Phytoestrogens in Common Herbs Regulate Prostate Cancer Cell Growth in Vitro

Nader S. Shenouda, Christine Zhou, Jimmy D. Browning, Pete J. Ansell, Mary S. Sakla, Dennis B. Lubahn, and Ruth S. MacDonald

Abstract: Prostate cancer is an important public health problem in the United States. Seven phytoestrogens found in common herbal products were screened for estrogen receptor binding and growth inhibition of androgen-insensitive (PC-3) and androgen-sensitive (LNCaP) human prostate tumor cells. In a competitive ^H-estradiol ligand binding assay using mouse uterine cytosol, 2.5 μM quercetin, baicalein, genistein, epigallocatechin gallate (EGCG), and curcumin displaced > 85% of estradiol binding, whereas apigenin and resveratrol displaced > 40%. From growth inhibition studies in LNCaP cells, apigenin and curcumin were the most potent inhibitors of cell growth, and EGCG and baicalein were the least potent. In PC-3 cells, curcumin was the most potent inhibitor of cell growth, and EGCG was the least potent. In both cell lines, significant arrest of the cell cycle in S phase was induced by resveratrol and EGCG and in G2/M phase by quercetin, baicalein, apigenin, genistein, and curcumin. Induction of apoptosis was induced by all of the 7 compounds in the 2 cell lines as shown by TUNEL and DNA fragmentation assays. Androgen responsiveness of the cell lines did not correlate with cellular response to the phytoestrogens. In conclusion, these 7 phytoestrogens, through different mechanisms, are effective inhibitors of prostate tumor cell growth.

Introduction

Prostate cancer is an important public health problem accounting for more than 192,000 estimated new cases and 38,000 deaths in the United States in 2003. Prostate cancer is an excellent candidate disease for chemoprevention because it is typically slow-growing and is usually diagnosed in elderly males; therefore, even a modest delay in the neoplastic development achieved through pharmacological or nutritional intervention could result in a substantial reduction in the incidence of mortality (1).

Estrogen therapy, principally the use of diethylstilbestrol (DES), is a suggested practice in prostate cancer treatment (2,3). The primary mode of action for estrogens is through feedback on the anterior pituitary with suppression of gonadotropin secretion and subsequent decrease in testosterone production by leydig cells of the testes. This causes a decrease in androgen required for growth and proliferation of hormone-dependent tumor cells. However, direct effects of DES through prostate estrogen receptors (ERs) are also possible. Whether DES works through a classical ERα pathway, the ERβ pathway, or another ER-independent mechanism has not been fully determined.

The incidence of prostate cancer is lower in Asian populations and in vegetarians compared with populations consuming a more Western diet (4). Components of the Western diet most often cited as playing a role in increased prostate cancer risk are animal products (5). The most consistent correlation for prostate cancer prevention is consumption of fruits, vegetables, and grains, which are potential sources of phytoestrogens (6). Phytoestrogens are also found in many plants, which are commonly used in traditional medicine. Phytoestrogens may be either agonists or antagonists of ERs. Therefore, it has been proposed that phytoestrogens may influence prostate cancer cell growth, and herbal therapies have been developed that include these compounds (7). It is possible that phytoestrogens affect prostate cancer through similar mechanisms as DES. When male rats were fed diets containing genistein from conception, downregulation of androgen and ERs was observed in dorsolateral prostate in a dose-dependent manner (8). Similarly, male Sprague-Dawley rats fed diets containing 600 ppm isoflavones for 20 days had lower circulating levels of testosterone and smaller prostates than rats fed an isoflavone-free diet (9).

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In vivo and in vitro studies have shown that phytoestrogens may influence not only steroid hormone metabolism and biological activity, but also intracellular enzymes, growth factor action, protein synthesis, malignant cell proliferation, and angiogenesis, thus making them strong candidates as anticarcinogenic agents (10).

Quercetin, baicalein, apigenin, genistein, resveratrol, curcumin, and epigallocatechin gallate (EGCG) are biologically active plant compounds found in foods that possess estrogenic and antiestrogenic actions. Each of these compounds has been found to provide protection against cancer in a variety of animal models (5). Evidence suggests that some of these compounds influence not only steroid hormone metabolism, but also intracellular signaling cascades that regulate cell proliferation and apoptosis. Each compound has specific effects on these pathways; some may overlap and, therefore, generate a synergistic response.

The goal of our work was to study the effects of 7 commonly used herbal phytoestrogens on the growth and proliferation of both androgen-sensitive (LNCaP) and androgen-insensitive (PC-3) human prostate cancer cell lines. In addition, we evaluated potential mechanisms of action of these phytoestrogens at the cellular level, including cell cycle kinetics, the induction of programmed cell death, and the binding capacity of these phytoestrogens to ERs.

Materials and Methods

Quercetin, resveratrol, curcumin, and EGCG (>95% purity) were obtained from Sigma Chemical Co. (St. Louis, MO). Baicalein and apigenin (>97% purity) were obtained from Indofine Chemical Co. (Somerville, NJ). Genistein was obtained from LC Laboratories (Wobom, MA). These pure phytoestrogens were dissolved in DMSO and stored as 100 mM stocks at -20°C. The human reactive polyclonal antibodies for ERα (PAI-308) and ERβ (PAI-312) were purchased from Affinity Bio Reagents (Golden, CO). The unlabeled 17β estradiol and ICI 182,780 were obtained from Sigma Chemical Co. 3H-17β estradiol was obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture Experiments

Human prostate carcinoma (PC-3 and LNCaP) cells were obtained from ATCC (Manassas, VA) and cultured in complete RPMI 1640 medium (Gibco Rockville, MD) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (5 mM), NEAA (0.1 mM Non Essential Amino Acids), HEPES buffer (10 mM), and glucose (2.5 gm/L) in 5% CO2 at 37°C.

Effect of Dihydrotestosterone on Growth and Proliferation

The PC-3 and LNCaP cells were plated at 1 x 10^6 cells in 6-well culture dishes in RPMI 1640 media containing charcoal-stripped serum (10%), and cells were grown to 50% confluence. The culture media were replaced with media containing 1 or 10 nM dihydrotestosterone (DHT) and incubated for 5 days. The media were aspirated, and the cells were washed with phosphate buffered saline (PBS; 10 mM, pH 7.4), then NaOH (1 ml) was added to lyse the cells. The total cellular protein concentration was determined by the DC Bio-Rad assay.

Growth Inhibition Curve and IC50

PC-3 and LNCaP cells were plated at 1 x 10^4 cells per well in 24-well dishes with RPMI 1640 complete culture medium, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete media containing 0-150 µM of each phytoestrogen and incubated for 72 h. The media were aspirated, the cells were washed twice with PBS (10 mM, pH 7.4), and then 1 N NaOH (250 µl) was added to lyse the cells. The total cellular protein concentration was determined by DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). The IC50 (the concentration of the compound that is required to inhibit the tumor cell growth by 50%) for each phytoestrogen was calculated based on the growth inhibition after 72 h compared with the control.

Time Course Study

PC-3 and LNCaP cells were plated at 1 x 10^4 cells per well in 24-well dishes with RPMI 1640 complete culture medium, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete media containing the IC50 concentration of each phytoestrogen and incubated for 24, 48, or 72 h. Total cellular protein concentration was determined as a marker of cell protein by DC Bio-Rad assay.

Cell Cycle Kinetics

PC-3 and LNCaP cells were plated at 1 x 10^4 cells per well in 24-well dishes with RPMI 1640 complete culture medium, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete media containing the IC50 concentration of each phytoestrogen and incubated for 72 h. The cells were released with trypsin, washed twice with PBS, and centrifuged. The cell pellet was resuspended in 2.5 ml of cold ethanol (95%) for 1 h at 4°C, washed with PBS, and incubated with 5 µl RNase (20 µg/ml final concentration) at 37°C for 30 min. The cells were chilled over ice for 10 min, then stained with propidium iodide (50 µg/ml final concentration) for 1 h and analyzed by flow cytometry (CyAn Flow, Cytomation, Inc, Somerville, NJ).

Detection of Apoptosis

1-TUNEL assay: The PC-3 and LNCaP cells were plated at 1 x 10^6 cells in 100-mm culture dishes in RPMI 1640 complete media, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete...
media containing the IC₅₀ concentration of each phytoestrogen and incubated for 72 h. The adherent cells were released with trypsin, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by the use of an APO-Direct Apoptosis Kit obtained from Phoenix Flow Systems (San Diego, CA). The labeled cells were then analyzed by flow cytometry.

**2-DNA fragmentation assay:** The PC-3 and LNCaP cells were plated at 1 × 10⁶ cells in 100-mm culture dishes in RPMI 1640 complete media, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete media containing the IC₅₀ concentration of each phytoestrogen and incubated for 5 days. The cells were then washed twice with PBS (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, and 0.5% Triton X-100), chilled on ice for 15 min, and pelleted by centrifugation (14,000 g) at 4°C. The pellet was incubated with DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM NaCl, 1 mM EDTA, and 1% Triton X-100), proteinase K (0.1 mg/ml), and SDS 10% overnight at 37°C. The DNA was extracted using phenol/chloroform (1:1) and precipitated with 95% ethanol and 3M Na acetate, pH 7, for 2 h at -20°C. The DNA precipitate was centrifuged for 15 min, and the cells were washed with PBS (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA), and the pellet was air dried, then dissolved in 30–100 µl of DNA lysis buffer (10 mM Tris, pH 8.0, and 1 mM EDTA) and 3–5 µl of RNase One Ribonuclease (Promega, Madison, WI). The DNA was resolved over 1.8% agarose gels, containing 0.3 µg/ml ethidium bromide in 1× TBE buffer (pH 8.3, 89 mM Tris, 89 mM boric acid, and 2 mM EDTA). The bands were visualized under UV transilluminator (Model # TM-36, UVP Inc., San Gabriel, CA) followed by Polaroid photography (MP-4 photographic system, Fotodyne Inc., Hartland, WI).

**Effect of E2 (17β estradiol) and ICI 182,780 on Growth and Proliferation**

The PC-3 and LNCaP cells were plated at 1 × 10⁶ cells in 6-well culture dishes in RPMI 1640 media containing charcoal-stripped serum (10%), and cells were grown to 50% confluence. The culture media was replaced with media containing 1 or 10 nM 17β estradiol (E2) with or without 1 µM ICI 182,780 and incubated for 5 days. The media were aspirated, and the cells were washed with PBS (10 mM, pH 7.4); then NaOH (1 ml) was added to lyse the cells. The total cellular protein concentration was determined by the DC Bio-Rad assay.

**Binding Assays**

**Tissue Cytosol Preparation**

Female adult mice from our colony were euthanized and uteri immediately removed, stripped of adhering fat, wrapped in aluminum foil, quickly immersed in liquid nitrogen, and stored at -80°C. Tissues were homogenized (1 gm/10 ml) in TEG buffer (10 mM Tris-HCL, 1.5 mM EDTA, 10% glycerol, 3 mM sodium azide, pH 7.4) on ice, employing a Tissue Tearor (Biospec Products Inc., Racine, WI). The homogenized solution was centrifuged at 10,000 g for 15 min at 4°C and the supernatant centrifuged at 300,000 g for 2 h at 4°C. Resulting cytosol samples were stored at -80°C. Protein concentration (15 µg/µl) of cytosol extracts was determined using Total Protein diagnostic kit with bovine serum albumin (BSA) as the standard (Sigma Chemical Co.).

**Saturation Binding Analysis**

Uterine cytosol was incubated overnight at 4°C with 5 nM 3H-estradiol and 2.5 µM of each of the 7 phytoestrogens. Bound and free ligands were separated by dextran-coated charcoal, and an aliquot of bound radioactivity was measured by scintillation counting.

**Western Blotting for ER Detection**

PC-3 and LNCaP cells were grown in RPMI1640 complete media in 100-mm culture dishes to about 90% confluence. The media were aspirated, cells washed with cold PBS, then lysed in ice-cold buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 100 mM Na3VO4, 0.5% NP-40, 1% Triton X-100, 100 mM PMSF [pH 7.4]) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) on ice for 30 min. The cells were scraped, collected in microfuge tubes, and sonicated for 30 s. The cell lysate was cleared by centrifugation at 14,000 g for 15 min at 4°C, and the supernatant (total cell lysate) was used or immediately stored at -80°C. The total cellular protein concentration was determined by the DC Bio-Rad assay. For Western blotting, 20 µg protein was resolved over 8–12% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature, then with appropriate rabbit polyclonal primary antibody (1/1,000) overnight at 4°C, followed by incubation with anti-rabbit secondary antibody (1/10,000) horseradish peroxidase conjugate (Amersham Life Science Inc., Arlington Height, IL). Bands (64 KDa for ERα and 55 KDa for ERβ) were detected by chemiluminescence and autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY).

**Statistical Analysis**

Data in Tables 3 and 4 and in Figs. 1 and 4 were analyzed by one-way analysis of variance using the GLM procedure of SAS (Version 8, SAS Institute, Cary, NC). Post-hoc mean comparisons were made using the Duncan’s Multiple Range component. Means were considered significantly different at P < 0.05 or less.
Results

Proliferation of PC-3 and LNCaP cells was evaluated in the presence of 1 or 10 nM DHT over 5 days. PC-3 cells were unaffected by either 1 or 10 nM DHT over the 5 days compared with control (Fig. 1A). LNCaP cells were unaffected by 1 or 10 nM DHT on day 1, but by days 3 and 5, both concentrations of DHT induced significant cell proliferation (Fig. 1B). A similar response to DHT was observed when thymidine uptake was measured (data not shown).

The growth response to the varying concentrations of the 7 phytoestrogens are shown in Fig. 2A for the androgen-sensitive (LNCaP) cell line and in Fig. 2B for androgen-insensitive (PC-3) cell line. In PC-3 cells, curcumin was most effective in inhibiting cell growth. In LNCaP cells, both apigenin and curcumin were the most potent inhibitors of cell growth. The IC50 for each of the 7 phytoestrogens was calculated for the 2 prostate cancer cell lines (Table 1). In LNCaP cells, EGCG had no effect on cell growth below 90 µM (Fig. 2A). When 100 µM EGCG was added, however, cell protein was decreased to 54% of the control. This response likely reflects an interaction between EGCG and FBS (11,12) at lower concentrations. When cells were incubated in serum-free media, 50% growth inhibition was observed with 25-35 µM EGCG (data not shown), therefore suggesting reduced efficacy of EGCG in the presence of serum. Because of the limitations on growth of the cells in serum-free media, we choose to define IC50 for EGCG as 100 µM and used this concentration in subsequent experiments.

A time course of cell growth was performed in the 2 prostate cancer cell lines over 3 days using the IC50 concentration of each phytoestrogen. In PC-3 and LNCaP cells, all 7 phytoestrogens induced 50% inhibition of growth from day 1 through day 3 except baicalein, apigenin, and resveratrol, which induced 50% inhibition by day 3 only (Table 2).

In both prostate cancer cell lines, significant arrest of the cell cycle in S phase was induced by resveratrol and EGCG and

![Figure 1](image1.png)

**Figure 1.** A: PC-3 and B: LNCaP cells were plated at 1 x 10⁶ cells in 6-well dishes in RPMI 1640 media containing charcoal-stripped serum, and cells were grown to 50% confluence. The culture media were replaced with media containing 1 or 10 nM DHT and incubated for 5 days. Total cellular protein was measured using DC Bio-Rad assay. Values are means ± standard error of the mean for 3 individual experiments. Bars designated with asterisks are significantly different from the 0 DHT mean within each day, *P < 0.05.*

![Figure 2](image2.png)

**Figure 2.** A: LNCaP and B: PC-3 cells were plated at 1 x 10⁴ cells per well in 24-well dishes with RPMI 1640 complete culture medium, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete media containing 0-100 µM of each phytoestrogen and incubated for 72 h. The total cellular protein concentration was determined by DC Bio-Rad assay. The IC50 for each phytoestrogen was calculated based on the growth inhibition after 72 h compared with the control. Values are means ± standard error of the mean for 3 individual experiments.
in G2M phase by quercetin, baicalein, apigenin, genistein, and curcumin compared with the untreated cells (Table 3).

The 2 prostate cancer cell lines were treated with the IC50 concentration of each phytoestrogen for 5 days, and the percentage of apoptotic cells was determined by the TUNEL assay. All of the 7 phytoestrogens induced significant apoptosis in both cell lines (PC-3 and LNCaP) when compared with untreated cells (Table 4). In PC-3 cells, EGCG was the most potent apoptotic phytoestrogen, whereas in LNCaP cells, curcumin was the most potent. Apoptosis was confirmed, as phytoestrogens caused significant DNA smears and ladders in the treated cells compared with the untreated cells. Figure 3 demonstrates DNA laddering in PC-3 cells treated with genistein or curcumin.

The 7 compounds were tested for their potential to displace bound estradiol in mouse uterine cytosol using the competitive 3H-estriadiol ligand binding assay. At 2.5 μM, quercetin, baicalein, genistein, EGCG, and curcumin displaced >85%, and apigenin and resveratrol >40% of 3H-estradiol (Fig. 4).

Both cell lines expressed detectable amounts of ERα (Fig. 5A) and ERβ (Fig. 5B) by Western blot analysis. Because both cell lines contained ER, we determined if the treatment with an ER agonist (E2) or ER antagonist (ICI 182,780) affected cell proliferation. E2 (1 or 10 nM) with or without ICI 182,780 (1 μM) had no effect on the growth of PC-3 or LNCaP cells over 5 days (data not shown).

### Discussion

Interest in the physiological roles of bioactive compounds present in plants has increased dramatically over the last decade. Of particular interest because of their reported roles in human health is the class of compounds known as phytoestrogens. Phytoestrogens embody several groups of nonsteroidal estrogens, including isoflavones that are widely distributed within the plant kingdom (13). Phytoestrogens

### Table 1. IC50 (μM) of the 7 Phytoestrogens in PC-3 and LNCaP Cell Lines

<table>
<thead>
<tr>
<th>Phytoestrogen</th>
<th>PC-3</th>
<th>LNCaP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Baicalein</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Curcumin</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td>EGCG</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Genistein</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Quercetin</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

*a: IC50 of the 7 phytoestrogens was calculated from 3 individual experiments.

### Table 2. Time Course Study of Growth Inhibition in PC-3 and LNCaP Cell Line Using the ICso Concentrations of the 7 Phytoestrogens

<table>
<thead>
<tr>
<th></th>
<th>PC-3</th>
<th>LNCaP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>Apigenin</td>
<td>10.3 ± 0.7</td>
<td>25.2 ± 1.2</td>
</tr>
<tr>
<td>Baicalein</td>
<td>15.3 ± 0.8</td>
<td>30.7 ± 1.4</td>
</tr>
<tr>
<td>Curcumin</td>
<td>47.8 ± 2.7</td>
<td>49.8 ± 3.0</td>
</tr>
<tr>
<td>EGCG</td>
<td>47.3 ± 1.1</td>
<td>48.8 ± 3.5</td>
</tr>
<tr>
<td>Genistein</td>
<td>47.9 ± 2.1</td>
<td>48.8 ± 1.6</td>
</tr>
<tr>
<td>Quercetin</td>
<td>49.5 ± 2.3</td>
<td>50.1 ± 1.0</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>16.2 ± 0.7</td>
<td>30.6 ± 1.6</td>
</tr>
</tbody>
</table>

*a: Values are percent growth inhibition relative to untreated controls and are shown as mean ± standard error of the mean. N = 3 individual experiments. Abbreviation is as follows: EGCG, epigallocatechin gallate.

### Table 3. Cell Cycle Changes Induced by the Seven Phytoestrogens as Determined by Flow Cytometry

<table>
<thead>
<tr>
<th></th>
<th>PC-3</th>
<th>LNCaP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>S</td>
</tr>
<tr>
<td>Control</td>
<td>58.2 ± 1.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.1 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apigenin</td>
<td>58.6 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.1 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baicalein</td>
<td>59.7 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.5 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Curcumin</td>
<td>60.7 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.5 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG</td>
<td>48.6 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30.3 ± 3.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Genistein</td>
<td>57.3 ± 1.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.9 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quercetin</td>
<td>62.6 ± 1.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.0 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>47.7 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32.7 ± 1.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*a: Values are percent of cells in each phase of cell cycle and are shown as mean ± standard error of the mean. Values within each column with different letter superscripts are significantly different, P < 0.05. N = 3 individual experiments. Abbreviation is as follows: EGCG, epigallocatechin gallate.

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Table 4. Induction of Apoptosis by the 7 Phytoestrogens in PC-3 and LNCaP Cell Lines as Determined by the TUNEL Assay

<table>
<thead>
<tr>
<th>Phytoestrogen</th>
<th>LNCaP (Apoptotic Events)</th>
<th>PC-3 (Apoptotic Events)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>0.94 ± 0.08c</td>
<td>1.01 ± 0.10c</td>
</tr>
<tr>
<td>Positive Control</td>
<td>22.90 ± 1.41c</td>
<td>20.75 ± 1.02c</td>
</tr>
<tr>
<td>Apigenin</td>
<td>27.20 ± 1.19c</td>
<td>27.85 ± 0.86c</td>
</tr>
<tr>
<td>Baicalin</td>
<td>19.24 ± 0.66c</td>
<td>18.94 ± 1.09c</td>
</tr>
<tr>
<td>Curcumin</td>
<td>52.80 ± 3.05c</td>
<td>21.89 ± 1.18c</td>
</tr>
<tr>
<td>EGCG</td>
<td>49.20 ± 0.54c</td>
<td>40.67 ± 1.33c</td>
</tr>
<tr>
<td>Genistein</td>
<td>33.60 ± 1.67c</td>
<td>20.78 ± 1.17c</td>
</tr>
<tr>
<td>Quercetin</td>
<td>43.00 ± 1.47d</td>
<td>32.50 ± 1.42c</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>46.40 ± 1.21d</td>
<td>19.56 ± 1.03c</td>
</tr>
</tbody>
</table>

a: Values are percent of total events counted and are shown as mean ± standard error of the mean. Values within each column with different letter superscripts are significantly different, P < 0.05 or less. N = 3 individual experiments. Negative control is the untreated cells. Positive control is camptothecin-treated cells. Abbreviation is as follows: EGCG, epigallocatechin gallate.

bind to ER, with a preference for the more recently described ER β (14). In the present study, each of the 7 phytoestrogens (2.5 μM) displaced 

\[ ^3H \text{-estradiol} \] in a competitive ligand-binding assay using mouse uterine cytosol. Branham et al. (14) reported similar results for apigenin, baicalin, and genistein but found no binding activity for either quercetin or catechins as EGCG. Maggiolini et al (15) and Markaverich et al. (16) demonstrated that quercetin competed for \[ ^3H \text{-estradiol} \] binding to cytosol and nuclear type II sites in rat uterine preparations. In other reports, catechins, including EGCG, have been shown to bind to ER (17).

Both PC-3 and LNCaP cells express ERα and ERβ; however, cell proliferation was not affected by E2 (up to 10 nM) either in the presence or absence of ICI 182,780 (up to 1 μM). This suggests that the effect of the 7 phytoestrogens on the growth of these cells is independent of the ER pathway. Lieberman et al. (2) and Cox and Crawford (3) described the role of estrogen in the form of DES in the treatment of prostate cancer as working through a negative feedback on the anterior pituitary, resulting in decreased testosterone production required for hormone-dependent cancer. A direct effect for DES, whether through a classical ERα, ERβ, or another ER-independent mechanism, has not been defined. Furthermore, there is evidence that ER and androgen receptors are not essential for phytoestrogens or their metabolites to inhibit the growth of human breast and prostate cancer cells (18). Therefore, we decided to study these phytoestrogens to understand the mechanisms by which they regulate cell proliferation in prostate cancer cells.

The mechanisms by which androgens stimulate proliferation of prostate cancer cells are poorly understood. It has been proposed that androgen stimulation may induce the mitogen activated protein (MAP) kinase system leading to cellular proliferation. We confirmed that PC-3 cells are androgen-insensitive and LNCaP cells are androgen-sensitive, which agrees with previous reports (19-22). The different response of the 2 cell lines to DHT did not correlate with the growth inhibition pattern that was induced by the 7 phytoestrogens in these cells.

Each of the phytoestrogens used in our study has been shown to inhibit the growth and proliferation of several cancer cell lines and tumors in animal models; hence, they are good chemopreventive candidates. Agents that are capable of inducing selective cell cycle changes or apoptosis of cancer cells are receiving considerable attention for cancer prevention (23,24). In the current study, the androgen-insensitive (PC-3) cell line showed that the IC50 of the 7 phytoestrogens ranged from 25 to 1.25 DNA Standard (100bp).

2. Negative Control


4. Curcumin Treated Cells

5. Positive Control

6. DNA Standard (Kbp)

Figure 3. PC-3 cells were plated at 1 x 10⁴ cells in 100-mm culture dishes in RPMI 1640 complete media, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete media containing the IC50 concentration of genistein or curcumin and incubated for 5 days. Detection of DNA ladders and smears as markers for apoptosis were performed by DNA fragmentation assay. The untreated PC-3 cells were used as negative control, whereas camptothecin-treated (6 μg/ml) PC-3 cells were used as positive control.
Figure 4. Saturation Binding Analysis—uterine cytosol was incubated overnight at 4°C with \(^{3}H\)-estradiol (5 nM final concentration) and the aliquot of each of the 7 phytoestrogens (2.5 \(\mu\)M). Bound and free ligands were separated by dextran-coated charcoal, and an aliquot of bound radioactivity was measured by scintillation counting. Values are means ± standard error of the mean for 3 individual experiments. Bars designated with asterisks are significantly different from estradiol, \(P < 0.05\) or less.

100 \(\mu\)M concentration, with curcumin being the most effective compound. In androgen-sensitive (LNCaP) cells, the IC\(_{50}\) of the 7 phytoestrogens ranged from 20–100 \(\mu\)M, with apigenin, curcumin, and quercetin of equal potency. In both cell lines, EGCG was the least potent with regard to inhibition of cell growth. Because these experiments were performed in the presence of FBS, the effectiveness of EGCG may have been reduced (11,12). Growth inhibitory effects have been described for apigenin, genistein, EGCG, quercetin, and resveratrol in PC-3, LNCaP, breast, and some bladder cancer cell lines but at higher IC\(_{50}\) (25–28), whereas curcumin and baicalein induced growth inhibition in LNCaP cells at similar IC\(_{50}\) (29–31).
It is not presently known if concentrations of this magnitude could be achieved in human prostate tissues. Mice fed diets containing 1 gm/kg of genistein for 3 wk had a serum genistein concentration of 7.5 ± 0.6 μmol/L (32). Japanese men who consumed daily cereal bars containing 20 mg soy isoflavones for 1 yr were found to have 15.8 ± 0.8 μmol/L of genistein in genitourinary tissues (33). Some compounds, such as lycopene, do concentrate in the prostate (34); however, it has not been demonstrated that the concentrations tested could be achieved in vitro by dietary consumption.

Growth inhibition of the 2 prostate cancer cell lines could be a result of induction of programmed cell death that may be mediated by perturbation and arrest of the cell cycle (23). In our study, using the empirically determined IC₅₀, resveratrol and EGCG induced S phase arrest while baicalein, apigenin, genistein, quercetin, and curcumin caused G2M phase arrest in both cell lines. All of the 7 phytoestrogens induced apoptosis in both prostate cancer cell lines, as evidenced by TUNEL and DNA fragmentation assays. Cancer cell lines, including bladder, breast, and prostate, showed clear cell cycle changes and apoptosis induction by apigenin (23,27), genistein (24,26,27), curcumin (35), quercetin (26), and EGCG (36–38). In our study, curcumin and EGCG were the most potent apoptotic phytoestrogens in both prostate cell lines. However, the role of baicalein and resveratrol in induction of cell cycle changes and apoptosis in prostate cell lines has not been described before. The induction of cell cycle changes and apoptosis in prostate cells could be explained by induction of the cyclin kinase inhibitor WAF1/p21 (36) or alteration of BCL-2, BCL-X, and BAD expression in prostate cells (23).

In conclusion, we have shown that quercetin, baicalein, apigenin, genistein, resveratrol, curcumin, and EGCG inhibited the growth and proliferation of both androgen-sensitive and androgen-insensitive prostate cancer cells. These phytoestrogens altered cell kinetics and induced apoptosis. The results presented here indicate that these phytoestrogens are of potential value for the chemoprevention of prostate cancer.

Acknowledgments and Notes

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References


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