The nutritional herb *Epimedium grandiflorum* inhibits the growth in a model for the Luminal A molecular subtype of breast cancer

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Abstract. The Luminal A subtype of breast cancer expresses the estrogen receptor (ER)-α and progesterone receptor (PR), but not the human epidermal growth factor receptor (HER)-2 oncogene. This subtype of breast cancer responds to endocrine therapy involving the use of selective estrogen receptor modulators and/or inhibitors of estrogen biosynthesis. However, these therapeutic agents are frequently associated with long-term systemic toxicity and acquired tumor resistance, emphasizing the need to identify non-toxic alternative treatments for chemo-endocrine therapy responsive breast cancer. The present study utilized the human mammary carcinoma-derived, ER⁺/PR⁺/HER-2; MCF-7 cell line as a model of the Luminal A subtype of breast cancer to examine the growth inhibitory effect of the Chinese nutritional herb *Epimedium grandiflorum* (EG) and determine the mechanisms underlying this effect. MCF-7 cells maintained in a serum-depleted culture medium retained their ability to grow in response to 17β-estradiol (E₂). Treatment of the MCF-7 cells with EG resulted in dose-dependent inhibition of E₂-promoted growth. Mechanistically, EG inhibited E₂-promoted cell cycle progression through G₁ stage arrest and modulated the cellular metabolism of E₂, increasing the formation of the anti-proliferative metabolites 2-hydroxyestrone and estriol. Long-term treatment of MCF-7 cells with EG inhibited E₂-promoted anchorage independent growth, a surrogate *in vitro* biomarker of tumorigenesis. In conclusion, the results of the present study demonstrate the growth inhibitory effects of EG on MCF-7 cells and identified clinically relevant mechanistic leads for its anti-tumorigenic efficacy.

Introduction

Metastatic breast cancer is one of the leading causes of breast-cancer associated mortality in the United States, and the American Cancer Society (Atlanta, GA, USA) projections for breast cancer incidence and mortality rates estimate 246,660 newly diagnosed cases of invasive breast cancer and 40,450 metastatic breast cancer-associated mortalities in women in 2017 (1). These figures emphasize the requirement for a more precise molecular classification of breast cancer for subtype-specific targeted therapy, in addition to the necessity to identify novel, non-toxic and efficacious modalities as alternatives for cancer prevention and therapy.

The global gene expression profiling of breast cancer has led to the identification of molecularly distinct subtypes. Patients with the molecular subtype Luminal A express estrogen receptor-α (ER)-α and progesterone receptor (PR), and they lack the expression of human epidermal growth factor receptor-2 (HER-2). Patients with this subtype respond to endocrine-based targeted therapy, such as selective ER modulators and specific small molecule inhibitors of estrogen biosynthesis (2-4). However, long-term endocrine-based therapy has been associated with adverse systemic toxicity, acquired tumor resistance and the emergence of drug resistant cancer stem cells that compromise therapeutic efficacy and promote disease progression (4-6).

Complementary and alternative medicine using herbal products has been applied to endocrine therapy-responsive patients with breast cancer, as a potential method of reducing therapy-associated toxicity. Several nutritional herbs, including *Lycium barbarum* (LB), *Cornus officinalis* (CO) and *Epimedium grandiflorum* (EG), are essential components of herbal formulations that are used in traditional Chinese medicine as palliative and/or adjuvant treatment options for women with breast cancer (7,8). The majority of nutritional herbs display potent immunomodulatory and antiangiogenic properties *in vivo* (7). However, the underlying molecular signaling pathways and targets that are responsible for the growth inhibitory effects of these herbs on breast cancer cells remain to be systematically elucidated.
The human mammary carcinoma-derived cell line MCF-7 expresses ER-α and PR, and lacks HER-2 expression, thus representing an accepted pre-clinical cell model for the Luminal A molecular subtype of breast cancer (2-4). Previous studies using MCF-7 cells as a model for the Luminal A breast cancer subtype have demonstrated the direct growth inhibitory efficacy of extracts from Chinese nutritional herbs, such as LB and CO, that function through distinct mechanistic pathways (9,10). The present study utilized MCF-7 cells to investigate the direct growth inhibitory effects of the Chinese nutritional herb EG and identify the potential molecular mechanisms underlying its efficacy.

Materials and methods

Cell culture. The ER-α+/PR+/HER-2 human mammary carcinoma-derived cell line MCF-7 was obtained from the Michigan Cancer Foundation (Detroit, MI, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (DMEM/F-12) supplemented with heat inactivated 7% fetal calf serum, 2 mM L-glutamine, 1% penicillin-streptomycin mixture (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10 μg/ml insulin (Ely Lilly and Company, Indianapolis, IN, USA) and 1 μM dexamethasone (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), following published protocols (9-11).

In the present study, MCF-7 cells were adapted for long-term growth in serum-depleted medium through maintaining the cells in DMEM/F-12 supplemented with 0.7% fetal calf serum [17β-estradiol (E2) <1 nM] for ≥5 passages. Stock cultures were routinely maintained in DMEM/F-12 supplemented with 0.7% fetal calf serum in a humidified atmosphere with 5% CO2 at 37°C. Cells were sub-cultured in a 1:4 ratio once they reached 80% confluence.

Growth parameters. Cell population doubling time was determined during the exponential growth phase. Viable cell counts were performed 24, 48, 72 and 96 h post-seeding of 1x10^5 cells using a trypan blue exclusion cell viability assay kit (Sigma-Aldrich; Merck Millipore), following the protocol supplied by the vendor. The data are expressed as the mean of the four time points. Similarly, saturation density was determined by the viable cell count on day 7 post-seeding, using the trypan blue cell viability assay kit. The experiment for anchorage independent (AI) growth was performed using 12-well/treatment group. AI growth was evaluated by determining the number of AI colonies that had developed in the EPICS® 752 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA), equipped with a 488 nm excitation filter and a 630 nm longpass filter. Cell cycle phase distribution was analyzed using MultiCycle MPLUS 2.0 software (Phoenix Flow Systems, San Diego, CA, USA). Results are expressed as the percentage of cells in G1, S and G2/M phases of the cell cycle was monitored using the EPICS® 752 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA), equipped with a 488 nm excitation filter and a 630 nm longpass filter. Cell cycle phase distribution was analyzed using MultiCycle MPLUS 2.0 software (Phoenix Flow Systems, San Diego, CA, USA). Results are expressed as the percentage of cells in G1, S and G2/M phases of the cell cycle. In addition, the G2+S+G3/M ratio of the number of cells in each phase is presented. This assay was performed using 3 flasks/treatment group.

Cellular metabolism of E2. To examine the effect of EG on the cellular metabolism of E2, on day 1 post-seeding, 1x10^6 MCF-7 cells were treated with 20 nM E2 (Sigma-Aldrich; Merck Millipore) alone (control) or with 20 nM E2+1.0% EG were analyzed using fluorescence-activated cell sorting, following an optimized protocol (12,13). Briefly, trypsinized cell cultures from the control and treatment groups were stained with propidium iodide (Calbiochem; Merck Millipore), and the percentage of the cell population in G1, S and G2/M phases of the cell cycle was monitored using the EPICS® 752 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA), equipped with a 488 nm excitation filter and a 630 nm longpass filter. Cell cycle phase distribution was analyzed using MultiCycle MPLUS 2.0 software (Phoenix Flow Systems, San Diego, CA, USA). Results are expressed as the percentage of cells in G1, S and G2/M phases of the cell cycle. In addition, the G2+S+G3/M ratio of the number of cells in each phase is presented. This assay was performed using 3 flasks/treatment group.

Dose-response analysis. To determine the growth inhibitory effects, and the minimum effective and maximum cytostatic concentrations of EG, the MCF-7 cells were treated with increasing concentrations of EG (0.5, 1.0 and 2.0%). The experiments for the dose-response of EG were performed using 6 flasks/treatment group. Viable cell counts were determined on day 7 post-seeding of 1.0x10^5 cells using the trypan blue exclusion cell viability assay as described above. Dose-response analysis identified the half maximal inhibitory concentration (IC50), the maximum cytostatic concentration (IC90) and the toxic concentration of EG for growth inhibition. The IC50 was defined as the highest dose of EG that resulted in a viable cell count ≥ the initial seeding density. The toxic concentration was defined as the lowest dose of EG that resulted in a viable cell count < the initial seeding density.

Aqueous extract of EG. To prepare an aqueous extract, 20 g of leaves and stems from EG were boiled in 200 ml of deionized water until the supernatant had reduced to 100 ml, and the supernatant was subsequently collected (extract 1). The resultant residue was further boiled in 100 ml of water until the volume had reduced to 50 ml (extract 2). The two extracts (100 and 50 ml) were combined and further concentrated through boiling until the volume had reduced to 25 ml. These combined extracts were centrifuged at 500 x g at room temperature for 10 min. The resulting supernatant (20 ml) was collected and stored in 5 ml aliquots at -20°C. The stock solution (100%) was serially diluted in DMEM/F-12 to obtain working solutions of 2.0, 1.0 and 0.5% for subsequent experiments.

Statistical analysis. Statistical analyses were performed using Student’s t-test and one-way analysis of variance,
followed by a Dunnett’s Multiple Range Test (α=0.05) where appropriate. All tests were performed using GraphPad Prism® software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. The threshold of α=0.05 was selected for comparison of multiple treatment groups using the Dunnett’s Multiple Range Test.

Results

Growth-promoting effects of E₂. Results of the investigation into the effects of E₂ on MCF-7 cells adapted for growth in 0.7% serum (E₂, <1 nM) vs. those in 20 nM E₂ are presented in Table I. MCF-7 cells treated with 20 nM E₂ demonstrated a 17.6% decrease in the population doubling time, a 91.9% increase in the saturation density and a 1.2-fold increase in the number of AI colonies compared with the control group that had been grown in 0.7% serum (E₂, <1 nM). The statistical significance of the data was analyzed by one-way analysis of variance and Dunnett’s Multiple Range Test (α=0.05).

Dose-response to EG. Results of the investigation into the growth inhibitory effects of EG on MCF-7 cells are presented in Fig. 1. A 7-day treatment with 20 nM EG resulted in a viable cell count of 26.1±4.7x10⁶, a 26-fold increase compared with the initial seeding density (α=0.05). Furthermore, treatment with 20 nM EG+0.5% EG and 20 nM E₂+1.0% EG resulted in a significant decrease in the viable cell counts compared with the 20 nM E₂ alone-treated control group (20 nM E₂+0.5% EG, 12.8±2.3x10⁶; 52.6% decrease; α=0.05) and (20 nM E₂+1.0% EG, 2.4±0.4x10⁶; 87.0% decrease; α=0.05). This dose-response analysis identified that EG had an IC₅₀ of 0.49±0.18% and an IC₅₀ of 1.03±0.17% (data not shown). An EG concentration of 2.0% was toxic to MCF-7 cells.

Effect of EG on cell cycle progression. Data from the investigation into the effect of E₂ and E₂+EG on the cell cycle progression of MCF-7 cells are presented in Table II. The data demonstrates that treatment with 20 nM E₂ induced an increase in the percentage of cells in the S and G/M phases, with a simultaneous decrease in the percentage of cells in the G₁ phase, compared with the serum-treated control group. Furthermore, treatment with 20 nM EG and the IC₅₀ concentration of EG (1.0%) induced pronounced G₁ cell cycle arrest with a simultaneous decrease in the percentage of cells in the S and G/M phases, compared with the E₂ alone-treated control. Compared with the serum-treated control group, treatment with E₂ resulted in a 54.3% decrease (α=0.05) in the G₁; S+G/M ratio of cells. In addition, treatment with EG+E₂ resulted in a 76.2% increase in the G₁; S+G/M ratio of cells compared with the E₂ alone-treated control group (α=0.05). These results indicate that E₂ accelerates cell cycle progression and that EG can attenuate the effect of E₂.

Effect of EG on the cellular metabolism of E₂. Data from the investigation into the effect of EG on E₂ metabolism are presented in Table III. The results demonstrate that the E₂+EG group had a significantly increased formation rate of E₁ (P=0.01), 2-OHE₁ (P=0.006) and E₆ (P=0.001) compared with the E₂ alone-treated control group. EG induced a 3.3-fold increase in E₁ formation, a 5.0-fold increase in 2-OHE₁ formation and a 9.0-fold increase in E₆ formation compared with the E₂ alone-treated control group. Additionally, the primary data were further analyzed and expressed as the 2-OHE₁:16α-OHE₁ and E₆:16α-OHE₁.
The Luminal A molecular subtype of clinical breast cancer, defined by the expression of ER-α and PR, is typically treated with selective ER modulators and/or aromatase inhibitors, with or without cytotoxic drugs (3-6). These treatments are frequently associated with systemic toxicity and acquired tumor resistance, compromising therapeutic efficacy. Relatively non-toxic nutritional herbal products are used as therapeutic alternatives in traditional Chinese medicine. However, the long-term clinical safety, efficacy and potential interaction of nutritional herbs with traditional therapies remain unclear. The present study utilized the human mammary carcinoma-derived MCF-7 cell line (ER+/PR+/HER-2) as a preclinical cell model of the Luminal A molecular subtype of breast cancer, in order to investigate the growth inhibitory effects of the nutritional herb EG and to identify possible molecular mechanisms for its efficacy.

MCF-7 cells that were maintained in a medium supplemented with 0.7% serum (E2, <1 nM) retained their responsiveness to physiologically relevant concentrations of E2. This response was demonstrated by an increase in the number of viable cells following treatment with 20 nM of E2 compared with the serum-treated control group. In addition, at this physiological concentration E2 decreased cell population doubling time, increased saturation density and enhanced AI growth compared with the corresponding control groups. These data are consistent with a recent study, where E2 concentrations within the physiological range induced progressive increases in viable cell number (14). These results indicate that the method used in the current study produced an optimized E2-responsive cell culture model for the Luminal A molecular subtype of breast cancer.

Dose-response analysis demonstrated that E2-promoted cell growth was inhibited by EG in a dose-dependent manner, with an IC50 of 0.49±0.18% and IC90 of 1.03±0.17%. EG at a concentration of 2.0% demonstrated a cytotoxic response, indicated by a significantly reduced viable cell count, relative to the initial seeding density. These data indicate that EG antagonizes the growth-promoting effect of E2.

The growth-promoting effect of E2 on MCF-7 cells was demonstrated when investigating the effect of E2 on cell cycle progression. These was a reduction in the G0/S+G2/M ratio of cells in the E2-treated group compared with the serum-treated control group. Treatment of MCF-7 cells with E2 resulted in a decreased percentage of cells in the G1 phase, with a simultaneous increase in the percentage of cells in the S phase of the cell cycle. At its IC90 concentration, EG inhibited the E2-induced cell cycle progression of MCF-7 cells, as demonstrated by a pronounced G1 stage arrest and simultaneous decrease in the number of cells in the S+G2/M phases of the cell cycle. This alteration in cell cycle progression was associated with an increase in the G1; S+G2/M ratio and suggests that the cytostatic effect of EG is predominantly due to the inhibition of E2-promoted cell cycle progression.

Data from the present study on the cytostatic effect of EG at a relatively low dose are consistent with previous studies investigating the effects of extracts from other nutritional...
herbs, such as Tabebuia avellandae (TA), CO and LB, on MCF-7 cells (9,10,13,14). The signaling pathways susceptible to extracts from TA, CO and LB include cell proliferation, cellular apoptosis, ER, xenobiotic metabolism and cell cycle progression through the modulated expression of specific genes (9,10,13,14). Additionally, these extracts affect the cellular metabolism of E2, as demonstrated through the increased formation of specific anti-proliferative metabolites of E2 (9-11,13,14). These results suggest that low-dose treatment with extracts from nutritional herbs is associated with an acceptable toxicity profile for future animal experiments and clinical trials.

The organ-selective growth-promoting effects of E2 have been well established in cell culture and in vivo in animal models. Previously, E2 has been demonstrated to promote the growth of ER-positive MCF-7 cells in vitro, and promote tumor development in vivo (15). In addition to the effects of E2 on ER-dependent cellular signal transduction pathways (5,6), specific cellular metabolites of E2 have been demonstrated to modulate the growth of mammary carcinoma-derived cells (16,17). The intermediate metabolite E1 functions as a common precursor for the formation of the pro-mitogenic 16α-OHE1 through the C16α-hydroxylation pathway, or for the formation of anti-proliferative 2-OHE1 through the C2-hydroxylation pathway (16-21). The results of the present study demonstrated that treatment with EG significantly increased the formation of E1, 2-OHE1 and E2, while the formation of 16α-OHE1 remained essentially unaltered, when compared with the E2-treated control group. Since the C2- and C16-hydroxylation pathways represent two divergent metabolic pathways generating distinct E2 metabolites, and since E2 represents a proximate metabolite of 16α-OHE1, the relative ratios of these metabolites serve as important endocrine biomarkers for carcinogenic risk (9,10). In the present study, E2 metabolite ratios demonstrated that in response to the treatment with EG, the 2-OHE1:16α-OHE1 and the E2:16α-OHE1 ratios were substantially increased compared with the E2-treated control group. This indicates that EG selectively induces increased formation of 2-OHE1 and accelerates conversion of 16α-OHE1 into E2. Notably, similar modulation of the cellular metabolism of E2 by extracts from CO and LB has been revealed (9,10,14).

AI growth represents a specific and sensitive in vitro surrogate end-point biomarker for tumorigenic phenotypes (9-11,14,20-22). In the current study, E2 promoted the formation of AI colonies by MCF-7 cells. In response to treatment with EG, the number of AI colonies decreased in a dose-dependent manner, supporting the potential of EG as an anti-tumorigenic agent.

The results of the present study provide mechanistic evidence for the growth inhibitory effect of EG in a cellular model of ER-α+/PR+/HER-2 Luminal A subtype breast cancer. A recent study compared the efficacy of several Chinese nutritional herbs on ER non-functional (ER-NF) and ER functional (ER-F) phenotypes of breast cancer (14), where EG demonstrated comparable growth inhibition in ER-NF and ER-F phenotypes, suggesting potential efficacy in ER-α- and ER-α+ breast cancer. In addition, an ongoing study into the growth inhibitory effects of EG in a ER-α+/PR+/HER-2 triple-negative MDA-MB-231 breast cancer cell model has elucidated the potential molecular mechanisms underlying the cell cycle arrest and induction of cellular apoptosis (Telang et al., unpublished results). These results suggest that EG is an anti-tumorigenic agent in ER-α- and triple-negative breast cancer, in addition to the Luminal A subtype investigated in the present study.

It is well known that traditional herbal medicine combines the use of several herbal preparations and that these herbs contain several bioactive agents. Traditionally, herbs are boiled in water and consumed by patients as herbal tea (7,8,23-25). Therefore, to simulate patient consumption, non-fractionated aqueous extracts from EG were used as the test agent in the present study. Despite the growth inhibitory effects of the non-fractionated aqueous extract of EG demonstrated in the current study, the identity of the water-soluble bioactive agent(s) in this extract remain unclear. Methanol extracts from EG containing icarin and icaritin have been demonstrated to effect multidrug resistance in HepG2/ADR cells (26). In contrast, the same methanol soluble bioactive agents induce growth promoting effects through the upregulation of epidermal growth factor receptor/mitogen-activated protein kinase

Figure 2. Effect of EG on the AI growth of MCF-7 cells. Results are presented as the mean ± standard deviation (n=12/treatment group). E2 promoted AI growth of the cells, represented by the number of AI colonies, decreased in response to treatment with EG in a dose-dependent manner. Serum vs. E2 20 nM (serum vs. E2 α=0.05), E2 20 nM vs. E2 20 nM + EG 0.5% (E2 vs. EG α=0.05), E2 20 nM vs. E2 20 nM + EG 1.0% (E2 vs. EG α=0.05). Data was analyzed using one-way analysis of variance and Dunnett's test (α=0.05). E2, 17β-estradiol, EG, Epimedium grandiflorum; AI, anchorage independent.
signaling pathways in a model of HER-2\(^+\) breast cancer (27). Furthermore, organic solvent fractionated extracts from *Epimedium brevicornum* containing the prenylflavone breviflavone B have been demonstrated to exhibit inhibitory effects on ER-\(\alpha\) expression, in addition to on the growth of ER-\(\alpha\)+ breast cancer cells in *vitro* and *in vivo* (28-30). The growth inhibitory effect of non-fractionated aqueous extract from EG in the present study and the growth modulatory effects of organic solvent fractionated extracts from different *Epimedium* species in previous studies, suggest that EG has a pleiotropic effect, which is likely due to the presence of distinct constitutive bioactive components. In addition to the MCF-7 cell line representing a model of the Luminal A subtype of breast cancer, MCF-7 cells have provided a model for endocrine therapy-resistant cancer stem cells (31). Investigations into the response of cancer stem cell models to EG may identify potential therapeutic targets for stem cell therapy. A study using a similar approach has identified the antitumorigenic efficacy of the phytochemicals quercetin and sulforaphane in pancreatic cancer stem cells (32).

In conclusion, the present study has identified distinct phenomenological and mechanistic evidence for the antitumorigenic efficacy of EG in the Luminal A molecular subtype of breast cancer. The results of the present study validate a mechanism-driven cell culture approach to prioritize efficacious herbal medicinal products for the treatment of patients with the ER\(^+\) Luminal A molecular subtype of breast cancer.

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