

**PHYTOESTROGENS MAY INHIBIT PROLIFERATION OF MCF-7 CELLS, AN
ESTROGEN-RESPONSIVE BREAST ADENOCARCINOMA CELL LINE**

A Thesis

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Abstract

After menopause, a woman's production of 17β -estradiol, the predominant female sex hormone, declines. This change is associated with increased risk of osteoporosis/osteopenia and atraumatic bone fracture, cardiovascular disease, and breast and ovarian cancers. Phytoestrogens are non-steroidal compounds isolated from plants that have antagonistic, weak agonistic, or super-agonistic estrogenic effects in mammalian tissues; they have emerged as a potential therapeutic to alleviate post-menopausal symptoms. While some epidemiological evidence indicates that dietary consumption of phytoestrogens can alleviate post-menopausal health risks, other research suggests that phytoestrogens may not be completely safe.

The research presented in this thesis indicates that a high concentration and sustained dose of phytoestrogens may be necessary to achieve antiestrogenic effects. MCF-7 cells, an estrogen-sensitive breast adenocarcinoma cell line, were used as a model system, and proliferating cell nuclear antigen (PCNA) was used as a marker of cell proliferation. Immunoblotting shows that genistein, a commercially purified phytoestrogen, promotes cell proliferation when administered for 24 hours, but may reduce proliferation when cells were treated for 48 hours. Genistein and estrogen have an additive effect on cells that were treated simultaneously with both hormones for 24 hours. In contrast, Promensil™, an over-the-counter phytoestrogen dietary supplement, was able to abolish expression of PCNA after 48 hours, and at high concentrations prevented estrogen-induced upregulation of PCNA after 48 hours. The clinical significance of these findings is that phytoestrogens may reduce the risk of breast cancer, but only after sustained high doses, which may be difficult if patient non-compliance is at issue.

Additionally, because cell proliferation and not cell survival was investigated, we cannot say whether phytoestrogens are cytotoxic to breast cancer cells, only that they reduce proliferation.

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Introduction

In North America, breast cancer is the most frequent malignancy diagnosed in women, with 200,000 new cases diagnosed and 50,000 mortalities occurring every year (Maggiolini *et al.*, 2001). 23,000 new cases of ovarian cancer are diagnosed and 14,000 deaths occur annually, making ovarian cancer the fifth most common malignancy and fifth leading cause of cancer mortality (Holschneider and Berek, 2000). Additionally, ovarian cancer is often not diagnosed until it has metastasized, giving it the highest fatality-to-case ratio of all gynecological cancers (Holschneider and Berek, 2000). A woman's risk for developing both of these malignancies increases with age, possibly due to menopause (Liede and Narod, 2002). Other health concerns associated with menopause include osteoporosis, and increased risk of cardiovascular disease. Therefore, methods to ameliorate or abrogate the changes associated with menopause offer an appealing therapy.

The current treatment option to alleviate post-menopausal symptoms is hormone replacement therapy (HRT), in which a woman's declining estrogen levels are augmented with estrogen or an estrogen agonist. Because estrogen is such a potent mitogen (Pratt and Pollak, 1993), progesterone is often administered concomitantly to reduce the risk of breast or endometrial cancer. Recently, the use of HRT has come under scrutiny. Some studies indicate that, despite the use of progesterone, estrogen supplementation is associated with an increased risk of breast and endometrial cancer. In fact, one aspect of the Women's Health Initiative—one of the largest clinical trials to examine the efficacy of estrogen supplementation—was terminated prematurely because the group receiving both hormones presented an increased incidence of breast cancer (Hays *et al.*, 2003).

These trends have prompted increased research to find a selective estrogen receptor modulator (SERM), which would ideally provide the beneficial effects of estrogen without the increased risk of breast and endometrial cancer. Many epidemiological studies (Carusi, 2000; Chiechi, 1999; Diel *et al.*, 2000; Knight and Eden, 1995; Lissin and Cooke, 2000) have examined the difference in morbidity of breast cancer, endometrial cancer, and osteoporosis between American women and Asian women. These studies implicate phytoestrogens—non-steroidal estrogen-like compounds from plants—as the model SERM. Phytoestrogens are consumed as part of a diet rich in soy and plant material; this diet is typical of East Asian countries, such as Japan. The incidence of breast cancer in Japan, for example, is 20% that in the United States (Liede and Narod, 2002) and this difference may be due to dietary factors.

This thesis sought to evaluate the effects of a commercially purified phytoestrogen, genistein, and compare genistein to an over-the-counter (OTC) phytoestrogen dietary supplement. MCF-7 cells, a well-characterized estrogen responsive breast adenocarcinoma cell line, were chosen as the model system. This tumor model has been used in other studies (Pratt and Pollak, 1993; Maxwell and van den Berg; 1999, Qin *et al.*, 1999) as a model of estrogen sensitive breast epithelial cells. We found that estrogen does up-regulate proliferating cell nuclear antigen (PCNA) levels compared to an ethanol-treated vehicle, although not in the expected dose-responsive manner. Genistein may inhibit growth of MCF-7 cells, but only at very high, sustained concentrations, which may not be achievable through dietary consumption of phytoestrogen-rich foods. It remains uncertain if genistein is able to reduce the proliferative effect of estrogen; at the doses tested, we conclude that low levels of

genistein exacerbate estrogen-induced proliferation. OTC phytoestrogen supplements, in contrast, seem to abrogate expression of PCNA. At high doses, these supplements can preclude estrogen's proliferative effect, and compete with estrogen in a dose-dependent manner. These results lead us to believe that high levels of phytoestrogens, which may require supplementation in order to attain the desired effects, can be effective in prevention and treatment of breast cancer.

Literature Review

Post-menopausal Health Risks

A variety of changes happens to a woman's body during and after menopause. The most significant of these changes is the decreased production of the female sex hormone estrogen as ovulation ceases. In addition to declining fertility, menopausal and postmenopausal women also become prone to a number of health problems which can be grouped into two categories: cardiovascular disease, and osteoporosis.

The cardiovascular diseases pertinent to post-menopausal women are coronary artery disease, atherosclerosis, and hypercholesteremia (Lissin and Cooke, 2000). All of these conditions are a result of decreased production of estrogen. Estrogen is implicated in maintaining the function of the smooth vascular endothelium (Mihmanli *et al.*, 2002). It suppresses expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), reducing the ability of platelets and clotting factors to adhere to the vascular endothelium (Lissin and Cooke, 2000; Mendelsohn and Karas, 1999). Estrogen also reduces the susceptibility of low density lipoprotein (LDL) to oxidation, which reduces the risk of atherogenesis. The mechanism behind this event is the maintenance of stable ApoB-100 (Brunelli *et al.*, 2000), a predominant structural component of LDL particles. Amino acids in apoB-100, which are susceptible to oxidation/reduction, increase the risk of oxidation of nearby LDL molecules, which is atherogenic (Tikkanen *et al.*, 1998). Estrogen protects apoB-100 from oxidation/reduction. Estrogen also causes vascular endothelial cells to rapidly release nitric oxide, which relaxes vascular smooth muscle and prevents platelet activation

(Mendelsohn and Karas, 1999). Other effects that estrogen has on vascular function are to reduce plasma homocysteine and angiotensin-converting enzyme, promote vascular relaxation, and change the concentration of clotting factors (Brunelli *et al.*, 2000).

The most visible symptom of decreased estrogen production is the appearance of the “dowager’s hump.” With decreased estrogen production, a woman’s bones become thinner, less dense, and less able to support her weight, leading to compression and curvature of the spine. Also as a result of reduced estrogen levels, bone-resorption increases, and there is a general increase in bone turnover, leading to the condition commonly known as osteoporosis. This leaves post-menopausal women at risk for atraumatic fractures (Evans and Turner, 1985).

One study (Hoerger *et al.*, 1999) estimates that of the \$187 billion in 1997 dollars spent annually on women over age 45, \$60.4 billion were spent on cardiovascular disease, \$12.9 billion on osteoporosis, and \$5 billion were spent on breast and gynecological cancers. While there is no unambiguous causal link between osteoporotic fractures and death, there is an increased risk of death associated with these fractures. It is estimated (Johnell *et al.*, 2004) that possibly 23% of deaths occurring after a hip fracture may be causally related to the fracture.

Hormone Replacement Therapy

Hormone replacement therapy is the most popular treatment for the alleviation of post-menopausal symptoms. This method consists of augmenting a woman’s declining production of estrogen with synthetic estrogen (estrogen replacement therapy), most often 17 β -estradiol (**Figure 1**). While successful in alleviating some symptoms—hot flashes, osteoporosis, change in lipid profile, and others—the administration of estrogen

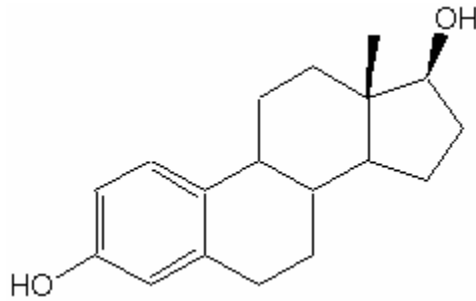


Figure 1. 17 β -estradiol.

is clearly linked to an increased risk of estrogen responsive breast and endometrial cancers (Hays *et al.*, 2003). Furthermore, in women with a history of coronary artery disease, there is an increased risk of a second cardiovascular event (Lissin *et al.*, 2000).

Estrogen's proliferative effect on breast epithelial tissue has been established since the 1960's, when the oral contraceptive pill was developed. Recent research has investigated the mechanism through which estrogen can cause cells to become tumorigenic. In addition to a strong proliferative effect in responsive tissues, estrogen has been shown (Kyo *et al.*, 1999) to upregulate telomerase activity. This enzyme acts as a reverse transcriptase to maintain and elongate the telomeric DNA; the shortening of telomeres is implicated in the aging and eventual senescence of replicating cells. Although telomerase activity is not an infallible marker of malignancy, and is not an oncogene, it is up-regulated in many tumor cells (Kyo *et al.*, 1999).

The Estrogen Receptors (ERs) are nuclear transcription factors that, upon binding estrogen, dimerize, and form transcription complexes (Jordan *et al.*, 2001). There are two types of estrogen receptors, ER α and ER β , and their differential distribution may partially explain estrogen's—and anti-estrogens'—different effects on different tissues. For example, the anti-estrogen tamoxifen (**Figure 2**) exerts an anti-proliferative effect on

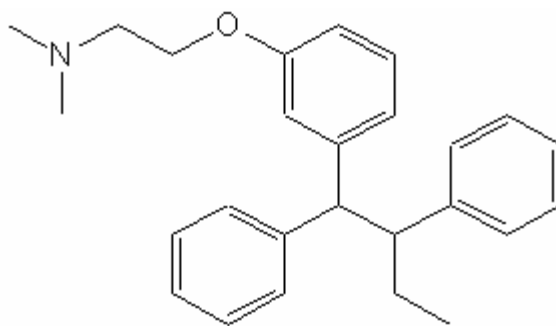


Figure 2. Tamoxifen

breast cancer cells, but exerts a proliferative effect on uterine tissue (Jordan *et al.*, 2001; Abdelrahim *et al.*, 2002).

The regulation sites controlled by the ER are the estrogen response element (ERE), and AP1 (Paech *et al.*, 1997). **Figure 3** diagrams a model of the ERE signaling pathway. Activation at AP1 also requires the transcription factors Fos and Jun (Paech *et al.*, 1997); Sp-1 is another transcription factor influenced by the ER (Gruber *et al.*, 2004). It may be more useful to describe whether compounds have transcriptional or anti-transcriptional effects. Estrogen and estrogen agonists have positive transcriptional effects when acting on the ERE through ER α , but estrogen antagonists down-regulate transcription. Conversely, estrogen antagonists induce transcription when acting on the AP1 element through ER β (Paech *et al.*, 1997). AP1 and the ERE are implicated in regulating expression of heat shock proteins (HSPs), and glucose-related proteins (GRPs), both of which are upregulated in transformed cells and tumors. Stress response proteins are also believed to enhance resistance to chemotherapy and radiation therapy (Zhou and Lee, 2001). The ER activates kinases such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) before nuclear translocation

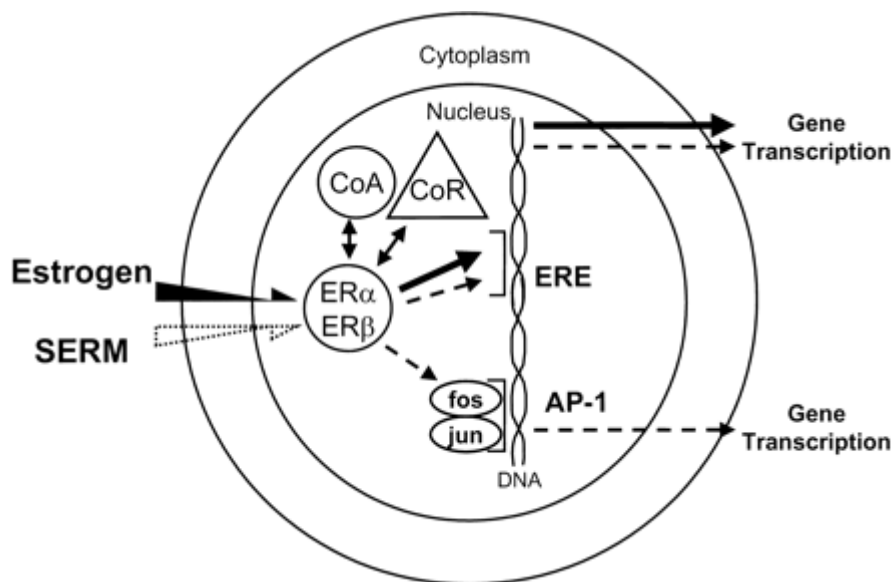


Figure 3. Model of ERE/AP-1 Signal Transduction. Estrogen, or selective estrogen response modulators (SERMs), after diffusing through the plasma membrane binds either ER. The E_2 /ER complex then recruits coactivators (CoA) to form a complete transcription complex. Anti-estrogens/SERMs interfere with this process by altering conformation of the ER-ligand complex, leading to recruitment of corepressor (CoR) molecules instead. Additionally, ER-ligand complexes can also interact with fos/jun, and then AP-1 sites (Jordan *et al.*, 2001)

(Duan *et al.*, 2002). These kinases in turn upregulate the expression of *c-fos*, a potential protooncogene. Cyclin D1 plays a role in cell-cycle advancement from G1 to S phase, and is influenced by estrogen (Castro-Rivera *et al.*, 2001).

To combat these risks, a woman's HRT regimen can be modified. Progesterone (Figure 4) can reduce the risk of uterine hyperplasia or endometrial cancer, but also limits the improvement in lipid profile (Turner *et al.*, 1995). Progesterone acts through two distinct nuclear receptors, Progesterone Receptor A and B (PRA and PRB, respectively). Ligand bound PRA exerts a dominant negative effect on expression of the estrogen receptor gene, and enhances degradation of ER proteins (Dai *et al.*, 2002). It may also abrogate ER-induced transcription events. Independent of estrogen, receptor-bound progesterone activates the tumor suppressor genes p21 and p27, and PRB down-regulates expression of the cellular adhesion molecules fibronectin, integrin $\alpha 3$ and $\beta 1$

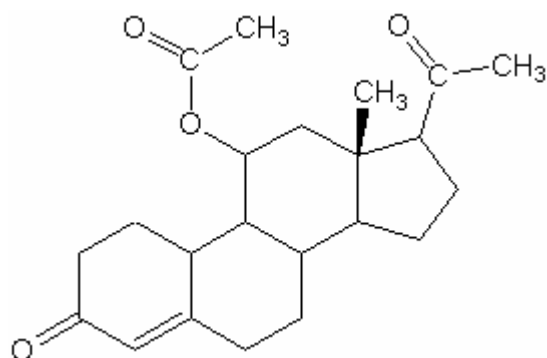


Figure 4. Progesterone

(Dai *et al.*, 2002). These molecules not only function as cellular adhesion molecules, but also play a role in signal transduction, affecting proliferation, differentiation, and apoptosis. Additionally, progesterone down-regulates cadherin 6, which is implicated in promoting tumorigenesis (Dai *et al.*, 2002). Newer selective anti-estrogens, and selective estrogen receptor modulators (SERMs), such as raloxifene (**Figure 5**) can help maintain bone density and cardiovascular function, while having minimal estrogenic effects in uterine tissue (Jordan *et al.*, 2001)

Recently, HRT has come under scrutiny due to the Women's Health Initiative (WHI), a clinical study designed to assess the effects of estrogen alone versus estrogen

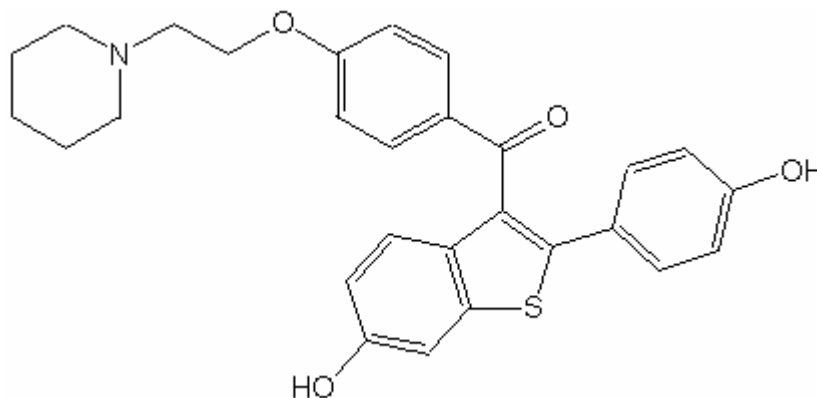


Figure 5. Raloxifene.

plus progestin (progesterone) on health-related quality of life for postmenopausal women. The investigation of women receiving combined therapy was stopped 2 years earlier than anticipated due to increased risk for cardiovascular disease and breast cancer. The overall conclusions currently available from this study indicate that combination therapy provides no improvement in quality of life for postmenopausal women, and may increase the risk of breast cancer (Hays *et al.*, 2003).

Anti-estrogens

Because HRT has come under scrutiny for breast and endometrial cancer, it is sometimes augmented with anti-estrogens, such as tamoxifen or raloxifene. These two chemotherapeutics are prescribed to fight breast cancer, and sometimes to prevent breast cancer in women with a high risk. Raloxifene has the additional benefit of being estrogenic in bone tissues. Both these drugs can combat breast cancer, but do have some estrogenic effects in uterine tissue (Jordan *et al.*, 2001). For this reason, other therapeutics are being investigated to relieve post-menopausal health concerns. Phytoestrogens, plant compounds that have estrogenic effects in mammalian tissues, may provide a solution to this problem.

Phytoestrogens

The term “phytoestrogen” is used loosely to describe any plant-derived compound that exerts estrogen-like effects on mammals; these effects can be antagonistic, agonistic, or even super-agonistic. The general structure of this diverse group of compounds consists of at least one phenolic ring in a non-steroidal poly-cyclic system. Along with

phytoestrogens, mycoestrogens, estrogen-like compounds produced by fungi, may have potential therapeutic value in the treatment of post-menopausal conditions. However, some studies suggest phytoestrogens may have a cancer inducing effect (Lissin *et al.*, 2000). Phytoestrogens were first discovered as causing “red clover” disease. This condition causes infertility in sheep which graze on red clover, a plant high in phytoestrogens. The same effect has been seen in captive-bred cheetahs fed a diet high in plant matter. It is proposed (Hsu *et al.*, 1999a) that phytoestrogens may be a form of plant defense, limiting predation of plant species by causing long-term infertility, increased risk of postnatal mortality, and other adverse reproductive effects on grazing herbivores.

In the 1970’s, phytoestrogens were first investigated as a treatment for post-menopausal symptoms. The synthetic isoflavone ipriflavone was developed to treat women at risk for osteoporosis without increasing the risk of breast or endometrial cancer. This drug did not affect vaginal maturation, an indicator of estrogenic activity, and did not induce endometrial hyperplasia (Carusi, 2000).

Phytoestrogens promote bone maturation and mineralization (Carusi, 2000; Cheichi 1999). Increased alkaline phosphatase activity occurs in rat femoral bone as a result of exposure to genistein (**Figure 6**) or daidzein (Carusi, 2000), two phytoestrogens,

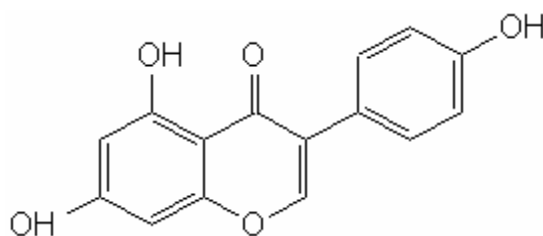


Figure 6. Genistein.

indicating maintenance of bone density/health. Administration of genistein to ovariectomized rats was able to prevent bone loss (Fanti *et al.*, 1998).

Proposed Mechanisms

Phytoestrogens can exhibit both estrogenic and anti-estrogenic activity in different—or even the same—tissues. This dichotomy has two possible causes: phytoestrogens act as agonists at low concentrations but act as antagonists at high concentrations, or phytoestrogens have different affinities for the two estrogen receptors (ER α /ER β), which themselves are expressed in different tissues. Distribution studies of the two ER's show that ER α has a much broader, systemic expression. In contrast, ER β is expressed more discretely, most notably in the ovaries, lungs, and in males in the prostate and epididymis (Morito, *et al.*, 2001). Possibly, ER β acts as a negative regulator of the estrogen response element (Paech *et al.*, 1997). Diel *et al.* (2001) show a mixed estrogenic/anti-estrogenic effect on the expression of progesterone receptor, and ER α mRNA in MCF-7 cells. The compounds used were tested at only one concentration—0.1 nM for E₂, and 1 micromolar for phytoestrogens genistein, daidzein, and coumestrol. The physiological serum concentration of estrogen is between 0.1 and 10 nM (Mihmanli *et al.*, 2002). Other research indicates that over a wide concentration range, phytoestrogens such as genistein have biphasic effects (Tanos *et al.*, 2002). At low concentrations—below 10 μ M—genistein is an estrogen agonist, while above that concentration genistein exerts antagonistic effect on MCF-7 cells. The suggested explanation for this discrepancy is that at low concentrations, genistein acts through the ER, while at high concentrations, it acts through a different pathway. Genistein has a higher affinity for ER β , and will preferentially bind this subtype, although a high

concentration is required to induce estrogenic transcription (Morito *et al.*, 2001). A similar effect was seen by Hsu *et al.* (1999) using biochanin A, another phytoestrogen found predominantly in legumes.

It is relevant to examine the absorption of dietary phytoestrogens. Most phytoestrogens are found as glycosides in plants; the phytoestrogen is covalently bound to a sugar molecule (Allred *et al.*, 2001). Enteric bacteria—and to a lesser extent salivary, hepatic, and intestinal enzymes—deglycosylate the compounds into an aglycone form, which then enters circulation.

The maximum physiological concentration of genistein achieved through dietary consumption is 18.5 μM (Maggiolini *et al.*, 2001). However, anti-cancer effects are not reached below concentrations of 10 μM (Allred *et al.*, 2001). For example, the IC_{50} for genistein to inhibit DNA synthesis in MCF-7 cells is approximately 52 μM (Hsu, *et al.*, 1999b). It is believed that below this threshold, phytoestrogens act as estrogen agonists; at concentrations as low as 200 nM genistein is a potent estrogen agonist in MCF-7 cells (Allred *et al.*, 2001).

Because phytoestrogens are such a large and diverse group of compounds, their mechanism of action in preventing breast cancer and endometrial cancer is unclear. Phytoestrogens bind human estrogen receptor with an affinity 10 to 1,000 fold lower than estrogen (Morito *et al.*, 2001). Additionally, phytoestrogens may or may not have different effects depending on their oxidation state; some research suggests that these compounds have different effects depending on whether or not they are present in the bloodstream as glycosides, aglycones, or in fully reduced form (Morito, *et al.*, 2001).

The conversion of glycosides to aglycones by enteric bacteria may be necessary for phytoestrogens to be absorbed through the intestines (Lissin and Cooke, 2000).

The physiological effects of phytoestrogens on cardiovascular health are attributed to the increased secretion of bile acids and an alteration of hepatocyte metabolism, which lead to improved uptake of LDL (Tikkanen *et al.*, 1998). Experiments with LDL receptor⁻ mouse models suggest that phytoestrogens increase removal of LDL by its receptor (Lissin *et al.*, 2000). Atherosclerosis is prevented when phytoestrogens block the expression of ICAMs and VCAMs, which stops monocytes/macrophages from binding and developing into foam cells. Foam cells are a differentiated subpopulation of monocytes/macrophages present at the site of atherosclerotic plaques. While foam cells perform the beneficial role of scavenging extracellular lipoproteins, they also secrete an array of cytokines that attract activated T cells, which then cause lesions to rupture, resulting in a vessel occlusion (Okazaki *et al.*, 2002).

Many mechanisms have been proposed for how phytoestrogens exert antiproliferative and anti-tumor effects. Genistein is believed to inhibit tumorigenesis by competing for the estrogen receptor, or inhibiting protein tyrosine kinase by competing for the ATP-binding site (Chen *et al.*, 2003). Other studies (Hsu *et al.*, 1999b) suggest that regulation of a proto-oncogene such as *c-myc* may be involved. Inhibition of angiogenesis (Fotsis *et al.*, 1993), anti-oxidative maintenance of DNA, inhibition of DNA-topoisomerase II, S6 kinase or phosphoinositide 3-kinase, and synthesis of heat-shock proteins (Maggiolini *et al.*, 2001) are other proposed mechanisms. Phytoestrogens are also believed to down-regulate expression of insulin-like growth factors (IGFs), and

IGF binding proteins (Maxwell and van den Berg, 1999). IGF binding proteins (IGFBPs) are believed to maintain a high local concentration of IGFs, which can lead to proliferation of malignant cells.

Cancer Pharmacology

The most promising outcome of phytoestrogen consumption is the potential prevention of the development of breast cancer. *In vitro* systems have displayed an anti-proliferative effect of phytoestrogens on both ER⁺ and ER⁻ breast cell lines (Chen *et al.*, 2003; Schmitt *et al.*, 2001). This suggests that the anti-proliferative effect is due to some mechanism other than binding or blocking the estrogen receptor. However, the concentrations required for phytoestrogens to exhibit these growth inhibitory effects is relatively high, and may not be attainable by normal dietary intake. The plasma concentration of isoflavones, a class of phytoestrogens abundant in soybeans, does not exceed 13 μ M in humans, even among Japanese, who typically eat a diet rich in soy (Murphy *et al.*, 1997). It is also uncertain whether the form of a phytoestrogen will affect its bioavailability. Some studies indicate that oxidation/glycosylation may influence whether phytoestrogens act as estrogen agonists or antagonists (Murphy *et al.*, 1997; Allred *et al.*, 2001).

Epidemiological Data

There is a large volume of contradictory evidence regarding the effects of phytoestrogens on human health, much of which comes from epidemiological study. The incidence and morbidity of breast cancer is drastically different between women in North

America and women in East Asia (Carusi, 2000). The higher consumption of plant matter is implicated in explaining this discrepancy (Lissin and Cooke, 2000). Typically, studies have examined the diets of Japanese women, American women, and Western European women.

In terms of breast cancer, there are conflicting results. Some studies indicate a decreased risk of breast cancer in countries such as China and Singapore, where a traditional diet includes more plant-material (Lissin and Cooke, 2000). Other studies show that there is no correlation between phytoestrogen consumption and development of breast cancer (Carusi, 2000).

Studies into the effects on bone density are unclear. Bone mass density can be improved in long bones with increased consumption of phytoestrogens, but this effect may not apply to other bones such as lumbar vertebrae (Carusi, 2000). A high dose of a phytoestrogen supplement can improve the effect on short bones, but this does not correlate to a systemic improvement in bone density. The main flaw in these studies (Kardinaal *et al.*, 1998, reviewed in Carusi, 2000), is that no study can be double-blind; subjects are always aware whether or not they are eating a phytoestrogen-rich diet. A study of more than 30,000 women in Nagasaki and Hiroshima, for example, found no correlation between dietary soy intake and later development of breast cancer (Key *et al.*, 1999), while other studies suggest that phytoestrogens are effective in alleviating post-menopausal symptoms such as hot flashes (Key *et al.*, 1999).

The cardiovascular profile of subjects consuming a high-soy diet shows improvement in low-density lipoprotein levels (LDL), but no change in other aspects of lipid profile (Carusi, 2000). However, a more profound effect is seen when

phytoestrogens are consumed along with soy protein. This suggests that a pill or pure phytoestrogen dietary supplement may not be effective. Improved functioning of the vascular endothelium is believed to be a result of reduced production of Vascular Cellular Adhesion Molecules (VCAMs), and Intercellular Adhesion Molecules (ICAMs), possibly through an anti-oxidant effect.

Insulin-Like Growth Factor Binding Proteins

Insulin-like growth factors play an important part in the development of malignant breast tumors (Maxwell and van den Berg, 1999). It is believed that insulin-like growth factor binding proteins (IGFBPs) have two functions; in some cases, they retain a high local concentration of insulin-like growth factors, or they bind IGF-I and inhibit its function (Maxwell and van den Berg, 1999). IGFBP-3 and -4 are the most relevant IGFBPs to the study of breast cancer. IGFBP-3 is inhibitory to both ER⁺ and ER⁻ breast cancer cells. Antimitogenic compounds, such as the antiestrogen raloxifene, induce IGFBP-3 expression by breast cancer cells (Qin *et al.*, 1999). Because IGFBP-3 expression correlates to growth hormone production, it is implicated in maintaining bone density; IGFBP-3 is also believed to promote Vitamin D production, which is another indicator of bone density (Kelley *et al.*, 1996). In contrast, IGFBP-4 is up-regulated in ER⁺ breast cancer cells in response to estrogen treatment; thus it indicates whether a compound acts through the ER (Qin *et al.*, 1999). IGFBP-4 is also believed to promote growth (Kelley *et al.*, 1996).

Materials and Methods

Cell Culture

MCF-7 (HTB-22) cells were purchased from American Type Culture Collection (Manassas, VA), and maintained between 5×10^5 and 1.5×10^6 cells/ml in Dulbecco's Modified Eagle's Medium supplemented with 0.1 mL non-essential amino acids (Gibco, Grand Island, NY), and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA). Penicillin/streptomycin/amphotericin (Gibco, Grand Island, NY) were used at a concentrations of 100 units/ml, 100 μ g/ml, and 250 ng/ml, respectively (DMEM/FBS/AA/PSA). Cells were routinely maintained in T75 flasks (Becton Dickinson, Franklin Lakes, NJ) in a 37°C 5% carbon dioxide incubator and were passaged every 7 days by trypsinization (0.25% trypsin/1 mM EDTA, Gibco, Grand Island, NY).

IGFBP-4/PCNA Bioassay

For experimental use, cells were trypsinized from T75 flasks, and re-plated in a 24 well plate (Costar Corning Incorporated, Corning NY) at 6×10^5 cells/ml, 0.5 ml/well (85-99% confluence) in DMEM/ FBS/AA/PSA. After at least 24 hours, cells were changed to phenol red-free, serum free DMEM. Media was changed daily thereafter, and cells were kept in serum-free media for at least 48 hours before starting hormone treatment. After this time, the media was changed and test compounds—17 β estradiol, genistein (Sigma-Aldrich, St. Louis, MO), or Promensil™ over-the-counter phytoestrogen supplement (Novogen Inc, Stamford, CT)—were administered at the indicated concentrations in a final ethanol concentration of 1% (vol/vol).

The concentration of phytoestrogens was determined as follows. According to manufacturer packaging, each tablet contains “plant estrogens (isoflavones), 40 mg (as red clover extract).” Two tablets were pulverized, and dissolved in 10 ml of ethanol by heating to 80°C for two hours. Using the molecular weight of genistein, 270.2 g/mol, this gives a concentration of:

$$(0.080 \text{ g})/(270.2 \text{ g/mol})=2.96 \times 10^{-4} \text{ mol}; (2.96 \times 10^{-4} \text{ mol})/(0.010 \text{ L})= 2.96 \times 10^{-2} \text{ M.}$$

This solution was then sterile-filtered through a 0.22 µm filter. Stock solutions were stored at 4°C.

Test compounds were administered to cells for 12-48 hours as indicated; as stated previously after 24 hours, medium was changed and new compound was added. Cells treated with an equal volume of ethanol served as negative controls. After hormone treatment, conditioned media was harvested and frozen at -80°C in the presence of a protease inhibitor cocktail (Upstate Cell Solutions, Lake Placid, NY), containing AEBSF (4-(2-Aminoethyl)benzenesulfonyl fluoride, 20 mM), EDTA (10 mM), bestatin (1.3 mM), E-64 (140 µM), Leupeptin (10 µM), and aprotinin (3 µM). Cell lysates were obtained by freezing cells overnight or longer at -80°C in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl (PBS) in the presence of protease inhibitors.

Immunoprecipitation

500 µl of conditioned media were incubated overnight at 4°C with 5 µl of 200 µg/ml rabbit anti-IGFBP-4 (Santa Cruz Biotechnology, Santa Cruz, CA). The immunocomplex was then captured with 100 µl of Protein A Agarose beads (Upstate Cell Solutions, Lake Placid, NY) at 4°C for at least 2 hours, with occasional agitation. Agarose beads were pelleted by pulse-centrifugation at 14,000 rpm, and the supernatant

discarded. Beads were washed with 800 μ l of PBS, and re-collected by pulse-centrifugation (3 times). Pelleted beads were assayed for IGFBP-4 by Western blot as described below.

Western Blotting

Cell lysates were assayed for total protein by the Bio-Rad assay (Hercules, CA), and were separated by SDS-PAGE on a 12% polyacrylamide gel under reducing conditions, according to the procedures in *Current Protocols in Molecular Biology*. Chemiluminescent molecular weight markers were obtained from Invitrogen. Samples were electrophoresed for approximately 3 hours at 40 mA, and then transferred to Immobilon-P polyvinylidene fluoride membrane (Millipore, Bedford, MA) at approximately 300 mA·hours. The membrane was blocked with 5% non-fat dry milk in 10 mM Tris-Cl, pH 7.4, 150 mM NaCl (TBS) for at least an hour at room temperature, or at 4°C for 2 hours or longer. TBS was used to briefly rinse the membrane, and then the 1° antibody, mouse anti-PCNA (Oncogene Research Products, San Diego, CA) at a concentration of 2.5 μ g/ml in blocking buffer or rabbit anti-IGFBP-4 at 0.5 μ g/ml, was applied. Proliferating cell nuclear antigen (PCNA) is a 37 kDa DNA Polymerase accessory protein; IGFBP-4 migrates between 28-32 kDa. After at least 8 hours at 4°C, the membrane was washed with TBS for 5 minutes, TBS/0.1% Tween for 5 minutes (twice), and again with TBS for 5 minutes. The membrane was incubated in 2° antibody, goat anti-mouse IgG·horseradish peroxidase at a 1:500 dilution or goat anti-rabbit IgG·horseradish peroxidase at a 1:1000 dilution (Upstate Cell Solutions, Lake Placid, NY) for 1-2 hours. The membrane was washed sequentially with TBS (once), TBS/0.1% Tween (twice), and TBS (once), 5 minutes each, before the substrate solution was added.

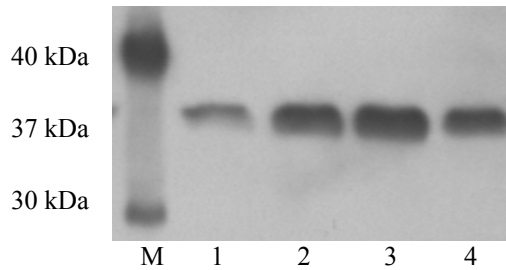
LumiGlo and peroxide substrates (Upstate Cell Solutions, Lake Placid, NY) were mixed, and added to the membrane for approximately one minute. X-ray film (BioMax MS Film, Kodak, Rochester, NY) purchased from Sigma-Aldrich (St. Louis, MO) was exposed for 5-10 seconds, followed by 5 minute development in GBX Developer solution, rinse, 3 minute fixation in Kodak Rapid Fixer Plus Hardener, and 5 minute wash all at ~25°C in a darkroom. Developed films were photographed with ALPHAIMAGER software, and quantified with Scion Image software (Scion Corporation, distributed through NIH). Data were quantified as arbitrary densitometric units and expressed as % control as indicated. For all experiments where $n > 2$, data are expressed as mean \pm S.E.M.

Results

Because there is such a large body of conflicting research regarding the effects of phytoestrogens on post-menopausal symptoms and health risks, the goal of this project was to evaluate the effect of OTC phytoestrogen supplements on the proliferation of a breast adenocarcinoma cell line (MCF-7). As a positive control, estrogen, which is a known mitogen in MCF-7 cells, was assayed at different concentrations from 10^{-11} to 10^{-7} M, since the physiological concentration of estrogen is approximately 10^{-9} M. Data from other researchers (Maxwell and van den Berg, 2000; Pratt and Pollak, 1993; Qin *et al.*, 1999) suggest that the proliferative effect of estrogen can be detected after 24 to 48 hours of hormone treatment. Therefore, estrogen was administered to cells as described in Materials and Methods for varying amounts of time.

Figure 7 shows an estrogen dose-response after 24 hours. Estrogen shows an inverse dose-response effect on PCNA expression, with the highest concentration— 10^{-7} M E_2 —causing reduced expression of PCNA compared to the ethanol-treated control, and the greatest stimulation occurs at 10^{-11} M. This dose-response was repeated for 12, 16, 36 (data not shown), and 48 hour treatments. No reproducible dose-responsive trend was observed at the 12 or 16 hour time points. The 36 hour exposure followed the trend of the 24 hour time point, with the greatest stimulation seen at the lowest dose tested, 10^{-11} M. However, as shown in **Figure 8**, 48 hours of treatment produced a different response. In contrast to the data obtained after 24 hours, all treatments were proliferative after 48 hours. While both 10^{-9} and 10^{-11} M estrogen increased proliferation to a greater extent than 10^{-7} M, at 48 hours the greatest increase was seen at 10^{-9} M. These two

A



B

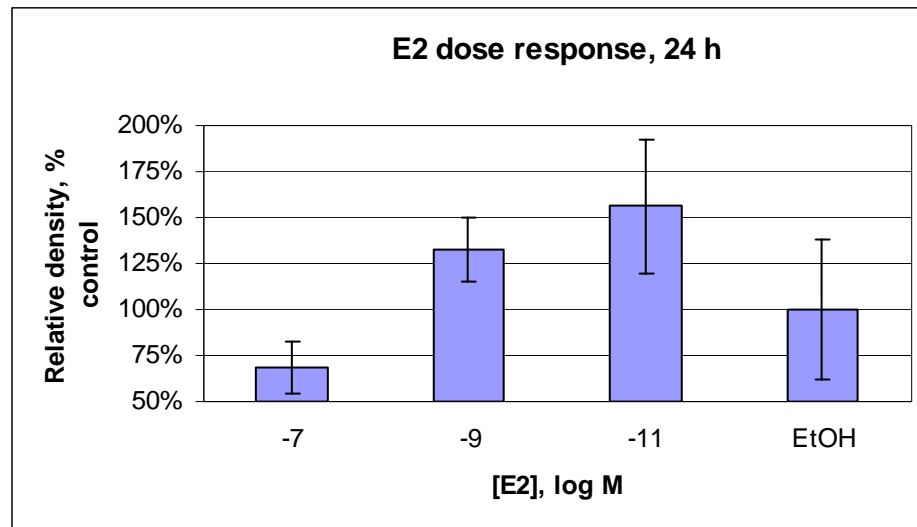


Figure 7. Effect of Estrogen (E₂) on PCNA Expression After 24 Hours. Cells were treated with E₂ for 24 hours as described in Materials and Methods, and PCNA was detected by immunoblotting. **A.** A representative immunoblot of cells treated with E₂ for 24 hrs. Lane M: chemiluminescent molecular weight marker; Lane 1: 10⁻⁷ M E₂; Lane 2: 10⁻⁹ M E₂; Lane 3: 10⁻¹¹ M E₂. **B.** Densitometric quantification of E₂ 24 hour dose-response assays, obtained as described in Materials and Methods. Data are expressed as percent of EtOH-treated control, n=6, mean ± SEM.

figures indicate that 10⁻⁹ M E₂ is able to raise PCNA levels compared to the ethanol treated control, and this change can be detected after 24 hours.

To investigate the effect of a known phytoestrogen on cell proliferation, cells were treated with genistein for 24 hours, and the cell lysates were assayed for PCNA (**Figure 9**). Genistein had the greatest proliferative effect at 10⁻¹¹ M, and exhibited a dose-dependent decrease in PCNA expression up to 10⁻⁵ M. At 10⁻² M, genistein

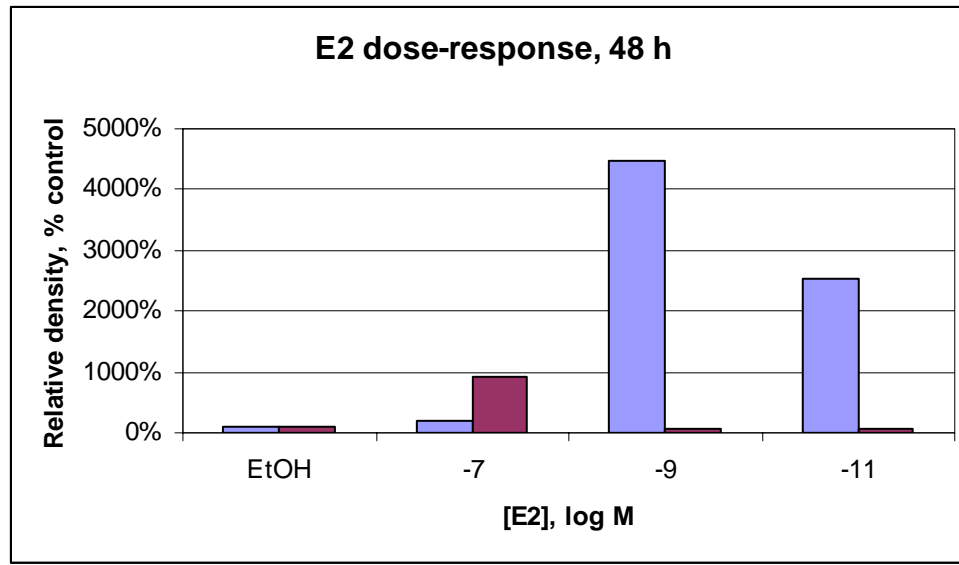


Figure 8. Effect of E₂ on PCNA Expression After 48 Hours. Cells were treated with E₂ at the indicated concentrations for 48 hours, and PCNA was detected by Western blotting with immunodetection as described in Materials and Methods (n=2, both trials shown).

increased PCNA levels more than at 10⁻⁵ M or 10⁻⁸ M, but not to the same extent as seen at 10⁻¹¹ M.

While the data are not statistically significant, they seem to indicate the potential that genistein has a biphasic effect on cell proliferation. When cells were treated with genistein for 48 hours (**Figure 10**), the lowest concentration tested, 10⁻⁸ M, stimulated proliferation, although to a lesser extent than at 24 hours. The higher two concentrations reduced PCNA expression somewhat, relative to ethanol treated controls. This suggests that 24 hours of hormone treatment is not a long enough exposure for genistein to exert an antiproliferative effect on MCF-7 cells.

To determine whether the proliferative effects of estrogen and genistein at 24 hours would be cumulative, genistein at varying concentrations was added to cells in the presence of 10⁻⁹ M E₂. **Figure 11** shows that estrogen and genistein do appear to have an additive effect on expression of PCNA. The percent increase at all doses of genistein was

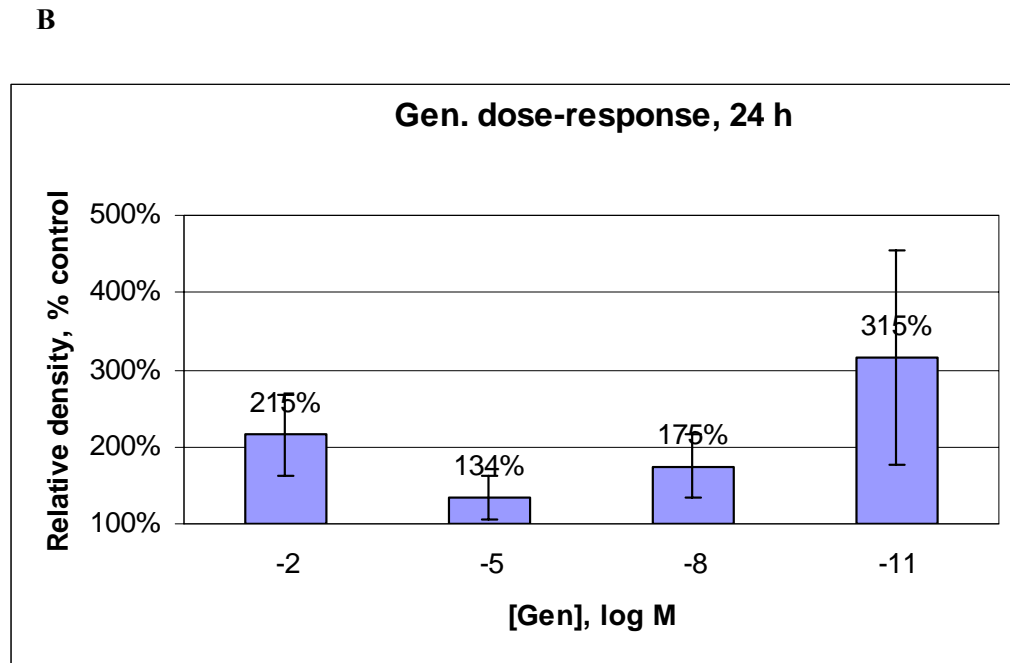
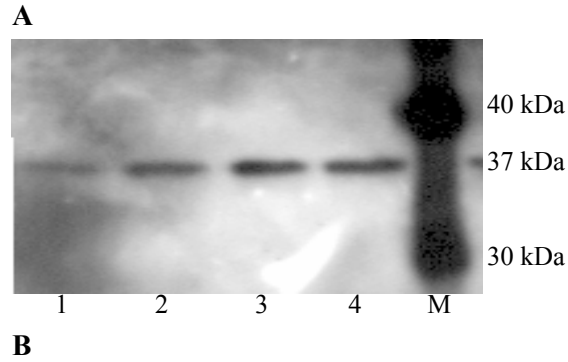


Figure 9. Effect of Genistein on PCNA Expression After 24 Hours. The commercially purified phytoestrogen genistein was administered to cells at the concentrations indicated for 24 hours. **A.** Representative Western blot. Lane 1: 10^{-2} M G; Lane 2: 10^{-5} M G; Lane 3: 10^{-8} M G; Lane 4: 10^{-11} M G; Lane M: Molecular weight marker. **B.** Quantification of 24 hour genistein dose-response assays. Data are expressed as percent of EtOH-treated control (not shown), mean \pm SEM, n=11.

higher than that for estrogen alone at 10^{-9} M at 24 hours. A definite relationship cannot be determined, however at all concentrations, genistein and estrogen together have a greater proliferative effect than either compound has alone.

The ultimate goal of this project was to ascertain the effect of over-the-counter phytoestrogen supplements. To that end, Promensil™, an OTC supplement, was dissolved in ethanol and administered to MCF-7 cells. Briefly, two tablets containing 80

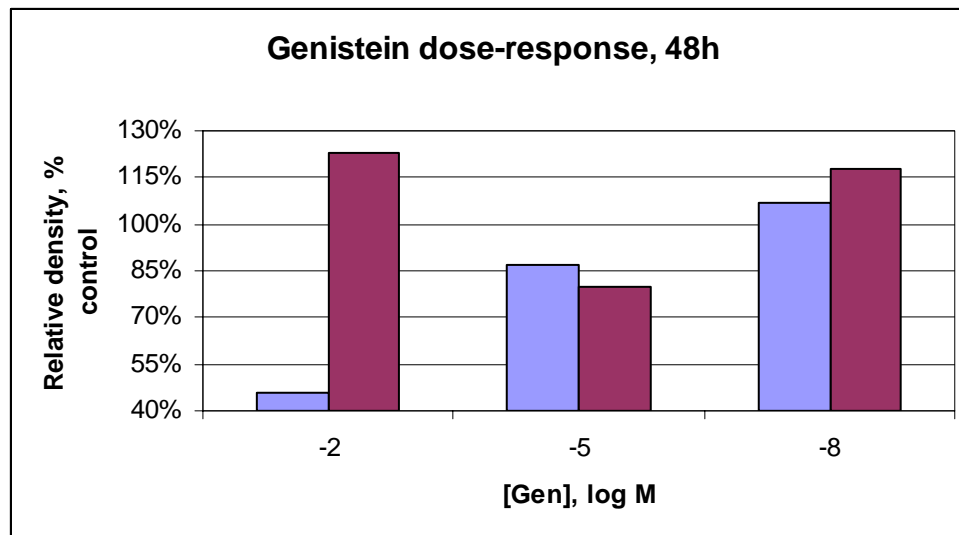
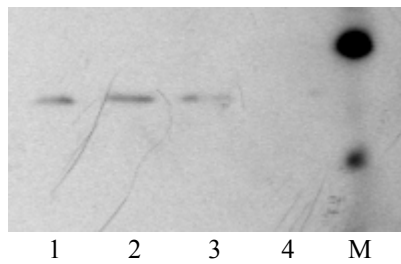


Figure 10. Genistein Dose-Response, 48 Hours. As described previously, cells were treated with the indicated concentrations of genistein for 24 hours, and PCNA was detected by immunoblotting. Data are expressed as mean percent of EtOH-treated control (not shown), n=2, both assays shown.

mg of isoflavones were dissolved in 10 ml of hot (80°C) ethanol. The molecular weight of genistein, 270.24 g/mol, was used to calculate the approximate molar concentration of phytoestrogens. 80 mg/10 ml corresponds to 29.6 mM. As shown in **Figure 12**, all concentrations of phytoestrogens reduce PCNA expression by themselves to undetectable levels after 48 hours. These data could not be quantified or compared to an ethanol-treated control because both the phytoestrogen-treated cells and the ethanol-treated cells had levels of PCNA that were nearly undetectable by immunoblot. Furthermore, it was found that at high concentrations, 10^{-4} M, phytoestrogens abolish proliferation in the presence of 10^{-9} M E_2 . It is worth noting that **Figure 12A** and **Figure 12B** are two Western blots of cells from the same plate; therefore the ethanol-treated samples in **Figure 12A** are the negative control for both sets, and the 10^{-9} M E_2 -treated cells in **Figure 12B** are the positive control for both sets. The quantification of this experiment

A



B

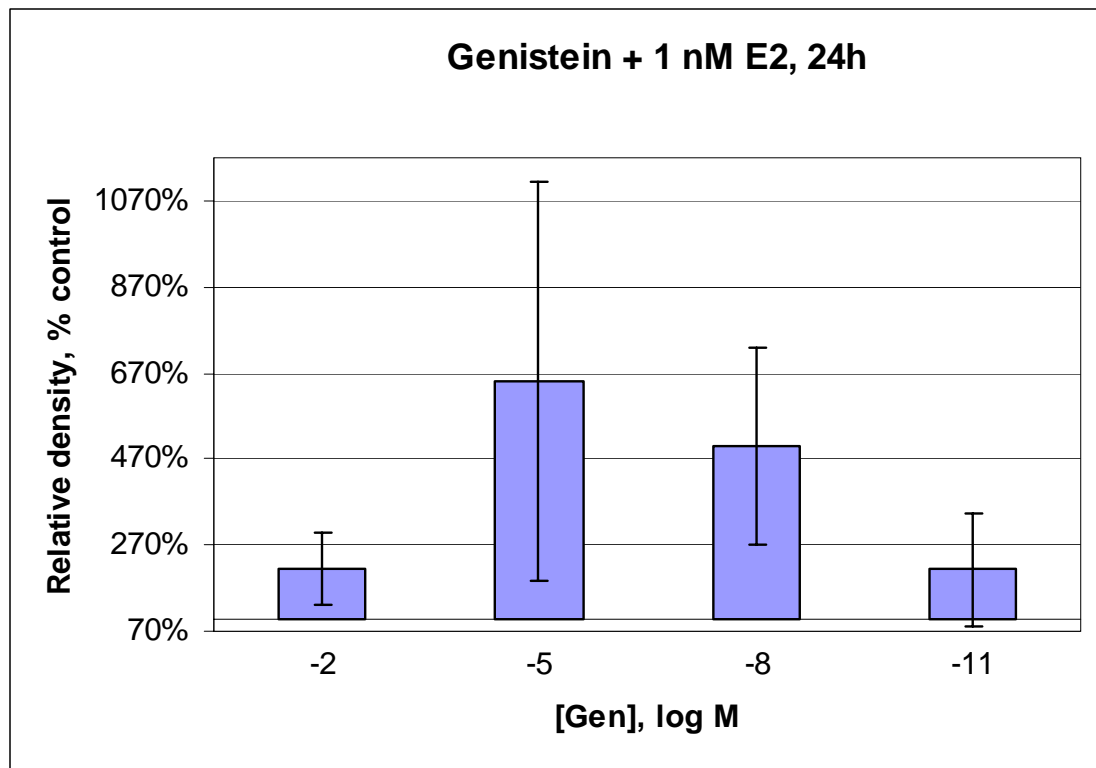


Figure 11. Combination of Genistein and E₂. Both 10⁻⁹ M E₂ and the indicated concentrations of genistein were administered to cells for 24 hours, after which PCNA was detected by Western blotting. **A.** Representative immunoblot. Lane 1: 10⁻² M G; Lane 2: 10⁻⁵ M G; Lane 3: 10⁻⁸ M G; Lane 4: 10⁻¹¹ M G; M: Molecular weight marker. **B.** Densitometric analysis of **A.** Data are expressed as percent of EtOH-treated control (not shown), mean ± SEM, n=4.

(**Figure 12C**) was therefore made in comparison to 10⁻⁹ M E₂-treated control, because the negative control cells expressed no detectable PCNA. The results of this experiment

indicate that independently-administered phytoestrogens are able to block proliferation of breast cancer cells, and high concentrations of phytoestrogens may abrogate E₂-induced proliferation.

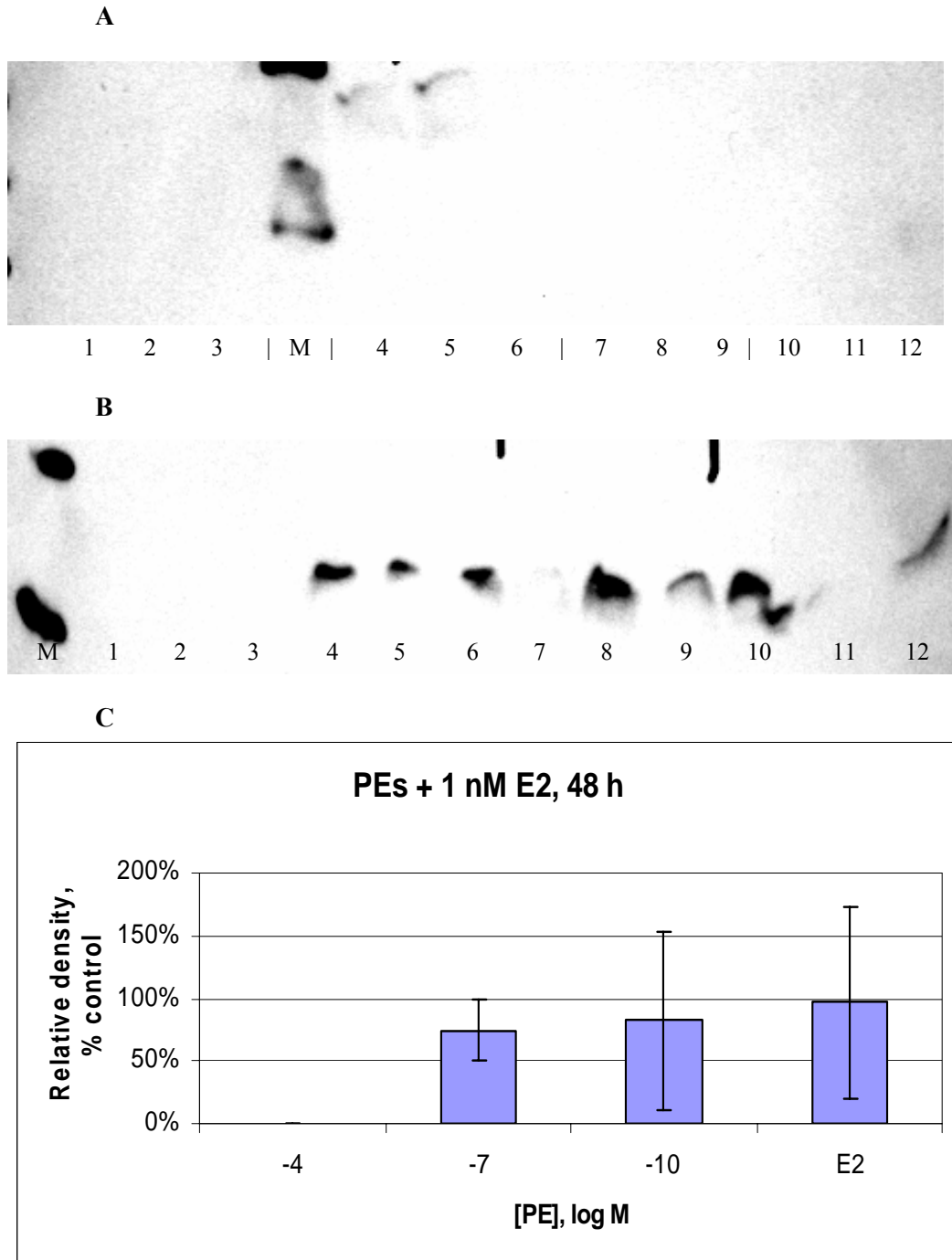


Figure 12. OTC Phytoestrogens Reduce PCNA Expression. **A.** Cells were treated with phytoestrogen extracts as described before for 48 hours. Lanes 1-3: 10^{-4} M PEs; Lane M: molecular weight marker; Lanes 4-6: 10^{-7} M PEs; Lanes 7-9: 10^{-10} M PEs; Lanes 10-12: 10^{-12} M PEs; EtOH-treated control. **B.** Cells were treated with both phytoestrogens and 10^{-9} M E₂. Lanes are as in **A**, except Lane 12: EtOH/ 10^{-9} M E₂ treated control. **C.** Quantification of **B**. Data are expressed as percent of the E₂-treated control (n=3), mean \pm SEM.

Discussion & Conclusions

The goal of this project was to determine if MCF-7 cell proliferation, as monitored by PCNA expression, is influenced by purified phytoestrogens or phytoestrogens extracted from OTC dietary supplements. To that end, immunoblotting was used to detect and measure relative amounts of PCNA in hormone-treated breast adenocarcinoma cells. We hypothesized that genistein would have a biphasic effect on PCNA levels, as reported in the literature using other indices of cell growth; at high concentrations, genistein would be growth inhibitory, but would stimulate proliferation at low concentrations (Allred *et al.*, 2001). OTC phytoestrogens were expected to produce the same result. MCF-7 cells have been used as a standard model for breast cancer cells by many researchers, and are particularly well-characterized. PCNA is a reliable indicator of cell growth in cancer cell lines, including MCF-7 cells (Biesterfeld *et al.*, 1998 and references therein).

As expected, cells treated with estrogen at concentrations comparable to physiological concentrations, between 10^{-10} and 10^{-8} M, responded by increasing expression of PCNA. This is consistent with the effects of estrogen reported in the literature. However, it was not expected that this effect would be most pronounced only after 48 hours. Likewise, it was surprising that at both 24 hours and at 48 hours, estrogen did not seem to elicit a dose-responsive increase in PCNA expression; this may be an artifact of the high variability in these data. Another possible explanation is that at the highest concentration tested, 10^{-7} M, estrogen displays some high-end toxicity, or that high concentrations of estrogen down-regulate ER expression, saturate the ER, or reduce ER or ERE sensitivity. However, none of these effects have previously been reported in

the literature (Pratt and Pollak, 1993). 10^{-9} M E_2 is closer to the physiologic range (10^{-8} to 10^{-10} M), and this concentration has a pronounced proliferative effect at 48 hours. The lowest concentration tested also increased PCNA expression, but not to the same extent as 10^{-9} M. These two data points are more in accordance with the reported dose-dependent effects of estrogen on MCF-7 cells. Testing more concentrations between 10^{-7} and 10^{-11} M may make any dose-responsive trend more apparent, as well as replicating the 48 hour assays. This may be because steroid hormones have a longer half-life *in vivo* compared to peptide hormones (Hadley, 1988).

The data from this project indicate that the phytoestrogen genistein is not growth-inhibitory to MCF-7 cells under the conditions tested here; this is contrary to many published studies (such as Chen *et al.*, 2003; Zhou *et al.*, 2001). The high variability in our data makes it difficult to say with confidence that the data presented here dispute these studies. The biphasic effect of genistein, and other phytoestrogens, may partially explain this discrepancy. As stated before, genistein acts as an estrogen agonist in breast cancer cells at low concentration (Allred *et al.*, 2001) but as an antagonist at high concentrations. The results from this project indicate that genistein stimulates proliferation of breast cancer cells when treated for 24 hours. When genistein was administered for 48 hours, however, it reduced the expression of PCNA. This may be because only after 48 hours was a high enough dose achieved to trigger the anti-proliferative effect. Further replication of the 48 hour time-course may help substantiate this idea.

The results from this project indicate that genistein and estrogen act synergistically. However, the data obtained only extended the treatment for 24 hours. At

this time point, unopposed genistein administration promotes estrogen-induced cell growth. It is therefore not surprising that genistein and estrogen cooperatively elevate PCNA expression. There are, however, no published reports that indicate any time-dependency in the growth-inhibitory effect of phytoestrogens. It remains to be seen whether MCF-7 cells treated with a high concentration of genistein for at least 48 hours, in the presence of physiologically relevant concentration of estrogen, would proliferate. It is plausible that after a sufficient duration, genistein would interfere with or possibly abolish the estrogen-induced up-regulation of PCNA. It is important to remember that PCNA is a marker of cell proliferation; absence or reduction of PCNA implies a cytostatic effect, not a cytotoxic effect. While genistein may inhibit estrogen-induced proliferation after a sustained dose, there may remain some initial proliferative effect due to genistein. If genistein is not administered for a sufficient amount of time, no antiproliferative effect may be attained. Furthermore, there may be some enhancement of neoplastic potential. Clinically, this raises the issue of patient non-compliance.

The effect of OTC phytoestrogen dietary supplements on breast cancer cell replication was a major focus of this project. We hypothesized that if these supplements contain bioavailable phytoestrogens, at high concentrations they would impede proliferation; this is what happened. At all concentrations, unaccompanied phytoestrogens reduced PCNA levels below the detection limit of our assay. This is especially significant because genistein alone, even at 100-fold greater concentration, was never able to abolish PCNA expression. This may indicate that the presence of multiple phytoestrogens has a cumulative benefit greater than any single compound.

When cells were treated with OTC phytoestrogens in conjunction with 10^{-9} M estrogen, the expected biphasic effect was seen. At the highest concentration of phytoestrogens tested, 10^{-4} M, no PCNA could be detected. As the concentration of phytoestrogens was reduced, PCNA appeared to increase relative to the ethanol treated control. This is what the literature predicts, both for purified phytoestrogens *in vitro*, as well as epidemiological effects. In this experiment, hormone treatment was carried out for 48 hours, which may explain why the data better reflect other research in the literature regarding *in vivo* administration of phytoestrogens (Ashby *et al.*, 1999; Bowers *et al.*, 2000).

The results of this project indicate that PCNA expression can be used to assess the proliferative effect of estrogen and phytoestrogens on MCF-7 cells. Genistein by itself has a dichotomous effect on cell growth; at low doses, it will promote cell proliferation, while at high doses it will inhibit proliferation. Furthermore, a high enough dose to demonstrate the anti-proliferative effect cannot be achieved in 24, or possibly less than 48 hours. This idea is supported by the result seen when cells are treated with only OTC phytoestrogens. Because of this, we conclude that low doses of genistein combine with simultaneously administered estrogen to enhance PCNA expression. It is predicted that high concentrations of genistein administered for more than 24 hours will reduce the stimulatory effect of estrogen. The basis for this hypothesis is the result of treating MCF-7 cells with OTC phytoestrogens in the presence of estrogen. This experiment showed that at high concentrations, phytoestrogens prevent the proliferative effect of estrogen. Our data suggest that phytoestrogens are suitable to treat post-menopausal symptoms and reduce the risk of—or possibly combat—breast cancer.

Although PCNA does demonstrate the proliferative or antiproliferative effects of phytoestrogens, additional indices of cell proliferation may help reduce the variability in our data. Additionally, PCNA expression does not indicate if phytoestrogens act through the estrogen receptor and estrogen-response element, or another cell-signaling pathway. IGFBPs are implicated as an indicator of a compound's estrogenicity (Pratt and Pollak, 1993; Maxwell and van den Berg, 1999; Qin *et al.*, 1999), and may help elucidate if phytoestrogens act through the estrogen receptor. It was not possible to obtain enough data to fully investigate this. However, preliminary data (**Figure 13**) indicate that this may be an appropriate way to focus on phytoestrogens' mechanism of action in this model system.

Phytoestrogens, as already mentioned, are a very diverse group of compounds, and there is no consensus structural requirement to classify a specific compound as a phytoestrogen. Investigating the composition of OTC phytoestrogen dietary supplements would have two benefits. First it would establish the composition of these supplements, which are not regulated by the Food and Drug Administration. Secondly, it would be interesting to look at co-administering more than one type of phytoestrogen, to determine if different compounds interfere constructively or destructively. A structure-activity relationship study may help narrow down not only the mechanism of action of phytoestrogens, but could also explain why different phytoestrogens have different effects.

Because estrogen is such a pleiotropic hormone, it is worthwhile to explore the effects of phytoestrogens in different cell model systems. Two of the goals of estrogen/hormone replacement therapy are to maintain cardiovascular health, and to

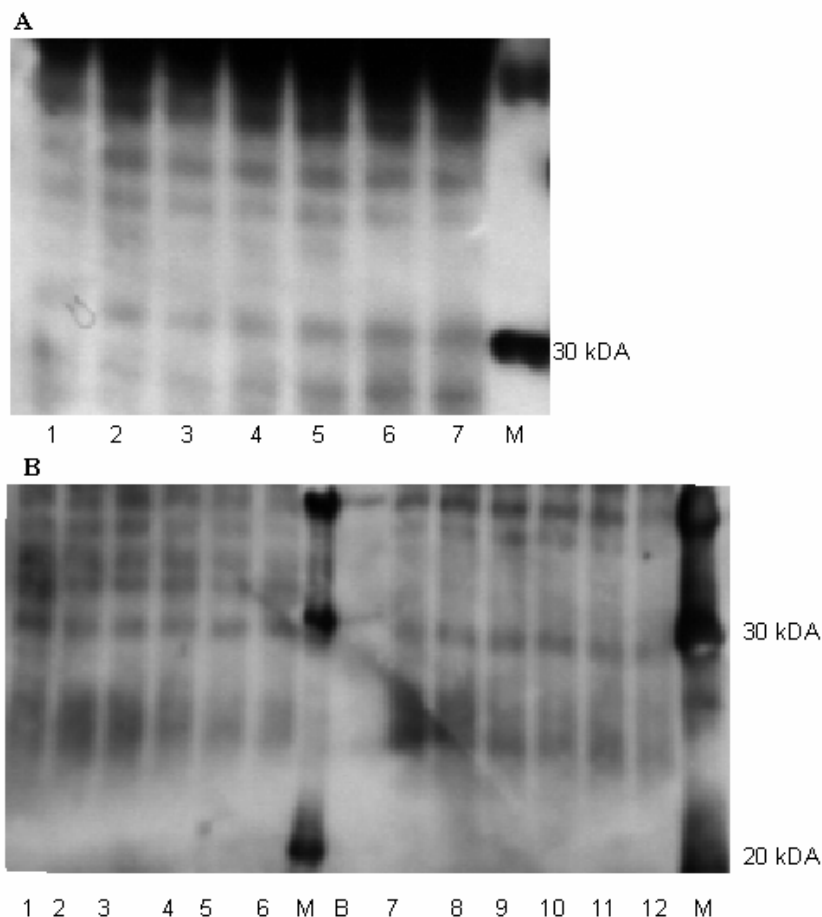


Figure 13. Two representative IGFBP-4 Immunoblots. As described in Materials and Methods, conditioned media were assayed for IGFBP-4 by immunoblot. IGFBP-4 migrates at approximately 30 kDa. **A.** Genistein and 10^{-8} M E_2 . Lane 1: EtOH control (-G/- E_2); Lane 2: 10^{-2} M G; Lane 2: 10^{-3} M G; Lane 3: 10^{-4} M G; Lane 4: 10^{-5} M G; Lane 5: 10^{-6} M G; Lane 6: 10^{-2} M G (- E_2); Lane 7: 0 G, 10^{-8} M E_2 . **B.** Phytoestrogen-treated cells. Lane 1: EtOH control; Lane 2: 10^{-4} M PE; Lane 3: 10^{-5} M PE; Lane 4: 10^{-6} M PE; Lane 5: 10^{-7} M PE; Lane 6: EtOH control; Lane M: molecular weight marker; Lane B: blank/empty lane; Lanes 7-12: same as Lanes 1-6.

maintain bone density. The human osteoblast cell line hFOB 1.19, and primary mouse/rat osteoblast and osteoclast cultures would be useful model systems. The human embryonic vascular endothelial cell lines HUV-EC-C would be indicated to assess the effect of phytoestrogens on vascular tissue. Since alterations in hepatocyte metabolism are implicated in estrogen's mechanism to promote cardiovascular health, human hepatoma cells—HepG2 or C3A—may also be worth examination.

The clinical implications of this project support the idea that phytoestrogens may prevent and treat breast cancer. However, it is important to note that physiologically, the maximum reported plasma concentrations reached by dietary consumption of phytoestrogens was 13-18 μM (Murphy *et al.*, 1997). This is only slightly lower than the maximum concentration of OTC phytoestrogens tested in this project; thus concentrations of phytoestrogens high enough to prevent breast cancer may not be attainable through normal diet. Therefore, sustained dietary supplements may be necessary to achieve phytoestrogens' beneficial effects. As mentioned earlier, phytoestrogens may require a sustained dose in order to achieve anti-cancer effects; it is pertinent for any clinicians to consider the issue of patient non-compliance. Additionally, the purity of the OTC supplements utilized in this project could not be determined. It is possible that the antiproliferative effects of these supplements may be due to coordinated activity of multiple phytoestrogens, and that other OTC supplements, containing different phytoestrogens, will have different effects.

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