Preventive Efficacy of the Chinese Nutritional Herb Epimedium grandiflorum in a Preclinical Cell Culture Model for Luminal A Molecular Subtype of Breast Cancer

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Abstract

**Background:** The Luminal A molecular subtype of clinical breast cancer expresses estrogen receptor (ER) and progesterone receptor (PR), but lacks the expression of HER-2 oncogene. This cancer subtype responds to endocrine therapy involving the use of selective estrogen receptor modulators or inhibitors of estrogen biosynthesis. Conventional therapeutic options are frequently associated with acquired tumor resistance and systemic toxicity, and therefore, emphasize a need for identification of promising new non-toxic agents for secondary prevention. Nutritional substances that do not incur long-term toxicity represent ideal candidates. Nutritional herbs have been used in traditional Chinese medicine for a variety of health related issues, including prevention of breast cancer.

**Methods:** The present study utilized the human mammary carcinoma derived ER⁺/PR⁺/HER-2⁻ MCF-7 cells as a model for the Luminal A breast cancer to examine the preventive efficacy of non-fractionated aqueous extract from Epimedium grandiflorum (EG), a popular Chinese nutritional herb. Anchorage dependent growth, cell cycle progression, cellular metabolism of 17β-estradiol (E₂), and anchorage independent colony formation represented the quantitative end point biomarkers for preventive efficacy.

**Results:** Maintenance of MCF-7 cells in a chemically defined serum depleted culture medium (serum 0.7%, E₂<1 nM) retained their cellular growth promoting response to the physiologically relevant concentration range of 1 nM to 20 nM E₂, exhibiting a 10.3% to a 91.9% increase in the viable cell number, respectively. A 7 day treatment to MCF-7 cells with EG resulted in a dose dependent inhibition of E₂ promoted growth (EG IC₅₀: 0.49%). At its maximum cytostatic concentration, EG inhibited cell cycle progression via G₁ arrest, resulting in a 1.6 fold increase in the G₁:S+G₂/M ratio, and modulated the cellular metabolism of E₂ in favor of formation of anti-proliferative metabolites 2-hydroxyestrone (2-OHE₁) and estriol (E₃), exhibiting a 5.1 and a 7.6 fold increase, respectively. In addition, EG produced a favorable 3.9 fold increase in the 2-OHE₁: 16α-OHE₁ ratio, an endocrine biomarker of breast cancer risk. A 21 day treatment of MCF-7 cells with EG produced a dose dependent inhibition in anchorage independent growth (EG IC₅₀: 0.49%; IC₉₀: 1.03%).

**Conclusions:** These data demonstrate the growth inhibitory effects of EG and identify clinically relevant mechanistic leads for its preventive efficacy. The present approach promises to facilitate identification of new efficacious herbs for the secondary prevention of the Luminal A subtype of clinical breast cancer.
Study Rationale I

• Global gene profiling of differentially expressed genes in clinical breast cancer provides a refined molecular classification of cancer subtypes (1).

• Tumors expressing estrogen receptor (ER) and progesterone receptor (PR) but lacking the expression of human epidermal growth factor receptor-2 (HER-2) are classified as the Luminal A subtype (2).

• The human carcinoma derived MCF-7 cells are ER+, PR+ and HER-2- and therefore, represent a cell culture model for the Luminal A molecular subtype of clinical breast cancer.
• Luminal A breast cancer represents an endocrine therapy responsive cancer subtype (1, 2).

• This subtype of clinical breast cancer responds to conventional systemic therapy comprising of selective estrogen receptor modulators or aromatase inhibitors with or without cytotoxic chemotherapy (3, 4).

• Conventional chemo-endocrine therapy is associated with acquired tumor resistance and long-term systemic toxicity (4).
Study Rationale III

• Nutritional herbs have been widely used in Chinese medicine for a variety of health conditions, including cancer in women (5-8).

• Naturally occurring phytochemicals and herbal products exhibit little systemic toxicity but interact with conventional chemo-endocrine therapy to enhance their efficacy and reduce toxicity (9).

• Epimedium grandiflorum is a nutritional Chinese herb with no documented toxicity, and may therefore be suitable for long-term prevention of breast cancer
Experimental Model

- The estrogen dependent ER\(^+\) MCF-7 cell line represents a well-established cell culture model for hormone responsive clinical breast cancer (10).

- MCF-7 cells adapted to grow in a chemically defined, serum depleted culture medium retain their responsiveness to 17\(\beta\)-estradiol (E\(_2\)), and exhibit a positive growth inhibitory response to several mechanistically distinct herbal extracts (11-16).
Preparation of Non-fractionated Extract from Epimedium grandiflorum (EG)

- **Extract # 1**: 20 g of EG boiled in 200 ml of distilled water to reduce the volume to 100 ml.
- **Extract # 2**: Residue from extract # 1 boiled in 100 ml of distilled water to reduce the volume to 50 ml.
- Extract # 1 and Extract # 2 combined (Total volume 150 ml) and boiled to reduce the volume to 25 ml.
- The combined extracts centrifuged (5,000 rpm at room temp.) to collect the supernatant (20 ml). This aqueous supernatant served as the stock solution (100%).
- The stock solution diluted with the culture medium to obtain 2%, 1%, 0.5%, 0.05%, 0.02% and 0.01% concentrations of EG.
- The diluted working solutions used to determine the IC$_{50}$ and maximum cytostatic concentrations of EG on MCF-7 cells.
Mechanistic End point Biomarkers

- Cytostatic growth arrest (number of viable cells)
- Cell cycle progression ($G_1$: S+$G_2$/M ratio)
- Cellular metabolism of $17\beta$-estradiol (2-OHE$_1$: 16α-OHE$_1$ and $E_3$: 16α-OHE$_1$ ratios)
- Anchorage independent growth (number of anchorage independent colonies)
**Figure 1**

Dose Response of 17β-estradiol ($E_2$) on human mammary carcinoma derived MCF-7 Cells

<table>
<thead>
<tr>
<th>Initial seeding Density</th>
<th>Serum 0.7%</th>
<th>1 nM</th>
<th>Serum 0.7% + $E_2$</th>
<th>5 nM</th>
<th>10 nM</th>
<th>20 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E$_2$&lt;1 nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 1**
Growth promoting effects of 17β-estradiol (E₂) on human mammary carcinoma derived MCF-7 cells

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>0.7% serum (&lt;1 nM E₂)</th>
<th>0.7% serum+ 20 nM E₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population doubling (hr.) a</td>
<td>33.0±1.9</td>
<td>27.2±1.6</td>
</tr>
<tr>
<td>Saturation Density (x10⁵) b</td>
<td>13.6±4.5</td>
<td>26.1±4.7</td>
</tr>
<tr>
<td>Anchorage independent (AI) Colonies c</td>
<td>16.5±1.5</td>
<td>37.2±2.1</td>
</tr>
</tbody>
</table>

a determined from exponential growth phase. b determined at day 7 post-seeding of 1.0x10⁵ cells. c determined at day 21 post-seeding of 1000 cells.
Figure 2
Dose response of Epimedium grandiflorum (EG) on human mammary carcinoma MCF-7 cells

Viable Cell Number (x10^5)

EG IC_{50}: 0.49%; EG IC_{90}: 1.03%
### Table 2
Inhibition of cell cycle progression by Epimedium grandiflorum (EG) in human mammary carcinoma MCF-7 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>% G&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;a, b&lt;/sup&gt;</th>
<th>% S+G&lt;sub&gt;2/M&lt;/sub&gt; &lt;sup&gt;a, b&lt;/sup&gt;</th>
<th>G&lt;sub&gt;1&lt;/sub&gt;: S+G&lt;sub&gt;2/M&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.7%</td>
<td>82.3±8.2</td>
<td>17.8±1.8</td>
<td>6.0±0.6</td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>20 nM</td>
<td>67.3±6.7</td>
<td>32.5±3.2</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;+EG</td>
<td>20 nM+1.0%</td>
<td>78.6±7.8</td>
<td>21.4±3.8</td>
<td>3.7±0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> determined from flow cytometry based cell cycle analysis.

<sup>b</sup> Mean ± SD, N=3 per treatment group.
Study Rationale IV

- Oxidative metabolism of 17β-estradiol (E₂) impacts on carcinogenesis of endocrine responsive target organs (17-19).

- E₂ metabolites exhibit pleotropic growth modulatory effects on breast carcinoma derived cells (16, 19-21).

- Altered E₂ metabolism provides an endocrine biomarker for carcinogenic risk and for effective prevention/therapy of breast cancer(14).
Cellular Metabolism of 17β-estradiol (E₂)

Cells from control and treatment groups cultured for 48hr. Culture medium processed for GC-MS analyses for detection of select E₂ metabolites. Data expressed as individual metabolites (ng/10⁶ cells), and as E₂ metabolite ratio.
Table 3
Modulation of estradiol metabolism by Epimedium grandiflorum (EG) in human mammary carcinoma MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>E₂ Metabolite (ng/ 10⁶ cells) b</th>
<th>a, b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E₁</td>
<td>2-OHE₁</td>
</tr>
<tr>
<td>E₂</td>
<td>20 nM</td>
<td>2.7±0.1</td>
<td>0.7±0.3</td>
</tr>
<tr>
<td>E₂+EG</td>
<td>20 nM+1.0%</td>
<td>11.7±0.6</td>
<td>4.2±0.3</td>
</tr>
</tbody>
</table>

a determined from GC-MS based metabolite analysis of the spent culture medium.
b Mean ± SD, N=3 per treatment group.
Figure 3
Up-regulation of estradiol metabolite ratio by Epimedium grandiflorum (EG) in human mammary carcinoma MCF-7 cells

2-OHE$_1$: 16α-OHE$_1$ Ratio

<table>
<thead>
<tr>
<th></th>
<th>20 nM</th>
<th>20 nM+1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_2$</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>$E_2$+EG</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

$E_3$: 16α-OHE$_1$ Ratio

<table>
<thead>
<tr>
<th></th>
<th>20 nM</th>
<th>20 nM+1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_2$</td>
<td>0.15</td>
<td>0.6</td>
</tr>
<tr>
<td>$E_2$+EG</td>
<td>0.3</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Figure 4
Effect of Epimedium grandiflorum (EG) on Anchorage independent growth in human mammary carcinoma MCF-7 cells

Number of Anchorage independent (AI) Colonies

<table>
<thead>
<tr>
<th>Serum</th>
<th>$E_2$</th>
<th>$E_2 + $EG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7% ($E_2 &lt; 1$ nM)</td>
<td>20 nM</td>
<td>20 nM + 0.5%</td>
</tr>
</tbody>
</table>
Study Outcome I

Experimental Model

- MCF-7 cells maintained in a serum depleted culture medium exhibit progressive growth in response to physiological levels of $E_2$.

- MCF-7 cells exhibit decreased population doubling, increased saturation density and increased anchorage independent growth in response to $E_2$. 
Study Outcome II

Response to Epimedium granflorum (EG)

- Increased number of cells in the $G_1$ phase with corresponding decrease in the number of cells in the $S + G_2/M$ phase of the cell cycle, relative to the $E_2$ treated control.

- Increased formation of anti-proliferative $E_2$ metabolites $2\text{-}OHE_1$ and $E_3$.

- Decreased number of $E_2$ promoted anchorage independent colonies.
Conclusions

• Cell culture model for the Luminal A subtype of clinical breast cancer retains responsiveness to estradiol.

• The Chinese nutritional herb Epimedium grandiflorum (EG) exhibits anti-proliferative effects via inhibition of cell cycle progression and modulation of cellular metabolism of estradiol.

• The present data suggest that as a nutritional herb, EG represents a promising agent for long-term prevention of breast cancer.

• The present approach may facilitate identification of efficacious herbs targeted towards the prevention or treatment for Luminal A subtype of clinical breast cancer.
Acknowledgements and Dedication

Major funding for this study was provided by philanthropic contributions to the American Foundation for Chinese Medicine by

- Peter Cheney
- Suzanne Hoyt
- The family of Hakan and Marie Ledin
- The family of Daniel and Kathleen Mezzalingua
- The Issac and Laura Perlmutter Fund
- The Social Visionary Foundation

This study is dedicated to the memory of Laurie Mezzalingua (1968-2009). Laurie fought gallantly against her breast cancer from 1993 to July 4, 2009. During that period she also selflessly and generously devoted herself to helping many others suffering from breast cancer.
References