next examined the effect of baicalein on E2-promoted cell adhesion.
Baicalin prevents E2-induced GPR30 signal transduction. The transmembrane receptor, GPR30, is able to mediate estrogen responsiveness in ER-positive or ER-negative breast cancer cells. Here, we aimed to determine whether or not baicalin has inhibitory effects on GPR30 expression or its signal transduction. We observed that GPR30 expression remained unchanged after exposure to E2 with or without baicalin in both the MCF-7 and SK-BR-3 cells (Fig. 5A). Previous studies have indicated that GPR30 activation triggers EGFR transactivation by tyrosine phosphorylation in breast cancer cells (23,24), thus we sought to evaluate the influence of baicalin in EGFR phosphorylation upon E2 stimulation. When cells were treated with baicalin in the presence of E2, there was a significant decrease in EGFR phosphorylation (Tyr 1173) at 10 and 15 µM (Fig. 5B). GPR30 triggers EGFR transactivation leading to the phosphorylation of ERK1/2 (24).
and activation of the PI3K/Akt transduction pathway (25). Thus, it was essential to analyze the phosphorylation status of ERK1/2 and Akt. Consequently, E2-induced phosphorylation of ERK1/2 at Thr 202/Tyr 204 and Akt at Ser 373 were repressed in the presence of baicalein at 10 and 15 µM (Fig. 5B). In addition, GPR30 agonist G1 was used to further confirm whether baicalein interferes with GPR30 signal transduction. Indeed, treatment with baicalein at 15 µM prevented G1-induced EGFR, ERK and Akt phosphorylation (Fig. 5C).

**Baicalein suppresses E2-induced GPR30-mediated gene expression.** Previous reports have shown that CYR61 and CTGF are the target genes which mediate the stimulatory effects triggered by GPR30 activation (26). The potential of baicalein to regulate GPR30-mediated expression of both target genes was detected by real-time PCR. E2 upregulated CYR61 and CTGF mRNA levels in the 2 cell lines. The downregulation of the 2 genes in the MCF-7 and SK-BR-3 cells (Fig. 6A) was observed after exposure to baicalein, when compared with the E2-treated groups. In addition, the upregulation of CYR61 and CTGF mRNA levels induced by G1 in the MCF-7 and SK-BR-3 cells was also inhibited in the presence of baicalein at 15 µM (Fig. 6B).

**Discussion**

Estrogens are essential for the growth and development of mammary glands (27). However, prolonged exposure to endogenous or exogenous estrogens is a major risk factor for the development of breast cancer (28). Estrogens have been confirmed to play a key role in breast cancer metastasis (29,30). Tumor metastasis consists of a series of highly relevant biological processes including disruption of cell-cell and augmentation of cell-ECM adhesion capacity, induction of cellular motility, invasiveness to the basement membrane and ECM constituents, angiogenesis, and formation of a metastatic lesion (31). In the present study, baicalein treatment inhibited cell migration in response to E2 stimulation. Furthermore, it suppressed E2-enhanced cell adhesion to Matrigel. Moreover, it significantly decreased E2-promoted invasive ability across a Transwell membrane coated with Matrigel. These results imply that baicalein may possess anti-estrogenic ability to suppress E2-induced migration, adhesion and invasion of MCF-7 and SK-BR-3 cells in vitro.

GPR30 is independent of the ER status in breast cancer cells and tissue samples, and its action differs from the classical nuclear ERs, ERα and ERβ (7). Recent studies have demonstrated that GPR30 mediates E2-enhanced cell migration and invasion in triple-negative or ERα-negative breast cancer cells (32,33). Furthermore, we recently found that GPR30 mediates E2-induced calpain activation, which is correlated with the metastatic phenotype of breast cancer, in both ERα-positive and -negative cells (34). We also showed that tamoxifen, an ER antagonist but a GPR30 agonist, could not suppress E2-promoted migration but enhanced migration in breast cancer cells and induced truncation of cyclin E.
via GPR30 (35). It appears that GPR30, regardless of ERα expression, plays an important role in E2-enhanced migration and invasion of breast cancer cells. Our results showed that baicalein suppressed E2-promoted migration and invasion in GPR30-positive/ERα-negative SK-BR-3 cells and also in GPR30/ERα-positive MCF-7 cells. Although baicalein had little effect on GPR30 expression, it markedly inhibited the E2-induced GPR30 signal activation, by decreasing EGFR phosphorylation and activation of ERK1/2 and Akt. Meanwhile, baicalein suppressed G1-induced upregulation of the phosphorylation of EGFR, ERK1/2 and Akt. The results indicate that the inhibitive effect of baicalein on the response of MCF-7 and SK-BR-3 cells to estrogen may derive partly from interfering with GPR30 signal transduction.

CYR61 and CTGF, also known as CCN1 and CCN2, belong to the CCN protein family, which exerts their biological functions by binding and activating cell surface integrins to stimulate cell growth, adhesion, matrix production and migration, and by regulating angiogenesis and tumorigenesis (36). CYR61 and CTGF expression is significantly associated with tumor size and lymph node metastasis, suggesting that they play a role in the progression of breast cancer (20). CYR61 is a survival and proangiogenic factor and induces metastasis and drug resistance in breast cancer cells (37). CTGF enhances migratory/invasive processes in human breast cancer by activating the integrin-αβ3-ERK1/2-dependent upregulation of S100 calcium-binding protein A4 (S100A4) (38). The two genes related to breast cancer metastasis are the target genes of GPR30. Here, we found that baicalein significantly inhibited E2- or G1-induced upregulation of CTGF and CYR61 gene expression. These results suggest that baicalein may prevent E2-induced migration, invasion and adhesion of MCF-7 and SK-BR-3 cells also by suppressing GPR30-dependent gene transcription.

Flavonoids possess a chemical structure similar to estrogen, and some have been considered to exert an anti-estrogenic effect and reduce the risk of breast cancer. Genistein, which is the main ingredient responsible for the cancer chemopreventive activity of soy-derived foods, has garnered much research. However, it displays a biphasic effect on the proliferation and death of MCF-7 cells and displays estrogenic activity to transactivate ERα at low concentrations. Although baicalein is an isomer of genistein, it does not have a biphasic effect on ER, which suggests that it could be a better chemopreventive agent than genistein (14). Moreover, genistein can stimulate GPR30 to mediate c-fos upregulation in breast cancer cells (39) and induce the proliferation of thyroid cancer cells (40). In the present study, we found that baicalein suppressed E2 or G1-induced GPR30 signal activation indicating a different activity from genistein. The precise difference between the two compounds with similar chemical structure remains to be resolved in future studies. The relationship between the structure of flavonoids and the inhibition of E2 events warrants further study. To fully investigate the mechanism, we need to explain the detailed molecular mechanisms with which baicalein influences GPR30 and downstream signaling, as well as crosstalks with other receptors. In addition, whether baicalein has dual effects on both ERα and GPR30 still needs further investigation.

In conclusion, baicalein attenuates E2-induced migration, adhesion and invasion of MCF-7 and SK-BR-3 breast cancer cells through interference with GPR30 pathway activation. This indicates that baicalein may be a promising agent for the treatment of GPR30-positive breast cancer metastasis.

Acknowledgements

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