

***Lycium Barbarum* Inhibits Growth of Estrogen Receptor Positive Human Breast Cancer Cells by Favorably Altering Estradiol Metabolism**

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Selective estrogen receptor modulators represent accepted therapy for estrogen receptor positive (ER⁺) breast cancer, exhibit adverse side effects, and reduce patient compliance. The use of phytoestrogen containing herbal medicines is limited because of efficacy and safety concerns. The ER⁺ MCF-7 model examined growth inhibitory effects of the medicinal herb *Lycium barbarum* (LB) and identified mechanistic leads for its efficacy. The MCF-7 cells maintained in 0.7% serum (17 β -estradiol, E₂ < 1 nM) exhibited 11%–87% increased growth after treatment with 1nM to 20 nM E₂. Growth promotion with 20 nM E₂ exhibited 5.2-fold increased estrone (E₁), 35.7% increased 2-hydroxyestrone (2-OHE₁), 15.4% increased 16 α -hydroxyestrone (16 α -OHE₁), and eightfold increased estriol (E₃) formation. Treatment of E₂ stimulated cells with LB exhibited a dose-dependent growth inhibition of 9.5%–42.8% at Day 3 and 33.9%–83.9% at Day 7. The 3-day inhibitory response to 1% LB (maximum cytostatic concentration) exhibited 84.8% increased E₁, 3.6-fold increased 2-OHE₁, 33.3% decreased 16 α -OHE₁, and 9.2-fold increased E₃ formation. Thus, MCF-7 cells retain their mitogenic and metabolic response to E₂ and LB down-regulates E₂-stimulated growth via the formation of antiproliferative 2-OHE₁ and accelerated conversion of mitogenic 16 α -OHE₁ to antimitogenic E₃.

INTRODUCTION

Clinical breast cancer represents one of the major causes of morbidity and mortality in the United States. The American Cancer Society estimated 182,460 new breast cancer cases and 40,480 breast cancer related deaths in 2008 (1).

Although dietary modification has provided convincing epidemiological and experimental evidence for modulation of breast cancer development (2), currently acceptable management of clinical breast cancer continues to be either chemotherapy, or chemo-endocrine therapy, depending on the estrogen receptor (ER) status of the tumor (3,4). The conventional therapeutic strategy is frequently associated with adverse side effects that decrease patient compliance.

As an alternative to conventional therapeutic intervention, herbal medicinal products have received increasing attention in the management of estrogen related health issues; thus, intervention with herbal medicines either as independent agents or as an adjuvant to conventional chemo-endocrine therapy may represent a less toxic alternative for treatment of breast cancer (5,6). Although the use of *Lycium barbarum* (LB), also known as Wolfberry or Gouqizi, has been well documented in traditional Chinese herbal medicine, there are only a few scientifically robust mechanistic studies that have addressed efficacy, safety, and toxicity of LB in preventive or therapeutic intervention of cancer. Evidence in the literature indicates that the polysaccharide component of LB sensitizes tumor cells against radiotherapy in the transplanted Lewis lung cancer model (7), enhances the clinical efficacy of LAK/IL 12-based immunotherapy in cancer patients (8), inhibits proliferation and induces apoptosis in human hepatoma QGY 7703 cell line in culture (9), inhibits proliferation of PC-3 cells in culture (10), and exhibits immuno-modulatory and antitumor activity (11,12).

The human breast carcinoma-derived MCF-7 cell line represents a widely accepted preclinical cell culture model for hormone responsive, ER⁺, breast cancer. This model has been extensively utilized both as an in vitro cell culture model as well as an in vivo xenotransplant model to evaluate the efficacy of synthetic selective estrogen receptor modulators (SERMs) and also of inhibitors of estrogen biosynthesis (13).

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Studies on tumorigenically transformed breast epithelial cells have demonstrated that regardless of the nature of the transforming agent, the cells treated with chemical carcinogens, expressing murine mammary tumor virus, or transfected with oncogenes uniformly exhibit altered cellular metabolism of 17β -estradiol (E_2) in favor of the $C16\alpha$ -hydroxylation pathway (14–16,20). Furthermore, several mechanistically distinct chemopreventive agents modulate the metabolism of E_2 in favor of the $C2$ -hydroxylation pathway in the initiated mammary epithelial cells that are at increased risk for carcinogenesis (17–19). Specific intermediate metabolites of these pathways, 16α -hydroxyestrone (16α -OHE₁) and 2-hydroxyestrone (2-OHE₁), have opposing effects on the growth of breast epithelial cells (16,20–22); thus cellular metabolism of E_2 represents a modifiable endocrine biomarker for carcinogenesis and chemoprevention (15,16,23).

The experiments in the present study have utilized the MCF-7 cell culture model to examine the growth inhibitory effects of the medicinal herb LB and to identify possible mechanistic leads for its efficacy. The data generated from the present study have demonstrated that LB at relatively low concentrations produces a cytostatic growth arrest in MCF-7 cells due to specific favorable alterations in the cellular metabolism of E_2 . Thus, these experiments identify possible mechanism(s) for the efficacy of LB and thereby validate the present MCF-7 cell culture model as a rapid mechanism-based approach to evaluate the therapeutic efficacy of medicinal herbs for clinical breast cancer.

MATERIALS AND METHODS

Cell Line

The ER⁺ human breast carcinoma MCF-7 cell line was originally obtained from the Michigan Cancer Foundation (Detroit, MI). These cells were cultured in DME/F12 medium supplemented with 7% heat inactivated fetal calf serum and recommended additives (13,17).

For the present experiments, MCF-7 cells were adapted for growth in serum-depleted medium by maintaining the cultures in the medium supplemented with 0.7% serum for at least 5 passages. These stock cultures were routinely maintained in DME/F12 medium supplemented with 0.7% serum in an humidified atmosphere of 95% air: 5% CO₂ at 37°C and were subcultured at 1:4 split at about 80% confluency.

Aqueous Extract of LB

To prepare the LB extract, 20 g of LB fruits were boiled in 200 ml of deionized water until the volume was reduced to 100 ml, and the supernatant was collected (aqueous Extract #1). The resultant residue of LB was further boiled in 100 ml of water until the volume was reduced to 50 ml (aqueous Extract #2). The two supernatants (Extract #1 100 ml and Extract #2 50 ml) were combined and concentrated by boiling until the volume was reduced to 25 ml. These combined extracts were

centrifuged at 5,000 rpm at room temperature for 10 min. The resultant supernatant (20 ml) was collected and stored as stock solution at –20°C in 5 ml aliquots. The stock solution of LB was appropriately diluted in DME/F12 medium to obtain the working concentrations of LB.

Effect of E_2 on Serum-Depleted MCF-7 Cells

To examine the response of E_2 , MCF-7 cells were seeded at the initial density of 1.0×10^5 cells/25cm² in T-25 tissue culture flasks. After a 24 h attachment period, the cultures were treated with 1, 5, 10, and 20 nM E_2 . This treatment was continued up to Day 7 postseeding, with a medium change every 48 h. At the end of the treatment, cultures were trypsinized, stained with trypan blue, and trypan blue excluding viable cells were counted.

Dose Response of LB

For the dose-response experiments, MCF-7 cells were seeded at the initial density of 1.0×10^5 cells/25 cm² in T-25 flasks. LB treatment at 0.05%, 0.1%, 0.25%, 0.5%, 1.0%, and 2.0% doses was initiated at Day 1 postseeding and continued up to Day 7 postseeding, with a medium change at every 48 h. At the end of the treatment schedule, the cultures were trypsinized, and trypan blue excluding viable cell counts were obtained. The data from the viable cell counts were used to determine minimum effective and maximum cytostatic doses of LB.

Sample Preparation for Cellular Metabolism of E_2

The MCF-7 cells at Day 1 postseeding were treated with appropriate concentrations of LB in the presence of 20 nM E_2 for 48 h, and the medium was analyzed for E_2 metabolites following published methods (24–27). Briefly, a 5 ml aliquot of the medium was diluted 1:1 with sodium acetate buffer (pH 4.65) and 20 μ l of β -glucuronidase (110,200 units/ml; Sigma Chemical, St. Louis, MO). This solution was incubated at 37°C for 24 h to deconjugate the steroids. After the addition of deuterated E_2 as an internal standard (25), each sample was thoroughly vortexed. Two volumes of chloroform were added to the samples, and the resulting mixture was vortexed and centrifuged. The chloroform layer was removed and reduced to dryness using a vacuum equipped centrovap console (Labconco, Inc., St. Louis, MO).

Each sample was derivitized by adding 10 μ l of dry pyridine and 40 μ l of bis (trimethylsilyl) trifluoroacetamide (BSTFA), vortexed, and allowed to react at room temperature overnight. One μ l of each sample was injected into the GC-MS apparatus without further treatment.

GC-MS Conditions for Analysis of E_2 Metabolites

Select E_2 metabolites were analyzed on an Agilent 6980N gas chromatograph equipped with an Agilent 5973 mass selection detector, an Agilent 7683 injector, and an HP GI701CA MSD Chemstation (Agilent Technologies, Newcastle, DE).

The injection port was equipped with a split/splitless capillary inlet system and a silanized glass insert. The temperature of the injection port was maintained at 300°C. The GC-MS interface was maintained at 270°C, and the ion source was maintained at 280°C. The ionization energy was 70 eV. The carrier gas was helium at a flow rate of 1 ml/min. Separations of metabolites were carried out using a Hewlett-Packard Ultra 2 capillary column with cross-linked 5% phenylmethyl silicone (25 m × 0.2 mm × 0.33 μm film thickness). The oven temperature was increased from 60°C to 260°C at 40°C/min then at 1°C/min to 280°C (24,26,27).

Under selected ion monitoring, the following mass ions and GC elution times of trimethylsilylated estrogens were routinely monitored: E₁ m/z 342, 15.90 min; E₂ m/z 416, 16.40 min; deuterated E₂ m/z 420, 16.40 min; 2-OHE₁ m/z 430, 18.47 min; 4-OHE₁ m/z 430, 18.92 min; 16α-OHE₁ m/z 286 and 430, 19.37 min; and E₃ m/z 504 and 345, 20.76 min. The other E₂ metabolites 2-OHE₂ and 4-OHE₂ were monitored using the mass and base ions m/z 504 and m/z 373 at 19.06 min and 20.15 min, respectively, in a second run using the same parameters as above. The [2, 4, 16α, 16β-²H₄] deuterated estradiol (d₄ E₂) was synthesized in our laboratory according to the method of Dehennin et al. (25) and was used as an internal standard. The individual metabolites were quantified using a 6-point calibration curve (range: 1–100 ng). The data were expressed as ng metabolite per 10⁶ cells.

Statistical Analysis

The dose response experiments with E₂ and with LB were performed using *n* = 3 flasks per treatment group. The experiment for E₂ metabolism was also performed using *n* = 3 flasks per treatment group.

The significance of differences between the control and experimental data points for individual experiments was analyzed using a 2-sample *t*-test, 2-way ANOVA and 1-way ANOVA with Dunnett's Multiple Range Test (α = 0.05) as a posttest where appropriate.

RESULTS

Effects of E₂ on Serum-Depleted MCF-7 Cells

To demonstrate persistent E₂ response, the effect of physiological levels of E₂ was evaluated on the growth of MCF-7 cells maintained in culture medium supplemented with 0.7% serum. The number of viable cells at Day 7 postseeding represented the quantitative endpoint. The data presented in Table 1 demonstrated a progressive E₂ dose-dependent increase in the viable cell number, ranging from 11% with 1 nM E₂ to 87% with 20 nM E₂, relative to that in cells maintained in medium supplemented with 0.7% serum, representing the controls.

The statistical significance of these data confirmed E₂ dose-dependent increases in the viable cell number as analyzed by the Dunnett's multiple comparison test (α = 0.05).

TABLE 1
Growth promotion of serum-depleted human mammary carcinoma MCF-7 Cells by 17β-estradiol (E₂)

Treatment	Concentration (nM)	Viable Cell Number (× 10 ⁵) ^a	Increase (% Control)
Serum	0.7%	10.0 ± 0.3 ^b	—
Serum + E ₂	1	11.1 ± 0.3 ^c	11.0
	10	14.3 ± 0.4 ^d	43.0
	20	18.7 ± 0.3 ^e	87.0

^aDetermined from number of viable cells at 7-day postseeding of 1.0 × 10⁵ cells.

^{b–e}Mean ± SD, *n* = 3 per treatment group.

^{c>b, d>b, e>b}Data analyzed by Dunnett's multiple comparison test (α = 0.05).

E₂ Metabolism in Serum-Depleted MCF-7 Cells

The cellular metabolism of E₂ plays a critical role in growth modulation of MCF-7 cells. This metabolic competence was evaluated by measuring the formation of E₁, 2-OHE₁, 16α-OHE₁, and E₃. The data presented in Table 2 reveals that in cells incubated with 20 nM E₂ as the enzyme substrate, formation of E₁, 2-OHE₁, 16α-OHE₁, and E₃ were increased by 5.2-fold (*P* = 0.03), 35.7% (*P* = 0.04), 15.4% (*P* > 0.05, NS), and eightfold (*P* = 0.01), respectively, relative to that in the cells grown in the medium supplemented with 0.7% serum.

The statistical significance of E₂ treated cells, relative to the controls maintained in medium supplemented with 0.7% serum, was analyzed by the 2-sample *t*-test.

Effect of LB on E₂ Promoted Growth in Serum-Depleted MCF-7 Cells

The dose response experiment with LB identified 0.1% LB as minimum effective (growth inhibition: 33.9%) and 1.0% LB as the maximum cytostatic dose (growth inhibition: 83.9%) at Day 7 postseeding. The experiment presented in Table 3 was designed to evaluate the efficacy of LB to downregulate growth promotion by E₂.

The treatment with 20 nM E₂ resulted in a 31.2% increase in the viable cell numbers at Day 3 and a 68.0% increase at Day 7 postseeding, respectively, relative to that in cells maintained in the medium supplemented with 0.7% serum without E₂. This latter group served as a control.

The treatment of cells with 0.1%, 0.5%, and 1.0% LB in the presence of 20 nM E₂ resulted in a progressive 9.5%, 23.8%, and 42.8% decrease in the viable cell number at Day 3, respectively, and 33.9%, 64.3%, and 83.9% decrease in the viable cell number at Day 7 postseeding, respectively. The cells maintained in the presence of E₂ served as the control.

For the statistical analysis of these data, a 2-way ANOVA with Dunnett's multiple comparison test (α = 0.05) was used. The 2-way ANOVA compared 4 levels of concentrations as the row factor and the 2 time points as the column factor. For

TABLE 2
Metabolism of 17 β -estradiol (E₂) in serum-depleted human mammary carcinoma MCF-7 cells^a

Treatment	Concentration (nM)	E ₂ Metabolite ^b (ng/10 ⁶ cells)			
		E ₁	2-OHE ₁	16 α -OHE ₁	E ₃
Serum	0.7%	1.4 \pm 0.8 ^c	1.4 \pm 0.1 ^e	3.9 \pm 0.3	0.05 \pm 0.03 ^g
Serum + E ₂	20	8.7 \pm 0.9 ^d	1.9 \pm 0.3 ^f	4.5 \pm 0.1	0.45 \pm 0.07 ^h

^aAbbreviations are as follows: 2-OHE₁, 2-hydroxyestrone; 16 α -OHE₁, 16 α -hydroxyestrone; GC-MS, gas chromatography-mass spectrometry. Data analyzed by the 2-sample *t*-test comparing with the data from medium supplemented with 0.7% serum (control group).

^bDetermined at Day 3 postseeding by stable isotope dilution and GC-MS analysis.

^{c-h}Mean \pm SD, *n* = 3 per treatment group.

^{c,d}*P* = 0.03.

^{e,f}*P* = 0.04.

^{g,h}*P* = 0.01.

both concentration and duration, there was significant interaction (*P* < 0.001).

Effect of LB on E₂ Metabolism in Serum-Depleted MCF-7 Cells

The experiment presented in Table 4 was designed to examine whether the inhibitory effect of LB is associated with alteration in cellular metabolism of E₂. The treatment of the cells with LB at the maximum cytostatic dose of 1.0%, induced about an 84.8% increase in E₁, a 3.6-fold increase in 2-OHE₁, and a 9.2-fold increase in E₃, whereas 16 α -OHE₁ exhibited a modest 33.3% decrease relative to that observed in the control cultures treated with E₂ alone.

The statistical significance of these data was analyzed by 1-way ANOVA and Dunnett's multiple comparison test (α = 0.05).

TABLE 3
Dose response of *lycium barbarum* (LB) on serum-depleted human mammary carcinoma MCF-7 cells

Treatment	Concentration	Viable Cell Number ($\times 10^5$) ^a	
		Day 3	Day 7
E ₂	20 nM	4.2 \pm 0.1 ^b	16.8 \pm 0.5 ^f
E ₂ + LB	0.1%	3.8 \pm 0.1 ^c	11.1 \pm 0.1 ^g
	0.5%	3.2 \pm 0.1 ^d	6.0 \pm 0.2 ^h
	1.0%	2.4 \pm 0.2 ^e	2.7 \pm 0.1 ⁱ

^aUntreated control viable cell number 3.2 $\times 10^5$ (Day 3) and 10.0 $\times 10^5$ (Day 7). Initial seeding density: 1.0 $\times 10^5$ cells per 25 cm².

^{b-e,f-i}Mean \pm SD, *n* = 3 per treatment group.

^{b>c,b>d,b>e,f>g,f>h,f>i}Data analyzed by 2-way analysis of variance (ANOVA) and Dunnett's multiple comparison test (α = 0.05). For ANOVA, concentration \times duration exhibited a significant interaction (*P* < 0.001).

Modulation of E₂ Metabolism by LB

To evaluate the effect of LB on global metabolism of E₂ in serum-depleted MCF-7 cells, the primary data obtained for individual metabolites was analyzed as ratios between 2-OHE₁:16 α -OHE₁ and 16 α -OHE₁:E₃. The data presented in Table 5 demonstrated that LB at the maximum cytostatic concentration of 1.0% produced a 5.9-fold increase in the 2-OHE₁:16 α -OHE₁ ratio and a 94% decrease in the 16 α -OHE₁:E₃ ratio as analyzed by Dunnett's test correcting for multiple comparisons (α = 0.05).

DISCUSSION

Treatment of choice for clinical ER⁺ breast cancer involves the use of the antiestrogen SERM tamoxifen independently or in combination with chemotherapeutic agents (27–29). These treatment options are frequently associated with adverse systemic toxicity that limits patient compliance. Alternate strategy involving medicinal herbs or natural phyto-chemicals in an adjuvant setting may lead to a dose reduction and/or enhanced efficacy of chemotherapeutic agents resulting in a favorable toxicity profile (5,6).

The human mammary carcinoma MCF-7 cell line represents a widely accepted preclinical model for ER⁺ breast cancer. The MCF-7 cells are dependent on the ovarian steroid hormone E₂ for growth in vitro and tumorigenicity in vivo (13). ER functions as a ligand-inducible transcription factor that uses E₂ as a ligand and initiates a cascade of molecular signaling pathways to induce cellular mitogenic response (28–30). Routinely, MCF-7 cells are maintained in the culture medium supplemented with 7%–10% serum (13,17). The presence of serum represents a major variable source of several mitogenic hormones and growth factors, including E₂. Traditional short-term approach to control the variable influences of serum derived mitogenic steroid hormones involves transient maintenance of the cells in phenol red-free medium supplemented with charcoal-dextran stripped serum. This approach, although excellent for short-term

TABLE 4
Effect of *lycium barbarum* (LB) on estradiol (E₂) metabolism in human mammary carcinoma MCF-7 cells^a

Treatment	Concentration	E ₁	E ₂ Metabolite ^b (ng/10 ⁶ Cells)		
			2-OHE ₁	16α-OHE ₁	E ₃
E ₂		4.6 ± 0.9	1.9 ± 0.3 ^c	4.5 ± 0.2 ^e	0.45 ± 0.09 ^g
E ₂ + LB%	0.1	6.8 ± 2.2	1.6 ± 0.4	4.3 ± 0.4	2.1 ± 0.1 ^h
	0.5	7.0 ± 2.4	1.8 ± 0.9	4.4 ± 0.3	6.5 ± 1.0 ⁱ
	1.0	8.5 ± 0.4	8.7 ± 0.4 ^d	3.0 ± 0.3 ^f	4.6 ± 0.7 ^j

^aAbbreviations are as follows: 2-OHE₁, 2-hydroxyestrone; 16α-OHE₁, 16α-hydroxyestrone; GC-MS, gas chromatography-mass spectrometry.

^bDetermined at Day 3 postseeding by stable isotope dilution and GC-MS analysis.

^{c-j}Mean ± SD, n = 3 per treatment group.

^{c<d,e>f,g<h,i,g<j}Data analyzed by 1-way analysis of variance and Dunnett's multiple comparison test (α = 0.05).

molecular/biochemical experiments, was not optimal for longer term experiments that involve frequent subculturing and expansion of cells for the ongoing studies on long-term effects of other herbal medicinal extracts. The experiment in this study was therefore designed to refine the model by long-term adaptation of the cells to grow in medium supplemented with 0.7% serum and to preserve their response to E₂. The data generated from these experiments demonstrated a positive growth regulation of serum-depleted MCF-7 cells in the presence of physiologically relevant levels of E₂, possibly via ER-dependent mechanism. Several experimental approaches have been documented to examine the impact of E₂ responsiveness on breast carcinogenesis. Thus, stable independent transfections of MCF-7 cells with select oncogenes such as Ras (31,32) and HER-2 (33,34) have been reported to minimize E₂ dependence or modulate the susceptibility of carcinoma cells to the selective estrogen receptor modulator tamoxifen. Similarly, in spontaneously im-

mortalized nontumorigenic murine mammary epithelial cells, targeted expression of Ras (16,35) or myc (36) oncogenes results in ER downregulation, serum-derived growth factor independence, and subsequently tumorigenic transformation relative to that in parental or vector treated cellular phenotypes. These observations taken together with the present observed persistent response of serum depleted MCF-7 cells to E₂ suggest that serum-depleted MCF-7 cells may represent a facile, specific, and sensitive approach to evaluate experimental modulations of E₂ responses. Specifically, transient downregulation of functional ER by limiting the availability of E₂ via serum depletion provides a facile isogenic approach to examine the specific effects of herbal medicinal products on biochemical and cellular effects of E₂ on cellular phenotypes with transiently functional or nonfunctional ER.

In addition to the ER-dependent growth responses, cellular metabolism of E₂ via C17-oxidation, C2-hydroxylation, and C16α-hydroxylation pathways generate E₁, 2-OHE₁, 16α-OHE₁, and E₃. E₁ functions as a common precursor for the formation of 2-OHE₁ or 16α-OHE₁ via the 2-hydroxylation or 16α-hydroxylation pathways, respectively, and E₃ represents the proximate metabolite of 16α-OHE₁ (15,37,38). These intermediate metabolites have distinct growth regulatory effects on MCF-7 cells. Thus, 2-OHE₁ and E₃ exhibit antimetogenic effects, whereas 16α-OHE₁ exhibits promitogenic effects in mammary carcinoma cells (21,22). In this context, it is noteworthy that serum-depleted MCF-7 cells in the presence of E₂ exhibited increases in E₁, 2-OHE₁, 16α-OHE₁, and E₃ formation. The positive growth regulation with E₂ and a persistent competence for cellular metabolism of E₂, taken together, represent a major refinement in the MCF-7 cell culture model for ER⁺ breast cancer.

The alteration in the cellular metabolism of E₂ represents one of the mechanisms for growth inhibition in murine or human breast cancer cells (14–16). Upregulation of the C2-hydroxylation:C16α-hydroxylation ratio, predominantly due to increased formation of 2-OHE₁, has been noted in response to the treatment with several synthetic chemopreventive

TABLE 5
Modulation of 17β-estradiol (E₂) metabolism by *lycium barbarum* (LB) in serum-depleted human mammary carcinoma MCF-7 cells^a

Treatment	Concentration	E ₂ Metabolism ^b	
		2-OHE ₁ :16α-OHE ₁	16α-OHE ₁ :E ₃
E ₂	20 nM	0.42 ± 0.10 ^c	10.0 ± 0.5 ^e
E ₂ + LB	(%)		
	0.1	0.37 ± 0.10	2.0 ± 0.2 ^f
	0.5	0.41 ± 0.33	0.7 ± 0.3 ^g
	1.0	2.90 ± 0.75 ^d	0.6 ± 0.4 ^h

^aAbbreviations are as follows: 2-OHE₁, 2-hydroxyestrone; 16α-OHE₁, 16α-hydroxyestrone.

^bDetermined from the concentrations of individual metabolites.

^{c-h}Mean ± SD, n = 3 per treatment group.

^{c<d,e>f,g<h,e>h}Data analyzed by Dunnett's multiple comparison test (α = 0.05).

agents as well as with several natural phyto-chemicals that inhibit mammary carcinogenesis (14–19). The experiments designed to evaluate the growth inhibition of MCF-7 cells have demonstrated that LB was capable of effectively inhibiting the E_2 -mediated growth in a dose- and time-dependent manner.

Our previous studies have shown that several mechanistically distinct chemopreventive agents such as indole-3-carbinol, tamoxifen, and 4-OH tamoxifen effectively downregulate the growth of MCF-7 cells by increasing 2-OHE₁ formation and thereby favorably altering the cellular metabolism of E_2 (17,18). The experiment designed to identify possible mechanism(s) responsible for the growth inhibitory effects of LB revealed that corresponding with the LB dose, formation of antimitogenic 2-OHE₁ increased, that of promitogenic 16 α -OHE₁ remained essentially unaltered, whereas that of E_3 increased. The data on the status of the 16 α -hydroxylation pathway suggest that LB may accelerate the conversion of 16 α -OHE₁.

Global metabolism of E_2 is also known to produce other catechol estrogens such as 4-OHE₁, 2-OHE₂, 4-OHE₂, as well as their proximate methoxy derivatives. Amongst these catechol estrogens, 4-OHE₁ and 4-OHE₂ have documented genotoxic effects and function as putative procarcinogenic agents (23,30). Furthermore, it is noteworthy that a comparison of intratumoral E_2 metabolism in clinical breast cancer and normal breast tissue has shown that 2-OHE₁:16 α -OHE₁ ratio was decreased, whereas 4OHE₂:2-OHE₂ ratio was increased (23), suggesting that 16 α -OHE₁ and 4-OHE₂ might be procarcinogenic, whereas 2-OHE₁ and 2-OHE₂ might be anticarcinogenic. In our experiments, however, the 4-OHE₂ and 2-OHE₂ metabolites were undetectable (data not shown). It is to be noted that in response to LB treatment, the 2-OHE₁:16 α -OHE₁ ratio increased due to an increase in 2-OHE₁ formation, whereas the 16 α -OHE₁: E_3 ratio decreased due to increased E_3 formation. In accord, our previous studies have demonstrated distinct antimitogenic effects of 2-OHE₁ and E_3 and promitogenic effects of 16 α -OHE₁ in the cell culture models in vitro as well as in the xenotransplant model in vivo (16,17,19,39).

Thus, all the observations discussed above taken together suggest that LB alters the cellular metabolism of E_2 in a manner that is favorable to upregulation of the 2-hydroxylation pathway and/or to enhance the conversion of 16 α -OHE₁ to E_3 . Since both of these pathways are associated with the production of antiproliferative metabolites of E_2 , specific alterations in cellular metabolism of E_2 may represent a possible mechanism responsible for antiproliferative effects of LB.

Altered cellular metabolism of E_2 in response to treatment with nonfractionated aqueous extract of LB are intriguing and difficult to interpret at the present time. It is likely that the aqueous extract may contain several water soluble components with varying biological activities. These data raise the possibility that relative proportion of various biologically active water soluble components of LB may interact to either specifically enhance the formation of antimitogenic 2-OHE₁ and/or to accelerate

the conversion of promitogenic 16 α -OHE₁ to its inert proximal metabolite E_3 .

Taken together, the data generated from the present study have identified a novel mechanistic lead for the effect of LB and thereby have validated a human breast cancer-derived preclinical cell culture model for rapid mechanism-based screening of medicinal herbs for their efficacy toward hormone responsive breast cancer.

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