**Abstract.** Estrogen performs an important role in the growth and development of breast cancer. There are at least three major receptors, including estrogen receptor (ER)α and β, and G protein-coupled receptor 30 (GPR30), which mediate the actions of estrogen through using transcriptional and rapid non-genomic signaling pathways. Flavonoids have been considered candidates for chemopreventive agents in breast cancer. Baicalein, the primary flavonoid derived from the root of *Scutellaria baicalensis* Georgi, has been reported to exert an anti-estrogenic effect. In the present study, the effects of baicalein on 17β-estradiol (E2)-induced cell invasion, and matrix metalloproteinase-9 (MMP-9) expression and activation were investigated. Furthermore, its effects were compared with that of the active form of the ER modulator tamoxifen 4-hydroxytamoxifen (OHT) and the GPR30 antagonist G15 in ERα- and GPR30-positive MCF-7 breast cancer cells. The results demonstrated that OHT failed to prevent E2-induced cell invasion, upregulation and proteolytic activity of MMP-9. However, baicalein was able to significantly suppress these E2-induced effects. Furthermore, E2-stimulated invasion, and MMP-9 expression and activation were significantly attenuated following G15 treatment. In addition, baicalein significantly inhibited G-1, a specific GPR30 agonist, induced invasion, and reduced G-1 promoted expression and activity of MMP-9, consistent with effects of G15. The results of the present study suggest that baicalein is a therapeutic candidate for GPR30-positive breast cancer treatment, and besides ERα targeting the GPR30 receptor it may achieve additional therapeutic benefits in breast cancer.

**Introduction**

It is well known that the steroid hormone estrogen performs a role in the pathogenesis of breast cancer, and the majority of breast cancer types involve estrogen signaling pathways in cancer initiation, progression and metastasis (1). The majority of estrogen-associated biological actions are traditionally attributed to activate classic estrogen receptors (ERs), ERα and ERβ (2). Thus, endocrine therapies that interfere with ER functions are currently applied in patients with ER-positive breast cancer. Although targeted inhibition of ERα is a successful approach, numerous patients fail to respond (de novo resistance) or relapse despite an initial response (acquired resistance) to anti-estrogen therapy (3). The selective ER modulator tamoxifen, which antagonizes estrogen-induced genomic-nuclear ERα activity, is widely used in the treatment of ERα-positive breast cancer. It has significantly reduced the mortality risk among patients, however, not all patients achieve beneficial effects as disease recurrence occurs in ~25-30% of patients (4).

The identification and characterization of the G protein-coupled receptor 30 (GPR30) represents an additional mechanism of estrogen effects, which mediate a wide range of estrogenic responses, leading to changes in gene expression and relevant biological responses (5). It has been revealed that GPR30 expression is associated with clinical and pathological biomarkers of poor outcome in breast cancer (6). In addition, GPR30 is overexpressed in invasive breast cancer and positively correlated with the development of distant metastases, furthermore, mouse mammary tumor virus-polyoma middle T (MMTV-PyMT) mice deficient in GPR30 expression exhibit a substantially decreased incidence of metastasis compared with GPR30 wild-type (6-8). These observations suggest that GPR30 is implicated in breast cancer metastasis and...
may provide a novel potential target for treatment. Although tamoxifen exhibits antagonistic properties with respect to ERα, it performs as a GPR30 agonist (9), which demonstrates the complex physiological and therapeutic actions it possesses.

Tumor metastasis consists of a series of relevant biological processes including cell adhesion, migration, and invasion. Degradation extracellular matrix (ECM) of tumor cells is a hallmark of tumor metastasis and an essential step for invading distant organs (10). The expression and activation of matrix metalloproteinases (MMPs) facilitate tumor cells to invade the ECM underlying their basement membrane and stroma (11). Among the MMPs, MMP-2 and MMP-9, two zinc-dependent endopeptidases, perform essential roles in ECM degradation. Overexpression of MMP-2 and MMP-9 were associated with high potential of metastasis and they are poor prognostic factors in patients with breast cancer (12). A positive association between GPR30 and MMP-9 expression was identified in malignant ovarian endometriotic cysts and epithelial ovarian cancer (13,14). Furthermore, E2 and selective GPR30 agonist G-1 increased the expression and proteolytic activity of MMP-9 in ovarian cancer cells, which could be inhibited by small interfering RNA targeting GPR30 or G protein inhibitor pertussin toxin (15), indicating a possible linkage between GPR30 and MMP-9. Nevertheless, the contribution of GPR30 in the regulation of MMP-9 in breast cancer cells remains unclear.

The incidence of breast cancer is much higher in Western countries compared with in Asian countries. Certain epidemiological studies contribute one factor of these differences to dietary flavonoids (16-18). Furthermore, as their polyphenolic ring is similar to the steroid nucleus of 17β-estradiol (E2), certain flavonoids have been considered to exert an anti-estrogenic effect and are candidates for chemopreventive agents to reduce the risk of breast cancer (19). Baicalein (5,6,7-trihydroxyflavone) is the primary flavonoid derived from Scutellaria baicalensis Georgi, whose structure is composed of a three-ring flavone backbone with phenolic hydroxyl at the 5', 6', and 7' position. Several studies have revealed that baicalein possesses antitumor activity in breast cancer and exhibits anti-estrogenic activity (20-22). In our previous study, it was demonstrated that baicalein is able to suppress E2 enhanced migration, adhesion and invasion of breast cancer cells and interferes with E2 or G-1 induced GPR30 signaling activation (23). However, whether this compound could inhibit E2 promoted MMP-9 upregulation and activation, as well as take effects via GPR30 remains unclear.

In the present study, the role of GPR30 in the regulation of the invasion process was evaluated, and the effects of baicalein on E2-induced cell invasion, MMP-9 activity and expression were investigated in ERα/GPR30-positive MCF-7 human breast cancer cells. In addition, the effects of baicalein with the active form of the selective ER modulator tamoxifen 4-hydroxytamoxifen (OHT) and the GPR30 antagonist G15.

Materials and methods

Reagents and antibodies. Baicalein (purity >98%) was provided by Professor Qinglong Guo and Zhiyu Li (China Pharmaceutical University, Nanjing, China). It was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 0.1 M and stored at -20°C. E2 (cat. no. E2758) and OHT (cat. no. H7904) were dissolved in DMSO as a stock solution at 10⁻¹ M and stored at 4°C (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). G-1 (cat. no. 10008933) and G15 (cat. no. 14,673) were purchased from Cayman Chemical Company (both from Ann Arbor, MI, USA) and dissolved in DMSO as a stock solution at 10⁻¹ M and stored at -20°C. Matrigel (cat. no. 356237) was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Primary antibodies directed against MMP-9 (W680) (cat. no. BS1241; polyclonal rabbit anti-human; dilution 1:1,000), and GAPDH (1A6) (cat. no. MB001; monoclonal mouse anti-human; dilution, 1:4,000) were purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA). The anti-mouse (cat. no. sc-2005) or anti-rabbit (cat. no. sc-2030) ImmunoglobulinG horseradish peroxidase-conjugated secondary antibodies (dilution, 1:3,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell culture and treatment. The human breast cancer MCF-7 cell line from the Kunming Cell Bank of the Chinese Academy of Sciences (Kunming, China) was maintained in DMEM (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Sigma-Aldrich; Merck KGaA). The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. Prior to the indicated treatments, cells were pre-cultured for 24 h at 37°C in phenol red (PR)-free DMEM (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) without serum to remove endogenous estrogen. Subsequently, cells were treated accordingly in PR-free DMEM (HyClone; GE Healthcare Life Sciences). Control cells were incubated in DMEM (0.01% v/v). The concentrations of each drug were used as follows: E2 (1 nM), OHT (1 µM), G1 (1 µM), G15 (1 µM) and baicalein (10 µM).

Invasion assay. Invasive ability of cells was measured using an assay in a Transwell chamber (EMD Millipore, Billerica, MA, USA) containing membranes with an 8-µm pore size, coated with Matrigel as previously described (24). Following treatment, cells were trypsinized and suspended at a final concentration of 5x10⁵ cells/ml in serum-free PR-free DMEM. The cell suspension was then added into each 10-mm upper chamber and PR-free DMEM with 10% FBS was added into the bottom chamber as a chemo-attractant. Subsequent to a 24 h incubation at 37°C in a humidified atmosphere with 5% CO₂, the upper surfaces of the membranes were swabbed to remove non-invasive cells and the cells attached onto the lower surface were fixed in 100% methanol, stained with hematoxylin and eosin (Beyotime Institute of Biotechnology, Haimen, China), and counted under an inverted light microscope equipped (Eclipse 50i) with a color camera (DS-Fi2) (both Nikon Corporation, Tokyo, Japan) at magnification, x200. A total of 5 randomly selected fields were analyzed from each group. Values represent the percentage of invasive cells relative to the controls (as 100%).

Western blot analysis. Cells were collected and lysed in lysis buffer (Beyotime Institute of Biotechnology). The lysates were separated using centrifugation at 4°C for 15 min at 13,000 x g.
Protein concentration was determined using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.) with a Varioskan multimode microplate spectrophotometer (Thermo Fisher Scientific, Inc.). Total protein (30 µg/lane) was separated using 12% SDS-PAGE and transferred to a PVDF membrane (EMD Millipore). The membrane was blocked using 5% non-fat milk at 37°C for 1 h, and subsequently incubated with aforementioned appropriate antibodies at 37°C for 1 h. Blots were visualized using an enhanced chemiluminescence kit (EMD Millipore). Digital images of blots were captured using a ChemiDoc XRS™ system and analyzed using Image Lab™ software (version 5.2) (both Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Gelatin zymography assay. The activity of MMP-2 and MMP-9 were analyzed using a gelatin zymography assay as previously described (25). Cells (2x10⁵ cells/ml) were seeded (2 ml) into a six-well plate. When 80% confluence was achieved, the cells were treated as indicated. Following treatment, the conditioned medium was collected and centrifuged at 112 x g for 5 min at 4°C to remove the dead cell debris. The conditioned medium with sample buffer (0.5 M Tris-HCl pH 6.8, buffer with 30% glycerin, 0.05% Bromophenol blue and 6% SDS, v/v at 2:1) was subjected to 10% SDS-PAGE containing 0.1% gelatin. Following electrophoresis, the gels were washed twice with rinsing buffer (50 mM Tris-HCl pH 7.6, with 5 mM, CaCl₂, 1 µM ZnCl₂ and 2.5% Triton X-100) to remove the SDS and incubated for 36 h at 37°C in incubating buffer (50 mM Tris-HCl pH 8.0, buffer with 5 mM CaCl₂, 1 µM ZnCl₂). The gels were subsequently stained with 0.1% Cooamassie Brilliant Blue R250 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 1 h at room temperature, followed with destaining with using 10% acetic acid and 10% methanol. The images of the gels were captured using the ChemiDoc XRS™ system and analyzed using Image Lab™ software (version 5.2).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted following treatment using an RNA Extraction kit (cat. no. 9767; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. cDNA was synthesized using a strand complementary DNA synthesis kit (cat. no. 6210A; Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. mRNA expression was measured using SYBR Green PCR Core reagents (cat. no. RR820A, Takara Biotechnology Co., Ltd.) according to the manufacturer’s protocol. mRNA expression was measured using SYBR Green PCR Core reagents (cat. no. RR820A, TaKaRa). The reaction were conducted in 96-well plate and the reaction volume for each well was 20 µl, including 2 µl (100 ng) cDNA, 10 µl SYBR Premix Ex Taq II, 0.8 µl (10 µM) of both forward and reverse primers and 6.4 µl ddH₂O. The reaction was performed with a CFX Connect™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). The following primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and used in the present study: MMP-9 forward, 5’-GTGGGGATTACATGGCAC-T3’ and reverse, 5’-AAGCTATTTTGCCAGGAC-3’ (26); β-actin forward, 5’-AGTTGCGTTACA CCGTTTTC-3’ and reverse, 5’-CCCTCCACGTCCTCCAG TT-3’ (27). The thermocycling conditions maintained were as follows: 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 65-95°C to detect a single gene-specific peak and verify the absence of primer dimer peaks. The MMP-9 mRNA level was quantified relative to β-actin mRNA expression using the 2^−ΔΔCq method (28).

Statistical analysis. Data are expressed as the mean ± standard error of the mean following ≥3 independent experiments. Statistically significant differences were calculated using one-way analysis of variance followed by the Bonferroni’s post hoc test for multiple-group comparisons by using SPSS Statistics v.17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Unlike OHT, baikalein is able to prevent E2-induced invasion and MMP-9 expression and activity in MCF-7 human breast cancer cells. To assess the effects of anti-estrogenic agents tamoxifen and baikalein on E2-induced invasion, estrogen-sensitive MCF-7 breast cancer cells were treated with OHT or baikalein in the presence of E2 for 24 h. It was observed that OHT failed to suppress E2-induced invasion, while baikalein was able to significantly suppress this effect compared with the E2-treated control group (Fig. 1A). MMPs perform essential roles in processes associated with tumor invasion and metastasis, when cells become invasive they often produce proteolytic enzymes, which can degrade the majority of the extracellular matrix (29). Next, the enzyme activity of MMP-2 and MMP-9 was addressed following the above treatment. It was demonstrated that E2 or E2 plus OHT promoted MMP-9 activity, while baikalein exerted a significant suppression of E2-enhanced MMP-9 activity compared with the E2-treated group (Fig. 1B). However, no significant differences were identified among the activity of MMP-2 across all treatment groups (Fig. 1B). Additionally, western blot analysis and RT-qPCR analyses were performed to further confirm the changes observed in MMP-9 activity. The protein and mRNA expression levels were upregulated following E2 or E2 plus OHT treatment compared with the untreated control group. However, the two expression levels were significantly downregulated in the presence of baikalein compared with the E2-treated group (Fig. 1C and D).

GPR30 mediates cell invasion and MMP-9 activity and expression induced by E2. Since tamoxifen acts as an ERα antagonist but agonist for the GPR30, it was hypothesized that the mechanism by which estrogen induced invasiveness occurs through GPR30 activation. To validate the potential role of GPR30 in cell invasion, and MMP-9 activity and expression, MCF-7 cells were pretreated with the GPR30 antagonist G15 prior to stimulation with E2. The results demonstrated that G15 effectively suppressed E2-induced cell invasion compared with the E2-treated group (Fig. 2A). G15 significantly reduced MMP-9 activity compared with the E2-treated group (Fig. 2B). Furthermore, MMP-9 protein expression in G15 pretreated groups was significantly lower compared with that in the E2-induced group (Fig. 2C). Similar changes in MMP-9 mRNA expression were observed (Fig. 2D). This suggests that the effects of E2 on cell invasion and MMP-9 are mediated through the activation of GPR30.
Similar to the effects of G15, baicalein inhibits G‑1 induced cell invasion and MMP‑9 activity and expression in MCF‑7 cells. To confirm whether baicalein exerted its effects by interfering with GPR30 activation, the action of baicalein on GPR30 agonist G‑1 induced invasion and MMP‑9 activity and expression was investigated, whereby G15 was used as a positive control. As expected, treatment with G‑1 resulted in an increase in cell invasion, which was significantly suppressed by G15 or baicalein pretreatment (Fig. 3A). In addition, it was revealed that treatment with baicalein led to significant suppression of G‑1‑induced MMP‑9 activation (Fig. 3B), similar to the effects of G15. In addition, the upregulation of MMP‑9 protein and mRNA expression induced by G‑1 was significantly inhibited following baicalein treatment (Fig. 3C and D).

Discussion

While anti-estrogenic agents have been used successfully, breast cancer remains the leading cause of cancer-associated mortality among females worldwide (30). Additionally, aromatase inhibitors, which depress E2 synthesis, are more efficacious and produce significantly lower recurrence rates when compared with tamoxifen (31), indicating that solely targeting ERα to inhibit the action of estrogen may be suboptimal. GPR30 is independent of ERα status in breast cancer cells and tissue samples, and its action differs from the classical nuclear ERs, ERα and ERβ (32). Upregulation and activation of GPR30 promotes the progression of breast cancer, and GPR30 is considered a biological target for innovative therapeutic strategies (33). Research has demonstrated that overexpression of GPR30 in primary tumors is positively correlated with the metastatic phenotype of breast cancer (6,7), and GPR30 knockout MMTV-PyMT mice possess less aggressive tumors and fewer metastases (8). In the present study, an ERα- and GPR30-positive MCF-7 breast cancer cell line was used as a model system. It was demonstrated that the inhibition of GPR30 activation by its specific antagonist G15 significantly suppressed E2- or G‑1‑induced invasion, which...
is the initial stage of metastasis. Furthermore, GPR30 inhibition significantly reduced the E2- or G1-induced increase in MMP-9 expression and proteolytic activity, suggesting that GPR30 regulates metastasis by enhancing cell invasive ability.

Tamoxifen was initially designed as an anti-estrogenic agent, but has also been demonstrated to partially induce estrogenic activity (34). It has been revealed to enhance the migration and invasion abilities of ERα-negative SK-BR-3 breast cancer cells (35), and promotes the proliferation and invasion of endometrial cancer cells, similar to the effects of E2 (36). Furthermore, previous studies have revealed that tamoxifen can increase invasiveness and the expression of MMPs in MCF-7 cells in vitro (37,38). In the current study, it was demonstrated that although OHT could inhibit the function of ERα, it failed to prevent E2-induced cell invasion, and MMP-9 activation and upregulation. However, the E2-stimulated activation and upregulation of MMP-9 were significantly inhibited by treatment of cells with the GPR30 antagonist G15.

In addition, tamoxifen could perform as a GPR30 agonist. The results of the present study indicate that E2 promoted MMP-9 upregulation and expression through GPR30, instead of ERα. Furthermore, these results suggest that GPR30 performs an important role in maintaining the responsiveness of breast cancer cells to E2 in the pharmacological blockade of ERα. Therefore, antagonistic action on ERα and GPR30 may be a rational strategy for the treatment of ERα-positive breast cancer.

Emerging evidence suggests that administering endocrine agents, including tamoxifen has effects beyond their initially described mechanism of action. For example, tamoxifen has been demonstrated to promote the induction of epidermal growth factor receptor, human epidermal growth factor receptor-2 and insulin-like growth factor I (IGF-I) receptor, which subsequently activates various cellular kinase cascades, and elicits tamoxifen resistance (39). In addition, previous studies demonstrated that GPR30 is involved in the development...
of tamoxifen resistance and appearance of metastasis (40,41). GPR30 expression is significantly increased in tamoxifen resistant tumor tissue compared with primary tumors from the same patients (40). Furthermore, Mo et al (41) revealed that the expression of GPR30 was significantly increased in metastatic tumor compared with their corresponding primary tumor samples from 53 GPR30-positive patients with tamoxifen recurrence and that the GPR30 antagonist was able to reverse tamoxifen-induced resistance. Thus, it is suggested that inhibiting GPR30 signaling activation may provide an alternative therapeutic strategy for treating tamoxifen-resistant patients with breast cancer.

A previous study demonstrated that baicalein exhibits effective inhibitory activities against E2/IGF-1-induced cellular proliferation and colony formation in human breast carcinoma cells (42). In addition, the binding ability of baicalein to the ER was confirmed through a competitive ligand-binding assay (43,44). Notably, baicalein antagonized the estradiol-induced estrogen responsive elements response in a dose dependent manner (21). Unlike genistein, an isomer of baicalein, it does not exhibit a biphasic effect on ERα, thus there is no exhibition of estrogenic activity to transactivate ERα at low concentrations (21). These investigations propose that baicalein exerts anti-estrogenic activity and inhibitory effects on ERα transcriptional function. Recently, the authors of the current study demonstrated that baicalein suppresses E2-promoted migration and invasion in MCF-7 (GPR30/ERα-positive) and SK-BR-3 (GPR30-positive/ERα-negative) breast cancer cells (23). Furthermore, it was revealed that baicalein significantly inhibited E2- or G1-induced GPR30 signal activation and GPR30 target genes, cysteine-rich 61, and connective tissue growth factor upregulation (23).

In the present study, it was demonstrated that baicalein significantly suppressed E2- or G1-induced cell invasion, and MMP-9 upregulation and activation, exhibiting a similar effect to G15, but a reverse effect compared with OHT. This indicates that baicalein exerts a different activity to tamoxifen, and may possess dual inhibitory effects on ERα and GPR30 signaling. However, this hypothesis requires further investigation. To further investigate the underlying mechanism of baicalein, a detailed explanation of the signaling pathway by which GPR30 regulates MMP-9 expression and activation.

Figure 3. Baicalein suppresses GPR30 agonist G-1-induced cell invasion, and MMP-9 activation and expression. MCF-7 cells were pretreated with G15 (1 µM) or baicalein (10 µM) for 30 min and followed by treatment with G-1 (1 µM) for 24 h. (A) Invasive ability of the cells was measured using a Transwell chamber assay (magnification, x200, scale bar=500 µm). (B) The activity of MMP-9 was measured using a gelatin zymography assay. (C) The expression of MMP-9 was determined using western blotting. (D) The mRNA expression of MMP-9 was detected using reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean ± standard error of the mean, and normalized as a percentage of the control. *P<0.05 vs. the G-1 treated group. GPR30, G protein-coupled receptor 30; MMP, matrix metalloproteinase; cont, control; bai, baicalein.
is required, as well as the molecular mechanisms by which baicalein influences GPR30 signaling. In addition, \textit{in vivo} studies are warranted to confirm the effects observed in the current study.

In conclusion, baicalein, but not OHT, significantly attenuated E2-induced invasion, and MMP-9 upregulation and activation in MCF-7 breast cancer cells, which may be due to their different actions on GPR30. As cell invasion and MMP-9 activation positively correlates with cancer metastasis, it could be suggested that the inhibition of GPR30 activation may be a promising approach to reduce metastasis, and improve the efficacy of anti-estrogens. Targeting ERz and GPR30 receptors may achieve additional therapeutic benefits for the treatment of patients with breast cancer.

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