## Nutrition and Cancer

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

Go Li [a]; Daniel W. Sepkovic [b]; H. Leon Bradlow [b]; Nitin T. Telang [a]; George Y. C. Wong [a]
[a] Strang Cancer Prevention Center, New York, New York, USA [b] Hackensack University Medical Center, University of Medicine and Dentistry, Newark, New Jersey, USA

## PLEASE SCROLL DOWN FOR ARTICLE

**Routledge**
Taylor & Francis Group

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

**Go Li**
*Strang Cancer Prevention Center, New York, New York, USA*

**Daniel W. Sepkovic and H. Leon Bradlow**
*Hackensack University Medical Center, University of Medicine and Dentistry, Newark, New Jersey, USA*

**Nitin T. Telang and George Y. C. Wong**
*Strang Cancer Prevention Center, New York, New York, USA*

**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 (17ß-estradiol, $E_2 < 1$ nM) exhibited 11%–87% increased growth after treatment with 1nM to 20 nM $E_2$. Growth promotion with 20 nM $E_2$ exhibited 5.2-fold increased estrone ($E_1$), 35.7% increased 2-hydroxyestrone (2-OHE$_1$), 15.4% increased 16$\alpha$-hydroxyestrone (16$\alpha$-OHE$_1$), and eightfold increased estriol ($E_3$) formation. Treatment of $E_2$ 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 $E_1$, 3.6-fold increased 2-OHE$_1$, 33.3% decreased 16$\alpha$-OHE$_1$, and 9.2-fold increased $E_3$ formation. Thus, MCF-7 cells retain their mitogenic and metabolic response to $E_2$ and LB downregulates $E_2$-stimulated growth via the formation of antiproliferative 2-OHE$_1$ and accelerated conversion of mitogenic 16$\alpha$-OHE$_1$ to antimitogenic $E_3$.**

## 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).

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 17ß-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, 16$\alpha$-hydroxyestrone (16$\alpha$-OHE$_1$) and 2-hydroxyestrone (2-OHE$_1$), 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% $CO_2$ at 37$^\circ$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 $-20^\circ$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 $1.0 \times 10^5$ cells/25cm$^2$ 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 $1.0 \times 10^5$ cells/25 cm$^2$ 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 $\mu$l of ß-glucuronidase (110,200 units/ml; Sigma Chemical, St. Louis, MO). This solution was incubated at 37$^\circ$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 $\mu$l of dry pyridine and 40 $\mu$l of bis (trimethylsilyl) trifluoroacetamide (BSTFA), vortexed, and allowed to react at room temperature overnight. One $\mu$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% phenyl-methyl silicone (25 m × 0.2 mm × 0.33 $\mu$m 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: $E_1$ m/z 342, 15.90 min; $E_2$ m/z 416, 16.40 min; deuterated $E_2$ m/z 420, 16.40 min; 2-OHE$_1$ m/z 430, 18.47 min; 4-OHE$_1$ m/z 430, 18.92 min; 16$\alpha$-OHE$_1$ m/z 286 and 430, 19.37 min; and $E_3$ m/z 504 and 345, 20.76 min. The other $E_2$ metabolites 2-OHE$_2$ and 4-OHE$_2$ 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, 16$\alpha$, 16ß-$^2$H4] deuterated estradiol (d$_4$ $E_2$) 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 $10^6$ cells.

## Statistical Analysis

The dose response experiments with $E_2$ and with LB were performed using $n = 3$ flasks per treatment group. The experiment for $E_2$ 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 ($\alpha = 0.05$) as a posttest where appropriate.

## RESULTS

### Effects of $E_2$ on Serum-Depleted MCF-7 Cells

To demonstrate persistent $E_2$ response, the effect of physiological levels of $E_2$ 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 $E_2$ dose-dependent increase in the viable cell number, ranging from 11% with 1 nM $E_2$ to 87% with 20 nM $E_2$, relative to that in cells maintained in medium supplemented with 0.7% serum, representing the controls.

The statistical significance of these data confirmed $E_2$ dose-dependent increases in the viable cell number as analyzed by the Dunnett's multiple comparison test ($\alpha = 0.05$).

TABLE 1
Growth promotion of serum-depleted human mammary carcinoma MCF-7 Cells by 17ß-estradiol ($E_2$)

| Treatment | Concentration (nM) | Viable Cell Number ($\times 10^5$)[a] | Increase (% Control) |
|---|---|---|---|
| Serum | 0.7% | $10.0 \pm 0.3^b$ | — |
| Serum + $E_2$ | 1 | $11.1 \pm 0.3^c$ | 11.0 |
| | 10 | $14.3 \pm 0.4^d$ | 43.0 |
| | 20 | $18.7 \pm 0.3^e$ | 87.0 |

[a]Determined from number of viable cells at 7-day postseeding of $1.0 \times 10^5$ cells.
$^{b-e}$Mean $\pm$ SD, $n = 3$ per treatment group.
$^{c>b,d>b,e>b}$Data analyzed by Dunnett's multiple comparison test ($\alpha = 0.05$).

### $E_2$ Metabolism in Serum-Depleted MCF-7 Cells

The cellular metabolism of $E_2$ plays a critical role in growth modulation of MCF-7 cells. This metabolic competence was evaluated by measuring the formation of $E_1$, 2-OHE$_1$, 16$\alpha$-OHE$_1$, and $E_3$. The data presented in Table 2 reveals that in cells incubated with 20 nM $E_2$ as the enzyme substrate, formation of $E_1$, 2-OHE 1, 16$\alpha$-OHE $_1$, and $E_3$ 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 $E_2$ 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 $E_2$ 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 $E_2$.

The treatment with 20 nM $E_2$ 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 $E_2$. 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 $E_2$ 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 $E_2$ served as the control.

For the statistical analysis of these data, a 2-way ANOVA with Dunnett's multiple comparison test ($\alpha = 0.05$) 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 17ß-estradiol (E$_2$) in serum-depleted human mammary carcinoma MCF-7 cells[a]

| Treatment | Concentration (nM) | E$_1$ | E$_2$ Metabolite[b] (ng/10$^6$ cells) | | |
| | | | 2-OHE$_1$ | 16$\alpha$-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 | $8.7 \pm 0.9^d$ | $1.9 \pm 0.3^f$ | $4.5 \pm 0.1$ | $0.45 \pm 0.07^h$ |

[a] Abbreviations are as follows: 2-OHE$_1$, 2-hydroxyestrone; 16$\alpha$-OHE1, 16$\alpha$-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).
[b] Determined 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 E$_2$ 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 E$_2$. The treatment of the cells with LB at the maximum cytostatic dose of 1.0%, induced about an 84.8% increase in E$_1$, a 3.6-fold increase in 2-OHE$_1$, and a 9.2-fold increase in E$_3$, whereas 16$\alpha$-OHE1 exhibited a modest 33.3% decrease relative to that observed in the control cultures treated with E$_2$alone.

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

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% | $2.4 \pm 0.2^e$ | $2.7 \pm 0.1^i$ |

[a] Untreated 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 cm$^2$.
[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 ($\alpha = 0.05$). For ANOVA, concentration $\times$ duration exhibited a significant interaction ($P < 0.001$).

## Modulation of E$_2$ Metabolism by LB

To evaluate the effect of LB on global metabolism of E$_2$ in serum-depleted MCF-7 cells, the primary data obtained for individual metabolites was analyzed as ratios between 2-OHE$_1$:16$\alpha$-OHE$_1$ and 16$\alpha$-OHE$_1$:E$_3$. 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-OHE$_1$:16$\alpha$-OHE$_1$ ratio and a 94% decrease in the 16$\alpha$-OHE$_1$:E$_3$ratio as analyzed by Dunnett's test correcting for multiple comparisons ($\alpha = 0.05$).

## 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 E$_2$ for growth in vitro and tumorigenecity in vivo (13). ER functions as a ligand-inducible transcription factor that uses E$_2$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 E$_2$. 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 ($E_2$) metabolism in human mammary carcinoma MCF-7 cells[a]

| Treatment | Concentration | $E_1$ | $E_2$ Metabolite[b] (ng/$10^6$ Cells) | | |
| --- | --- | --- | --- | --- | --- |
| | | | 2-OHE$_1$ | 16$\alpha$-OHE$_1$ | $E_3$ |
| $E_2$ | | $4.6 \pm 0.9$ | $1.9 \pm 0.3^c$ | $4.5 \pm 0.2^e$ | $0.45 \pm 0.09^g$ |
| $E_2$+ LB% | 0.1 | $6.8 \pm 2.2$ | $1.6 \pm 0.4$ | $4.3 \pm 0.4$ | $2.1 \pm 0.1^h$ |
| | 0.5 | $7.0 \pm 2.4$ | $1.8 \pm 0.9$ | $4.4 \pm 0.3$ | $6.5 \pm 1.0^i$ |
| | 1.0 | $8.5 \pm 0.4$ | $8.7 \pm 0.4^d$ | $3.0 \pm 0.3^f$ | $4.6 \pm 0.7^j$ |

[a]Abbreviations are as follows: 2-OHE$_1$, 2-hydroxyestrone; 16$\alpha$-OHE$_1$, 16$\alpha$-hydroxyestrone; GC-MS, gas chromatography-mass spectrometry.

[b]Determined at Day 3 postseeding by stable isotope dilution and GC-MS analysis.

[c−j]Mean $\pm$ SD, n = 3 per treatment group.

[c<d,e>f,g<h,g<i,g<j]Data analyzed by 1-way analysis of variance and Dunnett's multiple comparison test ($\alpha = 0.05$).

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 $E_2$. 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 $E_2$, possibly via ER-dependent mechanism. Several experimental approaches have been documented to examine the impact of $E_2$ 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 $E_2$ dependence or modulate the susceptibility of carcinoma cells to the selective estrogen receptor modulator tamoxifen. Similarly, in spontaneously im-

TABLE 5

Modulation of 17ß-estradiol ($E_2$) metabolism by *lycium barbarum* (LB) in serum-depleted human mammary carcinoma MCF-7 cellsa

| Treatment | Concentration | $E_2$ Metabolism[b] | |
| --- | --- | --- | --- |
| | | 2-OHE$_1$:16$\alpha$-OHE$_1$ | 16$\alpha$-OHE$_1$:$E_3$ |
| $E_2$ | 20 nM | $0.42 \pm 0.10^c$ | $10.0 \pm 0.5^e$ |
| $E_2$+ LB | (%) | | |
| | 0.1 | $0.37 \pm 0.10$ | $2.0 \pm 0.2^f$ |
| | 0.5 | $0.41 \pm 0.33$ | $0.7 \pm 0.3^g$ |
| | 1.0 | $2.90 \pm 0.75^d$ | $0.6 \pm 0.4^h$ |

[a]Abbreviations are as follows: 2-OHE$_1$, 2-hydroxyestrone; 16$\alpha$-OHE$_1$, 16$\alpha$-hydroxyestrone.

[b]Determined from the concentrations of individual metabolites.

[c−h]Mean $\pm$ SD, $n = 3$ per treatment group.

[c<d,e>f,e>g,e>h]Data analyzed by Dunnett's multiple comparison test ($\alpha = 0.05$).

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 $E_2$ suggest that serum-depleted MCF-7 cells may represent a facile, specific, and sensitive approach to evaluate experimental modulations of $E_2$ responses. Specifically, transient downregulation of functional ER by limiting the availability of $E_2$ via serum depletion provides a facile isogenic approach to examine the specific effects of herbal medicinal products on biochemical and cellular effects of $E_2$ on cellular phenotypes with transiently functional or nonfunctional ER.

In addition to the ER-dependent growth responses, cellular metabolism of $E_2$ via C17-oxidation, C2-hydroxylation, and C16$\alpha$-hydroxylation pathways generate $E_1$, 2-OHE$_1$, 16$\alpha$-OHE$_1$, and $E_3$. $E_1$ functions as a common precursor for the formation of 2-OHE$_1$ or 16$\alpha$-OHE$_1$ via the 2-hydroxylation or 16$\alpha$-hydorxylation pathways, respectively, and $E_3$ represents the proximate metabolite of 16$\alpha$-OHE$_1$ (15,37,38). These intermediate metabolites have distinct growth regulatory effects on MCF-7 cells. Thus, 2-OHE$_1$ and $E_3$ exhibit antimitogenic effects, whereas 16$\alpha$-OHE$_1$ 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 $E_2$ exhibited increases in $E_1$, 2-OHE$_1$, 16$\alpha$-OHE$_1$, and $E_3$ formation. The positive growth regulation with $E_2$ and a persistent competence for cellular metabolism of $E_2$, taken together, represent a major refinement in the MCF-7 cell culture model for ER$^+$ breast cancer.

The alteration in the cellular metabolism of $E_2$ represents one of the mechanisms for growth inhibition in murine or human breast cancer cells (14–16). Upregulation of the C2-hydroxylation:C16$\alpha$-hydroxylation ratio, predominantly due to increased formation of 2-OHE$_1$, has been noted in response to the treatment with several synthetic chemopreventive

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-OHE$_1$ 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-OHE$_1$ increased, that of promitogenic 16$\alpha$-OHE$_1$ remained essentially unaltered, whereas that of $E_3$ increased. The data on the status of the 16$\alpha$-hydroxylation pathway suggest that LB may accelerate the conversion of 16$\alpha$-OHE$_1$.

Global metabolism of $E_2$ is also known to produce other catechol estrogens such as 4-OHE$_1$, 2-OHE$_2$, 4-OHE$_2$, as well as their proximate methoxy derivatives. Amongst these catechol estrogens, 4-OHE$_1$ and 4-OHE$_2$ 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-OHE$_1$:16$\alpha$-OHE$_1$ ratio was decreased, whereas 4OHE$_2$:2-OHE$_2$ ratio was increased (23), suggesting that 16$\alpha$-OHE$_1$ and 4-OHE$_2$ might be procarcinogenic, whereas 2-OHE$_1$ and 2-OHE$_2$ might be anticarcinogenic. In our experiments, however, the 4-OHE$_2$ and 2-OHE$_2$ metabolites were undetectable (data not shown). It is to be noted that in response to LB treatment, the 2-OHE$_1$:16$\alpha$-OHE$_1$ ratio increased due to an increase in 2-OHE$_1$ formation, whereas the 16$\alpha$-OHE$_1$:E$_3$ ratio decreased due to increased $E_3$ formation. In accord, our previous studies have demonstrated distinct antimitogenic effects of 2-OHE$_1$ and $E_3$ and promitogenic effects of 16$\alpha$-OHE$_1$ 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$\alpha$-OHE$_1$ 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-OHE$_1$ and/or to accelerate

the conversion of promitogenic 16$\alpha$-OHE$_1$ 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.

## REFERENCES

1. Jemal AR, Siegel R, Ward E, Murray T, Xu J, and Thun MJ: Cancer Statistics, 2007. *CA Cancer J Clin* **57,** 43–66, 2007.
2. Surh YJ: Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* **3,** 768–780, 2003.
3. Early Breast Cancer Trialists Group (EBCTCG): Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15 years survival: an overview of the randomized trials. *Lancet* **365,** 1687–1717, 2005.
4. Lippman ME: Efforts to combine endocrine and chemotherapy in the management of breast cancer: Do two and two equals three? *Breast Cancer Res Treat* **3,** 117–127, 1983.
5. Tindle HA, Davis RB, Phillips RS, and Eisenberg DM: Trends in the use of complementary and alternative medicine by US adults: 1997–2002. *Altern Ther Health Med* **11,** 42–49, 2005.
6. Molassiotis A, Scott JA, Kearny N, and Kearney N: Complementary and alternative medicine use in breast cancer patients in Europe. *Support Care Cancer* **14,** 206–267, 2006.
7. Lu CX and Cheng BQ: Radiosensitizing effects of Lycium barbarum polysaccharides for Lewis lung cancer. Zhong. Xi. Yi. Jie. He. Za. Zhi. **11,** 611, 1991. [PMID: 1806310, PubMed-Indexed for MEDLINE].
8. Cao GW, Yang WG, and Du P: Observations of the effects of LAK/IL-2 therapy combining with Lycium barbarum polysaccharides in treatment of 75 cancer patients. Zhonghua. Zong. Liu. Za. Zhi. **16,** 428–431, 1994. [PMID: 7720497, PubMed-Indexed for MEDLINE].
9. Zhang M, Chen H, Huang J, Li Z, Zhu C, and Zang S: Effect of Lycium barbarum polysaccharides on human hepatoma QGY 7703 cells: inhibition of proliferation and induction of apoptosis. *Life Sci* **18,** 2115–2124, 2005.
10. Liu XL, Sun JY, Li HY, Zhang L, and Qian B: Extraction and isolation of active component for inhibiting PC-3 cell proliferation in vitro from the fruit of Lycium barbarum L. Zhongguo Zhong Yao Za Zhi **25,** 481–483, 2000. [PMID: 12515210, PubMed-Indexed for MEDLINE].
11. Gau L, Hua, ZS, LIang, YX, and Bi XH: Immunomodualtion and anti-tumor activity by a polysaccharide-protein complex from Lycium barbarum. *Int Immunopharmacol* **4,** 563–569, 2004.
12. Gong H, Shen P, Jin L, Xing C, and Tang F: Therapeutic effects of Lycium barbarum polysaccharide on irradiation or chemotherapy-induced myelo-suppressive mice. *Cancer Biother Radiopharm* **20,** 155–162, 2005.
13. Lippman ME, Osborne CK, Knazek R, and Young N: In vitro model systems for the study of hormone dependent human breast cancer. *N Engl J Med* **296,** 154–159, 1977.
14. Suto A, Bradlow HL, Wong GY, Osborne MP, and Telang NT: Persistent estrogen responsiveness of ras oncogene transformed mouse mammary epithelial cells. *Steroids* **57,** 262–268, 1992.

15. Fishman J, Osborne MP, and Telang NT: The role of estrogens in mammary carcinogenesis. *Ann NY Acad Sci* **768,** 91–100, 1995.

16. Telang NT: Oncogenes, estradiol metabolism and mammary carcinogenesis. *Ann NY Acad Sci* **784,** 277–287, 1996.

17. Suto A, Bradlow HL, Kubota T, Kitajima M, Wong GY, Osborne MP, and Telang NT: Alteration in proliferative and endocrine responsiveness of human mammary carcinoma cells by prototypic tumor suppressing agents. *Steroids* **58,** 215–219, 1993.

18. Tiwari RK, Guo L, Bradlow HL, Telang NT, and Osborne MP: Selective responsiveness of human breast cancer cells to indole-3-carbinol, a chemopreventive agent. *J Natl Cancer Inst* **86,** 126–131, 1994.

19. Telang NT, Katdare M, Bradlow HL, and Osborne MP: Cell cycle regulation, apoptosis and estradiol metabolism: novel end point biomarkers for human breast cancer prevention. *J Clin Ligand Assay* **23,** 130–137, 2000.

20. Telang NT, Suto A, Wong GY, Osborne MP, and Bradlow HL: Induction by estrogen metabolite 16$\alpha$-hydroxyestrone of gentoxic damage and aberrant proliferation in mouse mammary epithelial cells. *J Natl Cancer Inst* **84,** 634–638, 1992.

21. Schneider J, Huh MM, Bradlow HL, and Fishman J: Antiestrogen action of 2-hydroxyestrone on MCF-7 human breast carcinoma cells. *J Biol Chem* **259,** 4840–4845, 1984.

22. Lottering ML, Haag M, and Seegers JC: Effects of 17ß-estradiol metabolites on cell cycle events in MCF-7 cells. *Cancer Res* **52,** 5926–5932, 1992.

23. Castagnetta LM, Granata OM, Cocciadiferro L, et al.: Sex steroids, carcinogenesis and cancer progression. *Ann NY Acad Sci* **1028,** 233–246, 2004.

24. Sepkovic DW, Bradlow HL, Michnovcz J, Murtezani S, Levy I, and Osborne MP: Catechol estrogen production in rat microsomes after treatment with Indole-3-Carbinol, Ascorbigen, or ß-napthaflavone: a comparison of stable isotope dilution gas chromatography-mass spectrometry and radiometric methods. *Steroids* **59,** 318–323, 1994.

25. Dehennin L, Reiffsteck A, and Scholler R: Simple methods for the synthesis of twenty different highly enriched deuterium labeled steroids suitable as internal standards for isotope dilution mass spectrometry. *Biochem Mass Spectrometry* **7,** 493–499, 1980.

26. Raju U, Sepkovic DW, Dixon, JM, Bradlow HL, and Levitz M: Estrone and estradiol metabolism in vivo in human breast cysts. *Steroids* **65,** 883–888, 2000.

27. Jellnick PH, Makin HLJ, Sepkovic DW and Bradlow HL: Influence of indole carbinols and growth hormone on the metabolism of 4-androstenedione by rat liver mocrosomes. *J Steroid Biochem Mol Biol* **46,** 791–798, 1993.

28. Fisher B, Constantino JP, Wickerman DL, Redmond CK, Kavanah M, et al.: Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 study. *J Natl Cancer Inst* **90,** 1371–1388, 1998.

29. Jordan VC: Historical perspective on hormonal therapy of advanced breast cancer. *Clin Ther* **24**(**A**Suppl), A3–A16, 2002.

30. Yager JD and Davidson NE: Estrogen carcinogenesis in breast cancer. *N Engl J Med* **354,** 270–282, 2006.

31. Kasid A, Lippman ME, Papageorge AG, Lowy Dw, and Gelman EP: Transfection of v-ras[H] DNA in to MCF-7 cells bypasses their dependence on estrogen for tumorigenicity. *Science* **228,** 725–728, 1985.

32. Sommers CL, Papageorge AL, Wilding G, and Gelman EP: Growth properties and tumorigenesis of MCF-7 cells transfected with isogenic mutants of ras[H]. *Cancer Res* **50,** 67–71, 1990.

33. Kurukava H, Lenferink AE, Simpson JE, Pisakane PI, Sliwkowski MX, et al.: Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER-2 overexpressing, tamoxifen-resistant breast cancer cells. *Cancer Res* **60,** 5887–5894, 2000.

34. Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathi D, et al.: Esrtrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res Treat* **24,** 85–95, 1992.

35. Telang NT, Narayanan R, Bradlow HL, and Osborne MP: Co-ordinated expression of intermediate biomarkers for tumorigenic transformation of RAS-transfected mouse mammary epithelial cells. *Breast Cancer Res Treat* **18,** 155–163, 1991.

36. Telang NT, Osborne MP, Sweterlisch L, and Narayanan R: Neoplastic transformation of mouse mammary epithelial cells by deregulated myc expression. *Cell Reg* **1,** 863–872, 1990.

37. Schneider J, Kinne D, Frachhia A, Pierce V, Anderson KE, et al.: Abnormal oxidative metabolism of 17ß-estradiol in women with breast cancer. *Proc Natl Acad Sci USA* **79,** 3047–3051, 1982.

38. Fishman J, Schneider J, Herschkopf RJ, and Bradlow HL: Increased estrogen 16$\alpha$-hydroxylase activity in women with breast and endometrial cancer. *J Steroid Biochem Molec Biol* **20,** 1077–1081, 1984.

39. Suto A, Telang NT, Tanino H, Takeshita T, Ohmiya H, et al.: In vitro and in vivo modulation of growth regulation in the human breast cancer cell line MCF-7 by estradiol metabolites. *Breast Cancer* **6,** 87–92, 1999.