FLAVONOIDS AND STEROID HORMONE-DEPENDENT CANCERS

by

Rachel Stacey Rosenberg Zand

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Nutritional Sciences
University of Toronto

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FLAVONOIDS AND STEROID HORMONE-DEPENDENT CANCERS
DOCTOR OF PHILOSOPHY 2001
RACHEL STACEY ROSENBERG ZAND
GRADUATE DEPARTMENT OF NUTRITIONAL SCIENCES
UNIVERSITY OF TORONTO

ABSTRACT

Steroid hormone-dependent cancers, including breast and prostate, are leading causes of cancer morbidity and mortality in North America. In Asian countries, these diseases are much less common. Nutritional and lifestyle factors are associated with differences in incidence, with an emphasis on consumption of plant foods, in particular soy. Flavonoids are one class of compounds present in these plant foods. With over 4000 identified members, these compounds have been hypothesized to have several important chemopreventive effects.

The purpose of this project was to assess the steroid hormone activities of plant foods, with a reference to soy, in three forms: pure flavonoids, plant extract (nutraceutical), and whole (functional) food. After developing an in vitro bioassay, soy isoflavones and other flavonoids were evaluated for steroid hormone activities, defined as their ability to induce or inhibit production of estrogen- (pS2) and androgen/progestin- (prostate-specific antigen, PSA) regulated proteins. Genistein and biochanin A demonstrated highest estrogenic activity of the compounds tested, which was dose-response down to $10^{-8}$ M and $10^{-7}$ M, respectively. These soy isoflavones also demonstrated significant anti-androgen activity (76% and 98%, respectively at $10^{-5}$ M).

Natural products and nutraceuticals were then tested for steroid hormone activities. As with pure isoflavones, the commercial soy and red clover extracts had
high estrogenic activity. Soy extract demonstrated 63% anti-androgen activity at the highest strength tested.

Finally, we determined the steroid hormone potentials of one functional food, soy, in healthy subjects. By using biological fluids obtained from individuals in a soy feeding study, we found that short-term feeding did not increase agonist potentials. Moreover, non-significant reductions in estrogenic and androgenic activities in women and men, respectively, may indicate that long-term feeding of this functional food may reduce risk factors for steroid-hormone dependent cancers.

These results indicate that soy isoflavones and other flavonoids have steroid hormone activities. These activities may act in vivo to modulate activities of potentially carcinogenic endogenous hormones. Further research is needed to determine the physiological importance of flavonoids in prevention and/or management of hormone-dependent cancers, and how to make best use of natural products, nutraceuticals and functional foods in individuals at risk.
ACKNOWLEDGEMENTS

I would like to first and foremost acknowledge and thank my supervisors, Drs. David Jenkins and Eleftherios Diamandis, for their mentorship, assistance and patience with me over the past 5 years. They have taught me many lessons, both scientific and life, and will remain major influences in my life's path. Thank you to my advisors Drs. Venket Rao and Tibor Heim, for always being there when I needed their guidance, or just their ear.

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And finally, to my husband, Frank Zand, thank you for putting up with all my day-to-day worries, concerns, joys, and frustrations that made up this experience. May we share only successes in the years to come.
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<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AF</td>
<td>Activation function</td>
</tr>
<tr>
<td>AIN</td>
<td>American Institute of Nutrition</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARE</td>
<td>Androgen response element</td>
</tr>
<tr>
<td>BaP</td>
<td>Benzo(a)pyrene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum antigen</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMAB</td>
<td>3,2'-Dimethyl-4-aminobiphenyl</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>ECG</td>
<td>Epicatechin gallate</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>F</td>
<td>Fischer</td>
</tr>
<tr>
<td>FAD</td>
<td>Focal area of dysplasia</td>
</tr>
<tr>
<td>GCDFP</td>
<td>Gross cystic disease fluid protein</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid response element</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High density lipoprotein cholesterol</td>
</tr>
<tr>
<td>hK2</td>
<td>Human glandular kallikrein 2</td>
</tr>
<tr>
<td>HRE</td>
<td>Hormone response element</td>
</tr>
<tr>
<td>17β-HSD</td>
<td>17β-Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>17β-HSOR</td>
<td>17β-Hydroxysteroid oxidoreductase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein</td>
</tr>
<tr>
<td>NAF</td>
<td>Nipple aspirate fluid</td>
</tr>
<tr>
<td>NMU</td>
<td>Nitrosomethylurea</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PhIP</td>
<td>2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PRE</td>
<td>Progesterone response element</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>RIA</td>
<td>Radiometric imunoassay</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>SAMlg</td>
<td>Sheep anti-mouse immunoglobulin G</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>SPI</td>
<td>Soy protein isolate</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end buds</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-Tetradecanoyl phorbol 13-acetate</td>
</tr>
</tbody>
</table>
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Papers


Posters


CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Hormone-dependent cancers, including those of the breast and prostate, are leading causes of cancer morbidity and mortality of women and men in North America (1). Current medical therapies focus on hormone treatment, whether it be hormone antagonists, e.g. antiestrogens; strong hormone agonists, e.g. progestin derivatives; or drugs aimed at inhibiting the formation of more carcinogenic forms of hormones, e.g. 5α-reductase inhibitors to block production of dihydrotestosterone. Alternative therapies have looked towards natural products including foods and herbs to combat, or complement treatments of these diseases. Western medicine originally dismissed such alternatives as quackery. However, researchers have discovered that several plants and plant foods contain compounds very similar in structure to the anti-hormones and hormones given in pharmaceutical treatments, as well as endogenous steroid hormones. One group of these compounds are the flavonoids, a class of over 4000 members which are concentrated in plant foods, including fruits, vegetables, legumes, red wine and tea.

Although many epidemiological studies have related consumption of soy isoflavones to lower risks of steroid hormone-dependent cancers, very little is known about the mechanisms by which these reductions of risk occur. Therefore, the purpose of this research was to assess one mechanism by which flavonoids, in general, and soy isoflavones, specifically, may reduce incidences of these cancers. Through an in vitro bioassay, the steroid hormone activities of flavonoids were determined: as pure compounds, as extracts of natural products and nutraceuticals, and finally, as biological
fluids from subjects on soy feeding studies. These results may provide further insight into how flavonoids can be used in the prevention and/or management of steroid hormone-dependent cancers.

1.2 Literature Review

1.2.1 Flavonoids and Steroid Hormone-dependent Cancers

1.2.1.1 Introduction

Steroid-hormone dependent cancers, including those of the breast, prostate and colon, are leading causes of morbidity and mortality in western countries. In Asian countries, particularly rural areas, these diseases are relatively uncommon. Epidemiological studies contribute these differences in incidence to environmental causes, particularly those of diet and lifestyle. Dietary factors include low consumption of fruit, vegetables and legumes, particularly soy, in the west. Flavonoids are one component of these plant foods being investigated for their role in chemoprevention.

Flavonoids are phenolic compounds characterized by their diaryl nucleus (Figure 1.1). They are structurally similar to steroid hormones (Figure 1.2), particularly estrogens, and therefore have been studied over the past several years for their potential roles in prevention of hormone-dependent cancers. Over 4000 flavonoids have been characterized in edible plant foods, and are consumed in highest amounts in soy, apples, red wine, tea and onions (Table 1.1)(1). Human consumption of all flavonoids has been estimated to be a few hundred milligrams to 1 g per day (2).
Figure 1.1. **Structures of Flavonoids.** General structures of flavones, isoflavones, flavanones and flavans.
Figure 1.2. Structures of Steroid Hormones and Antagonists
Table 1.1. Food Sources and Structures of Specific Flavonoids

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Food Source</th>
<th>Structure</th>
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</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>Celery</td>
<td><img src="image1.png" alt="Structure" /></td>
</tr>
<tr>
<td>Biochanin A</td>
<td>Soy</td>
<td><img src="image2.png" alt="Structure" /></td>
</tr>
<tr>
<td>Catechin</td>
<td>Tea</td>
<td><img src="image3.png" alt="Structure" /></td>
</tr>
<tr>
<td>Coumestrol</td>
<td>Soy</td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
<tr>
<td>Daidzein</td>
<td>Soy, other legumes</td>
<td><img src="image5.png" alt="Structure" /></td>
</tr>
</tbody>
</table>
| Equol       | Soy, other legumes | \[
\begin{align*}
\text{HO-} & \text{O-} \\
\text{HO} & \text{O-} \\
\text{OH} & \\
\end{align*}
\] |
|------------|-------------------|---|
| Genistein  | Soy, other legumes| \[
\begin{align*}
\text{HO-} & \text{O-} \\
\text{HO} & \text{O-} \\
\text{OH} & \\
\end{align*}
\] |
| Hesperetin | Orange, lemons    | \[
\begin{align*}
\text{HO-} & \text{O-} \\
\text{OH} & \text{OCH}_3 \\
\end{align*}
\] |
| Kaempferol | Broccoli, tea     | \[
\begin{align*}
\text{HO-} & \text{O-} \\
\text{HO} & \text{O-} \\
\text{OH} & \\
\end{align*}
\] |
| Luteolin   | Celery            | \[
\begin{align*}
\text{HO-} & \text{O-} \\
\text{OH} & \text{O-} \\
\text{OH} & \\
\end{align*}
\] |
This review shall present the current knowledge and research available in the field of flavonoids and steroid hormone-dependent cancers. Several mechanisms will be discussed, from associations made through epidemiology, through in vitro studies to animal models and human trials. Much work is still needed to firmly establish the chemopreventive applications of flavonoids, and the foods in which they are contained.

1.2.1.2 Epidemiological Evidence

The original associations between flavonoids and steroid hormone-dependent cancers were made through several ecological studies looking at the low incidences of breast, colon and prostate cancers in Asian countries compared to western ones. Soy consumption is very high in Asia compared to North America and Europe, and it was found that this food could be negatively correlated with cancer incidence (3-5). Since
these initial studies, many other plant foods have been studied for their associations with these cancers.

Most epidemiological studies have concentrated on whole foods, not components present within them. Through these studies, many interesting associations have been made between vegetable consumption and various cancers. Messina et al have extensively reviewed the in vivo and in vitro data of soy, showing protective effects in both animal models and epidemiologic studies (6). Steinmetz and Potter (7) have reviewed over 200 case-control and cohort studies, finding that over the majority of those investigating cancers of the breast, colon, cervix, endometrium and ovary have shown statistically significant decreases in incidence with vegetable and/or fruit consumption (8-60). The only site for which such a relationship was not found was the prostate (60-64). However, a Seventh-day Adventist study, involving over 34,000 vegetarians and omnivores, found that vegetarians had significantly reduced risks of colon and prostate cancer rates.

Epidemiological studies looking specifically at certain flavonoids are becoming more important, as databases and techniques for quantifying consumption of these compounds are improving (61-66). Flavonoids present in highest amounts in the human diet include the soy isoflavones genistein, daidzein and biochanin A, flavonols quercetin, myricetin and kaempferol, and the flavones luteolin and apigenin (2,71). One case-control study used this new database (70) to look at 83 prostate cancer patients and 107 age-matched controls. They found that after adjustments for total calories, greater consumption of most phytoestrogens, including isoflavones and other flavonoids, had a slightly protective effect on prostate cancer risk. Moreover, genistein,
daidzein, and coumestrol (a structurally-related compound) showed the strongest protective associations. However, the overall intake of the isoflavones was very low compared to Japanese men (1.2 mg versus 39.2 mg/day). Some studies have reported weak protective effects of flavonoids from fruits and vegetables and prostate and colon cancers (71,72). Others have shown no associations between flavonoid intake and cancer mortality or incidence (73,74). At present, relationships between whole foods, i.e. vegetables and soy, show much stronger chemopreventive associations than the flavonoids that they contain.

1.2.1.3 In Vitro and Animal Models

Over the last few years, various mechanisms by which flavonoids may prevent, or even treat steroid hormone-dependent cancers, have been investigated. These include activities at the tissue, cellular (Table 1.2), and even sub-cellular levels. We will concentrate here on steroidal activities, proliferation, antioxidant activities, signal transduction, and tumorigenic pathways.

**Estrogenic Activity**

Estrogenic effects of flavonoids were first discovered through determining the cause of infertility in Australian sheep eating red clover. The leaves contain up to 5% by dry weight of the soy isoflavones biochanin A and formononetin (75,76). Of the flavonoids, the soy isoflavone genistein has remained the model "phytoestrogen". Genistein is structurally similar to estradiol, other steroid hormones (testosterone,
Table 1.2. Cell Lines Used

<table>
<thead>
<tr>
<th>Type</th>
<th>Cell Line</th>
<th>Steroid Receptor Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>MCF-7</td>
<td>ER, PR (+)</td>
</tr>
<tr>
<td></td>
<td>BT-474</td>
<td>ER, PR, AR (+)</td>
</tr>
<tr>
<td></td>
<td>T47-D</td>
<td>ER, PR, AR (+)</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-468</td>
<td>ER, PR (-)</td>
</tr>
<tr>
<td>Prostate</td>
<td>LNCaP</td>
<td>AR (+)</td>
</tr>
<tr>
<td></td>
<td>VeCaP</td>
<td>AR (-)</td>
</tr>
<tr>
<td></td>
<td>PC-3</td>
<td>AR (-)</td>
</tr>
<tr>
<td>Colon</td>
<td>HT-29</td>
<td>none (VDR (+))</td>
</tr>
<tr>
<td></td>
<td>Caco-2</td>
<td>none</td>
</tr>
<tr>
<td>Ovary</td>
<td>OVCAR-5</td>
<td>ER (+)</td>
</tr>
</tbody>
</table>

progesterone), and synthetic inhibitors including tamoxifen. This isoflavone possesses 1/1000 the estrogenic activity of estradiol (77-79). Circulating concentrations in individuals consuming moderate amounts of soybeans are nearly 1000-fold higher than peak levels of endogenous estradiol in premenopausal women (80-82). Genistein and other soy isoflavones have been shown to compete with radiolabeled estradiol for binding to the estrogen receptor (83). This may be because of the structural similarity between the steroid nucleus of 17β-estradiol and the polyphenolic ring structure of isoflavones. Since other plant-derived flavonoids also possess this structure, they too have become relatively well-studied for estrogenic and anti-estrogenic activities. In contrast, androgenic activities of flavonoids have only recently started being investigated. The data obtained from these studies support important applications for these compounds and the foods in which they are found, in prostate cancer prevention.

Steroid hormone activities are important because they are mechanisms by which cells communicate with their environment. Direct agonistic activities consist of, in simplified terms, a ligand (estrogen, E or androgen, A) binding to a nuclear receptor
(estrogen, ER or androgen, AR), resulting in the dimerization of the nuclear receptor. This ligand-nuclear receptor complex then binds to a hormone response element (HRE, either estrogen, ERE or androgen, ARE) on estrogen- or androgen-dependent genes, respectively, leading to its transcription. The mRNAs are then translated, and proteins are produced, exerting biological responses (Figure 1.3). Beato et al describe this mechanism in much greater detail (84). The effects of steroid hormones in the carcinogenesis process are very complex. Ovariectomy is the oldest treatment for breast cancer (85). Surgical castration is still an option for prostate cancer (86), and chemical castration is a common strategy for managing its aggressiveness today (87-89). Timing of estrogens may also be important. Some studies indicate that while exposure to diethylstilbestrol (DES) in utero may predispose children to adult cancers several decades later (90-94), prepubertal estrogens may be protective (95). During adulthood, these hormones are once again linked to carcinogenicity.

The steroid hormone pathways described above are utilized in the development of assays to determine the agonistic and antagonist steroid hormone activities of compounds. This is accomplished either through measurement of estrogen-or androgen-regulated proteins, reporter proteins such as chloramphenicol acetyl
Figure 1.3. Regulation of Steroid Hormone-Regulated Genes. When a ligand diffuses into the cell, it will bind to its respective steroid hormone receptor in the nucleus of the cell. The receptor is then activated through dimerization and phosphorylation, and the steroid hormone-steroid hormone receptor complex may then bind to a hormone response element (HRE) in the promoter region of a steroid hormone-dependent gene. The gene is then transcribed to mRNA, and further translated to protein. This protein may remain in the cell, or may be secreted into the supernatant surrounding the cell. Several techniques are currently used to measure steroid hormone activity, based upon this mechanism.
transferase (CAT) and luciferase, with ERE or ARE promoters, or Southern (using cDNA) and Northern blots.

Zava et al have determined estrogenic and anti-estrogenic activities of several flavonoids, including genistein, equol, kaempferol and hesperetin using two methods in ER(+) and ER(-) breast cancer cell lines (96). The first involved incubating ER(+) cells (MCF-7) in tubes containing both $[^{125}]$estradiol and flavonoid for 5 minutes at 4°C, aspirating the supernatant, and measuring the $[^{125}]$estradiol remaining in the nuclei, using a gamma counter. Results were then expressed as percent $[^{125}]$estradiol binding to ER in the nucleus in the absence or presence of increasing concentration of flavonoid, from 100 pM to 10 μM. This technique demonstrated specific binding of test compound to ER, and thereby, anti-estrogenic activity. The second technique, that to measure estrogenic activity, involved measuring the estrogen-regulated, secreted protein, pS2, after incubating the cells with flavonoid at the above concentrations for 72 hours. The assay used here was a two-site enzyme-linked immunosorbent assay. Genistein and equol, the metabolite of the soy isoflavone daidzein, were found to have the highest binding affinities, 0.1 that of estradiol. Next came kaempferol (0.012), and finally quercetin (0.001). Hesperetin and β-naphthoflavone did not have blocking activity. All of the compounds found to bind to the ER in the previous study stimulated pS2 production, in the same order as their binding activity. Blocking of pS2 production by tamoxifen further confirmed that binding to the ER was the mechanism observed. Similar results for genistein and other flavonoids were seen through measuring pS2 and/or cathepsin-D transcription (83,97-99). Our group (100) has evaluated estrogenic activity of over 70 flavonoids and structurally-related compounds using pS2
concentrations in the supernatant as the endpoint. Structure-function relationships were discussed in this study, with the diphenolic structure, and positions of hydroxyl groups residing in the C-7 or 4’ positions (Figure 1.1) being most important for estrogenic activity. Flavonoids with hydroxyl groups at the C-3 position, such as quercetin, hinders binding, as is reflected by the low estrogenic/binding affinity in both studies. At least two other studies, using different systems, concur with these results. Miksicek assessed estrogenicity of flavonoids (at 1 μM) using HeLa cells transfected with wild-type, recombinant estrogen cDNA expressed from the plasmid pER-18, and an estrogen-responsive reporter plasmid system measuring production of CAT (pERE-TK-CAT). Genistein displayed highest activity, with significant activities also being seen with kaempferol, apigenin, biochanin A and daidzein. The importance of hydroxyl groups at carbon positions 4’, 7 and/or 6 was shown here, as all compounds determined to have estrogenic activity possessed hydroxyl groups at one or more of these positions (101). Binding to the ER was confirmed through competition assays with [3H]estradiol. Le Bail et al used a stably-transfected MCF-7 cell line with a luciferase reporter gene and ERE to determine estrogen agonist and antagonist activities. Significant agonist activities were seen for compounds containing 7 and/or 4’ hydroxyl groups at concentrations as low as 1 μM. Moreover, they determined that phenolic hydroxyl groups in positions 4’ and 7 could be considered to be equivalent to the position 3 and 17-hydroxyl groups of estradiol (102). These results were also confirmed using a recombinant yeast strain stably transfected with the human ER gene, with upregulation of this gene being the measured endpoint (103). Finally, resveratrol, a red wine polyphenol, not a flavonoid, but possessing a similar structure, has also been shown to
bind to ER in the \([^{125}\text{I}]\)estradiol competition assay, and to induce luciferase production in both MCF-7 and T47-D cell lines transfected with ERE-tk109-luc or ERE2-tk109-luc plasmids. Resveratrol also increased progesterone receptor mRNA expression and pS2 expression in MCF-7 cells at 10 \(\mu\)M concentrations (104).

Several researchers have investigated the androgenic and anti-androgenic activities of flavonoids. None have found these compounds to have agonist androgenic activities, but most have observed anti-androgenic (105) and/or inhibition of androgen-mediated activities. Our group evaluated inhibition of prostate-specific antigen (PSA) production in an ER (+), AR (+) breast cancer cell line (BT-474) and prostate cancer cell transfected with the human AR (PC-3(AR)2). 18 of the 72 flavonoids and related compounds tested demonstrated such inhibition of PSA production at concentrations of 10 \(\mu\)M. Several compounds, including genistein and biochanin A, had blocking effects on PSA production at concentrations as low as 0.1 \(\mu\)M (106,107). Davis et al examined modulation of PSA expression in the LNCaP (androgen-dependent) VeCaP (androgen-independent) prostate cancer cell lines by genistein. This isoflavone had differential effects on PSA expression in the two cell lines. PSA mRNA and protein expression and secretion were suppressed in the LNCaP cell line at all concentrations, while only high concentrations of genistein inhibited PSA expression in VeCaP. Inhibition of cell proliferation in VeCaP was independent of PSA signaling pathways, leading the authors to conclude that the anti-proliferative effects of genistein were irrespective of androgen responsiveness in this androgen-refractory cell line (108).

Resveratrol, in concentrations of 50, 100 and 150 \(\mu\)M, was given to LNCaP cells in the presence or absence of 1 nM mibolerone, a non-metabolized, synthetic androgen,
to evaluate expression of two androgen-regulated proteins, PSA and human glandular kallikrein 2 (hK2), as well as AR. The cells were transfected with either a PSA promoter-luc, hK2 ARE/minimal thymidine kinase promoter/CAT or an AR promoter-luc construct. Resveratrol treatment completely abolished androgen-induction of the PSA promoter, hence inhibiting production of any PSA. Transfection with triple ARE constructs demonstrated similar results. Western blotting of AR protein levels indicated a dose- and time-dependent inhibition of AR with resveratrol, and ARA70, a co-activator, was found through Northern blotting to decrease, with a maximum reduction using 100 µM resveratrol. These results indicate that AR-mediated gene expression and cell processes are affected by resveratrol, and, moreover, repression of expression of ARA70 may further enhance inhibitory effects of this red wine polyphenol on androgen action (109). Green tea polyphenols have been shown to have similar repressive effects on androgen action in mice (110).

Several enzymes responsible for steroid hormone production and metabolism can be inhibited by flavonoids. These include aromatase, sulfatases, hydroxylase enzymes, 5α-reductase, and 17β-hydroxysteroid oxidoreductase enzymes (Figure 1.4). Aromatase is a cytochrome P450 complex responsible for conversion of androgen to estrogen. Inhibition of this enzyme may thus reduce estrogen-dependent cancers. Jeong et al tested 28 flavonoids against partially purified aromatase prepared from human placenta. Over 50% of the flavonoids tested significantly inhibited aromatase activity, with greatest activity seen with apigenin, chrysin and hesperetin (111). In preadipocytes, the flavonoid chrysin has been shown to have similar potency and effectiveness against aromatase as aminoglutethimide, a pharmaceutical aromatase
Figure 1.4. Main Biosynthetic and Inactivating Pathways of Estrogens and Androgens. DHEA – dehydroepiandrosterone; DHEA-S – DHEA-sulfate; E₁ – estrone; E₂ – estradiol; DHT – dihydrotestosterone; Test – testosterone.
inhibitor (112). Other flavonoids with anti-aromatase activity include luteolin and kaempferol (113). Using radioactive labeling and HPLC, various flavonoids were shown to have inhibitory effects on both aromatase and 17β-hydroxysteroid dehydrogenase. A hydroxyl group at C-7 was essential for anti-17β-hydroxysteroid dehydrogenase activity. Flavonoids with 7-methoxy or 8-hydroxyl groups on the A ring showed only aromatase activity (114).

Inhibition of estrone sulfatase activity, a route for estrogen synthesis, is another mechanism for breast cancer prevention. Quercetin, kaempferol, and naringenin were found to inhibit this enzyme at concentrations of 10 – 150 µM in human liver microsomes (115). These compounds were found to dose-dependently inhibit estradiol metabolism in concentrations of 10 to 500 µM. No influence on estrone formation was observed, while these compounds demonstrated potent inhibition of estriol formation. The proposed mechanism for these results was described to be inhibition of the cytochrome P450III/A4 enzyme, which reversibly hydroxylates 17β-estradiol → estrone → estriol (116). Similarly, 17β-hydroxysteroid oxidoreductase enzymes (17β-HS0Rds) catalyze the reversible reaction between weak estrone (oxidized form) and highly active estradiol. One study (117) examined whether flavonoids inhibit reduction of estrone to 17β-estradiol by one of these enzymes, 17β-hydroxysteroid dehydrogenase (17β-HSD). This enzyme is expressed in steroidogenic cells including ovarian granulose cells and target tissues of estrogen action, including normal and malignant breast and endometrium, and in the epithelium of prostatic urethra and collecting ducts (118-121). By measuring conversion [3H]estrone and [3H]estradiol, genistein and coumestrol were found to dose-dependently block 17β-HSD. This inhibition was NADPH-dependent.
Similarly, in P. testosteronii, this enzyme was competitively inhibited by daidzein and genistein (122). 5α-reductase has also been inhibited by isoflavones in a Sprague Dawley (SD) rat model (123). These results indicate that flavonoids may exert some of their biological effects by modulating the activities of enzymes responsible for metabolizing steroids critical to hormonal functions. By doing so, dietary flavonoids may help prevent steroid hormone-dependent cancers.

**Cell Growth and Signal Transduction**

Many studies have investigated proliferative effects of flavonoids, and mechanisms by which stimulation or inhibition of cell growth may occur. Several experiments have examined the biphasic effects of genistein and other isoflavones, which in ER(+) MCF-7 and T47-D cells stimulate cell growth at concentrations of 10 nM – 10 μM, and inhibit proliferation at concentrations of 50 – 100 μM (96,98,102,113,124,125).

Over 30 flavonoids have been tested for effects on cell proliferation and potential cytotoxicity, in a variety of cell lines, including colon cancer cell lines Caco-2 and HT-29, and the breast cancer cell line MCF-7, for their influence on proliferation and apoptosis, measured through caspase-3. EC$_{50}$ values ranged between 40 and 200 μM. In almost all cases, inhibition by flavonoids occurred in the absence of cytotoxicity. Only baicalein, myricetin and flavone were able to induce apoptosis in Caco-2 and HT-29 cells. No alterations of cell growth have been seen for taxifolin, hesperitin or catechin in the HT-29 cell line (126,127). In the breast cancer cell lines tested, 7-hydroxyflavone, 7,8-dihydroxyflavone and kaempferol did not alter proliferation either (102).
Other flavonoids that are inhibitory at high concentrations in both ER (+) and ER (-) breast cancer cell lines include quercetin, luteolin, biochanin A, coumestrol, daidzein, kaempferol and apigenin (99,124,128). None were shown to work through cytotoxic mechanisms. However, when MCF-7 cells were exposed to 50 or 100 µM genistein continuously for up to 10 days, DNA synthesis was strongly inhibited from Day 1-4, after which time cytotoxicity occurred (124). In T47-D breast cancer cells, 20 µM genistein, quercetin and kaempferol markedly inhibited growth. Only quercetin was inhibitory over the entire concentration range of 100 nM – 20 µM. At the highest concentrations, extensive chromatin fragmentation was observed, suggesting apoptosis (96). This is similar to short-term effects of flavone, which has been observed, through semi-quantitative RT-PCR, to inhibit cell proliferation through apoptosis. Flavone was highly selective towards transformed cells only, and thus may be important for chemotherapeutic uses in colon cancer (127).

No obvious structure-function relationships have been observed for effects on proliferation, either for type of flavonoid (isoflavone, flavone, flavanone) nor hydroxylation pattern (102,126).

At concentrations greater than 25 µM, the soy isoflavones genistein, biochanin A, equol, and to some extent daidzein have been shown to modulate the proliferative activity of environmental carcinogens, including o,p'-DDT, 4-nonylphenol and 5-octylphenol. Genistein was the most potent, with an IC₅₀ of 25 – 33 µM (129). Therefore, diet-derived flavonoids may be protective against environmental xenoestrogens.
Mechanisms for inhibition of proliferation involve signal transduction pathways, including effects on protein kinases, epithelial growth factor (EGF) and vitamin D receptors, insulin-like growth factor (IGF-1) and transforming growth factor (TGF-β) (Figure 1.5).

Kinases are enzymes involved in signal transduction, responsible for conversion of 1-phosphatidylinositol to inositol 1,4,5-triphosphate (IP₃). Increased levels of IP₃ have been found in clinical samples human carcinomas and tissue culture cell lines, including cancers of breast and ovary, compared with normal cells (130). Of 14 flavonoids evaluated in one study, myricetin was found to be the most potent inhibitor of IP₃-kinase activity, with an IC₅₀ of 1.8 µM. Apigenin, fisetin and quercetin were also effective. Structure-function analysis of flavonoid-induced inhibition of IP₃-kinase, indicated the importance of position, number and substitution of hydroxyl groups on the B-ring, and saturation of the C-2 – C-3 bond (131). Weber et al found that quercetin and genistein down-regulated IP₃ in both a time- and dose-dependent fashion. This led to marked reduction of IP₃ concentration, then to apoptosis (132).

Tyrosine kinases are ubiquitous enzymes involved in many processes, including those responsible for growth and differentiation. Several studies have investigated the effects of flavonoids on this group of enzymes. Genistein has been found to be the strongest inhibitor of tyrosine kinases in several cell lines (133,134). Apigenin and kaempferol have also been demonstrated to dose-dependently inhibit both EGF receptor kinase autophosphorylation and mitogen activated protein (MAP) kinase (131,135), and to decrease phosphotyrosine content (136). However, other studies
Figure 1.5. **Signal Transduction Pathways influenced by Flavonoids.** $IP_3$ – inositol triphosphate. Pathways found to be influenced by flavonoids are in bold, blue print.
have conflicting results, in both human breast cancer (137), and prostate cancer cell lines (138). These studies concluded that a more distal event in the EGF receptor-mediated signal transduction cascade was involved for cell growth inhibition.

Experiments with the tea catechins, epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) demonstrated that low concentrations these compounds may activate MAP kinase, leading to the expression of survival genes c-fos and c-jun. At suprapharmacological concentrations, these flavonoids induce non-specific necrotic cell death (139). Genistein, on the other hand, has been found to down-regulate EGF-induced c-fos transcription in MDA-MB-468 human breast cancer cells (134). Various flavonoids and metabolites have also been shown to modulate cytochrome P450s responsible for site-specific 7α-, 6β- and 2α-hydroxylation of testosterone (140).

Inhibition of cell proliferation by genistein and other flavonoids, have been shown to be associated with specific arrest of the cell cycle. Genistein treatment of MCF-7 cells (10 μM) causes reversible arrest at the G2/M phase of the cell cycle (141). Quercetin arrests the cell cycle at G1 and S-phase boundary (Table 1.3). Moreover, the two flavonoids together have been shown to synergistically inhibit growth in OVCAR-5 ovarian cancer cells (142). One mechanism of cell cycle arrest and apoptosis by apigenin, luteolin and quercetin has been shown to be through wild type p53 accumulation (143). Synergism has observed in cell growth inhibition, cytotoxicity and reduction of IP3 concentrations with tamoxifen and genistein treatment of MDA cells (144). These results may be of interest in clinical treatment of breast and ovarian cancers.
Table 1.3. Cell Cycle

<table>
<thead>
<tr>
<th>Phase</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>G1 (and G0)</td>
<td>Gene expression and protein synthesis. Regulated primarily by external stimuli. Allows cell to grow and produce proteins needed for next phase. Cell may enter G0 instead and remain quiescent.</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis – doubling of cell’s DNA.</td>
</tr>
<tr>
<td>M</td>
<td>Mitosis – cell divides into two daughter cells.</td>
</tr>
</tbody>
</table>

Other mechanisms involved may include inhibition of other nuclear receptors, such as the vitamin D and retinoic acid (RXR-α) receptors. Downregulation of these two receptors has been shown with treatment of human keratinocytes with apigenin, quercetin and fisetin (145). Finally, induction of p21WAF/CIP1 expression may also be important. A 3 - 4-fold induction of this protein has been shown to precede apoptosis (125, 145-149).

Antioxidant Activities

Flavonoids have been shown to have both antioxidant and pro-oxidant activities in vitro, and in animal models. Activities and structural requirements for these activities have been determined by several investigators. However, conflicting potencies have been reported, dependent on the assay and methods used.

Using an oxygen radical absorbance capacity assay, and three different reactive species – a peroxyl radical generator, a hydroxyl radical generator, and Cu²⁺, a transition metal – Cao et al determined both antioxidant and pro-oxidant activities of
various flavonoids, and structure-function relationships. They found that both antioxidant and Cu$^{2+}$ pro-oxidant activities were dependent on the number of hydroxyl substitutions, with compounds that contained multiple hydroxyl groups showing anti-peroxyl radical activities several times stronger than Trolox, an α-tocopherol analogue. Dihydroxyl substitutions at C-3' and 4' were particularly important to peroxyl radical absorbing activity. O-methylation of hydroxyl groups inactivated both antioxidant and pro-oxidant activities (150). Flavonoids including naringenin, naringin, hesperetin and apigenin were also found to form pro-oxidant metabolites that oxidized NADH and glutathione upon oxidation by peroxidase/hydrogen peroxide. The implications of pro-oxidant activities of flavonoids is uncertain (151,152).

The importance of the 4' hydroxyl group and 5',7 dihydroxy was determined through examination of antioxidant activity in the aqueous phase through ABTS$^+$ total antioxidant activity assay (Figure 1.1). The order of reactivity for isoflavones in radical scavenging was genistein>daidzein, genistin, biochanin A, daidzin. No activity was seen for formononetin or ononin (153). Examining other flavonoids, quercetin, cyanidine, which contain 3',4'-dihydroxy substituents and conjugation between A and B rings have antioxidant activities four-fold greater than that of Trolox. Kaempferol, which does not have the ortho-dihydroxy substitution, and catechin and epicatechin, which do not possess the 2,3 double bond, have greater than 50% reduction of antioxidant activity. Free radical scavenging was highest with EGCG, then quercetin (50% less effective). Genistein, daidzein, hesperetin and naringenin were not effective (154).

Hydrogen donating ability of a range of phytoestrogens were assessed using electron spin resonance spectroscopy, the ferric-reducing ability of plasma assay and
Trolox equivalent antioxidant capacity. The ability of compounds to inhibit lipid peroxidation was also examined in vitamin E-deficient liver microsomes. Only kaempferol was found to have significant antioxidant activity in this study. Genistein had the highest activity of the isoflavones, however, isoflavones as a group were relatively poor hydrogen donors. Equol and coumestrol had higher potencies than isoflavones, but they too were weak (155,156). Inhibition of microsomal lipid peroxidation by isoflavones and metabolites were found to have the relative activities of isoflavan > isoflavanone > isoflavone (157). In 12-O-tetradecanoyl phorbol 13-acetate (TPA)-activated HL-60 cells, genistein had potent activity against hydrogen peroxide. Daidzein had moderate antioxidant effects, and apigenin and biochanin A had no effect. In contrast, genistein, apigenin and prunectin were equally potent in inhibiting superoxide anion generation by xanthine/xanthine oxidase. For both systems, hydroxyl substitution at 4' position was important (158).

Finally, rats gavage-treated with tangeritin, chrysin, apigenin, naringenin, genistein and quercetin were analyzed for effects on phase I and II enzymes in liver, colon and heart, and antioxidant enzymes in red blood cells. Glutathione-S-transferase was significantly induced by apigenin, genistein and tangeritin in heart, but not colon or liver. In red blood cells, chrysin, quercetin and genistein significantly decreased activities for glutathione reductase, catalase and glutathione peroxidase. Superoxide dismutase was only inhibited by genistein. Moreover, chrysin, quercetin, genistein and β-naphthoflavone were significantly protective against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-DNA adduct formation in the colon, suggesting a feedback mechanism (159). Black tea also strongly reduced PhIP-DNA adduct
formation (67%) in the colons of male Fischer (F) 344 rats (160). However, for many of these in vitro and animal studies, high concentrations of flavonoids were used. Several researchers have therefore questioned the physiological relevance of these antioxidant effects (161,162).

**Carcinogenesis**

Flavonoids have been demonstrated to reduce carcinogenesis in animal models and in vitro, and to modulate enzymes implicated in the carcinogenic process. Their effects on initiation and promotion stages of the carcinogenic process have been evaluated, and several mechanisms have been proposed, including influences on development and hormonal activities.

The initial animal work was performed by Troll et al, who demonstrated a significant reduction of mammary cancer by X-irradiation in SD rats fed a raw soybean diet, compared to those fed casein (44% developed tumors versus 74%). Although this protection was attributed to protease inhibitors present in soy, other compounds, including isoflavones could have been at least partially responsible (163). Barnes and Messina have conducted many animal studies using soy and isoflavones in chemical carcinogenesis models of mammary and other tumors. The majority of these show protective effects (6).

The soy isoflavones genistein, daidzein and biochanin A, as well as soy protein and soy milk have all been used to determine effects on mammary carcinogenesis. When female SD rats were fed a high fat basal diet containing 10% fermented soy milk or 0.02% or 0.04% isoflavone mixture during and after PhIP exposure, mammary
tumors were significantly smaller, and tumor multiplicity significantly lower than controls (164). Similarly, when SD rats were fed a 10% soybean or 10% miso supplemented diet, or were given 10 or 50 mg/kg body weight biochanin A for 18 weeks, tumor multiplicity was significantly decreased. The 50 mg/kg biochanin A dose also significantly reduced tumor incidence compared to control (32% versus 80%), and proliferation cell nuclear antigen labeling index. When MCF-7 cells or MDA-MB-468 cells were treated with 15, 30 or 45 μM genistein, then implanted into a nude mouse xenograft model, the tumorigenic potential of the cells was diminished (165). Not all studies agree with these results. SD rats fed one of 4 purified diets containing casein or soy with 0.03, 0.4 or 0.81 mg isoflavones for 2 weeks, then treated with 7,12-dimethylbenz[a]anthracene (DMBA) or peanut oil, showed only a trend (p<0.09) for an inverse relationship between tumor incidence and isoflavone intake (166). Cohen et al found no significant inhibition of nitrosomethylurea- (NMU) induced mammary carcinogenesis for rats taking soy protein-supplemented diets (167).

Timing of isoflavone treatment may be important. SD rats were exposed to 0, 25 or 250 mg genistein/kg AIN-76A diet from conception to Day 21 post-partum. At Day 50 post-partum, all were treated with 80 mg DMBA/kg body weight to induce mammary cancer. Once sacrificed, it was found that genistein feeding resulted in a dose-dependent protection against the development of mammary tumors, with fewer tumors per rat. Moreover, it was found that the mice given genistein had fewer terminal end buds (TEBs), which are mammary structures most susceptible to carcinogenesis. (168). These results were confirmed in a similar study (169). 500 μg/g body weight genistein given subcutaneously to prepubertal female SD rats prior to DMBA
administration at Day 50 also resulted in fewer TEBs and more lobules (170). It was concluded that genistein could suppress the development of chemically-induced mammary cancer without reproductive or endocrinological toxicities (171).

Genistein, daidzein, their glycosylated forms (genistin and daidzin), as well as soy products have also be protective against early stages of 3,2'-dimethyl-4-aminobiphenyl (DMAB)-induced prostate cancer (172), and azoxymethane (AOM)-induced colon cancer in rat models (173). Therefore, although not all studies show chemopreventive effects, the vast majority indicates that soy isoflavones and food sources may be useful in preventing steroid hormone-dependent cancer development.

Other flavonoids evaluated for anticarcinogenic effects in the colon include quercetin, rutin and hesperidin. Female CF1 mice were treated with 0.1, 0.5 or 2.0%/diet quercetin, or 1.0 or 4.0%/diet rutin for 50 weeks to assess inhibition of AOM-induced colonic neoplasia. No alterations were seen for mice fed flavonoid-supplemented diets without AOM treatment. However, among AOM-treated mice, both 2.0% quercetin and 4.0% rutin significantly inhibited hyperproliferation and the shift of S-phase cells to middle and upper portions of the crypts. Moreover, significantly fewer focal areas of dysplasia (FADs) were found with both of these treatments, and 2.0% quercetin reduced tumor incidence. 4.0% rutin showed a trend for lower tumor incidence (174). A second study performed by this group fed quercetin supplementation of 0.5, 2.0 or 5.0%, or 2.0% or 4.0% rutin with 5% or 20% corn oil to female CF1 mice for 9 weeks. 80% of quercetin-fed mice on the high fat diet remained free of FADs, compared to 29% of those on the high fat control diet (p<0.01). For rutin, the difference was bordering significance (p<0.08) (175). Similar results for AOM-
induced mice have been shown by other groups (176). Quercetin- and rutin-supplementation in normal mice have also been shown to increase numbers of colonic epithelial cells per crypt column, apoptotic index and redistribution of apoptotic cells along the crypt axis. Quercetin alone has also been observed to induce FAD in normal mice fed AIN-76A diets (175).

Hesperidin, the major flavanone in orange juice has been shown to inhibit chemically induced colon carcinogenesis (177), and double strength orange juice slows down DMBA-induced mammary cancer in rats (178). Based on these studies, Miyagi et al tested the hypothesis that not-from concentrate orange juice given to male F344 rats instead of drinking water could inhibit AOM-induced colon cancer. Cancer was initiated by 2 subsequent injections of AOM at 22 and 29 days of age, and on Day 35, half of the rats were put on orange juice + modified diet to equilibrate the macronutrient profiles between the two groups. At 33 weeks of age, the rats were sacrificed and colons analyzed. A 22% reduction in tumor incidence was found for the group fed orange juice compared to control (p<0.05), with a trend towards smaller average tumor burden (p=0.13). The labeling index and proliferation zone were lower, and enhanced cell differentiation was found in the bottom two-thirds of the crypt (179). Pure hesperidin and diosmin, also found in citrus fruits, were fed for 5 weeks (initiation treatment) or 28 weeks (post-initiation treatment) at levels of 1000 ppm each or in combination (900 + 100 ppm) to AOM-induced male F344 rats. At the end of 32 weeks, incidence and multiplicity of neoplasms with or followed by flavonoid treatment were significantly smaller than with AOM alone (p<0.001). The combination of the two flavonoids was not better than each alone. Both significantly inhibited development of aberrant crypt foci,
and were significantly associated with lower labeling index and other markers of carcinogenesis (177). These studies indicate that flavonoids, either purified or within foods, are chemopreventive against colon carcinogenesis.

Cyclooxygenase 2 (COX-2) and cytochrome P450s are enzymes involved in the carcinogenic process that can be modulated by flavonoids. Of 12 flavonoids tested in the DLD-1 human colon cancer cell line, quercetin was the most potent suppressor of COX-2 transcription, with an IC_{50} of 10.5 μM. Catechin and epicatechin also demonstrated weak inhibition of COX-2. Structure requirements for inhibition were found to be hydroxyl groups on the B ring and an oxo group at the 4-position of the C ring (180). In lipopolysaccharide-activated macrophages, apigenin, genistein and kaempferol all markedly inhibited transcriptional activation of this enzyme with IC_{50}s less than 15 μM, with apigenin being most potent (181). These observations are important because COX-2 is linked to the loss of growth control and apoptosis in various tumors (182). In colorectal tumors, over-expression of this enzyme occurs in almost 90% of cases (183), hence it is a key target of pharmacotherapy (184). In HT-29 cells exposed to 150 μM flavone for 48 hours, COX-2 mRNA levels were strongly reduced. Transcription of NF-κB, a transcription factor able to inhibit apoptosis in cancer cells was also inhibited by flavone. Inhibition of both of these proteins may help to reduce chemoresistance of cancer cells (127).

Finally, several cytochrome P450 enzymes induce carcinogenesis through metabolism of procarcinogens to ultimate carcinogens. Several studies have assessed the ability of various flavonoids to inhibit activation of benzo(a)pyrene (BaP) or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Analysis of 23 flavonoids found that flavonols or
flavones with free 5- or 7-hydroxyl groups are potent inhibitors of P450-mediated metabolism of BaP in liver S9 homogenate from rats (185). Genistein, daidzein, genistin and daidzin were found to block TCDD-induced CYP1A1 activation. This inhibition was correlated with the capacity of these isoflavones to prevent CYP1A1-mediated covalent binding of BaP metabolites to DNA (186). Inhibition of these enzymes may thus represent one mechanism by which flavonoids are protective against carcinogenesis.

1.2.1.4 Influences on Modulators of Risk for Steroid Hormone-Dependent Cancers

Few clinical trials have been conducted to determine the influences of flavonoids on risk factors for steroid hormone-dependent cancers. Most of those that have been conducted, have looked at potential modulation of breast cancer risk factors by soy isoflavones. The results of these studies are conflicting, and confusing.

Urinary excretion of isoflavones have been assessed in many studies as a marker of isoflavone consumption. To determine whether this method is reliable, several groups have correlated database calculations for isoflavones based on self-reporting and urinary isoflavone excretion. All have shown very significant correlations, along a broad range of intakes (187-190). Therefore the use of food frequency questionnaires for measurements of soy intake appear to be relatively reliable.

Risk factors that have been associated with decrease in risk of breast cancer include lower plasma concentrations of ovarian hormones, higher levels of sex hormone binding-globulin (SHBG), longer menstrual cycle, particularly the follicular phase, and high urinary isoflavone excretion. Therefore, many of the clinical trials conducted were
to determine the effects of soy feeding on these endpoints. In one of the initial soy feeding studies, Cassidy et al examined the effects of soy feeding on ovarian cycle. 60 g of soy protein, providing 45 mg isoflavones/day significantly (p<0.01) increased follicular phase length and/or delayed menstruation. Moreover, midcycle surges of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were significantly suppressed, and plasma estradiol concentrations in the follicular phase increased. These results were exciting, as similar responses have been seen with tamoxifen, when used as a prophylactic agent against breast cancer (191).

Since then, many other clinical trials have been conducted. Lu et al have examined the modulation of soy feeding on these parameters in several studies. In one study, 10 healthy, cycling premenopausal women were given 36 ounces of soy milk (equivalent to 113-207 mg isoflavones/day) for one month. A 25% reduction in estradiol, and a 45% reduction in progesterone, were found, both highly significant (p<0.01, p<0.0001, respectively). No changes in LH or FSH were found (192). However, in a randomized cross-over study, using soy protein powders with low isoflavone (64 mg/day), high isoflavone (128 mg/day) or control, for 3 cycles plus 9 days, the low isoflavone diet significantly decreased LH (p=0.003) and FSH (p=0.04), during the periovulatory phase. The high isoflavone diet also reduced free thyroxine and dehydroepiandrosterone sulfate (DHEAS) during early follicular phase. Besides estrone, which decreased during mid-follicular phase, no other significant changes in hormones or length of cycle were found (193). Several other feeding studies have shown similar conflicting results (194-196). Length of treatment and levels of soy do not seem to influence results.
Higher levels of sex hormone binding globulin have been associated with decreased breast cancer risk. This plasma protein binds to steroid hormones (estrogens and androgens), thereby "inactivating" them, i.e. only free steroids exert their effects. Therefore, SHBG is one mechanism through which steroidal activity can be modulated (197). Significant positive correlation between urinary excretion of phytoestrogens and plasma SHBG, and negative correlation between SHBG and excretion of 16α-hydroxyestrone (see below) and estriol have been found (198). These results suggested that isoflavones may affect uptake of sex hormones by regulation of plasma SHBG levels and influence biological activity. However, other studies, do not support this hypothesis (191, 199).

Higher levels of two putative carcinogenic metabolites of estradiol have also been implicated in breast cancer risk. These are 4-hydroxyestrogen and 16α-hydroxyestrogen. Lower amounts of the anticarcinogenic metabolite 2-hydroxyestrogen are also associated with greater breast cancer risk. In order to determine whether a soy diet may alter the metabolism of 17β-estradiol to these products, eight women were placed on the soy milk-supplemented diet described above for one complete menstrual cycle. After a four-month washout, they were then given an isoflavone-free soy milk supplement, providing less than 4.5 mg isoflavones/day. It was found that the isoflavone diet increased mean daily urinary excretion of 2-hydroxyestrone by 47% (p=0.03), but had no effect on 16α-hydroxyestrone production. Because the ratio of 2:16α also significantly increased, it was suggested that isoflavones, through increased metabolism of endogenous estrogens to the protective 2-hydroxy form, may be important in lowering 17β-estradiol levels (200).
The role of ethnicity has been a consideration for breast cancer development, as Asian women have significantly lower incidence. Hence, Wu et al placed 20 premenopausal women, 10 Asian-American, 10 non-Asian American, on a 7-month soy intervention study. Asian soy foods, including tofu, soy milk and green soybean peas, providing 32 mg isoflavones/day, were added to a basal diet for 3 menstrual cycles. The intervention was found to significantly reduce serum estradiol in the luteal phase in the Asian women only. Moreover, these women had higher excretion levels of isoflavones, compared to their non-Asian counterparts. It was concluded that perhaps genetics does play a role in the metabolism of these compounds, and thus modulation of breast cancer risk factors (201). Opposing this theory, is a study of 31 premenopausal Japanese women, fed 400 mL soymilk per day, providing 109 mg isoflavones, for 3 consecutive menstrual cycles. Although estrone and estradiol levels decreased 23% and 27%, respectively in the soymilk group, these were not statistically significant (202). Perhaps a longer study would have enabled these results to reach significance.

Another parameter that may be important in determining breast cancer risk is the profile of breast secretions. Petrakis et al hypothesized that long-term consumption of commercial soy protein products would alter features of nipple aspirate fluid (NAF) of non-Asian women to resemble those previously characterized in Asian women. 24 normal pre- and postmenopausal Caucasian women underwent this year-long study. For months 1-3 and 10-12, they did not eat any soy products. Between months 4 and 9, these women consumed 38 g of soy protein isolate, (SPI) containing 38 mg of genistein. NAF volume, gross cystic disease fluid protein (GCDFP-15) concentration and NAF
cytology were used as biomarkers of possible effects of soy protein isolate on breast. Other measurements taken were plasma concentrations of estradiol, progesterone, SHBG, prolactin, total cholesterol, HDL-C and triglycerides. Compliance was measured through urinary excretion of genistein and daidzein, and was excellent throughout the study (203).

Compared to months 1-3, there was a 2-6-fold increase in NAF volume in months 4-9 for all premenopausal women, and no change in postmenopausal subjects. No changes were seen in any of the plasma parameters for postmenopausal women. However, in the premenopausal group, plasma estradiol levels erratically increased during soy consumption, and there was a moderate decrease in GCDFP-15 levels. What was surprising to investigators, and of potential concern, was the cytological detection of epithelial hyperplasia found in 7 of 24 women (29.2%) during months of SPI consumption. These results indicate that prolonged consumption of SPI has stimulatory effects on premenopausal breast, suggestive of an estrogenic stimulus (203). These data were further supported by a randomized trial examining the effects of 60 g soy supplement on the proliferation rates of premenopausal, histologically normal breast epithelium of 48 women with benign or malignant breast disease. One group was given a normal diet, and the other given diet + soy supplement for 14 days. Biopsy samples of normal breast were labeled with [3H]-thymidine to detect the number of cells in S-phase, and were measured for the proliferation antigen Ki67. The proliferation rate of the breast lobular epithelium significantly increased after the 14-day soy supplementation, when both day of cycle and age of patient were accounted for. PR levels also increased (204). Increases in pS2 concentrations in NAF have also been
found (205). These studies, taken together, demonstrate that both short-term and long-term soy feeding may provide a stimulus for proliferation in the breast. The implications of this remain unknown.

Very few studies have looked at men. We examined the effects of feeding 33 g/day soy, providing 86 mg isoflavones/2000 kcal/day, to 31 men and postmenopausal women. Ex vivo hormone activity showed no alterations in androgen (through PSA) or estrogen (pS2) activities (206). Many more studies must be done before we can decipher the data thus far, let alone determine the effects of other flavonoids in steroid hormone-dependent cancers.

1.2.1.4 Discussion

Many mechanisms have been proposed for how flavonoids may help prevent steroid hormone-dependent cancers and several have been validated in vitro and in animal models. These include modulation of steroid hormones, inhibition of proliferation, and anticarcinogenic and antioxidative activities. However, the physiological implications are questioned by most. For most animal models, doses tested are significantly higher than those that would exist in human plasma after consumption of whole foods. Nutraceutical or functional food administration has thus been suggested as possibilities for reaching such concentrations.

Randomized clinical trials are still in their infancy in this area. To date, only soy has been tested in this capacity, dealing specifically with breast cancer risk factors. Though several studies have been done, these are very conflicting. Little data exist on other flavonoids or other cancer types. At this point in time, we are still in the dark. We
cannot be sure that consumption of soy will have positive, neutral or even negative effects of breast cancer risk. Advising women at high risk for breast cancer, or those who have this disease and want alternate therapies is not feasible at present.

1.2.2 Steroid Hormone Receptors

Steroid hormone receptors, including ER, AR and PR, are members of the Nuclear Hormone Receptor Superfamily, which is made up the classic steroid receptors, thyroid receptors, vitamin D receptor and retinoic acid receptors – RAR and RXR. All of the members of this superfamily share common functional domains, lettered A to F. Starting from the N-terminus, these are the trans-activation, DNA binding, hinge and ligand binding domains (207,208 and references within).

A/B: Trans-activation Domain

This domain is weakly conserved among members of the superfamily, and is of variable length. It contains the autonomous activation function (AF) AF-1, and is involved with binding of specific and general transcription factors necessary for transcription of the nuclear receptor-regulated genes.
C: DNA-binding Domain

This domain, towards the carboxy terminus of the trans-activation domain, consists of 66-68 amino acids, and is the most highly conserved region among the steroid receptors. It contains 20 invariant amino acids, forming two zinc finger DNA binding motifs, which provides the basis for protein-DNA interactions with regulator sequences of target genes (209-215). Specifically, nine of these conserved amino acids are cysteine residues, of which 8 interact in a coordinated fashion to form two separate tetrahedral metal-binding complexes, or fingers. Four cysteine residues in each finger binds one Zn$^{2+}$ molecule, which permits interaction with DNA. The functional role of the second finger is not entirely clear, but studies suggest it aids in the stability of binding of the receptor to DNA (214).

Amino acids adjacent to these cysteines are also important, as they define the specificity of a given receptor binding to its respective response element. It has been shown through mutation studies of the ER, that substitution of glutamine, glycine and alanine, found in ER, by glycine serine and valine, contained by the glucocorticoid receptor (GR), results in a receptor that no longer recognized the ERE, but instead, the glucocorticoid response element (GRE). Therefore alterations of these three amino acids can alter or destroy receptor function.

D: Hinge Domain

This region is involved in homodimer formation, necessary for activation of nuclear receptor. It contains sequences responsible for nuclear localization of steroid
receptors, with studies indicating that signals for localization of the receptor into the nucleus can occur even in the absence of ligand binding (210-212).

**E: Ligand-binding Domain**

This multifunctional, carboxy-terminal half of the protein encompasses the ligand-binding domain, a second activation function, AF-2, a dimerization domain and a region involved in nuclear localization. The AF-2 autonomous activation domain is composed of an amphipathic α-helix that is highly conserved among nuclear receptors and is critical for transcription activation (216-219).

**F: Unknown Domain**

This final domain is absent in some receptors, including PR, RAR and RXR. It is quite variable and its function is yet unknown.

**1.2.3 Steroid Hormone Receptor Signaling**

In the absence of steroid ligand, a steroid hormone receptor resides as part of a larger molecular complex with heat shock proteins (HSPs) (220). Once bound by its respective ligand, the receptor becomes phosphorylated at several serine and tyrosine residues (221). Specific conformational changes are subsequently induced in the receptor, resulting in the dissociation of HSPs, receptor dimerization and localization of the receptor into the nucleus. The receptor-ligand complex then binds with a HRE within the promoters of target genes (222). Transcription factors then act to transcribe the gene to mRNA, which then leaves the nucleus and enters the endoplasmic
reticulum for translation into protein. The protein then performs its specific physiological function (See Figure 1.3 in Section 1.2.2) (84).

The AF-2 site may alternately be regulated by coactivators and/or corepressors (223,224) in a ligand-dependent manner. This mechanism supports the theory of "squelching", the transcriptional interference between steroid hormone receptors due to limiting common transcriptional cofactors (223-225). These mediators or coactivators are required to achieve efficient transcription (226-228). Details of these cofactors and mechanisms by which they act are beyond the scope of this project.

1.3 Hypothesis

Flavonoids may have steroid hormone activities, estrogenic, progestational and/or androgenic, and thereby may influence the regulation of steroid-hormone dependent genes. Other factors, including the vehicle in which they are found, i.e. natural or processed extract or food, may alter these activities.

1.4 Objectives

1. To develop the techniques and methodology necessary to determine steroid hormone activity.

2. To assess the agonist and antagonist activities of flavonoids and related compounds in vitro, and to relate their activity, where possible, to structural properties.
3. To evaluate the steroid hormone activities of several natural products and commercially prepared nutraceuticals being taken by the North American public, many of which are sold for hormonal purposes.

4. To determine the steroid hormone effects of one functional food, soy, in healthy subjects, using our model system.
CHAPTER 2: DEVELOPMENT OF TOOLS TO MEASURE STEROID HORMONE ACTIVITY
CHAPTER 2: DEVELOPMENT OF TOOLS TO MEASURE STEROID HORMONE ACTIVITY

2.1 Rationale

In order to evaluate the steroid hormone activities of flavonoids, related compounds and metabolites, the tools necessary for making these assessments had to first be developed and tested. These included cell lines, indicator proteins, the assays needed to measure these proteins, and the positive and negative controls – namely the steroids and steroid antagonists.

The cell line first chosen was the BT-474 human breast cancer cell line, because it was positive for all three receptors (estrogen, ER; androgen, AR; and progesterone, PR). Dr. Pascal Pujol, at the University of Montpellier, Villeneuve, France, determined through a competitive PCR technique (229) that the ER present in these cells was exclusively ERα. This distinction is important because ERβ has been shown to have a significantly greater affinity for genistein than ERα (230). The clinical implications of this difference is yet unknown.

A prostate cancer cell line (PC-3) was then added, which is a receptor-negative cell line that has been stably transfected with the human AR cDNA. This cell line was developed by Dr. Theodore Brown, Department of Obstetrics and Gynecology, Mount Sinai Hospital. The subclones PC-3(AR)₂ and PC-3(AR)₆ were tested for their upregulation of PSA and other androgen-responsive genes, and used for relevant studies.

The immunoassay for PSA, which is regulated by androgens and progestins, was already developed and tested in the lab, so the requirement was to develop an immunoassay to measure pS2, an estrogen-regulated protein. This pS2 assay is a
competitive, enzyme-linked immunosorbent (ELISA)-type assay, and uses a pS2 antibody (Ab), a biotinylated 28-mer pS2 peptide, and a sheep anti-mouse immunoglobulin G (SAMIg) secondary Ab. The pS2 Ab was purchased from Neomarkers, Union City, CA, and the pS2 peptide was a gift from Dr. Atul Tandon. After developing this assay, pS2 upregulation was measured after stimulation by estradiol and other steroids. This was necessary in order to qualify pS2 as a good marker of estrogen activity and regulation.

The final study in this area was to test the effectiveness of clinically-used antagonists to block steroid hormone activity in this system. The effectiveness of ICI 182,780 (faslodex, a pure antiestrogen) to inhibit pS2 production, and the antiprogestin RU 486 (mifepristone) to inhibit PSA production, was determined in the BT-474 human breast cancer cell line. The cross-reactivity of the two antagonists was then determined.

2.2 Development and Evaluation of a Competitive Time-Resolved Immunofluorometric Assay for the Estrogen-Regulated Protein pS2

2.2.1 Introduction

pS2 is a secreted (231), estrogen-regulated protein (232), classified as a member of the trefoil family of proteins (233). It is expressed in normal tissue, primarily in the stomach (234,235), but is also found in normal breast epithelium and other sites (236,237). Like human intestinal trefoil factor (hITF) and human spasmolytic polypeptide (hSP), the other two family members, the functions of pS2 have not been completely resolved. However, current research is focussing on its motogenic capabilities and its role in tissue healing and repair (238-242).
pS2 was first discovered in MCF-7 breast cancer cells by virtue of its estrogen-controlled regulation (232). The presence of this protein in breast tumors has been shown to be a favourable prognostic indicator (243). It is associated in many studies with estrogen (ER) and progesterone receptors (PR) (243). pS2 has also been used in-vitro to measure estrogenic activity of natural and synthetic compounds (244). To date, pS2 has been measured with Northern blot (245), immunohistochemistry (246), and with immunoradiometric assays (96). To our knowledge, only one IRMA-type assay is currently commercially available for this interesting protein.

The scarcity of quantitative assays for pS2 protein suitable for analysis of tissue culture supernatants and other biological samples prompted us to develop this method which, we then applied to practical applications. By using the developed assay we here show the mode of regulation of pS2 protein by steroid hormones in the breast carcinoma cell line BT-474.

2.2.2 Materials and Methods

BT-474 and MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. All steroids were from Sigma Chemical Co., St. Louis, MO, USA. Stock, $10^{-3}$ M solutions of steroids were prepared in absolute ethanol. The secondary antibody (sheep anti-mouse IgG, SAMlg) was purchased from Jackson ImmunoResearch, Westgrove, PA, USA. The pS2 mouse monoclonal antibody (catalog no. MS-111-PABX) was obtained from NeoMarkers, Union City, CA, USA. pS2 peptide was a gift from Dr. Atul Tandon. The sequence of the
peptide, which represents 28 amino acids of the carboxyterminus of native pS2 is NH$_2$-KGCCFDVTGRGPWCFYPNTIDVPEEEE.

Diflunisal phosphate (DFP) was synthesized in our laboratory. The stock solution of DFP was 0.01 mol/L in 0.1 mol/L NaOH. Alkaline phosphatase-labeled streptavidin (SA-ALP) was obtained from Jackson ImmunoResearch as a 1 g/L solution. Working SA-ALP solutions were prepared by diluting the stock solution 20,000-fold in a bovine serum albumin (BSA) diluent (described below). White, opaque, 12-well microtiter strips were obtained from Dynatech Labs., Alexandria, VA. The substrate buffer was a Tris buffer (0.1 mol/L, pH 9.1) containing 0.1 mol of NaCl and 1 mmol of MgCl$_2$ per liter. The substrate working solution (DFP, 1 mmol/L in substrate buffer) was prepared just before use by diluting the DFP stock solution 10-fold in the substrate buffer. The SA-ALP diluent was a 60 g/L solution of BSA in 50 mmol/L Tris buffer, pH 7.40, containing 0.5 g/L sodium azide. The wash solution was prepared by dissolving 9 g of NaCl, and 0.5 g of polyoxyethylene sorbitan monolaurate (Tween 20) in 1 L of a 10 mmol/L Tris buffer, pH 7.40. The developing solution contained 1 mol of Tris base, 0.4 mol of NaOH, 2 mmol of TbCl$_3$, and 3 mmol of EDTA per liter (no pH adjustment).

2.2.2.1 Instrumentation

A time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada), was used to measure Tb$^{3+}$ fluorescence in white microtiter wells. The procedure has been described in detail previously (247-249).
2.2.2.2 pS2 Standard Solutions

MCF-7 breast cancer cells were grown to confluency in 75 mL flasks with 50 mL of media (phenol-free RPMI with 10% fetal bovine serum, 10 mg/L insulin, 200 mmol/L L-glutamine (Gibco BRL, Gaithersburg MD, USA). The flasks were stimulated with $10^{-8}$ mol/L estradiol and incubated for 8 days at 37°C, 5% CO$_2$. The supernatants were then harvested and pooled. This stock solution was calibrated as described below.

2.2.2.3 Procedures

Coating of microtiter plates with pS2 mAb. We coated polystyrene microtiter wells by incubating overnight 500 ng/100 μL per well of sheep-anti-mouse immunoglobulin (SAMIg) in a 50 mmol/L Tris buffer, pH 7.80. The wells were then washed six times with the wash solution and incubated for 2 hours with 100 μL per well of pS2 mAb diluted 1:4,000 (25 ng/100 μL) in 6% BSA. The strips were then washed another six times.

Biotinylation of pS2 peptide. Biotinylation was performed with sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-LC-Biotin) obtained from Pierce Chemical Co., Rockford, IL. Approximately 1 mg of NHS-LC-LC-Biotin was reacted with 200 μg of the peptide in 1 mL of a 0.5 M carbonate buffer, pH 9.1. After biotinylation for 1 h at room temperature, the peptide was stored at 4°C and used without any further purification. The concentration of the stock biotinylated peptide solution was 200 μg/mL.

pS2 calibrators. MCF-7 supernatants were analyzed for pS2 concentration using an immunoradiometric kit (IRMA) (Cis-BIO, Gif-Sur-Yvette Cedex, France). pS2 calibrators of 0, 62, 125, 250, 500 and 1000 ng/mL were prepared by diluting the standard stock in
a 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA and 0.5 g of sodium azide per liter.

**pS2 assay.** Calibrators or samples (100 μL) were pipetted into coated microtiter wells and 50 μL of biotinylated peptide diluted 20,000-fold in 6% BSA was added (approximately 0.5 ng of peptide per well). The plates were incubated with mechanical shaking for 1 h at room temperature and then washed six times. To each well we then added 100 μL of SA-ALP conjugate diluted 20,000-fold in the SA-ALP diluent (approximately 5 ng of conjugate per well), incubated for 15 min as described above, and then washed six times. To each well we then added 100 μL of the 1 mmol/L DFP working substrate solution and incubated for 10 min as described above. We added 100 μL of developing solution to each well, mixed by mechanical shaking for 1 min, and measured the fluorescence with the time-resolved fluorometer essentially as described elsewhere (250). The calibration curve and data reduction were performed automatically by the CyberFluor 615 Immunoanalyzer.

### 2.2.2.4 Tissue Culture Experiments with Cell Lines

BT-474 breast cancer cells, positive for estrogen, progesterone and androgen receptors were grown to confluency in phenol-free RPMI media supplemented with 10% fetal calf serum, insulin, and L-glutamine at 37°C, 5% CO₂. They were then transferred to 24-well microtiter plates (Corning no. 25280), where they were grown to confluency in media containing charcoal-stripped fetal calf serum instead of the regular fetal calf serum. They were stimulated with either estradiol, aldosterone, dihydrotestosterone (DHT), dexamethasone, norgestrel, mibolerone at 10⁻⁷ M, or alcohol. They were
incubated for 7 days, at which time their supernatants were harvested and measured for pS2 concentration.

Time Course Study

The same protocol as above was used, with the exception of the incubation period being 1, 3 or 5 days.

Dose-Response Study

BT-474 cells were grown and plated as above. Cells were then stimulated with each one of six steroids at $10^{-12}$ to $10^{-7}$ M, or alcohol (control) and incubated for 7 days. At this time, supernatants were harvested and analyzed for pS2 concentration.

2.2.3 Results

2.2.3.1 Antibody Selection and Assay Optimization

Two mouse monoclonal antibodies and one rabbit polyclonal antibody were initially evaluated to develop the pS2 immunofluorometric assay. Among all possible assay configurations, as described elsewhere (251), we found that indirect immobilization of the primary antibody and use of the monoclonal antibody described above, along with a one-step protocol, gave the best overall results. Other variables, including incubation times and amounts of reactants, were optimized with a previously published optimization strategy (251). The final conditions are described in “Methods”.
2.2.3.2 Calibration Curve, Detection Limit and Precision

A typical calibration curve of the proposed assay is shown in Figure 2.1. The detection limit, defined as the concentration of pS2 detected with a precision of 20% is 16 ng/mL. Within-run and between-run precision was assessed at various pS2 concentrations of tissue culture supernatants between 30 and 300 ng/mL. CVs ranged from 3 to 12% within this range, which are typical for this type of assay.

2.2.3.3 Tissue Culture Experiments

In Figure 2.2 we present data showing conclusively that the pS2 protein expression is regulated by estrogens only. All other tested steroids had no detectable effect on pS2 regulation. Furthermore, as shown in Figure 2.3, pS2 secretion and accumulation into the tissue culture supernatant is time-dependent following stimulation with estradiol. The effect of estradiol was dose-responsive (Figure 2.4). The effect of estradiol on pS2 upregulation is evident at estradiol concentrations equal or higher than $10^{-11}$ M.

2.2.4 Discussion

We have developed a simple, convenient, and sensitive competitive immunoassay for quantifying pS2 protein. Unlike Northern blotting and immunohistochemistry, this method allows for secreted or extracted pS2 to be measured in tissue culture supernatants or in other fluids. The method is efficient since about 42 samples per plate can be analyzed within 2-3 hours.
Figure 2.1. Typical Calibration Curve for the pS2 Competitive Assay
Figure 2.2. **pS2 Production by Six Steroids and Alcohol.** All steroids were used at a concentration of $10^{-7}$ M. DHT, dihydrotestosterone; Aldo, aldosterone; Dexa, dexamethasone; Norg, norgestrel; Mibo, mibolerone.

Figure 2.3. **Time Course of pS2 Production by Estradiol**
Figure 2.4. Dose-Response of Steroids at Concentrations between $10^{-7} - 10^{-12}$ M. For abbreviations see Figure 2.
pS2 has been demonstrated in previous studies, and in this one to be an excellent marker of estrogenic activity. Estradiol significantly upregulated pS2 production among all of the steroids tested. pS2 production was shown to be suppressed by dexamethasone in other studies (252), as measured by Northern blot. However, this was not seen in the present study. Furthermore, pS2 expression was shown here to be both time- and estradiol dose-dependent.

A number of clinical applications have already been realized for pS2. This protein is associated with ER and PR presence in breast tumors (243). It has been shown that pS2 is a predictor of response to tamoxifen treatment (253). Patients with pS2-positive breast tumors show greater relapse-free (243), progression-free and overall survival rates (254,255). In patients with intermediate levels of ER and PR, pS2 has been able to predict tamoxifen effectiveness (256), allowing for therapy decisions to be made.

Research applications of pS2 analysis are now emerging in the literature. This protein has been used as a marker of estrogenic activity for phytoestrogens and xenoestrogens, compounds with estrogen-like structures found in plant foods and in the environment (96,245).

In conclusion, we have described a simple, quantitative assay for pS2 protein and have applied it to study the mode of regulation of the pS2 gene by steroid hormones in the breast carcinoma cell line BT-474. Previously, we have shown that in breast cancer cell lines, the PSA gene is upregulated by androgens and progestins (257). The combined use of pS2 and PSA as markers of hormonal activity in BT-474
cells may allow us to assess the biological activity of natural and xenobiotic compounds which are currently considered as putative endocrine disruptors.

2.3 Is ICI 182,780 an Anti-Progestin in Addition to being an Anti-Estrogen?

2.3.1 Introduction

ICI 182,780 (Faslodex) is a pure antiestrogen, used for the treatment of advanced breast cancer after failure of long-term adjuvant tamoxifen therapy (258,259). Like other antiestrogens, ICI 182,780 may eventually find applications in other aspects of breast cancer (e.g. prevention), as well as in other gynecological and nonmalignant conditions. This compound is a derivative of estradiol, and as such, has high affinity for estrogen receptors (ER). However, because of its long side chain at the 7α position (Figure 2.5), ICI 182,780 stearically hinders receptor dimerization. In the absence of dimerization, binding of the ER to estrogen response elements (EREs) may be abolished or attenuated (260). ICI 182,780 has also been shown to cause the destruction of ER (261,262). It has therefore been demonstrated that ICI 182,780, in vitro mediates virtually no transcription of ER (263).
Other compounds that interact with or affect ER function are also under investigation or are already used in breast cancer therapy. These include the non-steroidal antiestrogens (NSAE), of which tamoxifen is the classic example. Second generations NSAE, which have greater antagonistic and lesser agonistic activity than tamoxifen (264-269), include raloxifene and droloxifene. Progestins, such as R4020 and antiprogestins, such as RU-486 (mifepristone) are also being examined. Progestins are currently the most commonly used second-line endocrine therapy for advanced breast cancer (263). Antiprogestins have been in clinical trial for a few years (263). However, the question remains as to their precise mechanism of inhibiting receptor-positive tumours.

Studies, using cells transfected with ER and PR reporter genes, have indicated one mechanism of action of antiprogestins. They demonstrate extensive inhibitory cross-reactivity between ER and PR. Both progestins (R5020) and antiprogestins (RU-486) have been shown to act as potent ligand-dependent repressors of ER activity when
bound either isoform of PR (270). Moreover, RU-486 has been shown to stimulate growth of MCF-7 cells at $10^{-6}$ M, which can be blocked by either 4OH-tamoxifen and ICI 164,384, suggesting weak estrogenic activity (271). A recent study (272) has pointed to "reverse" cross-reactivity between these receptors, i.e. blocking of progestin-induced transcription by the antiestrogen ICI 182,780. In this study, cell lines devoid of endogenous hormone receptors were transfected with PR expression plasmids, and a luciferase reporter gene driven by progesterone response elements (PREs).

Reconstituted hormone action in cells devoid of endogenous receptors has advantages and disadvantages. Among the advantages is the simplicity of the system and the avoidance of "cross-talk" between various types of receptors. The incorporation of reporter genes with minimal promoter elements and hormone response elements (HREs) further facilitates quantification of semi-quantitative detection of the response. However, such systems may provide misleading or physiological non-relevant results, especially when multiple HREs are incorporated into the promoters of the reporter gene which then become "too-sensitive" reporters (273). Furthermore, physiologically relevant ratios of various hormone receptors, co-activators, and co-repressors and transcription factors are not present in cell lines naturally devoid of hormone receptors. It would be important to verify newly identified actions of hormonal or antihormonal agents in artificial systems by using cells containing natural receptors and endogenous genes which are regulated by such receptors. In this study we examine the recently identified antiprogestin action of ICI 182,780 by using the hormone receptor-positive breast carcinoma cell line BT-474 and the endogenously regulated genes pS2
(estrogen-regulated), prostate specific antigen (PSA) (androgen-progestin-regulated) and human glandular kallikrein (hK2) (androgen-progestin-regulated).

2.3.2 Materials and Methods

2.3.2.1 Materials

The BT-474 human breast carcinoma cell line was obtained from the American Type Culture Collection (ATCC), Rockville, MD. The levels of ER and PR are 29 and 389 fmol/mg, respectively. Although at present AR cannot be quantified, we know from Northern blot studies that this cell line contains AR (274). ICI 182,780 was purchased from Tocris Cookson, Inc., Ballwin, MO, and RU-486 (mifepristone) was a gift from Roussel UCLAF, Paris, France. All steroids used were obtained from Sigma Chemical Co., St. Louis, MO, and stock solution were prepared at $10^{-3}$ M in anhydrous alcohol.

2.3.2.2 Methods

BT-474 cells were grown to confluency in phenol-free RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 mg/mL insulin and 200 mM L-glutamine at 37°C, 5% CO$_2$. Once confluent, they were subcultured in 24-well microtiter plates using the same media, but with substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum. The cells were then stimulated with either a steroid (progesterone, norgestrel or estradiol) alone at a concentration of $10^{-8}$ M, blocker (ICI 182,780 or RU-486) at $10^{-8}$, $10^{-7}$, $10^{-6}$ or $10^{-5}$ M, alone, or both blocker and steroid together. In wells with both blocker and steroid, blocker was added first, then incubated for 1 hour prior to addition of steroid at the conditions specified above. The
plates were then incubated for 7 days, at which time the supernatants were harvested. They were then analyzed quantitatively for pS2, prostate-specific antigen (PSA) and human glandular kallikrein (hK2) as exemplified below.

**Assays**

*pS2 Assay.* We have used an ELISA-type competitive immunoassay for pS2 which was developed in-house. The details of this assay will be described elsewhere. In short, we coat microtiter wells with a monoclonal anti-pS2 antibody and perform a competition between a biotinylated 28-mer peptide derived from the N-terminal sequence of pS2 and endogenous pS2. After washing unreacted moieties, we detect bound biotinylated peptide with a streptavidin-alkaline phosphatase conjugate and time-resolved fluorometry, essentially as described elsewhere (248). The detection limit of this assay is ~20 ng/mL.

*PSA Assay.* Prostate specific antigen was quantified with an ELISA-type immunofluorometric procedure essentially as described elsewhere (275). The detection limit of this assay is ~1 ng/L.

*hK2 Assay.* An ELISA-type immunofluorometric assay was also used. The details of this assay have been described elsewhere (276). The detection limit of this assay is ~6 ng/L.

All measurements were performed in duplicate and all experiments described were performed at least twice.
2.3.3 Results

pS2 is an estrogen-regulated gene and the concentration of pS2 in the tissue culture supernatants reflects estrogenic activity. Estradiol stimulates pS2 production in a dose-dependent manner at concentrations from 10^{-5} M down to 10^{-11} M. Other steroids do not induce any pS2 upregulation (data not shown). Similarly, the concentration of PSA and hK2 in the tissue culture supernatants reflect progestational/androgenic activity. Both progesterone and norgestrel (and in addition testosterone and dihydrotestosterone, data not shown) stimulate PSA and hK2 production in a dose-dependent manner, from 10^{-5} M down to 10^{-11} M. Estrogens upregulate these two genes, but only at concentrations higher than 10^{-8} M (data not shown). All three endogenous reporter genes used (pS2, PSA, hK2) are secreted proteins and we found that 1 week post-single stimulation provide the best quantitative and reproducible data.

ICI 182,780 and mifepristone were tested at concentrations from 10^{-8} to 10^{-5} M for estrogenic (pS2) and progestational/androgenic activity (PSA and hK2). The data are shown in Table 2.1. ICI 182,780 did not show any agonistic activity at these concentrations for either receptor. This was as expected for a pure antiestrogen. RU-486 showed no estrogenic activity, but some progestational/androgenic activity was found (Table 2.1). This is in line with other reports of agonistic activity of this antiprogestin (277). Our controls worked as expected, i.e. induction of pS2 by estradiol and of PSA and hK2 by progesterone and about 100-fold more potently by the synthetic progestin, norgestrel (Table 2.1).
Table 2.1. Production of pS2, PSA and hK2 by Blockers and Steroids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>pS2 ng/mL</th>
<th>PSA ng/L</th>
<th>hK2 ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI 182,780</td>
<td>10⁻⁵ M</td>
<td>&lt;20</td>
<td>15</td>
<td>&lt;6</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>10⁻⁶ M</td>
<td>&lt;20</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>10⁻⁷ M</td>
<td>&lt;20</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>ICI 182,780</td>
<td>10⁻⁸ M</td>
<td>&lt;20</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>RU 486</td>
<td>10⁻⁵ M</td>
<td>&lt;20</td>
<td>617</td>
<td>1054</td>
</tr>
<tr>
<td>RU 486</td>
<td>10⁻⁶ M</td>
<td>&lt;20</td>
<td>1050</td>
<td>4800</td>
</tr>
<tr>
<td>RU 486</td>
<td>10⁻⁷ M</td>
<td>&lt;20</td>
<td>656</td>
<td>2428</td>
</tr>
<tr>
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<td>10⁻⁸ M</td>
<td>&lt;20</td>
<td>434</td>
<td>600</td>
</tr>
<tr>
<td>Estradiol</td>
<td>10⁻⁶ M</td>
<td>202</td>
<td>49</td>
<td>8</td>
</tr>
<tr>
<td>Progesterone</td>
<td>10⁻⁷ M</td>
<td>&lt;20</td>
<td>799</td>
<td>1943</td>
</tr>
<tr>
<td>Norgestrel</td>
<td>10⁻⁸ M</td>
<td>&lt;20</td>
<td>66253</td>
<td>162822</td>
</tr>
</tbody>
</table>

Blocking experiments demonstrated that ICI 182,780, at concentrations of 10⁻⁵ M to 10⁻⁷ M, showed 97-100% blocking of estradiol action. At 10⁻⁸ M, this blocking was minimal (only 16%). Interestingly, RU-486 showed 74-82% blocking of ER at all concentrations used (Figure 2.6). There was no dose-response for this effect, suggesting that the phenomenon does not operate directly through the estrogen receptor but by an alternative pathway, likely involving a cross-talk with the progesterone receptor.

RU-486 showed complete blocking of PR, measured through both PSA and hK2, when either progesterone or norgestrel was used as the progestin. For hK2, however, mifepristone decreased blocking ability at 10⁻⁷ M and below (Figures 2.7 and 2.8). This was not seen for PSA, where blocking was 100% throughout (Figures 2.9 and 2.10).
Figure 2.6. Percent Blocking of pS2 Production, by the Anti-estrogen ICI 182,780 and Anti-progestin RU 486. BT-474 cells were stimulated by $10^{-8}$ M estradiol.
Figure 2.7. Percent Blocking of Human Glandular Kallikrein 2 (hK2) Production by ICI 182,780 and RU 486. BT-474 cells were stimulated by $10^{-8}$ M progesterone.
Figure 2.8. Percent Blocking of hK2 Production by ICI 182,780 and RU 486. BT-474 cells were stimulated by $10^{-8}$ M norgestrel.
Figure 2.9. Percent Blocking of Prostate-Specific Antigen (PSA) Production by ICI 182,780 and RU 486. BT-474 cells were stimulated by $10^{-8}$ M progesterone.
Figure 2.10. Percent Blocking of PSA Production by ICI 182,780 and RU 486. BT-474 cells were stimulated by $10^{-8}$ M norgestrel.
2.3.4 Discussion

The mechanism of gene regulation by steroid hormones is quite complex and involves many molecules including the steroid hormone receptors, heat shock proteins, transcriptional factors, co-activators, co-repressors, protein kinases, etc. (226,278,279). The final result, regarding specificity and potency of response, will depend on the availability and concentration ratios of all these factors and may be heavily dependent on tissue type or, in in-vitro experiments, on type of cell line used. Reconstituted steroid hormone action in cells that are devoid of hormone receptors is possible by exogenously transfecting plasmids containing the coding sequences of the receptors and plasmids containing minimal promoters with hormone response elements in front of indicator genes like CAT or luciferase. These simple systems have enjoyed widespread use because of their simplicity, ease of use and avoidance of complicating cross-talks between various receptor pathways. On the other hand, these systems should be approached with caution since they may produce results that have no relevance to the physiological situation. For example, a plasmid containing three or four EREs is progressively much more sensitive to estrogen stimulation than a plasmid containing only two EREs (273). Moreover, Weaver et al have reported, for example, that the antiestrogen ICI 164,384 had disparate actions on expression of the endogenous gene pS2 and on transfected reporters containing the pS2 estrogen response element (280). Thus, caution should be exercised in interpreting data of reconstituted hormone action experiments until these are verified in cells which possess the steroid hormone receptor system and utilize endogenous genes as reporter systems.
The study of Nawaz et al (272) is important since they report for the first time progestin-induced inhibition of transcription of the pure antiestrogen ICI 182,780. Thus, they conclude that ICI 182,780 acts as an antiprogestin with potency presumably similar to that of the classical antiprogestin RU-486 (mifepristone). They found no intrinsic progestin activity of 182,780 and no antiandrogen or antiglucocorticoid activity. The IC$_{50}$ of ICI 182,780 was around 2 x 10$^{-7}$ M.

We set out to examine these data in a completely different experimental system. Our cell line (BT-474 breast carcinoma cell line) had endogenous hormone receptors. We also used endogenously regulated genes, i.e. pS2 (estrogen-regulated) and PSA and hK2 (androgen-progestin regulated). Quantitative analysis of the three secreted proteins in the tissue culture supernatants assures quantitative comparison of data.

The possible antiprogestational activity of ICI 182,780 and its comparison to that of RU-486 is important for three reasons. (a) This antiprogestational, in addition to its antiestrogenic activity of ICI 182,780 may be important in relation to its anti-cancer activity in-vivo; (b) This effect may allow us to better understand the clinical pharmacology of the drug and its possible therapeutic mechanisms, so that other compounds with similar activity are developed; (c) ICI 182,780 is used in in-vitro studies with cultured cells at concentrations around 10$^{-6}$ M and the effects observed are attributed to its antiestrogenic activity. A possible antiprogestational activity of the drug at these levels will complicate the interpretation of data.

We have first verified that ICI 182,780 has no detectable estrogenic, androgenic or progestin activity even at concentrations as high as 10$^{-5}$ M (Table 2.1). Further, we have shown that RU-486 has significant progestin agonist activity, as reported
previously (248) and that norgestrel, a synthetic progestin, is about 100-fold more potent than progesterone, also in accordance with previous data regarding affinities of norgestrel and progesterone for the PR (248). In addition, we verified the specific upregulation of the pS2 gene by estradiol (Table 2.1).

The potent antiestrogenic activity of ICI 182,780 is shown in Figure 2.6. At $\geq 10^{-7}$ M, ICI 182,780 inhibited $\geq 97\%$ of estradiol's action, as expected (Figure 2.6). We have further observed a strong antiestrogenic activity of the antiprogestin RU-486 (approximately 70-80% inhibition of estradiol's activity) which was not dose-responsive (Figure 2.6). This activity does not seem to be directly related to the ability of RU-486 to block the estrogen receptor, but rather, to be an indirect phenomenon associated with progesterone receptors liganded by RU-486. Such an effect has previously been published in the literature with progestins or antiprogestins demonstrating either inhibitory or stimulating estrogenic effects (270,271).

In Figure 2.7 we demonstrate, in agreement with the data of Nawaz et al, weak antiprogestational activity of ICI 182,780. The IC$_{50}$ of ICI 182,780 for progesterone is between $10^{-6}$ – $10^{-7}$ M, also in agreement with the previous report. However, the inhibition of the synthetic progestin norgestrel was only between 40-70% at an ICI 182,780 concentration of $10^{-5}$ M and not detectable at lower concentrations. Comparison of the inhibiting (antiprogestational) activity of ICI 182,780 to RU-486 clearly indicates that the latter is at least a 1000-fold more potent antiprogestin (Figures 2.7, 2.8, 2.9 and 2.10).

In conclusion, we have verified with a tissue culture system that contains endogenous receptors and with utilization of endogenous genes that indeed, ICI
ICI 182,780 has weak antiprogestational activity. However, this activity is far less than the activity of the synthetic antiprogestin RU-486. It seems unlikely that ICI 182,780 in-vivo, and at the concentrations used to treat breast cancer, will have any clinically significant antiprogestational activity in addition to its potent antiestrogenic activity. The weak antiprogestational activity of ICI 182,780 may complicate interpretations of tissue culture experiments in which the compound is used at concentrations $\geq 10^{-6}$ M and is considered a pure antiestrogen.
CHAPTER 3: STEROID HORMONE ACTIVITY OF FLAVONOIDS AND RELATED COMPOUNDS
CHAPTER 3: STEROID HORMONE ACTIVITY OF FLAVONOIDs AND RELATED COMPOUNDS

3.1 Rationale

After developing a tissue culture bioassay system to determine steroid hormone activities, our next objective was to determine the agonist and antagonist activities of pure isoflavones, other flavonoids, and structurally-related compounds. Our secondary objective was to relate activity of these compounds to structural components, including importance of the flavonoid diaryl nucleus, position of the B ring, and hydroxyl side groups. Dr. Vigi Bezwada of Indofine Chemical Co., Sommerville, NJ, a company that specializes in natural compounds, kindly provided us with 60 flavonoids and related compounds, including the soy isoflavones biochanin A, genistein and daidzein. Another 15 compounds were purchased from Sigma Co., St. Louis, MO.

Because of the relatively large number of compounds, we first screened them at concentrations of $10^{-5}$ M and $10^{-7}$ M for estrogenic and androgenic/progestational activity. The compounds that were found to be positive for activity were then tested in a dose-response manner at concentration of $10^{-5}$ to $10^{-6}$ M. The strongest estrogenic compounds were the soy isoflavones, genistein and biochanin A. The majority of the compounds that had activity were flavonoids, and had hydroxyl groups at positions 7, 6 or 4'. A few flavonoids also had progestational activity, with apigenin having the strongest. All of these had hydroxyl groups at positions 7 and 4'. None of the soy isoflavones possessed progestational activity, demonstrating that position of the B ring on carbon 2 was necessary.

Antagonist experiments were conducted similarly. The compounds were first screened at a concentration of $10^{-5}$ M. Antagonist activity was defined as the flavonoid
inhibiting production of pS2 or PSA by greater than 50% when stimulated by a steroid (10^-9 M estradiol or DHT, or 10^-10 M norgestrel). Compounds that demonstrated at least 50% blocking were then tested for dose-response activity at 10^-5 to 10^-8 M. ICI 182,780, mifepristone and nilutamide (antiandrogen) were tested similarly as controls. The most significant result of this study is that several of the compounds tested have antiandrogen activity. The initial observation was with the soy isoflavone genistein, which, until the scientific correspondence was submitted, was thought only as a potent phytoestrogen. Since then, at least one other study has confirmed this result (108). We have demonstrated that several flavonoids were able to inhibit PSA production in the BT-474 breast cancer and in PC-3(AR)_2 prostate cancer cell lines.

As potential natural antiandrogens, genistein and other flavonoids may play important roles in prostate cancer prevention and treatment. Research in this field has only started being pursued, and many more in vitro and in vivo studies must be conducted, for clinical relevance to be determined.

3.2 Agonist Activity of Flavonoids and Related Compounds on Steroid Hormone Receptors

3.2.1 Introduction

Many studies have focused on the soy isoflavones, particularly daidzein and genistein, and their possible anticarcinogenic properties. These compounds, have been demonstrated in-vitro and in-vivo to have estrogenic as well as antiestrogenic activity (83,97). Moreover, these compounds, have been shown in-vivo to lengthen the follicular phase of the menstrual cycle (191), reduce urinary excretion of 17β-estradiol, and favor 2-hydroxyestrone formation (281), all of which are associated with reduced
risk for breast cancer. More recently, there is increased interest on selective estrogen receptor modulators (SERMs) which hold promise of being used to prevent osteoporosis in post-menopausal women, and at the same time reduce the risk of developing breast cancer and atherosclerosis (282-285). Raloxifene is one SERM that is now in clinical trials (286). Others have put forward the idea that phytoestrogens may represent natural SERMs (287).

Isoflavones are members of a larger family of compounds, known as the flavonoids. This family also includes flavones, flavanones, and flavans, which, like isoflavones, contain a phenyl side-chain, with a variable number of hydroxyl or other groups (Figure 1.1). These compounds have some structural similarities to the natural estrogen estradiol, as well as other steroid hormones and steroid hormone antagonists (Figure 1.2).

The study of flavonoids and their possible role in preventing chronic diseases including heart disease, and breast and prostate cancers, has increased dramatically in the last decade. These compounds exist in relatively large amounts in our food supply, especially in fruits and vegetables, tea, red wine, and legumes. Intakes of these compounds are now estimated to be up to 45 mg/day (288), and a database of foods with known flavonoid content has been created (67).

In-vitro, flavonoids have been shown to act as antioxidants (289), arrest the cell cycle and inhibit topoisomerase II (290,291), and exert antiproliferative activities (292-295). Many of the observed effects are thought to play a role in preventing breast and/or prostate cancers. With the exception of soy isoflavones, few studies have focused on the estrogenicity of the flavonoids per se (102,103,296), and to date, to our
knowledge, only one study has examined the possible androgenic and progestational activities of flavonoids (297). Furthermore, examination of flavonoid structure and correlation to biological activity (e.g. estrogenicity) has been limited (101,298). Whether similar structure/function relationships exist for estrogen, androgen and progesterone receptor binding of these phytochemicals is unknown.

Therefore, we assessed the estrogenic and androgenic/progestational activities of 72 flavonoids and related compounds using a human breast cancer cell line system, utilizing two steroid hormone-regulated endogenous proteins. pS2 is an estrogen-regulated protein, and prostate-specific antigen (PSA) is regulated by androgens and progestins. We have developed immunoassays to measure these secreted proteins in tissue culture supernatants. The data allowed us to identify active compounds and compare activity to structure. These data may be useful for comparing relative potencies of each of the phytochemicals regarding their ability to bind and activate steroid hormone receptors.

3.2.2 Materials and Methods

3.2.2.1 Materials

The BT-474 human breast cancer cell line was purchased from the American Type Culture Collection, Rockville, MD. The levels of estrogen receptor (ER) and progesterone receptor (PR), as quantified by commercial ELISA assays (Abbott Diagnostics, Abbot Park, Chicago, IL) were 29 and 389 fmol/mg, respectively. Although the androgen receptor (AR) content was not quantified, Northern blot studies indicated that this cell line contains AR (299). All steroids used were from Sigma Chemical Co.,
St. Louis, MO, USA. Stock, $10^{-3}$ M solutions of steroids were prepared in absolute ethanol. Flavonoids and related compounds were obtained from Indofine Chemical Co., Inc., Summerville, NJ, and Sigma. We prepared $10^{-2}$ M stock solutions in absolute ethanol. Mifepristone (RU 486) and nilutamide (RU 56187) were gifts from Roussel-UCLAF, Romainville, France.

3.2.2.2 Methods

BT-474 cells were grown to confluency in phenol-free RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 mg/mL insulin and 200 mM L-glutamine at 37°C, 5% CO$_2$. Once confluent, they were subcultured in 24-well microtiter plates using the same media, but with substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum.

The cells were then stimulated with a flavonoid or related compound at $10^{-5}$ M and $10^{-7}$ M (final concentrations) and incubated for 7 days at the same conditions as above. Estradiol, norgestrel and dihydrotestosterone (DHT) at $10^{-8}$ M were used as positive controls and ethanol (solvent) as a negative control. After 7 days, the supernatants were harvested and analyzed for pS2 and PSA. Compounds found to stimulate production of pS2 or PSA were then tested for dose-response, in the range from $10^{-5}$ to $10^{-8}$ M.
**Assays**

*pS2 Assay.* pS2 protein was analyzed using an ELISA-type competitive fluorometric immunoassay. The details of this assay are described elsewhere (300). The detection limit is ~20 ng/mL.

*PSA Assay.* Prostate specific antigen was quantified with an ELISA-type immunofluorometric procedure essentially as described elsewhere (250). The detection limit of this assay is ~1 ng/L.

### 3.2.3 Results

The breast carcinoma cell line BT-474 is steroid hormone receptor-positive. Hall et al have demonstrated presence of estrogen (ER), androgen (AR) and progesterone (PR) receptor mRNAs in BT-474 cells (299). We have further confirmed presence of ER and PR by ELISA assays and of AR by immunohistochemistry (data not shown).

Our tissue culture system is based on the principle that the selected endogenous genes pS2 and PSA are directly up-regulated by steroid hormones. The pS2 gene contains estrogen response elements in its proximal promoter and its expression is increased after estrogen induction (251), but not after androgen or progestin induction (300). Similarly, the PSA gene contains multiple androgen response elements in its promoter/enhancer region (301) and is up-regulated by androgens and progestins but not estrogens (257). Thus, this tissue culture system is a sensitive indicator of hormone receptor activation and induction of transcription. The principles of transcriptional activation by steroid hormones have been described by Beato et al (84).
Of the 72 flavonoids and related compounds tested (Table 3.1, Appendix), 18 were found to have estrogenic activity. Six compounds of this subset were also found to have androgenic and/or progestational activity. Of these, one compound had strong activity (apigenin), two had significantly lower activity (fisetin, naringenin) and three had very weak activity (Table 3.1). The soy isoflavones, biochanin A, genistein and daidzein, demonstrated strong estrogenic activity; the same was true for luteolin and naringenin, two citrus flavonoids. Resveratrol, a trihydroxystilbene found in red wine, 6-bromo-2-napthol, several hydroxyflavanones and two hydroxyflavones had moderate estrogenic activity (Table 3.1). A dose-response relationship for the three soy isoflavones is shown in Figure 3.1. Detectable estrogenic activity was seen at isoflavone concentrations around $10^{-7} - 10^{-8}$ M. The estrogenic activity of the compounds of Table 3.1 below naringenin was detectable only at a concentration of $10^{-5}$ M.

The relatively large number of compounds tested allowed us to correlate the structure of these compounds with the potency of their activity. Below, we summarize our general observations.
<table>
<thead>
<tr>
<th>Compound with Estrogenic Activity</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>100</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>95</td>
</tr>
<tr>
<td>Luteolin</td>
<td>58</td>
</tr>
<tr>
<td>Daidzein</td>
<td>55</td>
</tr>
<tr>
<td>Naringenin</td>
<td>40</td>
</tr>
<tr>
<td>7-Hydroxyflavone</td>
<td>25</td>
</tr>
<tr>
<td>6-Hydroxyflavanone</td>
<td>23</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>22</td>
</tr>
<tr>
<td>6-Bromo-2-naphthol</td>
<td>20</td>
</tr>
<tr>
<td>Chrysin</td>
<td>18</td>
</tr>
<tr>
<td>Apigenin</td>
<td>16</td>
</tr>
<tr>
<td>6-Hydroxyflavone</td>
<td>15</td>
</tr>
<tr>
<td>Morin</td>
<td>15</td>
</tr>
<tr>
<td>Fisetin</td>
<td>12</td>
</tr>
<tr>
<td>Quercetin</td>
<td>10</td>
</tr>
<tr>
<td>7,8-Dihydroxyflavone</td>
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</tr>
<tr>
<td>4'-Hydroxyflavanone</td>
<td>8</td>
</tr>
<tr>
<td>7-Hydroxyflavanone</td>
<td>8</td>
</tr>
<tr>
<td>Compounds with Progestational Activity</td>
<td>Relative Activity (%)</td>
</tr>
<tr>
<td>Apigenin</td>
<td>100</td>
</tr>
<tr>
<td>Fisetin</td>
<td>0.8</td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.7</td>
</tr>
<tr>
<td>Chrysin</td>
<td>0.2</td>
</tr>
<tr>
<td>Luteolin</td>
<td>0.2</td>
</tr>
<tr>
<td>Morin</td>
<td>0.1</td>
</tr>
<tr>
<td>Compounds without Agonist Activity</td>
<td></td>
</tr>
<tr>
<td>Abrine</td>
<td>3,4-Dimethoxycinnamic acid</td>
</tr>
<tr>
<td>Andrographolide</td>
<td>7,8-Dimethoxyflavone</td>
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<td>Ascorbic acid</td>
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<td>Flavanone</td>
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<td>2',6'-Dimethoxyacetophenone</td>
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</tr>
<tr>
<td>2,3-Dimethoxybenzaldehyde</td>
<td>Karanjin</td>
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Figure 3.1. Estrogenic Activity of Three Isoflavones, Measured as ng of pS2 protein/mL in the Tissue Culture Supernatant. The activity was dose-dependent at the concentrations shown.
1. The flavonoid core structure (diaryl ring) is important for estrogenic and progestational activity. From the 18 identified active compounds (Table 3.1), 16 were flavonoids. Of the 31 non-flavonoids tested, only 2 showed weak estrogenic activity. These two compounds were a naphthol derivative (6-bromo-2-naphthol), and a trihydroxystilbene (resveratrol). All of the compounds demonstrating progestational/androgenic activity were flavonoids.

2. Hydroxyl groups appear to be crucial for activity. If hydroxyl groups are not present in the structure, no estrogenic or progestational/androgenic activity is observed. For example, we observed no activity for 5,6-benzoflavone, 7,8-benzoflavone, flavone and flavanone, while we detected activity for 7-hydroxyflavone, 6-hydroxyflavone, 7,8-dihydroxyflavone, 6-hydroxyflavanone, 4'-hydroxyflavanone, and 7-hydroxyflavanone.

3. When hydroxyl groups are methylated, activity is diminished. Examples include 7,8-dimethoxyflavone, which has no estrogenic activity, versus 7,8-dihydroxyflavone (active); 7-methoxyflavone (inactive) versus 7-hydroxyflavone (active), and 6-methoxyflavone (inactive) versus 6-hydroxyflavone (active). However, this observation did not hold true for biochanin A which is as potent as genistein, despite having a methoxy group in position 3'.
4. Isoflavones, in general, were found to be more potent estrogens than other flavonoids. The isoflavones Biochanin A, genistein, and daidzein showed estrogenic activity in a dose-dependent manner, from $10^{-7}$ M to $10^{-5}$ M (Figure 3.1). The other flavonoids had no activity below $10^{-6}$ M. Additionally, apigenin (a flavone) and genistein (an isoflavone) differ only in the position of the benzyl ring (3 position versus 2 position). This difference reduces apigenin's estrogenic activity by 84% in comparison to genistein.

5. Flavones and flavanones appear to have greater estrogenic potency than flavans (e.g. 7-hydroxyflavone > 7-hydroxyflavanone >> 7-hydroxyflavan).

6. The position of the hydroxyl groups appears to be important. Hydroxyl groups at positions 6, 7 or 4' confer more potent estrogenic activity than hydroxyls at positions 3, 5 or 2'. For example, 6-hydroxyflavone > 3-hydroxyflavone and 5-hydroxyflavone (inactive); 6-hydroxyflavanone > 2'-hydroxyflavanone (inactive); 7-hydroxyflavone > 3-hydroxyflavone and 5-hydroxyflavone (inactive); 7-hydroxyflavanone > 2'-hydroxyflavanone (inactive) and 4'-hydroxyflavanone > 2'-hydroxyflavanone (inactive). Remarkably, the top 6 compounds with estrogenic activity (Table 3.1) have a 7-position hydroxyl group and apigenin, the most potent progestational agent also possesses a hydroxyl group at position 7. Additionally, conjugation of the hydroxyl group at position 7 renders the
conjugate inactive, e.g. naringenin (active) versus naringin (inactive). See also point #3 above.

7. The most potent estrogenic compounds have between 2 and 4 hydroxyl groups. At least one is localized in the 7 position of ring A, and another one in the 4' position of ring B (e.g. 7,8-dihydroxyflavone is much less potent than daidzein, in which the second hydroxyl group is in the 4' position).

8. A hydroxyl or other group at position 3 or 8 interferes with agonist activity on estrogen and progesterone receptors (e.g. luteolin > quercetin and rutin; luteolin, apigenin > fisetin; 7-hydroxyflavone > 7,8-dihydroxyflavone).

9. Specificity of flavonoids for estrogen or progesterone/androgen receptors is dependent on the position of the B ring (e.g. apigenin has progestational activity versus genistein which is an estrogen). From all compounds that have progestational/androgenic activity, none is an isoflavone; all are either flavones or flavanones (Table 3.1). The importance of the 4'-hydroxyl on progestational activity is further demonstrated by comparing apigenin versus chrysin, apigenin versus luteolin (which has an extra hydroxyl) and apigenin versus morín (has two extra hydroxyls).
10. A double bond between carbons 2 and 3 is important for progestin activity (e.g. apigenin versus naringenin). A single bond at this site reduces activity by 99%.

3.2.4 Discussion

Flavonoids, as components of our diets, have been demonstrated to have protective effects on heart disease (302,303), cancer (304), and other conditions and diseases (305-307). The cardioprotective effects of isoflavones are thought to be primarily due to their estrogen-like activity. Like estrogen replacement therapy, soy isoflavones have been demonstrated to increase arterial compliance (308). Additionally, these compounds diminish the symptoms of menopause, including hot flashes (309). For these reasons, many women are reanalyzing the risks and benefits of hormone replacement therapy, and supplementing their diets with soy products instead. Commercial vendors have made available isoflavone tablets. Phytoestrogens are increasingly becoming a natural alternative to synthetic estrogens.

Other flavonoids (flavones, flavanones) have been examined mostly for their antioxidant capabilities. The Seven-Country Study was one of the first to link flavonoid consumption with decreased risk of heart disease (73). Phenolics found in red wine are often used to partially explain the “French Paradox” (310); antioxidants and flavonoids have been shown to reduce LDL oxidation (311). Moreover, their estrogenic capacity may be partially responsible for the increase in HDL, often observed in red wine drinkers (312). These two activities may work simultaneously, providing the consumer
with extra cardioprotective benefits. However, the issue of the "French Paradox" still remains controversial.

The role of flavonoids in cancer development, especially breast and prostate carcinomas has yet to be fully examined. Few epidemiological studies have been conducted (70). In-vitro studies show the antiproliferative, antioxidant and cytostatic effects flavonoids (289-293). Currently, herbal alternatives to drugs, including PC-SPES for prostate cancer management, are being sought (313,314). These products have been shown to be antiproliferative and antitumorigenic (315). However, the mechanism is not well understood, and controlled, prospective studies have not been conducted.

We have examined the estrogenic and progestational/androgenic activities of 72 flavonoids and related compounds in a human breast carcinoma cell line, by monitoring endogenously regulated genes (pS2 and PSA) in the presence of endogenous hormone receptors. Eighteen of the 72 flavonoids and related compounds showed estrogen and/or progestational activity. The soy isoflavones demonstrated activity down to $10^{-7}$-$10^{-8}$ M. However, most of the other flavonoids did not show activity beyond $10^{-5}$ M. These concentrations are approximately 10-fold higher than those reported by other groups, including Miksicek (101) and Le Bail et al (102). Their studies used transfected estrogen-responsive reporter plasmid systems, measured through chloramphenicol acyl transferase or luciferase enzymes. Our system, using endogenous genes and receptors, may be closer to the in-vivo situation, in comparison to data generated with transfected indicator genes and receptors. These synthetic systems are usually too
sensitive and may give distorted results. This issue was stressed recently by Xu et al. (315) for studies related to hormone receptor function.

We analyzed the structure of compounds with and without estrogenic and progestational activity and devised general guidelines, as has been done by Lien et al for phenolic antioxidants (316). Through the testing of a large number of compounds, we were able to establish the relative importance of flavonoid class (flavone, flavanone, isoflavone, flavan), the number and position of hydroxyl groups and double bonds on the biological activity of these compounds. It is clear that subtle changes in the structure of these compounds can bring about a large change in their biological activity and receptor specificity. Our results concur with those of Miksicek (101) and Le Bail et al (102) regarding the importance of the diaryl ring structure, as 16 of the 18 estrogenic compounds were flavonoids. Only resveratrol (a trihydroxystilbene) and 6-bromo-2-naphthol exhibited weak estrogentic activity at a concentration of $10^5$ M. Similarly, we agree with the importance of the 7 and 4’ hydroxyl positions. However, we found the 6 hydroxyl position to confer estrogenic activity as well (6-hydroxyflavone, 6-hydroxyflavanone). Similar to other studies, we found that a hydroxyl group at position 3 or 8, or a total of greater than 4 hydroxyl groups reduces the estrogenic activity of the flavonoid. Hydroxyl groups at positions 2’ or 5 did not significantly affect estrogenic activity, but methylation at the 7 or 4’ position (with the exception of biochanin A) decreased estrogenic activity.

We have also examined the structure/function relationship of flavonoids and related compounds for progestational/androgenic activity. All of the compounds with such activity were flavonoids, demonstrating that the diaryl ring is crucial. Similarly,
they all had hydroxyl groups at positions 7 and 4'. Finally, the presence of a double bond between carbons 2 and 3 appears to be highly important. Elimination of this bond (apigenin → naringenin) decreases this activity by 99%.

One of our significant observations is the specificity of the position of the B ring in relation to the flavonoid core; whether it is bound to position 2 or 3. Two compounds that have the same empirical formula and molecular weight, namely genistein and apigenin, display the strongest biological activity, one being estrogenic (genistein) and one progestational (apigenin). The only difference between these two compounds is the position of the B ring, which qualifies the compound as being either an isoflavone (genistein) or a flavone (apigenin). This structural difference may be exploited to develop compounds that target one receptor versus the other.

This study has demonstrated estrogenic and progestational/androgenic activity of several flavonoids and other compounds by using a screening system based on a human breast carcinoma cell line. These activities should be taken into consideration to explain as to how these compounds, and the natural products in which they are found, may play a role in the prevention of breast and/or prostate cancers. The identified structure/function relationships may aid in the development of drugs or derivatized natural products with greater activity and specificity.
3.3 Genistein: A Potent Natural Anti-androgen

Flavonoids and other polyphenolic natural compounds have attracted much attention as candidate cancer preventive (317-319) and anticarcinogenic agents (126,320,321), as well as antiatherogenic (73,303,308,322) and antioxidant compounds (155,158,323). Although the public now consumes these compounds in significant amounts from dietary sources, food supplements, and, more recently, as "nutraceutical" tablets (308), their actual mode of action is not fully defined. The most compelling hypotheses correlate the biological action of flavonoids to their ability to mimic natural estrogens (83,99,324), like estradiol, or to act as antioxidants (158,323). Indeed, genistein, a natural soy isoflavone, is among the most potent known phytoestrogens. The ability of flavonoids to act as androgen mimics or anti-androgens, has attracted much less attention. Recently, we have shown that apigenin, a natural flavone found in chamomile, olive leaves, and other plant sources, has potent progestational activity (100,297).

We have examined the possible anti-androgenic activity of genistein using the steroid hormone receptor-positive breast cancer cell line BT-474. This cell line, when stimulated by dihydrotestosterone (DHT), produces prostate specific antigen (PSA) which is then secreted into the tissue culture supernatant and can be measured quantitatively by immunoassay. Details of this system are given elsewhere (250,297). To study anti-androgenic activity, the cells are first exposed to the putative anti-androgen (10^{-5}-10^{-8} M) for 1 hour and then stimulated with DHT (10^{-9} M). Controls with only anti-androgen or only DHT were included in all experiments. Nilutamide was used as a control anti-androgen. Our data (Figure 3.2) clearly demonstrate potent anti-
Figure 3.2. Percent Blocking of DHT, as Measured by PSA Production, by Genistein, Quercetin and Nilutamide. BT-474 human breast cancer cells were grown to confluency in phenol-free RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 mg/mL insulin and 200 mM L-glutamine at 37°C, 5% CO₂. Once confluent, they were subcultured in 24-well microtiter plates using the same media, but with substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum. The cells were stimulated with either candidate blocker (genistein, quercetin or nilutamide), blocker and steroid (DHT) or steroid alone. Blockers were tested at concentrations of $10^{-5}$ to $10^{-8}$ M, and DHT was used at $10^{-9}$ M. For the cells stimulated with blocker and steroid, the blocker was added first, and incubated for one hour, then the cells were stimulated with steroid. Alcohol was used as a negative control. The plates were then incubated for 7 days, at which time the tissue supernatants were harvested (297). The supernatants were then analyzed quantitatively for PSA (250). Blocking activity was assessed by dividing the amount of PSA produced by the candidate blocker + steroid and by steroid alone and multiplying by 100. None of the candidate blockers induced any PSA production when added alone.
androgenic activity of genistein, which is dose-dependent and is detectable down to $10^{-7}$ M. Quercetin and a number of other flavonoids tested are devoid of such activity (data not shown).

These data clearly demonstrate for the first time that genistein exhibits potent anti-androgenic activity, in addition to its well-established estrogenic activity. Indeed, the therapeutic potential of this compound in prostate cancer patients may be related to its combined estrogenic and anti-androgenic properties. It will be interesting to examine large numbers of natural compounds for anti-androgenic activity, which may qualify them as candidate therapeutic and preventive agents for prostate, breast and possibly other hormonally-dependent cancers.

3.4 Flavonoids Can Block PSA Production by Breast and Prostate Cancer Cell Lines

3.4.1 Introduction

Prostatic carcinoma is the most commonly diagnosed cancer in North American men, and the second leading cause of cancer death in this population (1). The incidence of prostate cancer has risen sharply over the last decade (1,325-327). Much of this increase can be attributed to better screening techniques, including serum prostate-specific antigen (PSA) analysis (326,328). Other potential contributing factors include the aging population, increasing rates of obesity, high consumption of meat and fat, combined with reduced physical activity (329-332).

Since most prostate tumors are hormone-dependent, combined androgen blockade (CAB) is a common strategy for primary or secondary therapy (333-335).
Agents used for treatment include diethylstilbestrol (an estrogen agonist) (336-338), luteinizing hormone-releasing hormone (LHRH) agonists (339-341), and anti-androgens, including flutamide, nilutamide and cyproterone acetate (342-345). Many of these drugs have side effects including gynecomastia, impotence and hepatic toxicity (346-352). For these reasons, many patients are searching for "natural" alternatives or complements to traditional drugs (313,353-355).

Over the past few years, the beneficial effects of soy and soy isoflavones in the prevention of breast and prostate cancers, as well as of heart disease have been investigated (96,108,289,319,356-358). A body of epidemiological evidence associates consumption of soy, and vegetables in general, with lower prostate cancer risk in Asian populations versus Western countries (319,356,358). In vitro studies have demonstrated the partial estrogen/anti-estrogen activities of soy isoflavones and other flavonoids (38,39), as well as their antioxidant effects (96,102). Cytostasis and induction of apoptosis are two mechanisms by which the monoterpenne, perillyl alcohol (found in citrus fruit), and the synthetic flavone flavopiridol may be effective against prostate and other cancer cells (360-364). These compounds are currently in phase II clinical trials (365,366).

To date, few studies have investigated the ability of plant-derived compounds to block androgenic activities, such as expression of androgen-dependent genes. The soy isoflavone, biochanin A, was demonstrated to decrease testosterone-induced production of PSA in the LNCaP prostate cancer cell line, through upregulation of a catabolic enzyme (367). Resveratrol, the phytoalexin found in red wine, has been shown to have estrogenic and anti-estrogenic activity (104,368) and to downregulate
transcription of both androgen receptor (AR) and PSA genes in this cell line (109). We therefore undertook this study to determine whether various flavonoids and related compounds could inhibit PSA production, a protein regulated by androgens, through the AR.

3.4.2 Materials and Methods

3.4.2.1 Materials

The BT-474 human breast cancer cell line was purchased from the American Type Culture Collection (ATCC), Rockville, MD. The levels of ER and PR, as quantified by commercial ELISA assays (Abbott Diagnostics, Abbott Park, Chicago, IL) were 29 and 389 fmol/mg protein, respectively. Although the AR content was not quantified, Northern blot studies indicated that this cell line contains AR (299). The PC-3 cell line, PC-3(AR)2, transfected with the human full-length AR cDNA, was described elsewhere (369). AR concentration of this cell line is 491 fmol/mg protein. The parental line has been described as having both glucocorticoid and estrogen receptors, although the latter is controversial (370-372). All steroids used were from Sigma Chemical Co., St. Louis, MO. Stock, $10^{-2}$ M solutions of steroids were prepared in absolute ethanol. Flavonoids and structurally-related compounds were obtained from Indofine Chemical Co. Inc., Summerville, NJ and Sigma. We prepared $10^{-2}$ M stock solutions of these compounds in absolute ethanol. Nilutamide (RU 56187) was a gift from Roussel-UCLAF Romainville, France.
3.4.2.2 Methods

BT-474 cells were grown to confluency in phenol-free RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 mg/mL insulin and 200 mM L-glutamine at 37°C, 5% CO₂. Once confluent, they were subcultured in 24-well microtiter plates using the same media, but with substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum. PC-3(AR)₂ cells were grown under similar conditions, using supplementation of media with 5% fetal calf serum and 100 μg/mL hygromycin-B (Calbiochem-Novabiochem Corp., La Jolla, CA).

BT-474 cells were incubated with a flavonoid or related compound at 10⁻⁵ M and 10⁻⁷ M (final concentrations) for 1 hour, after which time the cells were stimulated with dihydrotestosterone (DHT) at 10⁻⁹ M. The cells were then incubated for 7 days at the same conditions as above. DHT was also tested alone (no blocker added) to determine maximum production of PSA, and candidate blockers were tested without steroid (DHT), to determine their androgen agonist activity. The blocking activity of nilutamide (an established anti-androgen) used at 10⁻⁷ M, served as a positive control for anti-androgen activity, and ethanol (solvent) was a negative control. After 7 days, the tissue culture supernatants were harvested and analyzed for PSA. Compounds found to have greater than 50% blocking activity of DHT-induced PSA production were then tested for dose-response activity, in the range from 10⁻⁵ to 10⁻⁸ M in both BT-474 and PC-3(AR)₂ cell lines. Estradiol was also tested, at concentrations of 10⁻⁷ to 10⁻⁹ M, to determine whether this steroid had blocking activity. Each experiment was repeated at least twice, and cells were assayed using trypan blue to monitor cell viability.
Assays

**PSA Assay.** PSA was quantified using an ELISA-type immunofluorometric procedure described elsewhere (250). The detection limit of this assay is ~1 ng/L. Total protein for all supernatants did not differ significantly (data not shown).

### 3.4.3 Results

Our tissue culture testing system is based on the principle that the selected endogenous gene, PSA, is up-regulated by steroid hormones, in this case, DHT. The PSA gene contains multiple androgen response elements (AREs) in its promoter/enhancer region (301). After binding of the AR to its respective ligand, a conformational change occurs, and the ligand-receptor complex subsequently binds to these AREs of the PSA gene promoter. If other transcription factors are also available, the gene is transcribed to mRNA, and then translated to PSA protein, which is secreted. If any of these steps is inhibited, PSA protein production and secretion decrease, and this will be reflected by the PSA concentration in the culture medium. Thus, this tissue culture system is a sensitive indicator of hormone receptor activation and induction of transcription. This system has been used successfully in gene regulation studies (84,257,373) and in identifying biological activity of candidate synthetic progestins (374). The principles of transcriptional activation by steroid hormones have been reviewed by Beato et al (84).

Inhibition of PSA production was defined as greater than 50% blocking of DHT-induced PSA production. Twenty-two of the 72 tested flavonoids and structurally-related compounds were found to significantly inhibit PSA production in the BT-474
breast cancer cell line (Table 3.2, Figure 3.3). These include the soy isoflavones, biochanin A and genistein, the red wine phytoalexin resveratrol, and naringenin, a flavanone found in citrus fruits. Eight of these compounds showed dose-response blocking of PSA production from $10^{-5}$ down to $10^{-7}$ M (Figure 3.4). Eleven of the 22 compounds were previously demonstrated to have estrogen activity, while the other 11 were found to be non-estrogenic (100). Estradiol also blocked PSA production from $10^{-7}$ down to $10^{-9}$ M in the BT-474 cell line.

In the PC-3(AR)$_2$ clonal line, several of these compounds showed no significant blocking of PSA production. These included genistein, biochanin A, chrysin, naringenin and 6-hydroxyflavone. Similarly, estradiol did not inhibit PSA production in this cell line (Table 3.2). The compounds listed in Table 3.3 did not alter PSA production in any of the two cell line systems.

3.4.4 Discussion

The purpose of this study was to evaluate inhibition of androgen-regulated gene expression by flavonoids and structurally-related compounds, measured as inhibition of DHT-induced PSA production in two cell line-based systems. Several studies have investigated estrogen activity of similar compounds and natural products containing these polyphenols (100,103,146). However, very few studies have examined androgenic or antiandrogenic activities (104,109,110,367,368).

Our study has demonstrated that several flavonoids can inhibit PSA production, including isoflavones (genistein, biochanin A), flavones (luteolin, chrysin), and flavanones (naringenin), which were hydroxylated (5-hydroxyflavone, 2'-
Table 3.2. Flavonoids and Related Compounds that Significantly Inhibited PSA Production in the BT-474 and PC-3(AR)2 Cell Lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Blocking of PSA Production in BT-474&lt;sup&gt;2&lt;/sup&gt;</th>
<th>% Blocking of PSA Production in PC-3(AR)&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Bromo-2-naphthol*</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2',5'-Dimethoxyacetophenone*</td>
<td>100</td>
<td>99-100</td>
</tr>
<tr>
<td>Flavone</td>
<td>100</td>
<td>80-85</td>
</tr>
<tr>
<td>5-Hydroxyflavone</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2'-Methoxyflavone</td>
<td>100</td>
<td>60-70</td>
</tr>
<tr>
<td>5-Methoxyflavone</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Harmol</td>
<td>99-100</td>
<td>80-85</td>
</tr>
<tr>
<td>4'-Methoxyflavone</td>
<td>99-100</td>
<td>100</td>
</tr>
<tr>
<td>2'-Hydroxyflavanone</td>
<td>98-100</td>
<td>90</td>
</tr>
<tr>
<td>Biochanin A*</td>
<td>98-95</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Mangostine</td>
<td>81-100</td>
<td>100</td>
</tr>
<tr>
<td>7-Hydroxyflavanone*</td>
<td>81-87</td>
<td>95</td>
</tr>
<tr>
<td>Curcumine</td>
<td>80-97</td>
<td>99-100</td>
</tr>
<tr>
<td>Genistein*</td>
<td>76-87</td>
<td>&lt;50</td>
</tr>
<tr>
<td>4'-Hydroxyflavanone*</td>
<td>74</td>
<td>98-100</td>
</tr>
<tr>
<td>Resveratrol*</td>
<td>72-99</td>
<td>70-75</td>
</tr>
<tr>
<td>Harmalol</td>
<td>67-91</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Naringenin*</td>
<td>65-67</td>
<td>&lt;50</td>
</tr>
<tr>
<td>6-Hydroxyflavone*</td>
<td>64-92</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Luteolin*</td>
<td>62-81</td>
<td>80-85</td>
</tr>
<tr>
<td>Chrysir*</td>
<td>57-91</td>
<td>55-60</td>
</tr>
<tr>
<td>6-Methoxyflavanone</td>
<td>55-89</td>
<td>90-95</td>
</tr>
<tr>
<td>Estradiol&lt;sup&gt;3&lt;/sup&gt;</td>
<td>80-90</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

<sup>1</sup> Compounds tested at 10⁻⁵ M final concentration.

<sup>2</sup> Compounds tested at least twice. Blocking expressed as range, where applicable.

<sup>3</sup> Estradiol was tested at 10⁻⁶ M.

*Compounds found to be estrogenic in a previous study (100).
Table 3.3. Flavonoids and Related Compounds That Did Not Inhibit PSA Production

<table>
<thead>
<tr>
<th>Compound</th>
<th>7,8-Dihydroxyflavone</th>
<th>7-Hydroxyflavone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abrine</td>
<td>2',6'-Dimethoxyacetophenone</td>
<td>Karajin</td>
</tr>
<tr>
<td>Andrographolide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>2,3-Dimethoxybenzaldehyde</td>
<td>5-Methoxyflavanone</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3,4-Dimethoxycinnamic acid</td>
<td>7-Methoxyflavanone</td>
</tr>
<tr>
<td>5,6-Benzoflavone</td>
<td>7,8-Dimethoxyflavone</td>
<td>7-Methoxyflavone</td>
</tr>
<tr>
<td>7,8-Benzoflavone</td>
<td>Ellagic acid</td>
<td>6-Methoxyflavone</td>
</tr>
<tr>
<td>Bixin</td>
<td>Embelin</td>
<td>L-Mimosine</td>
</tr>
<tr>
<td>4'-Bromoacetophenone</td>
<td>Fisetin</td>
<td>Morin</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Flavanone</td>
<td>Naringin</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Folic acid</td>
<td>Picrotin</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>Gallic acid</td>
<td>Piperine</td>
</tr>
<tr>
<td>Conessine</td>
<td>Gardenin</td>
<td>Pongamol</td>
</tr>
<tr>
<td>Daidzein</td>
<td>Harmaline</td>
<td>Quercetin</td>
</tr>
<tr>
<td>2',4'-Dihydroxyacetophenone</td>
<td>Harmine</td>
<td>Rutin</td>
</tr>
<tr>
<td>2',5'-Dihydroxyacetophenone</td>
<td>Salicylic acid</td>
<td>Theophylline</td>
</tr>
<tr>
<td>2',6'-Dihydroxyacetophenone</td>
<td>6-Hydroxyflavanone</td>
<td></td>
</tr>
<tr>
<td>2,5-Dihydroxy-1,4-benzoquinone</td>
<td>3-Hydroxyflavone</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3. Dose-Response Inhibition of PSA Production of Flavonoids and Related Compounds in the BT-474 Human Breast Cancer Cell Line. Significant inhibition is shown as percent blocking of DHT-induced PSA production. Compounds were tested at $10^{-8} - 10^{-5}$ M, estradiol was tested at $10^{-9} - 10^{-7}$ M, and DHT was used at $10^{-9}$ M. $2',5'$-DMAP = $2',5'$-dimethoxyacetophenone.
hydroxyflavanone), methylated (6-methoxyflavanone, 5'-methoxyflavone) or neither (flavone). Other compounds that showed significant PSA-blocking include nitrogen-containing (6-bromo-2-naphthol, harmalol, harmol), and carboxyl-containing (curcumine, 2'5'-dimethoxyacetophenone) phenols. Several of these compounds were shown to have estrogenic activity in a previous study (100).

The ability of flavonoids and other polyphenols in regulating androgen-regulated proteins is not well studied. Three recent papers have all used the human prostate cancer cell line LNCaP, derived from the lymph node metastasis of a prostate cancer patient. The study by Mitchell et al (109) demonstrated dramatic decreases in androgen-induced PSA and human glandular kallikrein (hK2) production in the presence of resveratrol (50-150 μM). Our data supports this finding, but we used much lower resveratrol concentrations (10 μM). Using a PSA-promoter fragment in front of a luciferase reporter gene, they showed that this phytoalexin abolished androgenic induction of the PSA promoter, resulting in significant decreases in transcription of both AR and androgen-regulated genes.

Similar results were seen with green tea polyphenols (GTP) (110). 20-60 μg/mL GTP, incubated with LNCaP cells 1 hour prior to treatment with testosterone, was found to significantly reduce production of ornithine decarboxylase, another androgen-regulated protein. Moreover, Northern blot analysis revealed that this almost complete inhibition occurred at the transcriptional level. Moreover, in vivo, when GTP was added to drinking water, at 0.2% w/v, a 40% inhibition of ornithine decarboxylase activity was found in castrated and sham-operated rats, compared to controls. These two studies indicate that anti-androgen activity of these compounds must occur through inhibition of
transcription of AR and/or androgen-controlled genes, blocking of the receptor, or a combination of these events.

A study conducted with flavonoids, also in LNCaP cells, found that biochanin A significantly induced activity of UDP-glucuronyl transferase, an enzyme responsible for metabolizing testosterone to inactive products. Through this mechanism, inhibition of PSA production was also found (367). In this study a slight increase in binding sites of the AR, and AR protein was noted, therefore, inhibition of AR transcription was not a plausible mechanism for the action of these flavonoids.

In conclusion, we found that several flavonoids and related compounds can significantly inhibit PSA production, an androgen-regulated protein. These plant-derived compounds may thus play a role in the prevention and/or management of prostate cancer.
CHAPTER 4: STEROID HORMONE ACTIVITY OF NATURAL PRODUCTS AND NUTRACEUTICALS
CHAPTER 4: STEROID HORMONE ACTIVITY OF NATURAL PRODUCTS AND NUTRACEUTICALS

4.1 Rationale

After determining the agonist and antagonist steroid hormone activities of pure compounds, the next objective was to evaluate such activities of natural products and nutraceuticals. These were all natural or processed products claimed to contain flavonoids and related compounds previously tested.

With the perception of a deteriorating health care system, coupled with increasing access to medical information via the media and the internet, many patients and healthy individuals are turning to natural products and over-the-counter nutraceuticals to treat or prevent diseases including breast and prostate cancers, and cardiovascular disease. Many claims have been made for these herbs, seeds, roots and their processed products. These include hormonal and anticarcinogenic activities. Soy extracts have gained much popularity as a source of natural estrogens and progestins, for use in menopausal women to suppress symptoms including hot flushes, and to prevent osteoporosis and/or heart disease. Other isoflavone preparations, including red clover extract is also being used for these reasons. Saw palmetto has been scientifically proven to aid in the treatment of urinary problems associated with benign prostatic hypertrophy (BPH), and is now being clinically tested for use in prostate cancer.

This study was undertaken to determine whether sources of isoflavones, i.e. red clover and soy extracts, demonstrate steroid hormone activity in the tissue culture system similar to observations made with the pure isoflavones. Moreover, this study
was to evaluate steroid hormone activity of other commonly used natural products and nutraceuticals that contain several of the flavonoids tested in the previous studies.

4.2 Effects of Natural Products and Nutraceuticals on Steroid Hormone-Regulated Gene Expression

4.2.1 Introduction

Recently, there is a trend for healthy individuals and cancer patients to use complementary and/or alternative medicines (375). These are largely unproven therapies (356) which, at present, have no government regulations. Self-prescribed herbal and natural products represent one of the most popular alternative therapies used by the North American public (375,376). Moreover, the majority of households from the NHANES III study (377) admits taking herbal supplements, many without reporting such practices to physicians or other health care workers (376-378).

Healthy men and women, and prostate and breast cancer patients report several hormonally-related reasons for taking herbal remedies. Saw palmetto (Serenoa serrulata), an extract from the saw palmetto berries, is commonly used for treating benign prostatic hypertrophy (BPH) and accompanying urinary tract problems (379-381), and is now being extended, through self-medication, to prostate cancer management (356). Pumpkin extract is also being used for BPH (379). Dong quai (Angelica sinesis) and Mexican wild yam root (Dioscorea villosa) are being used by menopausal and post-menopausal women as phytoestrogens and/or phytoprogestins (382-384). Isoflavone preparations are being sold on the market (e.g. Promensil™ by Novogen, Estro-Logic™ by Quest) to reduce risk of cardiovascular disease (205,386-
388), osteoporosis (387,388) and to combat menopausal symptoms such as hot flushes (389,390).

Other nutraceuticals being widely used by the public include echinacea (Echinacea purpurea, Echinacea angustifolia) to boost immune function (391,392), Saint. John's Wort (Hypericum perforatum) as an anti-depressant (393,394), cranberry extract for antibacterial and antioxidant activity (395,396), and grapeseed extract, for its antioxidant activity (397,398). Many of these natural products have been hypothesized to have anticarcinogenic activity (397-399), and are being used ad libitum by breast and prostate cancer patients (400,401).

Because many of these natural products presumably have hormonal activity, and others are being used by individuals with, or at risk of developing hormone-dependent cancers, we have undertaken this study to evaluate steroid hormone agonist and antagonist activities of these preparations, using an in-vitro tissue culture system. The BT-474 human breast cancer cell line, which is positive for estrogen (ER), androgen (AR) and progesterone (PR) receptors, was used. For assessing biological activity, we utilized secreted proteins, namely pS2, which is estrogen-regulated, and prostate specific antigen (PSA), which is under the control of androgens and progestins.

4.2.2 Materials and Methods

4.2.2.1 Materials

The BT-474 human breast cancer cell line was purchased from the American Type Culture Collection (Rockville, MD). The levels of ER and PR, as quantified by commercial ELISA assays (Abbot Diagnostics, Abbot Park, Chicago, IL) were 29 and
389 fmol/mg protein, respectively. Although the AR content was not quantified, Northern blot studies indicated that this cell line contains AR (299). All steroids used were from Sigma Chemical Co. (St. Louis, MO). Stock, $10^{-2}$ M solutions of steroids were prepared in absolute ethanol. Natural products and nutraceuticals were purchased from pharmacies and health food stores (Table 4.1), with the exception of Estro-Logic™, which was a gift from Bruce Chapman, Boehringer Ingelheim Self Medication. ICi 182,780 was purchased from Tocris Cookson, Inc., Ballwin, MO, and RU-486 (mifepristone), and Nilutamide (RU 56187) were gifts from Roussel-UCLAF (Romainville, France). Stock, $10^{-2}$ M solutions of antagonists were prepared in absolute ethanol.

4.2.2.2 Methods

Preparations of Natural Products and Nutraceuticals

All liquid extracts were diluted 1:10 in anhydrous ethanol. Contents of capsules were mixed with 2 mL ethanol and incubated overnight. The liquid phase was then separated by centrifugation. Gelcaps were broken and the liquid contents were diluted with 1 mL ethanol. Tablets were crushed and incubated with 2 mL ethanol overnight. The liquid layer was then separated by centrifugation. More dilute solutions were further prepared in ethanol.
Table 4.1. Natural Products and Nutraceuticals Tested

<table>
<thead>
<tr>
<th>Name</th>
<th>Active Component/Claim</th>
<th>Form</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black Tea</td>
<td>Theaflavins</td>
<td>Tea</td>
<td>Natural food store</td>
</tr>
<tr>
<td>Canadian Ginseng</td>
<td>Ginsenosides</td>
<td>Capsule</td>
<td>Natural food store</td>
</tr>
<tr>
<td>Carob Syrup</td>
<td>B vitamins</td>
<td>Syrup</td>
<td>Natural food store</td>
</tr>
<tr>
<td>Chamomile</td>
<td>Apigenin</td>
<td>Gel-tab</td>
<td>Jamieson Natural Sources</td>
</tr>
<tr>
<td>Cran-Max</td>
<td>Procyanidins</td>
<td>Tablet</td>
<td>Swiss Natural Sources</td>
</tr>
<tr>
<td>Dong Quai</td>
<td>Phyto-progestin</td>
<td>Extract</td>
<td>Swiss Natural Sources</td>
</tr>
</tbody>
</table>
| Echinacea          | Immune function, anti-
                      |        | Extract                    |
|                    | inflammatory                |        | Swiss Natural Sources      |
| Estro-Logic        | Soy isoflavones              | Capsule| Quest                      |
| Evening Primrose Oil | γ-Linolenic acid           | Gel-tab| Jamieson Natural Sources   |
| Garlic             | Allium extract               | Gel-tab| Swiss Natural Sources      |
| Grapeseed          | Procyanidins                 | Capsule| Swiss Natural Sources      |
| Green Tea          | Catechins                    | Tea    | Natural food store          |
| Promensil          | Red clover isoflavones       | tablet | Novogen Ltd.               |
| Prostate-Ease      | Saw palmetto, flaxseed,      | Gel-tab| Swiss Natural Sources      |
|                    | pumpkin seed extracts        |        |                            |
| Rosehips           | Vitamin C                    | Tablet | Swiss Natural Sources      |
| Saw Palmetto       | Alleviate BPH symptoms       | Extract| Herbs Etc.                 |
| Siberian Ginseng   | Ginsenosides                 | Capsule| Natural food store          |
| St. John’s Wort    | Anti-depressant              | Capsule| Swiss Natural Sources      |
| Watermelon Juice   | Prostate health              | Juice  | Natural food store          |
| Wild Yam Root      | Diosgenin                    | Extract| Gaia Herbs Inc.            |
**Definition of Biological Activity**

In this study, we have used the breast carcinoma cell line BT-474 as a biological testing system and the steroid hormone-regulated genes pS2 (estrogen-regulated) and PSA (androgen and progestin-regulated) as indicators of hormone action. Natural products that stimulated pS2 production were defined as having estrogenic agonist activity while those which stimulated PSA production were defined as having progestational/androgenic agonist activity. Discrimination between progestational and androgenic activity were made by blocking experiments with either mifepristone (anti-progestin) or nilutamide (anti-androgen). Blocking (antagonist) activity of the natural products was defined as their ability to block production of pS2 (stimulated by estradiol) or PSA (stimulated by either dihydrotestosterone or norgestrel). These definitions are descriptive of the final effect but do not imply any mechanistic aspects of this up or down regulation of the indicator genes.

**Cell Culture**

BT-474 cells were grown to confluency in phenol-free RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 10 mg/mL insulin and 200 mM L-glutamine at 37°C, 5% CO₂. Once confluent, they were subcultured in 24-well microtiter plates using the same media, but with substitution of charcoal-stripped fetal calf serum for the regular fetal calf serum.
**Agonist Activity Study**

The cells were stimulated with natural product at full strength (stock solution), and 10, 100 and 1000-fold dilutions. Estradiol, norgestrel and dihydrotestosterone (DHT) at $10^{-6}$ M were used as positive controls and ethanol (solvent) as a negative control. The cells were incubated with the stimulant for 7 days. The tissue culture supernatants were then harvested and quantified for pS2 and PSA proteins. Preparations that were found to stimulate PSA production were subsequently tested using mifepristone (RU-486, an anti-progestin) or nilutamide (an anti-androgen) to determine whether the effect was progestational or androgenic.

**Antagonist Activity Study**

The BT-474 cells were incubated with natural product at full strength, or at 10, 100 or 1000-fold dilutions for 1 hour, after which time the cells were stimulated with either estradiol, norgestrel or DHT at $10^{-9}$ M. The cells were then incubated for 7 days at the same conditions as above. Steroid was also tested alone (no candidate blocker added) to determine maximum production of pS2 and PSA. The blocking activity of faslodex (ICI 182,780, an anti-estrogen), mifepristone and nilutamide used at $10^{-7}$ M, served as positive controls for antagonist activity, and ethanol (solvent) was a negative control. After 7 days, the tissue culture supernatants were harvested and analyzed for pS2 and PSA. Percentage blocking was calculated by quantifying the concentration of pS2 or PSA produced by natural product + steroid, divided by the concentration of pS2 or PSA produced by steroid alone, and multiplying by 100. Products were defined as having antagonist activity if their blocking activity was $\geq 50\%$. 
Assays

pS2 Assay. We use an ELISA-type competitive immunoassay for pS2 which was developed in-house. The details of this assay are described elsewhere (300). The detection limit of this assay is ~20 ng/mL.

PSA Assay. PSA is quantified using an ELISA-type immunofluorometric procedure described elsewhere (250). The detection limit of this assay is ~1 ng/L. These methods have been useful in several studies we have conducted, using pure flavonoids and antiestrogens (100,402).

4.2.3 Results

Of the twenty products tested (Table 4.1), four demonstrated significant estrogenic activity at the highest concentration. These included the red clover isoflavone preparation, Promensil™, the soy isoflavone preparation Estro-Logic™, grapeseed extract, and chamomile extract. Data with the first two preparations are presented in Figure 4.1. Promensil™ exhibited cytotoxic activity in the undiluted form, similar to that observed with genistein at high concentrations (100). At lower concentrations, these was a dose-response relationship and the estrogenic agonist activity was detectable up to 1000-fold dilution (Figure 4.1). Estro-Logic™ did not have any cytotoxic effect at the dilutions tested, and also demonstrated a dose-response relationship down to 100-fold dilution (Figure 4.1). Promensil’s activity was about 7 times higher than Estro-Logic’s at their most potent dilutions. This activity was equivalent to estradiol’s estrogenic activity at $10^{-8}$ M.
Figure 4.1. Dose-Response Estrogen Activity of Promensil™ and Estro-Logic™. Dose-response activity was demonstrated for both isoflavone preparations; red clover isoflavones and soy isoflavones, respectively. The products were tested undiluted, and at 10, 100 and 1000-fold dilutions in anhydrous ethanol. The maximal activity seen with Promensil™ (10-fold dilution) was equivalent to the activity of $10^{-9}$ M estradiol (data not shown).
Grapeseed and chamomile extracts exhibited both weak estrogenic and weak progestational activity but only at the highest concentrations tested (~200 ng/mL pS2 concentration in tissue culture supernatant). None of the natural products tested exhibited androgenic activity (as assessed by PSA production by the cell line BT-474). On the other hand, several products exhibited anti-estrogenic and anti-androgenic activity at the highest concentration tested (Table 4.2). These included Prostate-Ease, wild yam root and dong quai (anti-estrogens) and rosehips, dong quai and Promensil™ (anti-androgens). Table 4.2 summarizes the activities of the products that have been tested positive with our screening method.

Table 4.2. Biological Activity of Natural Products as Detected by our Tissue Culture Screening Procedure

<table>
<thead>
<tr>
<th>Product</th>
<th>Agonist $^1$ Activity</th>
<th>Antagonist $^2$ Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estrogen</td>
<td>Progestin</td>
</tr>
<tr>
<td>Promensil™</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Estro-Logic™</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Grapeseed extract</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Chamomile extract</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Prostate-Ease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild yam root</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dong quai</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosehips</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. For dose-response curves see Figure 4.1.
2. In brackets we present % blocking under the specified experimental conditions.
3. X represents specified activity.
4.2.4 Discussion

The use of alternative and complementary products is rapidly expanding (356,375-378). As the North American population ages, hormonal events such as menopause and andropause are coming to the forefront of medical issues (403,404), and the incidences of steroid hormone-dependent cancers are continuing to increase (1). Many menopausal women are turning to natural alternatives such as soy isoflavones, wild yam root, and dong quai. Similarly, many men, are looking towards complementary or alternative therapies to treat BPH, or prevent prostate cancer (314,400,405). Saw palmetto and PC-SPES are among many appealing alternatives (314,379,381,405).

Promensil™ contains 40 mg isoflavones per tablet, primarily genistein, biochanin A, daidzein and formononetin (406), which is approximately equivalent to the isoflavone content of 2 cups of soy milk. Isoflavones have been found to be increase arterial compliance in postmenopausal women (308), thus, presumably reducing heart disease risk. Recently, it has also been clinically shown to maintain bone density (407). Our findings suggest a mechanism for these physiological effects. This isoflavone preparation showed significant estrogenic activity, equivalent to $10^{-8}$ M estradiol. This product also demonstrated anti-androgen activity, similar to that demonstrated for the soy isoflavones genistein and biochanin A in previous studies (106,107).

Grapeseed extract, which contains procyanidins, compounds structurally similar to flavonoids, and chamomile, which contains apigenin, a flavone, showed both estrogenic and progestational activity at the highest concentration tested. Procyanidins present in polyphenolic fractions of grapeseeds are strong antioxidants, and have been
demonstrated to have antiproliferative and anticarcinogenic activities in cell culture and animal studies, respectively (408-410). Apigenin has been shown in our previous studies to have weak estrogenic, and relatively strong progestational activities (100,297). This flavonoid is present in chamomile extract (411), and the activity of this natural product (Table 4.2) is likely due to apigenin.

Positive results were not found for all natural products tested. Dong quai, which has been made into tonics by Chinese women for centuries (383), and wild yam root, both used as sources of phytoestrogens and phytoprogestins, were found not to have significant estrogenic or progestational activity, but rather to have weak anti-estrogenic and/or anti-androgenic activities. A study conducted by Zava et al (412) looking at Mexican wild yam products containing diosgenin, found that this product did not bind to either ER or PR, and, moreover, this steroid precursor is not metabolized to a progestational form in the human body. Whether any true steroid hormone-related physiological benefit can be derived from either of these products has yet to be ascertained. At present, the one double-blinded clinical study looking at the effects of dong quai in postmenopausal women has found no benefit over placebo in alleviating menopausal symptoms (413).

Saw palmetto and prostate-ease, both used for prostatic pathologies, including benign prostatic hypertrophy and prostate cancer, did not show androgenic nor anti-androgenic activity. Prostate-ease did show weak anti-estrogen activity. For saw palmetto, these results suggest that its effects are likely not mediated by the steroid hormone receptor system. One suggested mechanism is anti-inflammatory, through $\alpha_1$-adrenoreceptors (414).
In conclusion, we have demonstrated, by using an in-vitro system, that several natural products and nutraceuticals exhibit weak steroid-hormone agonist and antagonist activity, while many others do not. These data may be useful to explain some of the in-vitro biological activities or the lack of biological activity of these preparations. Moreover, the eventual precise definition of the biological activities of these compounds may help in the more rational use of these as natural therapeutive or chemopreventive agents.
CHAPTER 5: ANALYSES OF IN VIVO EFFECTS OF FUNCTIONAL FOODS
CHAPTER 5: ANALYSES OF IN VIVO EFFECTS OF FUNCTIONAL FOODS

5.1 Rationale

After determining the in vitro steroid hormone activities of soy isoflavones in purified form and as an extract, it was important to examine their in vivo activities as part of functional foods. Therefore, this study was undertaken to determine the overall steroidal potential of biological fluids of subjects on diets high in this functional food. The following study used 24-hour urines of subjects on one specific soy study. Several other studies have also been conducted, evaluating steroid hormone potential of soy or high vegetable feeding, using sera and urine, respectively. The data from the high vegetable study ("Simian Study") has recently been published (see List of Publications Arising from this Work). Data from the other soy feeding studies are currently being analyzed.

In this work, biological fluids were not hydrolyzed prior to using them in the tissue culture system. In vivo, only ~5% of isoflavones are found in the aglycone form in the bloodstream and urine, with the large majority conjugated to glucuronides or sulfates (415). The unconjugated isoflavones have been thought to be the more active components. However, this has not always been shown to be the case. Morito et al have demonstrated that genistin, the conjugated form of genistein binds more weakly to ER receptors and induces transcription less than genistein. However genistin stimulates the growth of MCF-7 cells more strongly than genistein, which may be important for carcinogenicity (416).

Hydrolysis prior to administration of the biological fluids in this system would have allowed for determination of total isoflavone activity, i.e. in the aglycone form. This
has been done by others for chemical determination of isoflavone content of legumes and urine samples (417). Since the initial observations indicated that aglycone isoflavones are more active than the conjugated forms, hydrolysis would result in a gross overestimation of the biological activity of the circulating isoflavones. Therefore, in the present work, the samples tested were not hydrolyzed, and the ratios of aglycone:conjugated isoflavones were similar to those that human tissues would be exposed to in vivo. We consider our results to reflect more closely what occurs physiologically.

5.2 Effect of Soy Protein Foods on Low-Density Lipoprotein Oxidation and Ex Vivo Sex Hormone Receptor Activity

5.2.1 Introduction

It has been suggested that soy food consumption may be part of the reason for the low rates of cardiovascular disease and hormone-dependent cancer in the industrialized nations of the Orient (418). Soy isoflavones have been proposed as the components of soy most responsible for many of the suggested health benefits (418-421). They have been associated with the cholesterol-lowering action of soy (387,421-423), they have antioxidant activity in vitro (77,323,324,388,389), and, as phytoestrogens, they have been suggested to play a possible role in reducing the risk of breast and prostate cancer by a variety of mechanisms (96,245,418-421,424-426).

On the other hand, there are also those who believe that soy consumption poses potential risks to health (427-433) and that, via excessive phytoestrogens activity, soy consumption may enhance breast tumor growth, cause abnormal sexual development in infants and children, and have adverse effects on the central nervous system (427-
433). Although there have been many studies on soy, many of these issues have not been dealt with specifically. In the meantime, the New Zealand Ministry of Health has issued a warning in relation to thyroid function in infants on soy formulated milk substitutes (434). On the other hand, as of October 1999, the US Food and Drug Administration (FDA) has allowed health claims to be made for soy in regards to its lowering of serum cholesterol, a risk factor for cardiovascular disease (435).

5.2.2 Subjects and Methods

5.2.2.1 Subjects

Thirty-one hyperlipidemic subjects (19 men and 12 postmenopausal women) completed two 1-month metabolic diet periods separated by at least a 2-week washout period in a randomized crossover study. The study details and data relating to serum lipids, blood pressure, fecal short-chain fatty acids and fecal bile acids are reported elsewhere (436). The subjects' mean age was 56.5 ± 9.0 years (range, 31 to 70), with a body mass index of 24.6 ± 2.3 kg.m² (range, 20.8 to 29.1). All subjects had elevated serum low-density lipoprotein (LDL) cholesterol (4.1 mmol/L) and a triglycerides level less than 4.0 mmol/L at recruitment. None had clinical or biochemical evidence of diabetes or liver or renal disease and none were using hypolipidemic agents, with the exception of one man on lovastatin 20 mg/d throughout the study. One woman was on hormone replacement therapy, 2 women were taking levothyroxine, and 1 man and 1 woman were using vitamin E supplements (400 to 800 mg/d). Dosage levels of all medications and supplements were held constant for both study periods. Subjects were also instructed to maintain their habitual level of physical activity throughout. Blood
samples were obtained after a 12- to 14-hour overnight fast prior to the study and at the end of weeks 2 and 4 of each metabolic phase. Serum was stored at -70°C prior to analysis. Body weight was measured at the start and at weekly intervals on both metabolic phases. Twenty-four-hour urine collections were made on an outpatient basis at the end of each phase.

The study was approved by the Ethics Committee of the University of Toronto and St. Michael's Hospital. Informed consent was obtained from all subjects and their primary-care physicians.

5.2.2.2 Methods

Diets

The metabolic diets were designed in conformity with the National Cholesterol Education Program step 2 dietary principles, but the dietary intake of cholesterol was further reduced. The macronutrient profile of the metabolic diets on each phase is presented in Table 5.1. The test diet provided 86 mg isoflavones per 2,000 kcal daily, while no isoflavones were detected in the control diet (Table 5.2).

The test and control metabolic diets followed a 7-day rotating menu plan. The control diet was a lacto-ovo vegetarian diet with milk products that were low in fat, consisting of skim milk, 1% dairy fat yogurt, skim-milk cheese, and low-fat cottage cheese. Eggbeaters (Lipton's, Toronto, Ontario, Canada) were used rather than whole eggs to further reduce the cholesterol intake.
Table 5.1. Calculated Macronutrient Intake (mean ± SE) on the Test and Control Metabolic Diets (N=31)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kcal/d</td>
<td>2,519 ± 86</td>
<td>2,341 ± 88</td>
</tr>
<tr>
<td>MJ/d</td>
<td>10.5 ± 0.4</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>Total protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>121 ± 4</td>
<td>118 ± 4</td>
</tr>
<tr>
<td>%</td>
<td>19.2 ± 0.2</td>
<td>20.2 ± 0.1</td>
</tr>
<tr>
<td>Vegetable protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>27 ± 1</td>
<td>110 ± 4</td>
</tr>
<tr>
<td>%</td>
<td>4.3 ± 0.1</td>
<td>18.8 ± 0.1</td>
</tr>
<tr>
<td>Soy protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>0 ± 0</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>%</td>
<td>0.0 ± 0.0</td>
<td>5.7 ± 0.1</td>
</tr>
<tr>
<td>Available carbohydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>343 ± 12</td>
<td>318 ± 12</td>
</tr>
<tr>
<td>%</td>
<td>54.6 ± 0.3</td>
<td>54.3 ± 0.3</td>
</tr>
<tr>
<td>Total dietary fiber</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>36 ± 1</td>
<td>62 ± 3</td>
</tr>
<tr>
<td>g/1,000 kcal</td>
<td>14.3 ± 0.2</td>
<td>26.3 ± 0.2</td>
</tr>
<tr>
<td>Soluble fiber</td>
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<td></td>
</tr>
<tr>
<td>g/d</td>
<td>9 ± 0</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>g/1,000 kcal</td>
<td>3.4 ± 0.0</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>Total fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>71 ± 3</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>%</td>
<td>25.5 ± 0.2</td>
<td>25.5 ± 0.2</td>
</tr>
<tr>
<td>SFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>18 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>%</td>
<td>6.5 ± 0.0</td>
<td>6.0 ± 0.0</td>
</tr>
<tr>
<td>MUFA</td>
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<td></td>
</tr>
<tr>
<td>g/d</td>
<td>24 ± 1</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>%</td>
<td>8.4 ± 0.1</td>
<td>8.4 ± 0.1</td>
</tr>
<tr>
<td>PUFA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>24 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>%</td>
<td>8.7 ± 0.1</td>
<td>9.6 ± 0.1</td>
</tr>
<tr>
<td>Dietary cholesterol</td>
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<td></td>
</tr>
<tr>
<td>mg/d</td>
<td>76 ± 3</td>
<td>77 ± 4</td>
</tr>
<tr>
<td>mg/1,000 kcal</td>
<td>30.2 ± 0.4</td>
<td>33.0 ± 1.4</td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/d</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>%</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>
Abbreviations: SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.

Table 5.2. Isoflavone Content of Study Diets (mean of seven 1-day 2,000 kcal composites)

<table>
<thead>
<tr>
<th>Isoflavone (mg/d)</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzin</td>
<td>—</td>
<td>16.8 ± 2.3</td>
</tr>
<tr>
<td>Genistin</td>
<td>—</td>
<td>33.7 ± 4.7</td>
</tr>
<tr>
<td>Glycitin</td>
<td>—</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>Malonyl daidzin</td>
<td>—</td>
<td>11.3 ± 3.6</td>
</tr>
<tr>
<td>Malonyl genistin</td>
<td>—</td>
<td>13.4 ± 6.0</td>
</tr>
<tr>
<td>Malonyl glycitin</td>
<td>—</td>
<td>0.6 ± 0.6</td>
</tr>
<tr>
<td>Daidzein</td>
<td>—</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>Genistein</td>
<td>—</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>Glycetin</td>
<td>—</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td><strong>Total daily intake</strong></td>
<td><strong>0.0</strong></td>
<td><strong>86.0 ± 17.1</strong></td>
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</tbody>
</table>
In the test diet, 93% of the animal protein was replaced with vegetable protein from soy, other legumes, and cereal foods provided as easy-to-prepare meals or frozen dishes, meat substitutes, and vegetarian "cold cuts". Soluble fiber was increased by the inclusion of oats, barley, and legume dishes at breakfast cereals, soups and main dishes. The fatty acid profile and dietary cholesterol intake on both diets were balanced by inclusion of butter and whole eggs on the test (e.g. 1 egg per week on a 2,000-kcal/d diet). Test food items used in this study were all readily available and were obtained from either a supermarket (Too Good To Be True; Loblaw Brands, Toronto, Ontario; and Yves Veggie Cuisine, Vancouver, British Columbia, Canada) or a health food store (MGM Products, Cedar Lake, MI; and Fantastic Foods, Petaluma, CA).

We have reported the details of the dietary and analytical methodology previously (436). The diets were balanced for fatty acids and plant sterols, since these might alter serum lipids and antioxidant status. At each clinic visit, the dietitian assessed compliance using the menus. Subjects were asked to weigh all foods and to check them against the menu plan when eaten. Additional items were noted in a blank column opposite the prescribed diet. These data were used to calculate the dietary intake (Table 5.1). Body weight was measured at each clinic visit, and the results were used to adjust the total caloric intake. Complete diets were packed at a central location and delivered weekly by courier to each subject's home at a time convenient to them.
Analyses

We assessed estrogenic and androgenic activity in an ex vivo human tissue culture system using immunoassays for pS2 and prostate-specific antigen (PSA) produced after stimulation with the test materials (300,437). BT-474 breast cancer cells were grown to confluence and then subcultured in 24-well microtiter plates. Once they were confluent in the wells, the cells were stimulated with urine diluted 1:1,000 or estradiol or dihydrotestosterone (DHT) at $10^{-7}$ M, the latter two serving as standards. Anhydrous ethanol was used as a negative control. The plates were incubated for 7 days, and estrogenic activity was measured in the supernatant as the pS2 concentration using an immunoradiometric assay (CIS Bio. Gif-Sur-Yvette, Cedex, France). pS2 is an estrogen-regulated protein that is demonstrated in the literature to be a good marker for estrogenic activity (243). Androgenic activity was measured using an enzyme-linked immunosorbent assay for PSA, an androgen-regulated protein (257). The procedure for this assay has been described elsewhere (257,437). pS2 and PSA concentrations were converted to equivalents of estradiol and DHT, respectively, using pS2 and PSA values obtained in the same assay for the steroid standards. Final values were expressed in picomoles.

Dietary isoflavonoid levels were measured in freeze-dried 24-hour composites of the 7-day test and control diets by high-performance liquid chromatography (HPLC) (417,438) using a 600E multisolvvent delivery system with a photodiode array detector monitoring at 200 to 350 nm (Waters. Marlborough, MA) and a YMC-pack-ODS-AM 303 column (5 μm, 250 mm x 4.6 mm ID; YMC. Wilmington, NC) equipped with an AM direct-connect C18 guard column. Appropriate isoflavone standards were analyzed
(Table 5.2). Biochanin A was used as an internal standard with recovery values of 80 to 100%. Urinary isoflavone levels were measured by HPLC after acid hydrolysis (417). Chromatographs were obtained from the 3-dimensional array using a photodiode array detector at 258 nm to allow an assessment of the common regions of relatively high absorbance for daidzein, genistein and the added recovery standard, flavone.

**Statistical Analysis**

The results are expressed as the mean ± SE. The weight change is expressed as kilograms per month. The percentage treatment differences between the endpoint values for both diets were calculated for each subject, and the data were assessed by Student's t test (2-tailed) for paired data. A 2-sample t test was used for comparison of treatment effects between subgroups. The absolute difference between treatments was assessed using the General Linear Model procedure and SAS software (PROC GLM/SAS) (439) with the end-of-treatment value as the response variable and the following main effects: diet, sex, treatment order (sequence), diet x sex, sex x sequence, a random term representing the subject nested within the sex x sequence interaction, and the baseline value as a covariate where measured. Pearson correlation coefficients were used to assess the significance of linear associations (439). A subject group of 30 allowed detection of a 28% difference in urinary ex vivo estrogenic activity (assuming a 52% SD of the effect; with α=0.05 and β=0.8) (439).
5.2.3 Results

Of 31 subjects, 16 received the test diet first. The diets were well accepted and compliance was good. On the test diet, subjects consumed 95% ± 7% of the calories provided. The respective control figure was 96% ± 6%. The test diet provided 86 mg isoflavones per 2,000-kcal diet daily, with no isoflavones detectable in the control diet (Table 5.2). There was a significant weight gain over the 1 month of the control diet (0.2 ± 0.1 kg, P=0.043) and a nonsignificant weight loss over the 1 month of the test diet (0.1 ± 0.2 kg, P=0.484). The treatment difference approached significance (0.3 ± 0.2 kg, P=0.069).

5.2.3.1 Urinary Isoflavone Excretion

The 24-hour urinary volume on the test diet (1.84 ± 0.17 L) and control diet (2.01 ± 0.14 L) was similar. On the test diet, 0.8 ± 0.2 mg/d genistein and 3.0 ± 0.6 mg/d were excreted in the urine. On the control diet, no isoflavonoids were excreted in the urine except for very low levels of daidzein in one woman (0.1 mg/d) who had the highest level on the test diet (5.3 mg/d).

5.2.3.2 Ex Vivo Sex Hormone Receptor Activity

No significant difference was found between treatment in urinary sex hormone receptor activity either before or after creatinine adjustment, although the mean values on the test diet tended to be lower than the control diet (Figure 5.1). On the control diet, urinary estrogen equivalents were higher for women than men after creatinine correction (7.5 ± 2.0 versus 3.9 ± 0.6, P=0.045). This difference between the sexes
Figure 5.1. Creatinine-Corrected Urinary Estrogen (A) and Androgen Equivalents (B) (pmol/mmol) in Men (n=19) and Postmenopausal Women (n=12) on Test and Control Diets.
was no longer significant on the test, due to a reduction in estrogen levels in women (Figure 5.1).

5.2.4 **Discussion**

Our data indicate that readily available soy foods providing modest levels of soy protein reduce the indices of oxidized LDL without increasing urinary estrogen activity. This supports previous reports that soy isoflavone consumption may protect LDL cholesterol from copper-mediated oxidative damage in vitro (388,389). However, in previous studies, no direct measurements were reported on unmodified LDL (388,389). This study therefore demonstrates that an additional cardioprotective effect of soy in reducing oxidized LDL cholesterol levels at a dose of soy protein that reduces serum cholesterol and would qualify for an FDA cardiovascular disease risk reduction health claim. These effects were achieved with no increase in urinary estrogen activity. Our data therefore do not support prior concerns about the possible increased breast cancer risk in women or altered sexual development in children consuming soy at these levels (427-430).

Oxidized LDL is more readily taken up by the macrophages of the scavenger system in the arterial wall and may contribute to plaque formation (440,441). The consumption of antioxidant flavonoids in tea, fruit and vegetables, lycopene in tomato products and vitamin E as a supplement have all been associated with a reduced risk of coronary heart disease (442-445). Vitamin E and lycopene have powerful antioxidant properties, reducing LDL oxidation and oxidative damage to plasma proteins (442,443).
LDL concentrations were reduced on the soy-containing diets (436). Both the amino acid composition of soy proteins and their isoflavone content have been suggested to be responsible for the cholesterol-lowering action of soy (386,422,423,444,445), although not all studies have reported an isoflavone effect (308,446). Soy contains many other potentially active components, including saponins, plant sterols, and polyunsaturated fatty acids, all of which may contribute to cholesterol reduction. In the present study, the lipids and sterols were separated from the soy protein isolate used in the majority of the soy products consumed. Soy fatty acids and sterols were therefore unlikely to play a part in the lipid changes (436).

It is well recognized that plant-derived sex hormone analogs may have important physiological effects (191,447). However, despite the high plasma levels which may be achieved, compared with endogenous hormones, isoflavones have relatively low potency. Of even greater importance, they may act both as potential agonists and antagonists (418). Evidence for their blocking action includes studies in which soy isoflavones fed to young women resulted in significant lengthening of the menstrual cycle (191). In our study, the ratio of dietary isoflavones to protein of 2.6 mg/g was associated with a relatively low urinary output of genistein and daidzein (448) and a tendency, on soy, for a reduction in ex vivo sex hormone activity in the urine. These data indicate that major hormonal changes may not be found at modest levels of soy protein intake that reduce the risk factors for cardiovascular disease (436). Therefore, in relation to breast cancer (230,429), the concerns about unwanted increases in estrogenic activity at the moderate levels of soy intake appear unwarranted. Conversely, the tendency for sex hormone activity to be depressed on soy diets is in
line with current strategies to treat and possibly prevent hormone-dependent cancers with sex hormone-blocking agents (449,450).

We conclude that a moderate intake of soy foods reduces the concentration of oxidized LDL cholesterol, possibly due to the increased consumption of isoflavonoids associated with soy protein. These changes were achieved without a significant alteration in urinary ex vivo hormone activity, which has been a major concern for those who predict potentially harmful effects from soy food consumption. Currently, soy consumption is advocated based on its cholesterol-lowering ability alone (422). However, in view of the apparent success of antioxidant agents in preventing experimental arteriosclerosis (451) and the effect of dietary antioxidants in reducing the risk of cardiovascular disease in cohort studies (442,443), the antioxidant effect of soy may add to its potential value in coronary heart disease risk reduction with no apparent risk of adverse effects.
CHAPTER 6: GENERAL DISCUSSION

6.1 General Discussion

Several epidemiological studies have shown an inverse association between soy consumption and breast and prostate cancer incidences (452-455). Because most are ecological studies, examining risk across countries and regions, mechanism cannot be elucidated from them. Therefore, several in vitro, animal, and a handful of human studies have been conducted to examine various components of soy, and mechanisms through which it may be chemopreventive (191,192,456-458).

Several researchers have put forth theories that not only may soy not be beneficial in prevention of cancers, but may actually increase risk. Warnings have been given to mothers intending to use soy-based formulas that such feeding may hurt their babies several decades down the road (434). Others are concerned that soy consumption by men and women who are at high risk of prostate or breast cancer may further increase this risk (428,429). The main hypothesis behind this is that because the soy isoflavones genistein, daidzein and biochanin A are estrogenic, they may increase overall steroid hormonal activities in vivo, thus increasing cancer risk.

Therefore, the purpose of this project was to assess the steroid hormone potential of plant foods, with special reference to soy. This was done by examining soy isoflavones in three forms, pure compound, plant extract (nutraceutical), and whole (functional) food, and determining how the in vitro activities may translate in vivo to overall steroid hormone potential.

Steroid hormone activity throughout this project was defined as the ability to stimulate or inhibit estrogen- or androgen/progestin- regulated proteins, i.e. pS2 and/or
PSA in the tissue culture system we developed. Although direct binding of ER, PR or AR was not done, previous work conducted in our lab has shown that the proteins measured are not produced in cell lines (breast and prostate) that do not possess these receptors (302). Moreover, measuring estrogen-regulated pS2 has been correlated with direct ER binding (96).

After developing a tissue culture system and an assay to measure the estrogen-regulated protein pS2, soy isoflavones and other flavonoids were evaluated for steroid hormone activities, including estrogenic, progestational and androgenic. Of all compounds tested, genistein and biochanin A demonstrated highest estrogenic activities, which were dose-responsive down to $10^{-8}$ M and $10^{-7}$ M, respectively. Soy isoflavones did not possess progestational or androgenic activities. However, they did demonstrated significant anti-androgen activity (76% and 98%, for genistein and biochanin A, respectively at $10^{-5}$ M), defined as blocking dihydrotestosterone (DHT)-stimulated prostate specific antigen (PSA) production. As soy has been associated with reduction of prostate cancer risk (455), these results showed one potential mechanism of action.

Soy and red clover (another source of the "soy isoflavones") extracts were then tested in this system, as were several other natural products and nutraceuticals commonly used by the public for supposed steroid hormone potential. As with pure isoflavones, the commercial soy and red clover extracts had high estrogenic activities. The estrogenicity of the highest strength of red clover extract used was equivalent in the pS2 assay to that of estradiol at $10^{-8}$ M. The estrogenic activity of soy extract was significantly lower than red clover. Theoretically this would be more beneficial, as
reducing overall estrogenicity is the mechanism by which soy isoflavones are believed to be related to breast cancer prevention (191,192,457).

Soy extract also demonstrated 63% anti-androgen activity at the highest strength tested. This is similar to the result seen with pure genistein, and supports prostate cancer prevention data.

The final objective of this project was to determine how the in vitro data may translate in vivo to overall steroid hormone potential. By using biological fluids obtained from individuals in a soy feeding study in our tissue culture system, we found that short-term feeding did not increase agonist potentials. These results indicate that soy consumption does not increase overall estrogenic or androgenic potential in humans. Moreover, we found non-significant reductions in estrogenic and androgenic activities in women and men, respectively. Because the sample population was small (19 men and 12 women), the power was not high enough to reach statistical significance. However, these results may indicate that long-term feeding of this functional food may reduce risk factors for steroid-hormone dependent cancers, which corroborate with epidemiological studies (452-455), as well as several prospective trials looking at risk factors for breast cancer (191,192,457).

The observation that biochanin A had estrogenic activity similar to genistein in our system is not in agreement with results obtained by other groups, using direct binding and recombinant yeast assays (96,103,230). Furthermore, some of the structure-function relationships we determined do not coincide with those seen by others (103,230), such as the strong activity shown by biochanin A, which contains a methoxy group on C-4'. One possible explanation may be cellular metabolism. We did
not measure flavonoid metabolism by the breast cancer cells used in this system, however, it is possible that conversion of some compounds to more active metabolites, e.g. biochanin A to genistein, could have taken place. This would also explain the highly estrogenic activities seen with the red clover and isoflavone preparations, which have been shown to need significant metabolism to become estrogenic (415).

We did not hydrolyze the biological fluids prior to using them in our system. Because 95% of soy isoflavones are conjugated in biological fluids (415), by not hydrolyzing the urines and sera, only ~5% of isoflavones are free to act in our system. However, our objective was to ascertain the biological implications of soy feeding in healthy subjects, i.e. to examine the in vivo situation using an in vitro assay system. Hence, hydrolyzing the samples to give the maximum amount of free isoflavone was not the aim. Rather, our aim was to assess the steroid hormone activity of the soy isoflavones with a ratio of bound:free isoflavone similar to that seen in vivo. Therefore, the choice not to hydrolyze the biological fluids was one made early on, and the results must be interpreted in that context. Moreover, a recent study has shown that although the conjugated isoflavone genistin, binds more weakly to ER and induces transcription less than genistein, it stimulates growth of MCF-7 cells more than the aglycone isoflavone (416). More work is needed to determine the in vivo activities of conjugated isoflavones.

In conclusion, these results indicate that soy isoflavones and other flavonoids have steroid hormone activities. These activities may act in vivo to modulate potency of potentially carcinogenic endogenous hormones, thereby reducing overall steroid hormone potential. Further research is needed to determine the physiological
importance of flavonoids in prevention and/or management of hormone-dependent cancers, and, moreover how to make best use of natural products, nutraceuticals and functional foods in individuals at risk.

6.2 Summary

Through the studies described, the following objectives have been met:

1. Development of several techniques and methods to evaluate steroid hormone activities of pure compounds, products and metabolites within biological fluids. An ELISA for pS2 protein was established, and several cell lines were evaluated for their usefulness in our tissue culture system. Antagonists used for treatment of hormone-dependent cancers were determined to be good positive controls of steroid hormone blocking in our system.

2. Several flavonoids were found to possess weak estrogenic and/progestational activities in vitro. The soy isoflavones had only estrogenic activities. These activities were structurally-related, and were dependent on presence of the diaryl nucleus and position and number of hydroxyl groups. None of the compounds tested had androgenic activities, but several had antiandrogenic effects, as characterized by their ability to inhibit DHT-stimulated PSA production. No structure-function relationship could be established for this activity, but the dose-dependency of a few of the estrogenic flavonoids was similar to the antiandrogen effects seen for estradiol in the BT-474 cell line.
3. Soy and red clover extracts, both sources of "soy isoflavones", and other natural products and nutraceuticals were then tested for steroid hormone effects. Like pure flavonoids, the isoflavone-containing products showed significant estrogenic activities. The soy extract showed antiandrogenic activity similar to that seen with genistein. This study showed that steroid hormone activities seen with pure compounds are maintained in extracts of natural products.

4. Steroid hormone potential of the functional food, soy, was determined in healthy subjects. By using non-hydrolyzed biological fluids obtained from individuals in a soy feeding study, it was found that short-term feeding did not significantly alter steroid hormone activity, compared to controls. Non-significant reductions in estrogenic and androgenic activities in women and men, respectively, may indicate that long-term feeding of this functional food may reduce risk factors for steroid-hormone dependent cancers. This study demonstrated that in vitro estrogenic activity did not translate into in vivo estrogenic activity, but instead, soy may act physiologically in an antiestrogenic manner, to reduce overall estrogenic potential.

6.3 Future Directions

Future directions in this field should include work into the use of natural products, and functional foods. Further investigation into the steroid hormone activity of natural products and nutraceuticals should be conducted. Because these products are relatively new, and are currently under no specific regulations, studies must be carried
out to determine their activities in vitro, and in vivo. Aspects such as unwarranted effects and interactions should be investigated. Processed products should be tested for flavonoid content, with results available to the consumer. Moreover, best use of these products as alternative or complementary therapies in individuals at risk of steroid hormone-dependent cancers warrants further research.

The use of soy as a functional food, as well as other foods requires specific attention. This project has shown that soy does not increase overall steroid hormone potential in men and postmenopausal women. However, more work must be done to further confirm this. Moreover, the use of soy in reducing estrogenic and androgenic activity in vivo is important in prevention of cancer. Human trials with endpoints including steroid hormone activities, serum PSA, prostatic intraepithelial neoplasia (PIN), pS2, breast tissue proliferation, et cetera, should be conducted. Other foods including flaxseed, fruits and vegetables, and teas, which contain flavonoids and related compounds with steroid hormone activities should also be tested.

6.4 Conclusion

This project enabled for the examination one mechanism by which flavonoids, in general, and soy specifically, may act to prevent steroid hormone-dependent cancers. Moreover, this study demonstrates that observations made in vitro cannot necessarily be translated directly in vivo. Although soy isoflavones do have estrogenic activity, the physiological impact was not estrogenic, but in fact, showed an antiestrogenic tendency. The antiandrogenic effects of these isoflavones, a surprising finding, did translate in
vivo. How these results may be applied in the use of soy in functional foods and/or nutraceuticals will be interesting to see.

In conclusion, this project has been challenging, interesting and exciting throughout. Many fruitful results have been produced, leading to further questions on the importance of flavonoids in prevention and/or management of hormone-dependent cancers, and the use of natural products, nutraceuticals and functional foods in individuals at risk. These data are a starting point for research on the use of soy as a functional food, and will hopefully lead towards understanding of how soy functions in humans, and how best to exploit these health benefits.
CHAPTER 7: REFERENCES
CHAPTER 7: REFERENCES


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## APPENDIX

Chemical Structures of Flavonoids and Related Compounds

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<th>Structure</th>
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*Rutinose*