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THE EFFECTS OF PLANT- DERIVED COMPOUNDS ON SEX HORMONE RECEPTORS:
IMPLICATIONS FOR HORMONE-DEPENDENT CANCER DEVELOPMENT AND TREATMENT

by

Rachel Stacey Rosenberg

A thesis submitted in conformity with the requirements
for the degree of M.Sc.
Graduate Department of Nutritional Sciences
University of Toronto

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Breast and prostate cancers are leading causes of cancer morbidity and mortality of women and men in North America. This risk is significantly higher than in the Japanese population, with epidemiological studies suggesting the difference due to high flavonoid consumption in the latter group. The purpose was to examine effects of plant-derived compounds on androgen (AR) and progesterone receptors (PR). In vitro studies on BT-474 and T47-D breast cancer cells were performed and 7 synthetic antiprogestins and 45 plant-derived compounds were tested. Prostate-specific antigen (PSA) was measured as a marker of receptor activity. The strongest blockers of PR were chlorophylline (87 ± 7%), β-carotene (82 ± 3%), taxifolin (80 ± 5%), and chlorogenic acid (61 ± 2%). The most potent antagonists of AR were chlorophylline (94 ± 3%), hesperetin (79 ± 11%), syringic acid (72 ± 11%), and α-tocopherol (65 ± 5%). Apigenin had an agonistic effect on these receptors.
Many people have contributed to the inception, planning, and development of this thesis. I would first like to thank Dr David Jenkins for his support, guidance, and patience in all aspects of this project and other endeavors. I would also like to thank Dr Eleftherios Diamandis for his teachings and direction, without which I would still be struggling with lab technique and data interpretation.

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AR – androgen receptor
ER – estrogen receptor
PR – progesterone receptor
GR – glucocorticoid receptor
MR – mineralocorticoid receptor
RAR – retinoic acid receptor

E₂ – estradiol
DHT – dihydrotestosterone
MPA – medroxyprogesterone acetate
MA – megestrol acetate

ERE – estrogen response element
HRE – hormone response element
GRE – glucocorticoid response element

PSA – prostate-specific antigen
SHBG – steroid hormone-binding globulin
hsp – heat shock protein

CHD – coronary heart disease
CVD – cardiovascular disease
IHD – ischemic heart disease

Zn – zinc

NNK – nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NDEA – N-nitrosodiethylamine
NMBA – N-nitrosomethylbenzylamine
20-MC – 20-methylcholanthrene
CHAPTER ONE

INTRODUCTION

AND

LITERATURE REVIEW
1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The relationship between diet and cancer has become a topic for much research. Many issues have come into the forefront, including the impact of fat, meat, antioxidant vitamins, trace elements, and now flavonoids on cancer development, and prevention (Gregoire et al. 1991; Tonioli et al. 1994; Hunter et al. 1993; Rohan et al. 1993; Hirayama 1986; Giovannucci et al. 1993; McKeown-Eyssen et al. 1988; Mills et al. 1989; Le Marchand et al. 1994; Greiret et al. 1989). Epidemiological studies evaluating nutrient intakes demonstrate positive associations between meat and fat intake and cancer development, as well as negative associations between vitamin, mineral, and flavonoid consumption and carcinogenesis (Franceschi et al. 1996; Ishimoto et al. 1994; Garland et al. 1993; Tominaga et al. 1995, Simopoulos 1987; Paganini-Hill et al. 1987; Hsing et al. 1990a). Flavonoids may also be important. Research has expanded in terms of carcinogenesis, and is beginning to give us new insights into the possible benefits of plant foods in cancer prevention.

Research into the ability of flavonoids to alter hormonal balance is relatively new. Its earlier days appeared somewhat irrelevant to the human situation, i.e. reproductive failures in sheep eating clover from certain fields, and cheetahs fed soy products at the Cincinnati Zoo (Bennetts et al. 1946; Setchell et al. 1987), however such “phytoestrogenic” effects soon proved to be relevant to humans. The follicular phase of Japanese women were measured to be longer than their American counterparts, related
to lower breast cancer risk (Henderson et al. 1985). This was shown to be manipulatable, as feeding American premenopausal women diets high in soy increased follicular phases by 1-2 days (Cassidy et al. 1994).

Phytoestrogens as they relate to breast and prostate cancer prevention have since been studied in vitro and in vivo (Messina et al. 1994; Adlercreutz et al. 1992; Lee et al. 1991; Setchell et al. 1988). This work includes their inhibitory activities on enzyme systems, the estrogen receptor per se, and cytostasis (Pelissero et al. 1996; Adlercreutz et al. 1993; Makela et al. 1995; Miksicek et al. 1993; Kohlmeier et al. 1995; Martin et al. 1996). However, the effects of these compounds on progesterone and androgen receptors have not yet been studied. As hormone-dependent cancer development is related to an imbalance of steroid hormone production and metabolism, the possible effect on these hormones by flavonoids must be understood in order to determine the full potential of plant-derived compounds in prevention and/or treatment of these cancers.
1.2 Literature Review

1.2.1 Demographics and Epidemiology

In 1997, hormone-dependent (e.g. breast and prostate) cancers made up the most frequently diagnosed carcinomas in women and men, respectively. The incidences of both are on the rise for the aging population. At the present time, 1 in 9 women and men will develop these diseases, mainly after the age of 50 years, with 1 in 25 Canadian women, and 1 in 27 Canadian men dying from these tumors or from their metastases (NCIC 1997). These risks are 5-10 fold higher than those seen in Japan and China. Heredity accounts for 5-10% of these cancers (Skolnick et al. 1997), and studies show that within two generations after migration to a new country (e.g. Japanese in the United States), incidences of developing these cancers reach the same levels as the native population (Haenszel 1961; Buell et al. 1965; Haenszel et al. 1968; Tominaga 1985; Thomas et al. 1987). For these reasons, environmental explanations (including diet) are being pursued.

Dietary differences believed to be responsible include lower fat and higher fiber consumption by lower risk groups, high monounsaturated fat intake relative to saturated fat, less ingestion of animal protein, and diets high in vegetables and fruits. Wine and tea have also been associated with the lower incidence of hormone-dependent cancers in populations that make these beverages a regular staple (Block et al. 1992; Negri et al. 1994; La Vecchia et al. 1988; Hertog et al. 1993).
1.2.2 Carcinogenesis

Carcinogenesis is a multi-step process that takes place over 30 years or more from initial insult to tumor formation (Miller et al. 1981). Although progressive over time, carcinogenesis occurs through necessary steps occurring on a specific time course. These steps are known as initiation, promotion, and progression.

Initiation is the initial insult to DNA through endogenous or exogenous (chemical, ultraviolet, radiation) means. Usually this damage is repaired via excision of the error, or through apoptosis (programmed cell death) of the cell. However, if DNA replication and cell division occur before this repair or apoptosis takes place, the damage may be converted into a stable genomic error (Miller et al. 1981). This process of increased cell proliferation is known as promotion. Agents that are classified as good promoters tend to be non-genotoxic on their own, but act as irritants. Estrogens are known to be good promoters.

Over time, tumors become more aggressive and acquire increased malignant potential. The process by which this occurs is defined as progression. Increasing malignancy, characterized by rapid growth, invasiveness, and the ability to metastasize progresses slowly over many years. It is related to the sequential appearance of subpopulations of cells that differ with respect to several phenotypic attributes including invasiveness, rate of growth, metastatic ability, karyotype, hormonal responsiveness, and susceptibility to antineoplastic drugs. These cancerous cells become heterogeneous, which most likely results from multiple mutations that accumulate independently in different cells, thus generating subclones with different
characteristics from original cells, as well as from each other. Progression ultimately leads to invasion, metastasis, and death (Miller et al. 1981).

1.2.3 Treatment Modalities

Three treatment modalities are usually employed when dealing with breast and prostate cancer. They are surgery, radiotherapy, and chemotherapy, including hormone blockade.

Surgery has been used since 1890, where Halsted showed that breast cancer may not necessarily be fatal (Halsted 1890). Surgery is used today as one of several options for the management of breast and prostate cancers, with cure possible if all tumor cells are removed. In some patients, surgery may just postpone the inevitable mortality from metastases or from extremely large tumors. As with all treatments, the decision for surgery, is made based on patient factors, tumor characteristics and stage of disease.

Radiation is another treatment option for these cancers. Some consider it to be the most effective treatment modality currently available to control local disease (Rutqvist et al. 1990; Rutqvist et al. 1994). Large studies including the National Surgery Adjuvant Breast Program Trial (Fisher et al. 1989) and the Swedish Uppsala-Orebro Trial (Uppsala-Orebro 1990) have demonstrated radiotherapy to decrease the number of breast cancer recurrences.

Hormone blockade is a third option. For breast cancer, estradiol is the hormone usually blocked, and for prostate cancer, it is dihydrotestosterone. This is accomplished
through use of tamoxifen and finasteride, respectively. Up to 60% of ER-positive tumors respond to hormone therapy, but only 10% of ER-negative tumors respond (Allegra et al. 1980; Samaan et al. 1981; Williams et al. 1987). Diethylstilbestrol and flutamide have response rates of 50-60% as primary treatment in prostate cancer patients (Chang et al. 1996). The theory behind hormone blockade is discussed in the next section.

1.2.4 Sex Hormones

All steroid hormones have in common the 17-carbon cyclopentanoperhydrophenanthrene structure with four rings (A-D) (fig. 1.1). Additional carbons at positions 10, 13, or side chains attached to C_{17} are found, as number and type of substituted group, number and location of double bonds, and stereochemical configuration all allow for differences in function of the groups of steroid hormones. All mammalian steroid hormones are formed from cholesterol through reactions in the mitochondria or endoplasmic reticulum of adrenal cells (Granner 1996).

Estrogens are a family of hormones synthesized in a variety of tissues. 17\beta-estradiol is the primary estrogen, formed mainly by aromatization of testosterone, in the ovaries of women and in the adipose tissue of men. Estrone is formed from aromatization of androstenedione in adipocytes. Estrogens have several mechanisms of action by which they can induce growth of human breast and other tissues. They can directly stimulate growth of cells, or induce nuclear transcription factors to increase the expression of genes regulated by the estrogen response element (ERE). Alternately, in
Figure 1.1 Structure of Steroid Hormones

The three sex steroids (estrogen, testosterone, and estrogen) are all 17-carbon cyclopentanoperhydrophenanthrene structures with four rings. Additional carbons at positions 10, 13, or side chains attached to C\textsubscript{17} are found, as number and type of substituted group, number and location of double bonds, and sterochemical configuration allow for differences in function of hormone.
ER-positive breast cancer cells, estrogens can stimulate their proliferation independently of interactions with other cells or tissues (Rochefort 1994).

Tamoxifen is a triphenylethylene derivative that functions mainly as an estrogen antagonist, but may have other mechanisms of action as well. It competively binds to the ER, forming a complex recognized by the ERE similar to the estradiol- (E$_2$) ER complex (Kumar et al. 1988; Miller et al. 1986), but induces alternate conformations. Thus, transcription-activating factors are not activated, and RNA is not produced. Tamoxifen hence inhibits estrogen-stimulated cell division. Stilbestrol is an estrogen agonist used in the treatment of prostate cancer. It raises estrogen levels in men, hence diminishing the testosterone:estrogen ratio believed to be of major factor in the development and progression of this disease (Walsh 1975; Resnick et al. 1975).

Progesterone is produced and secreted by the corpus luteum of the ovaries, from pregnenolone (Granner 1996) and generally it decreases the proliferative activities of estrogens. Progestins usually require previous or concurrent presence of estrogens to function, and the two classes of steroid hormones may act synergistically or antagonistically, depending on tissue and particular function (Spicer et al. 1992). Very high doses of progestins are used in breast cancer as first- or second-line therapy, and have been demonstrated to be as effective (30% response rate) as tamoxifen in the treatment of advanced breast cancer (Sedlack et al. 1994; Canobbio et al. 1987; Howell et al. 1987; Lundgren et al. 1989; Gundersen et al. 1990; Parnes et al. 1991; Pronzato et al. 1990). They are well-tolerated, with relatively low toxicity (Henderson et al. 1989), and are currently experiencing a resurgence in use (McGuire et al. 1985; McGuire et al.
Antiprogestins are now also being used in the treatment of breast cancer. RU 486 (mifepristone) an 11β-substituted steroid derivative utilized clinically and experimentally (Weiss 1993), and has become a starting point for the synthesis of a second generation of drugs with similar antiprogesterogenic, but weaker antiglucocorticoid activity than RU 486 (Horowitz 1992).

Pregnenolone is also a precursor to testosterone, and is synthesized to such through either the progesterone or dehydroepiandrosterone (DHEA) pathway in adrenal glands, ovaries and testis. Dihydrotestosterone (DHT), a more potent androgen is then produced in men by the enzyme 5α-reductase in target tissues such as the prostate (Granner 1996). Finasteride (Proscar), a 5α-reductase inhibitor, is used clinically in the management and treatment of prostate cancer. As such, it inhibits the reduction of testosterone to the more active form DHT (CPS 1992). Androgen agonists have been successfully used in the treatment of breast cancer (Kennedy 1958; Co-operative Breast Cancer Group 1964; Gordon et al. 1973; Tormey et al. 1983), however, severe side effects were seen with this treatment, and hence it was withdrawn in the 1970s.

1.2.5 **Sex Hormone Receptors**

The androgen and progestin receptors are two members of a group known as the nuclear receptor superfamily. Other receptors within this class include the mineralocorticoid and glucocorticoid receptors, as well as the estrogen receptor, thyroid receptor, vitamin D₃ receptor, and various orphan receptors with unknown functions.
Nuclear receptors consist of at least three functional regions (Wahli 1991). Starting from the N-terminus of the protein, these regions are:

1.2.5.1 **Transactivation Domain: A/B Region**

This region is responsible for optimizing the transactivation capability of the receptor, i.e. regulating and activating transcription factors such as DNA-dependent RNA polymerase (Evans 1988; Beato 1989; O’Malley 1990; Fuller 1991; Wahli 1991). It also recognizes specific sequences in transcriptional areas of the genes to which it binds. Because this domain does not contain a conserved region, steroid receptors are able to interact and bind with other cellular proteins (Wahli et al. 1991). This region is promoter and cell-type specific.

1.2.5.2 **DNA binding Domain: C Region**

This 66-68 amino acid-long region recognizes specific DNA sequences known as hormone-response elements (HRE) (Evans 1988; Beato 1989; O’Malley 1990; Fuller 1991; Wahli 1991). These sequences are specific for the type or subfamily of receptor and are made up of a 15 nucleotide-long partially palindromic sequence, usually found at the 5'-flanking region of the gene. These 15 base pairs are arranged as two hexameric half-sites separated by three bases (fig. 1.2) (Ham et al. 1988; Cato et al. 1988).

The DNA-binding domain is cysteine rich and is well conserved among categories within the nuclear superfamily (Amero et al. 1992; Laudet et al. 1992). This
Consensus Hormone Response Element (PRE, ARE, GRE)

AGAAC\textit{Annn}TGTTCT
TCTTG\textit{Annn}ACAAGA

Estrogen Response Element (ERE)

AGGTC\textit{Annn}TGACCT
TCCAG\textit{Annn}ACTGGA

\textbf{Figure 1.2} \hspace{1em} Idealized Hormone Response Elements

The DNA binding domain of the nuclear receptor superfamily recognizes specific DNA sequences known as hormone-response elements (HRE). These sequences are specific for the type or subfamily of receptor and are made up of a 15 nucleotide-long partially palindromic sequence, found at the 5'-flanking region of the gene. Glucocorticoid-type receptors (including progesterone and androgen receptors) and estrogen receptors discriminate between each others HREs in vivo. This is accomplished through consensus elements that differ at two bases in each half site.
region is folded into two zinc "fingers", Zn coordination sites made up of two zinc ions each, tetrahedrally coordinated by 4 cysteines to stabilize two peptide loops and cap amino termini of two amphipathic α-helices. The first finger specifies the receptor's DNA recognition sequence, and the second allows for dimerization of two receptor molecules during their binding with DNA (Wahli et al. 1991).

Glucocorticoid-type receptors (including progesterone (PR), and androgen receptors (AR)) and estrogen receptors (ER) discriminate between each others HREs in vivo. This is accomplished through consensus elements that differ at two bases in each half site (Mader et al. 1989; Danielson et al. 1989; Umesono et al. 1989) (shaded areas in fig. 1.2). The HRE responsible for transcription of the PSA gene recognizes and binds only to the GRE motif. Hence, stimulation of any or all of these receptors increase transcription of this gene, leading to production of PSA. Moreover, as ER does not have this motif, PSA is unaffected directly by estrogen stimulation.

1.2.5.3 Hormone binding Domain: E Region

This region consists of 250 amino acids at the C-terminus of the steroid receptor and is responsible for ligand binding. Once bound by a compatible hormone, conformational changes are induced that rearrange important amino acid residues to a proper tridimensional position, activating the transcriptional activity function of the receptor. Other transcriptional factors may interact with the activated receptor, leading to RNA synthesis. When unbound, this region folds over the DNA-binding domain (C region), preventing any association between unbound hormone and DNA (Jensen et al.
1991). This process is necessary for proper binding to DNA.

1.2.5.4 **Mechanism of action**

In the absence of ligand, the latent receptor resides as part of a macromolecular complex containing heat shock protein (hsp) 90, hsp 70, p59, and other proteins, located in the nucleus of the cell (Smith et al. 1993). If a ligand for a steroid receptor (progestin or androgen) is present within the cytoplasm of the cell, it can passively diffuse into the nucleus and bind with its respective receptor(s). Once bound by ligand, alterations in the receptor structure occur. Steroid receptor agonists and antagonists induce distinct conformational changes within their specific receptors (Allan et al. 1992; Beekman et al. 1993). This change promotes the dissociation of the heat shock proteins, thereby permitting the interaction of the receptor with specific DNA sequences in the regulatory regions of target gene promoters (Martinez et al. 1989; Tsai et al. 1989; Kumar et al. 1988). After activation of the complex through phosphorylation, subsequent binding occurs with its HRE on the PSA gene. This stimulates and facilitates binding of transcription factors with their regions on the PSA gene, which results in transcription of the gene. Translation occurs soon after, and measurable amounts of PSA protein are produced. The estrogen receptor does not bind to the HRE of the PSA gene. Therefore, the presence of estrogen cannot contribute to the production of PSA. However, estrogen, in relatively large amounts, may contribute to inaccessibility (through competitively binding with androgen and progestin receptors, or hormonal balance manipulations) of ligands to these steroid receptors, which may
ultimately result in PSA not being produced (fig. 1.3).

1.2.6 **Plant-derived Compounds**

Flavonoids are a class of compounds possessing a diphenolic structure (fig. 1.4). They are found ubiquitously in plants, and are in highest concentrations in soy, vegetables, fruits, tea, and red wine (Hertog et al. 1993; Hertog et al. 1995). The class includes isoflavonoids, flavans, flavones, flavonones, flavonols, anthocyanins, and catechins (Miksicek 1995). Flavonoids are found in nature as glycosides. Upon consumption, they are conjugated in the gut, deconjugated in the blood, and reconjugated in the liver and kidney for excretion. The relative amounts of free to conjugated forms are unknown, but the total half-life of these compounds in the body is approximately 7 hours. These conjugates can be measured in urine, blood, bile and feces (Adlercreutz et al. 1995).

Many compounds with diphenolic structure are able to function as phytoestrogens, with agonistic or antagonistic activity (Coward et al. 1993). Although potencies and affinities vary tremendously from among groups within the phytoestrogen class, as well as within groups, on average their binding affinities are 100 to 10,000 times higher than diethylstilbestrol (DES) or estradiol (Farmakalidis et al. 1985). Their potencies, as measured through experiments with genistein, approximate $10^{-3}$ to $10^{-5}$ that of these estrogens (Kitts et al. 1980; Somjen et al. 1976; Mayr et al. 1992; Welshons et al. 1990; Newsome et al. 1980; Folman et al. 1969; Markiewicz et al. 1993; Adlercreutz 1990), allowing them to act as partial agonists and partial antagonists of the
Figure 1.3  PSA Production

In the absence of ligand, the nuclear receptor resides as part of a macromolecular complex in the nucleus of the cell. If a ligand for a steroid receptor (progestin or androgen) is present within the cytoplasm of the cell, it can passively diffuse into the nucleus and bind with its respective receptor(s). Once bound by ligand, the complex is phosphorylated and binds to its HRE on the PSA gene. Transcription factors bind to the gene, and transcription occurs. The mRNA is translated in the endoplasmic reticulum, producing PSA protein. Through blocking of receptor, phosphorylating enzymes, or transcription factors, PSA production may be blocked.
Flavonoids are a class of compounds possessing a diphenolic structure. The class includes isoflavonoids, flavans, flavones, flavanones, flavonols, anthocyanins, and catechins.
estrogen receptor. These structures are also quite similar to those of androgens, and hence may also function on these and other nuclear subfamily receptors.

Flavonoids have been demonstrated to have various activities in vitro and in vivo. Their antioxidant capacities have been a major focus of research, in terms of prevention of coronary heart disease (CHD). The "French paradox" may be partially explained by the high flavonoid concentration in red wine, and several epidemiological studies have shown that populations consuming high amounts of these polyphenols in their diets (e.g. Mediterranean diet) have lower incidence of CHD than populations who consume low amounts (Hertog et al. 1995).

In the cancer arena, flavonoids have been demonstrated to block many enzyme systems responsible for cell proliferation including aromatase and tyrosine kinase, arrest the cell cycle in various phases, cause apoptosis, inhibit tumorigenesis in vivo, scavenge free radicals, and manipulate estrogen concentrations in various ways.

1.2.7 **Components of Various Plant Foods**

The original work in the field of flavonoids arose through the discovery of isoflavones in soybeans and soya products. Estrogenic effects of these compounds were discovered by evaluating what was causing reproductive failure in female animals such as cheetahs and ewes fed clover or soy feed, later termed "clover disease" (Setchell et al. 1987; Adams et al. 1979a; Adams et al. 1979b; Bennetts et al. 1946). This occurred due to impaired transport of spermatazoa through the cervix of these animals (Lightfoot et al. 1967) caused by morphological changes of the reproductive
tract induced by isoflavones (Nwannenna et al. 1995). Such changes occur in many other species as well, including some strains of rats and mice treated with flavonoids during neonatal life (McEwen et al. 1972). Isoflavones may also function to defeminize females and demasculize males of some species, e.g. rats, by decreasing luteinizing hormone surges in the females, and retaining capacity of this surge in males (Herbst et al. 1972; Arai et al. 1968). Such problems do not occur among all species, including cattle, and do not occur in humans. It is believed that nonsusceptible populations convert isoflavones relatively more rapidly to inactive metabolites than those susceptible to reproductive and sexual effects, or lack metabolic pathways for activation of highly estrogenic metabolites (Cassidy et al. 1995).

1.2.7.1 Soya

Soya is a very rich source of flavonoids, containing 2 mg of biochanin A, genistein, and daidzein, per gram (defatted) (Coward et al. 1993). These compounds are not destroyed or removed by processing, except for concentrates prepared by alcohol extraction (Anderson et al. 1985). In prospective and case-controlled studies significant negative correlations were found between intake of isoflavones (from miso or tofu consumption) and breast cancer risk (Hirohata et al. 1985; Nomura et al. 1978; Hirayama et al. 1986; Lee et al. 1991). In at least one of these studies (Lee et al. 1991) the ratio of soya to total protein remained significant after control for other dietary variables.

The anti-estrogenic effects of isoflavones have also been demonstrated in
relation to reproductive stages in humans, namely menopause and menstrual cycle. Studies have been conducted to determine whether or not soya supplementation can reduce menopausal symptoms and increase cycle length (Cassidy et al. 1994). Japanese women have been shown to have follicular phases 2-3 days longer than American women. Moreover, unlike in Western societies, where menopause is associated with physiological, physical and emotional stresses, in rural Asian areas, where tofu consumption is abundant, this natural transition of life stage occurs without trauma (Murkies et al. 1995).

In one study on the effects of soya on menstrual cycle, it was found that 60 g per day (equivalent to 45 mg of isoflavones) given for 1 month significantly (p<0.01) increased follicular phase length and/or delayed menstrual cycles by 1-5 days. No change in luteal phase was observed. This lengthening of follicular phase has been related to decreased breast cancer risk. No significant changes in sex hormone-binding globulin (SHBG) were seen (Cassidy et al. 1994).

Supplementation of soy flour also significantly decreased the number of hot flashes in menopausal women over a 12-week period, with a significant response after 6 weeks. The negative control, wheat flour also resulted in decreases of these episodes, though not as significantly. This choice of control was obviously a faulty one, as wheat has been shown to contain other phyto-sex steroids, namely enterolactones (lignans). Therefore, these compounds were both shown to have estrogenic activity (Murkies et al. 1995). More studies, using a truly negative control, are necessary to confirm these results.
Phyto-sex steroids may prevent or be used to treat hormone-dependent cancers through different mechanisms. These include their effects on hormone metabolism and sex hormone receptors. Diets high in flavonoids, e.g. Asian-type diets have been associated with increased levels of SHBG and decreased levels of free estradiol. These compounds stimulate SHBG synthesis by the liver, resulting in less binding of estrogen receptors. This process is believed to be related to lower breast cancer risk (Mousavi et al. 1993; Murphy 1982). These compounds also competitively bind to ERs in breast cancer cells or to hormone-metabolizing enzymes. Their antiestrogenic effects can inhibit the growth and proliferation of hormone-dependent cancer cells. Many phyto-sex steroids are structurally similar to tamoxifen, which lowers risk of contralateral breast cancer in women with breast cancer and is currently under investigation as a primary preventive agent for high-risk women.

Many in vitro and animal studies have been conducted with soy isoflavones to determine their anticarcinogenic effects. In vitro effects of genistein at low concentrations (100-200 nM) in the absence of estrogenic steroids include increased growth of MCF-7 breast cancer cells (Martin et al. 1978; Zava et al. 1997). At higher concentrations (>2 μM), found often in human sera, genistein has been demonstrated in the MCF-7 breast cancer cell line to bind to the estrogen receptor, thus preventing binding by estradiol. Thereby this receptor cannot be translocated to the nucleus. Inhibition of cellular growth has also been seen (Zava et al. 1997; Peterson et al. 1996) in both ER positive and negative cell lines of breast and prostate cancer origin. This suggests more than one mechanism by which these compounds act, one that is similar
to estradiol's effects on signal transduction (Migliaccio et al. 1993). Genistein, along with the other flavonoids, has also been demonstrated to inhibit tyrosine phosphorylation by tyrosine protein kinases, which may regulate phosphorylation of estrogen receptors, essential for estrogen receptor activation (Ogawara et al. 1989).

Genistein can also act additively or synergistically with other antitumor agents (e.g. tamoxifen) to inhibit cancer cell growth or to induce differentiation. Furthermore, daidzein and equol, an isoflavan produced by intestinal microfloral activity on soy isoflavones, have been shown to have estrogenic and anticarcinogenic activity (Tang et al. 1980). Equol accounts for approximately 30% of total isoflavones in biological fluids (Axelson et al. 1984; Setchell et al. 1984), and hence may play a major role in hormone-dependent cancer resistance.

Genistein has also been demonstrated to arrest human gastric cancer (HGC-27) cells at the G2-M phase (Matsukawa et al. 1993) (fig. 1.5). Dose-dependent inhibition of growth is observed between 10 and 60 μM, with an 50% inhibition concentration (IC50) of genistein of 20 μM on day 4 in the HGC-27 cell line. This flavonoid may also cause a weak block in late S phase. A concentration of greater than 25 μM genistein was needed to alter cell cycle progression, with a maximum accumulation of cells at G2-M observed at 40 μM or more. Genistein is also cytostatic against human leukemic (HL-60) cells (Hirano et al. 1994). This may be achieved through blocking membrane Na+K+-ATPase of tumor cells (Suolinna et al. 1975; Kuriki et al. 1976; Belt et al. 1979).

Genistein has been shown to inhibit DNA topoisomerases I and II in a manner similar to anticancer agents and plant lignan etoposides (Marcovits et al. 1989). This
Many flavonoids have been demonstrated to be cytostatic at various phases of the cell cycle. Genistein arrests human gastric cancer cells at the G₂-M, blocking division of the parent cell to two daughter cells. Apigenin has similar activity in neuronal cells. Quercetin has been shown to inhibit gastric and colon cancer cells at the G₁-S boundary, thus preventing DNA replication.
isoflavone also inhibits ribosomal S6 kinase activity and phosphatidylinositol breakdown. These results can be observed at concentrations as low as 1 to 10 μmol/ml, and are seen in other cancer lines of human and rodent origins (Robinson et al. 1993). These inhibitions of activities act separately or in combination to induce DNA damage and apoptosis (Devasagayam et al. 1995).

Animal models have demonstrated the antitumorigenicity of soy isoflavones on mammary cancer. Genistein and daidzein have been shown to reduce tumor numbers in rats (Molteni et al. 1995; Coward et al. 1993). Female Sprague-Dawley rats treated on days 2, 4 and 6 postpartum with genistein and initiated with dimethylbenz(a)anthracene (DMBA) had mammary tumor development delayed by 37 days (p<0.001) compared to those treated with carcinogen alone (La Martiniere et al. 1995). Female rats treated neonatally with genistein had an incidence of mammary tumors 12% lower than controls, and had almost 50% fewer tumors (p<0.001) with no effect on body weight. This was attributed to morphological differences in ductal structures in the genistein-treated group, with more differentiation of cells observed (La Martiniere et al. 1995). Pretreatment with genistein and/or daidzein of ICR female rats initiated with DMBA or DMSO-reduced DNA adduct formation at least marginally, as well as reduced proliferation of bone marrow cells (Giri et al. 1995).

1.2.7.2 **Coffee**

In the past, the relationship between coffee and hormone-dependent cancers was quite a controversial subject (Minton et al. 1979; Minton et al. 1981). However,
recent studies have shown no association between the two (Schairer et al. 1987; Hsing et al. 1990b), and even a protective effect in some cases (Franceschi et al. 1995). The compounds present within this "hot" beverage are the xanthines, including caffeine, theobromine, and theophylline.

Caffeine is the chemical within coffee most studied in the literature. Retrospective studies in both premenopausal and postmenopausal women found no correlation between caffeine intake and breast cancer (Folsom et al. 1993; Smith et al. 1994). However, another study on postmenopausal women showed inverse associations between caffeine intake and bioavailable testosterone, and a direct association with plasma estrone. SHBG levels, binding free estrogen were also increased (Ferrini et al. 1996). These results suggest that caffeine may influence sex hormone concentrations, but with increased SHBG, should not increase breast cancer incidence. Caffeine was not associated with prostate cancer in a retrospective study either (Slattery et al. 1993). Theobromine, another xanthine found in coffee presented a different result to this study. It was found that men consuming the higher 2 tertiles of this compound had an increased risk of prostate cancer, caused by a biological mechanism similar to that of caffeine in women. The study concluded that further exploration into theobromine was necessary (Slattery et al. 1993).

1.2.7.3 **Tea and Red Wine**

Epidemiological studies have shown an association between tea and red wine consumption and protection against human cancers. Case-control studies have pointed
to reductions in at least gastric, if not other types of cancer (Wang et al. 1992; You et al. 1989; Buiatti et al. 1989; Kono et al. 1988).

Various tea and red wine flavonoids have been demonstrated in vitro to be cytostatic in several different cell lines, arresting the cell cycle at different phases. Quercetin has been shown to inhibit gastric and colon cancer cells at the G1-S boundary of the cell cycle (Yoshida et al. 1990; Hosokawa et al. 1990) (fig.1.5), and produces dose-dependent growth inhibition of MCF-7 breast cancer cells (Scambia et al. 1994). Apigenin is cytostatic in the neuronal cell line B104 (Sato 1994). At 15-60 μM it inhibited proliferation in a dose-dependent manner, with its IC50 being 12.5 μM on day 4. It arrests the cell cycle in the G2-M phase. This action was seen in B104 with other flavonoids as well, and such growth inhibition is believed to be independent of structural groups. The use of apigenin as a chemopreventive and antitumor drug is presently being investigated (Sato et al. 1994).

As phytoestrogens, quercetin and kaempferol have also been shown to decrease estrogen receptor concentration, and induce progesterone receptor-negative cells to express progesterone receptors (Scambia et al. 1993). This effect may optimize hormonal therapy in breast cancer treatment.

Much animal work has also been conducted using tea or its flavonoids (also found in red wine). Treatment with black or green tea water extracts reduced lung and gastric papilloma incidence and tumor size in female A/J mice treated with N-nitrosodimethylamine (NDEA) (Yang et al. 1994). 0.6% decaffeinated green tea or black tea given during treatment with nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-
butanone (NNK) as the sole source of drinking water, tumor multiplicity was reduced by 67% or 65%, respectively. When given after NNK treatment until the end of the experiment, 0.3% and 0.6% decaffeinated green tea reduced tumor multiplicity by 74% and 85%, respectively, and tumor incidence by 14% and 30%, respectively. Decaffeinated black tea decreased tumor multiplicity by 63%, but did not significantly reduce tumor incidence (Yang et al. 1994). These results showed that black tea, which contains much lower amounts of catechins than green tea, was just as effective as green tea in inhibiting tumorigenesis when given to mice during the carcinogen treatment. This is in line with Wang et al (Wang et al. 1988) who reported that green and black tea polyphenols inhibited benzo(a)pyrene metabolism in rat liver microsomes. Thus tea preparations may have the ability to inhibit activation of a variety of carcinogens. Other mechanisms suggested include induction of phase II enzymes (Sparnins et al. 1982; Khan et al. 1992) and trapping of ultimate carcinogens (Wang et al. 1989).

Administration of 0.6% decaffeinated green tea or black tea extract as sole source of drinking water to Sprague-Dawley rats treated by subcutaneous injection with NMBA (2.5 mg/kg) twice weekly for 5 weeks resulted in less esophageal tumor incidence and 70% less multiplicity. Decreases in esophageal papilloma sizes by 89% and 97% in the green and black tea groups, respectively, were seen (Han et al. 1990). These tea infusions also inhibited esophageal tumorigenesis caused by NMBA precursors (methylbenzylamine plus sodium nitrite) in rats, reducing tumor incidence by 80% to 95% (Xu et al. 1990).
Quercetin and luteolin have been demonstrated to be antitumorigenic agents. It was observed that administration of quercetin or luteolin to 20-methylcholanthrene (MC)-treated mice reduced tumor incidence 47% and 40%, respectively. Tumor volume was also significantly lower in these groups, as well as rutin- and (+)-catechin-treated animals than in those treated with carcinogen alone (Elangovan et al. 1994). There was also a significant delay in growth, and reduced death rate in the flavonoid-treated animals. Furthermore, inhibition of (³H)thymidine incorporation was found to be highest in animals treated with luteolin, followed by quercetin, rutin and (+)-catechin. This is important because incorporation of (³H)thymidine is reflective of cell division, and cancer is a disease of uncontrollable replication. Therefore one mechanism by which luteolin and quercetin reduce tumorigenesis is through their effect on DNA synthesis (Elangovan et al. 1994). Another mechanism is through inhibition or stimulation of enzyme systems. Animals treated with these quercetin and luteolin also showed a reduction in lipid peroxide and cytochrome P450, and an increase in glutathione-S-transferase (Elangovan et al. 1994).

Compounds such as quercetin with 3′,4′ dihydroxy substituents in the B ring and conjugation between the A and B rings, have antioxidant potentials four times that of Trolox, a vitamin E analogue. Removing the ortho-dihydroxy substitution, as in kaempferol, or the potential for electron delocalization by reducing the 2,3 double bond in the C ring, as in catechin and epicatechin, decreases the antioxidative potential by more than 50%, but these structures are still more effective than ascorbic acid or α-tocopherol (Morel et al. 1994). The iron-chelating ability of catechin and quercetin has
been investigated on iron-loaded hepatocyte cultures, measuring the prevention of iron-
increased lipid peroxidation and inhibition of intracellular enzyme release. Catechin was
more effective than quercetin in protecting these cells from damage. The IC₅₀s were
related to structural characteristics, and showed that these compounds function not only
as antioxidants, but as chelators as well, to be cytoprotective in normal cells (Liu et al.

Antioxidant properties of tea and red wine components may act as cytoprotective
agents by various mechanisms. These include guarding cells against lipid peroxidation,
DNA single-strand breakage, and formation of 8-hydroxydeoxyguanosine. These
insults may contribute to carcinogenesis at both initiation and post-initiation stages.
Catechin was reported to inhibit NNK-induced DNA single-strand breaks in rat
hepatocytes in vitro and in vivo (Bhimani et al. 1991), and (-)epigallocatechin-3-gallate
was shown to inhibit TPA-induced 8-hydroxydeoxyguanosine formation in human
epithelial HeLa cells (Xu et al. 1992). Oral administration of green tea was also shown
to inhibit formation of 8-hydroxydeoxyguanosine in mice (Verdeal et al. 1980). Such
mechanisms may help to explain the epidemiological evidence of red wine and tea
being inversely correlated with chronic diseases including CVD and cancer.

1.2.7.4 Fruits and Vegetables

The Canada Food Guide recommends that Canadians eat between 5 and 10
servings of fruits and vegetables a day. This recommendation is derived from studies
showing lower mortality rates in groups with high fruit and vegetable consumption (Key
et al. 1996), especially in regards to CVD and cancer. Many compounds are found in these foods attributable to the decreased morbidity and mortality rates, including fiber, antioxidant and other vitamins, and flavonoids. In case-control studies of two Chinese populations, it was found that green vegetable intake had a statistically significant inverse association with breast cancer risk (Yuan et al. 1995). A cohort study conducted over 6 years found tomato products, containing lycopene, inversely associated with risk of prostate cancer when consumed greater than 10 versus 1.5 times per week (Giovannucci et al. 1995). Moreover, another case-control study found a strong inverse association between total vegetable intake and breast cancer risk in premenopausal women, independent of vitamin C, \(\alpha\)-tocopherol, folic acid, dietary fiber, and \(\alpha\)-carotene components (Freudenheim et al. 1996).

The Netherlands Cohort Study, a large prospective cohort study, found a non-significant inverse relationship between fruit consumption and breast cancer risk (Verhoeven et al. 1997). Animal and in vitro studies have demonstrated antitumorigenesis and cytostasis by flavonoids found in fruits and vegetables. The breast carcinoma cell line MDA-MB-435 showed 50% inhibition of proliferation after treatment with hesperetin, naringenin, or quercetin, and even greater inhibition in 1:1 combinations. \(IC_{50}\)s observed were from 4.7 \(\mu\)g/mL quercetin plus hesperetin, and quercetin plus naringenin, to 22.5 \(\mu\)g/mL naringenin plus hesperetin. Tumor development was delayed in rats given orange juice or fed naringin-supplemented diets, compared with controls. Those given orange juice also had smaller tumors. The studies were concluded to demonstrate that citrus flavonoids, and other flavonoids
found in fruits and vegetables are effective inhibitors of breast cancer cell proliferation, especially when combined with quercetin (So et al. 1996).

Citrus flavonoids (naringin, hesperidin, tangeritin) have also been shown to elevate levels of glutathione transferase in animals treated with them. This effect may be due to antioxidant effects and may be one of the factors for their anticarcinogenic activity (Elangovan et al. 1994; Oganivara et al. 1989). Flavonoids and related tannic acid also act to significantly inhibit single-strand breaks in plasmid pBR322 DNA induced by singlet molecular oxygen. Myricetin showed highest protective ability, followed by tannic acid, (+)-catechin, rutin, fisetin, luteolin and apigenin, after 90 minute incubation. Protective effects were time and concentration dependent. At equimolar concentrations (100 μM) antioxidative activity of myricetin was better than that of the antioxidant vitamins α-tocopherol and β-carotene (Rice-Evans et al. 1995).

Through these mechanisms and others, flavonoids found in fruits and vegetables may act individually or in combination with other properties of these plant foods to reduce cancer initiation and progression.

1.2.8 Methods of Assessing Sex Hormone Activity in Vitro

In order to assess the activity of flavonoids on androgen and progesterone receptors, two breast cancer cell lines, and a marker protein were chosen.
1.2.8.1  Breast Cancer Cell Lines

1.2.8.1.1  T47-D

This breast carcinoma cell line was isolated by Keydar from a pleural effusion obtained from a 54-year-old female patient with an infiltrating ductal carcinoma of the breast. This differentiated epithelial sub strain contains receptors to 17 β-estradiol, androgens, progestins, and other steroids (Freake et al. 1981; Sher et al. 1981; Lamp et al. 1981).

1.2.8.1.2  BT-474

This line was isolated by Lasfargues and Coutinho (Lasfargues et al. 1978) from a solid, invasive ductal carcinoma of the breast obtained from a 60-year-old female patient. The cells vary greatly in size, and have large nuclei with one or more nucleoli. It has androgen, progesterone, and estrogen receptors.

1.2.8.2  Prostate-Specific Antigen

Prostate-Specific antigen (PSA) is a 33 kDa monomeric glycoprotein widely used today in the monitoring of prostate cancer (Oesterling 1993). It was first identified in seminal plasma by Hara et al in 1971 (Hara et al. 1971), and isolated from prostatic tissue in 1979 (Wang et al. 1979). It was first found in human serum by Papsidero et al in 1980 (Papsidero et al. 1980). Its half-life in serum is 2.2 to 3.2 days (Stamey et al. 1987; Oesterling et al. 1988). PSA is a kallikrien-like serine protease, produced primarily by epithelial cells lining the acini and ducts of the prostate gland. Its function is to lyse seminal coagulum (Lilja 1985). PSA has trypsin and chymotrypsin-like activity.
PSA is found in the serum of normal males at levels of less than 4 ng/ml. The clinical cut-off point of PSA concentrations as non-cancerous prostatic carcinoma (including benign prostatic hypertrophy, prostatitis, and prostatic infarction) is unclear. Some authorities consider it to be as high as 15 ng/ml (Mettlin 1993). Patients with biopsy-proven prostate cancer are unlikely to have extracapsular disease or metastasis if PSA is less than 20 ng/ml. PSA values of 40 ng/ml and above are usually indicative of metastatic prostate cancer, however, non-metastatic disease has been seen with PSA values of greater than 80 ng/ml. PSA is a helpful negative predictor of prostate cancer, with an accuracy rate of 94 to 96% (Mettlin 1993).

Scientists are now focussing on proteins produced by breast tumors to aid in diagnosis, prognosis and treatment. One of these proteins is PSA. It is now believed that prostate-specific antigen is a favorable prognostic indicator in breast cancer. PSA immunoreactivity was found in 30% of over 1200 female breast tumor cytosols at levels greater than 0.03 ng PSA/mg total protein. All of these tumors were estrogen and progesterone receptor-positive, whereas ER and PR-negative tumors were found not to produce this protein. PSA is associated with early clinical stage of the disease, and smaller tumor sizes. Furthermore, PSA-positive patients have been found to be less likely to relapse (greater than 60% less) or die than PSA-negative patients (Yu et al. 1995a).

PSA is measured in tumors and in vitro systems in supernatant or cell lysate as a 33 kDa protein. It can also be detected in serum, as a 100 kDa molecule,
corresponding to PSA-bound α₁-antichymotrypsin (Yu et al. 1994; Yu et al. 1995a).

PSA is also measurable in normal breasts of subjects taking progesterone-containing oral contraceptives (Yu et al. 1995b), in postpregnant women, and in the milk of lactating mothers (Yu et al. 1995c). It has also been detected in breast cyst and amniotic fluids (Yu et al. 1995d). PSA has been suggested to be in high amounts in fetuses with congenital abnormalities (Yu et al. 1995c). It is also found in other tumors including ovarian, liver, kidney, adrenal, colon parotid and lung (Levesque et al. 1995).

Such observations have led researchers to hypothesize that PSA may act similarly to other proteinases of the kallikrein family, and may play a role as a local regulator of uterine function (Yu et al. 1995a).

In vitro studies have shown that breast cancer cell lines MCF-7 and T47D can be stimulated by androgens and progestins to produce PSA. Maximum production occurs in response to 10⁻⁶ to 10⁻⁷ mol/L of these compounds. Estrogen can suppress PSA production by these steroids (Yu et al. 1994). Therefore, PSA may be used as a model and an indicator of hormone balance within the tumor or cellular system between estrogen and androgen/progestin. This model will allow us to assess the effects of plant-derived compounds as agonists and antagonists of the androgen and progesterone receptors. Agonism will be assessed by measuring amounts of PSA produced upon incubation of the cells with candidate compound alone. Antagonism will be determined by measuring PSA production upon incubation of the cells with candidate compound plus steroid, relative to PSA produced by steroid alone.
1.3 Research Hypothesis and Objectives

I hypothesize that certain natural products, which are components of plant foods or beverages, may have steroid hormone agonist or antagonist activity by interacting with progesterone and/or androgen receptors. This hypothesis will be initially tested by using a tissue culture system consisting of a steroid hormone receptor-positive breast carcinoma cell line. The activity of the candidate compounds will be assessed by monitoring the expression of the prostate-specific antigen gene which is known to be regulated by steroid hormones (Yu et al. 1994).

The main objectives are:

1. To determine whether the PSA assay system can be used to assess sex hormone activity by using a set of synthetic antiprogestins synthesized by the Southwestern Research Foundation.

2. To determine the agonistic activity of 45 plant-derived compounds on progesterone and androgen receptors by measuring PSA produced upon stimulation of BT 474 breast cancer cells with the plant-derived compounds.

3. To determine the antagonistic activity of 45 plant-derived compounds on PR and AR by assessing PSA production upon stimulation of T 47D breast cancer cells with plant-derived and steroid, relative to PSA production upon stimulation with steroid alone.
CHAPTER TWO

SYNTHETIC ANTIPROGESTINS
flask, then detached by trypsin-EDTA treatment to be split or subcultured in 48-well microtiter plates for experimentation. The subculture medium was the same as above, except charcoal stripped fetal calf serum was used instead of the regular fetal calf serum, as it is devoid of any steroid hormones. Cell clumps were minimized by passing cells and media through an 18-gauge syringe. Cells were grown as monolayers until confluency, with approximately $2 \times 10^5$ cells per well, and each well contained 1 ml of medium.

2.1.2 **Harvesting of Supernatant**

Supernatant was separated from the cells through aspiration, using plastic pipets. Supernatant was immediately transferred into labeled Eppendorf tubes, and either immediately used for PSA assay or stored at -20°C until analysis. PSA is a very stable molecule, able to be stored for long periods of time without degradation (Hoffman et al. 1996).

2.1.3 **Experimental Design**

2.1.3.1 **Screening for Potential Agonistic Activity**

BT-474 and T47-D breast cancer cells were grown to confluency in 48-well and 24-well plates, respectively, under standard conditions, and stimulated by seven novel antiprogestin compounds (table 2.1, encoded 0, 1, 2, 3, 5, 6, and 9) at final concentrations of $10^{-7}$ and $10^{-9}$ M. All compounds were diluted with pure anhydrous ethyl alcohol. Positive controls were norgestrel (a progestin), testosterone (an
androgen), and norgestimate (a progestin) (Sigma Chemical Co., St Louis, MO 63178). Alcohol was the negative control. Mifepristone (Roussel-UCLAF, Romainville, France) was used as a known antiprogestin with weak progesterogenic activity.

2.1.3.2 **Dose-Response of Agonistic Activity**

BT-474 cells were incubated with 1µL of synthetic antiprogestin encoded #0, 1, 2, 3, 5, 6, or 9 at final concentrations of 10^{-7} to 10^{-9} M in 1ml media. Norgestrel at same concentrations was used as a positive control. Alcohol was a negative control.

2.1.3.3 **Assessment of Antagonistic Activity**

T47-D cells were grown to confluency under standard conditions in 48-well microtiter plates. The cells were incubated with 1 µL in 1 mL media of compound #0, 1, 2, 3, 5, 6, or 9 for 1 hour at final concentrations of 10^{-7} or 10^{-9} M. Dihydrotestosterone, or norgestrel were added at final concentrations of 10^{-8} or 10^{-10} M, or norgestimate at 10^{-8} M to create triads of progestin blocker alone, blocker plus agonistic steroid, and steroid alone (fig. 2.1). Alcohol was a negative control. Incubation time was 7 days.

2.1.4 **PSA Assay**

PSA protein was measured with a modified highly sensitive immunofluorometric procedure that can measure PSA at levels of 1 ng/L or higher (up to 10,000 ng/L) with a precision of <10% (Ferguson et al. 1996). This assay uses a mouse monoclonal anti-PSA capture antibody coated to polystyrene microtiter wells, a biotinylated mouse monoclonal detection antibody, and alkaline phosphatase-labeled streptavidin.
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</table>

**Figure 2.1  Creation of Triads for Assessment of Antagonistic Activity**

Triads were created for the assessment of antagonistic activity for both synthetic antiprogestins and plant-derived compounds. The triads were blocker alone, blocker plus agonistic steroid, and steroid alone. Alcohol was used as the negative control.
100 μL of sample were incubated in polystyrene microtiter wells with 50 μL of biotinylated anti-PSA antibody diluted 1:1000 in PSA assay buffer and shaken for 1.5 hours. After 6 washings, 100 μL alkaline phosphatase-labeled streptavidin conjugate, diluted 1:1000 in 6% BSA, was added and shaken for 15 minutes, followed by another 6 washings. 100 μL 5-fluorosalicylate phosphate diluted 1:10 in substrate buffer was added and shaken for 10 minutes, then 100 μl Tb³⁺-EDTA ("developing solution") was added and the plate was shaken for a further 2 minutes. If PSA was present in the samples, a ternary fluorescent complex was formed between the released 5-fluorosalicylate, Tb³⁺, and EDTA. The amount of fluorescence was quantified through time-resolved fluorescence using a pulse nitrogen laser excitation source (Cyberfluor Inc., Toronto, ON), and correlated to PSA concentration in ng/L.

2.1.5 Statistics

All studies were conducted four times each, and expressed as mean ± SEM. For analysis of antagonism studies, percent blocking was calculated as the percentage of PSA produced compared to the means of steroid (DHT or norgestrel) alone for each separate plate. Coefficients of variation were calculated for all compounds. Analysis between groups was conducted using Student-Newman-Keuls multiple range test. Pearson correlation was used to assess the strength of association between the receptors.
2.2 Results

2.2.1 Screening for Potential Agonistic Activity

Three of the seven compounds tested at $10^{-7}$ M final concentration in both BT-474 and T47-D cells were found to have agonistic activity. These were compounds #2, 5 and 9. Compound #5 was found to have strong activity, followed by compound #9, and finally compound #2 with moderate activity. Compound #0 was found to have weak activity in the BT-474 cell line, but was negative in the T47-D line. All other compounds (#1, 3, and 6) were found to be negative for agonistic activity in our system (table 2.1).

These compounds at $10^{-9}$ M final concentration in the BT-474 line were found to have no agonistic activity. However, in the T47-D line, weak activity was seen for compounds #5, 2, and 9 at this concentration (table 2.2). This was attributed to the use of 24-well microtiter plates used in this experiment, allowing for a greater number (approximately double) of cells to be stimulated.

2.2.2 Dose-Response of Agonistic Activity

Dose-Response experiments for compounds #2, 5 and 9 revealed strong activity at the highest concentrations ($10^{-7}$ M) for all three compounds. Moderate activity for compound #2 and #9 at $10^{-8}$ M. No activity was seen at this concentration for compound #5. At final concentrations of $10^{-9}$ to $10^{-11}$ M for all three compounds, no agonistic activity was observed (fig. 2.2).
Seven synthetic antiprogestins were tested for agonistic and antagonistic activity on androgen and progesterone receptors. These are the structures, codes, and chemical names of these compounds.
Table 2.2  Agonistic Activity of Synthetic Antiprogestins in the BT 474 and T 47D Breast Cancer Cell Lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>Agonistic Activity BT 474</th>
<th>Agonistic Activity T 47D</th>
</tr>
</thead>
<tbody>
<tr>
<td>mifepristone</td>
<td>7 ± 1</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>0</td>
<td>5 ± 2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1</td>
<td>1 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>54 ± 1</td>
<td>119 ± 11</td>
</tr>
<tr>
<td>3</td>
<td>1 ± 0</td>
<td>4 ± 0</td>
</tr>
<tr>
<td>5</td>
<td>336 ± 98</td>
<td>373 ± 36</td>
</tr>
<tr>
<td>6</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>9</td>
<td>118 ± 28</td>
<td>191 ± 38</td>
</tr>
<tr>
<td>Norgestrel</td>
<td>843 ± 27</td>
<td>697 ± 24</td>
</tr>
<tr>
<td>alcohol</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
</tr>
</tbody>
</table>

| Mifepristone | 0 ± 0 | 2 ± 1 |
| 0            | 1 ± 1 | 0 ± 0 |
| 1            | 1 ± 1 | 1 ± 0 |
| 2            | 0 ± 0 | 2 ± 0 |
| 3            | 1 ± 1 | 0 ± 0 |
| 5            | 3 ± 2 | 5 ± 0 |
| 6            | 1 ± 1 | 1 ± 1 |
| 9            | 0 ± 0 | 2 ± 1 |
| Norgestrel   | 417 ± 80 | 92 ± 2 |
| alcohol      | 1 ± 1 | 2 ± 2 |

1 Mean values ± standard error, N=4.

Seven synthetic antiprogestins were first tested for agonistic activity. Norgestrel was used as a positive control and alcohol as a negative control. Mifepristone was used as an antiprogestin with weak agonistic activity. All compounds were tested four times. Compounds #0, 2, 5, and 9 were found to have agonistic activity at 10^-7 M. By 10^-9 M, this activity was no longer seen.
Synthetic antiprogestins encoded #2, 5, and 9 were found to have agonistic activity on androgen and progesterone receptors. This was seen at $10^{-7}$ M final concentration, and dropped precipitously to 0 by $10^{-9}$ M. This activity was weaker than that seen with norgestrel, a progestin.
2.2.3 Assessment of Antagonistic Activity

Compounds #0, 1, 2, 3, 5, 6, and 9 were first tested for antiprogestin activity. This was done using both norgestrel, at $10^{-8}$ M and $10^{-10}$ M, and norgestimate at $10^{-8}$ M final concentrations. For the $10^{-7}/10^{-8}$ pair (blocker/progestin), both experiments demonstrated that compounds #0 and 5 were the strongest blockers, with $98 \pm 2\%$ and 100\% blockade, respectively, followed closely by compound #2 at $93 \pm 3\%$ blockade. Compound #9 had a 100\% blockade using norgestimate, but 80\% with norgestrel. Compound #6 had the weakest antiprogestin activity ($<50\%$). Compound #3 was also weak in its antagonistic activity on the progesterone receptor. The $10^{-9}/10^{-10}$ M pairs (blocker/norgestrel) showed a similar pattern. Compound #0 showed complete (100\%) antiprogestin activity, followed by #5 with $97 \pm 4\%$ blockade. Compound #2 showed $95 \pm 6\%$ antagonistic activity. Compound #6 was the least effective in blocking the progesterone receptor (27\% blockade) at this concentration as well (table 2.3).

Antiandrogenic activity was tested using the synthetic antiprogestins with dihydrotestosterone in $10^{-7}/10^{-8}$ and $10^{-9}/10^{-10}$ M pairs (table 2.3). Similar results to antiprogesterogenic activity were seen. Least blocking was seen with compound #6 at both. This was followed by compounds #3 ($77 \pm 1$ and $37 \pm 12\%$) and #1 ($80 \pm 6\%$ and $41 \pm 15\%$). Greatest blocking of the AR was seen with compounds #5, 2, and #9, with percentage blocks being $95 \pm 4\%$, $91 \pm 5$ and $90 \pm 3$ at $10^{-7}$ M, and $73 \pm 9$, $83 \pm 4$ and $66 \pm 12\%$ at $10^{-9}$ M, respectively.
Table 2.3  Antagonistic Activity of Synthetic Antiprogestins

<table>
<thead>
<tr>
<th>Compound $10^{-7}/10^{-8}$ M Pair</th>
<th>%Block of Norgestrel</th>
<th>%Block of Norgestimate</th>
<th>%Block of Dihydrotestosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98 ± 2</td>
<td>100</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>1</td>
<td>71 ± 12</td>
<td>87</td>
<td>80 ± 6</td>
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<tr>
<td>2</td>
<td>93 ± 3</td>
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<td>91 ± 5</td>
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<td>3</td>
<td>44 ± 22</td>
<td>50</td>
<td>77 ± 1</td>
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<tr>
<td>5</td>
<td>100 ± 0</td>
<td>100</td>
<td>95 ± 4</td>
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<tr>
<td>6</td>
<td>39 ± 1</td>
<td>0</td>
<td>33 ± 33</td>
</tr>
<tr>
<td>9</td>
<td>83 ± 2</td>
<td>100</td>
<td>90 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound $10^{-9}/10^{-10}$ M Pair</th>
<th>%Block of Norgestimate</th>
<th>%Block of Dihydrotestosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 0</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>1</td>
<td>62 ± 15</td>
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<tr>
<td>2</td>
<td>95 ± 6</td>
<td>83 ± 4</td>
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<tr>
<td>3</td>
<td>48 ± 15</td>
<td>37 ± 12</td>
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<tr>
<td>5</td>
<td>97 ± 4</td>
<td>73 ± 9</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>21 ± 21</td>
</tr>
<tr>
<td>9</td>
<td>83 ± 2</td>
<td>66 ± 12</td>
</tr>
</tbody>
</table>

1 Mean values ± standard error, N=4.

The antiprogesterogenic and antiandrogenic activities of the synthetic antiprogestins were tested. Compounds #0, 2, 5, and 9 were shown to have the greatest blocking effects. This was true for both the PR, demonstrated with norgestrel and norgestimate (a progestin with no cross-reactivity for the AR), and the AR, demonstrated with DHT.
CHAPTER THREE

PLANT-DERIVED COMPOUNDS
3. PLANT- DERIVED COMPOUNDS

Once the breast cancer assay system was found to be appropriate for measuring sex hormone receptor activity, we proceeded with the assessment of agonistic and antagonistic activity of plant-derived compounds.

3.1 Materials and Methods

The breast cancer cell lines, procedures of growth and maintenance of cells, harvesting of supernatant, and PSA assay are the same as outlined in chapter 2.

3.1.1 Experimental Design

A wide range of flavonoids, antioxidants, vitamins (Sigma Chemical Co., St Louis, MO 63178; Indofine Chemical Co., Sommerville, NJ 08876), teas (health food store) and wine (Liquor Control Board of Ontario) were tested for agonistic and antagonistic activities (table 3.1, fig. 3.1). These compounds were chosen because of the epidemiological research around the foods/beverages in which they are found. The categories of foods that contain these compounds include soya, tea, wine, fruits, vegetables, and coffee. For some of these compounds, elaborate work has been conducted already to determine their mechanism of action in cancer prevention and treatment. However, for the great majority, little to no literature exists.
### Table 3.1 Plant-derived Compounds

<table>
<thead>
<tr>
<th>Food Source</th>
<th>Compound</th>
<th>Concentrations</th>
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</thead>
<tbody>
<tr>
<td>coffee</td>
<td>caffeine</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>coffee</td>
<td>caffeic acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>coffee</td>
<td>theobromine</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>coffee</td>
<td>theophylline</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>coffee</td>
<td>chlorogenic acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>antioxidant vitamin</td>
<td>ascorbic acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>antioxidant vitamin</td>
<td>α-tocopherol</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>antioxidant vitamin</td>
<td>β-carotene</td>
<td>$10^{-2} - 10^{-4}$ M</td>
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<td>vitamin</td>
<td>folic acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>associated with folic acid</td>
<td>homocysteine</td>
<td>$10^{-2} - 10^{-4}$ M</td>
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<td>vitamin B₁₂</td>
<td>methylcobalamin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>vitamin B₆</td>
<td>pyridoxine</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>all green plants and vegetables</td>
<td>chlorophylline</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>tea and red wine</td>
<td>rutin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>tea and red wine</td>
<td>morin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>tea and red wine</td>
<td>gallic acid</td>
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<td>quercetin</td>
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<tr>
<td>tea and red wine</td>
<td>taxifolin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>tea and red wine</td>
<td>m-coumaric acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
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<td>tea and red wine</td>
<td>p-coumaric acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
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<td>tea and red wine</td>
<td>ferrulic acid</td>
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<td>vanillic acid</td>
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</tr>
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<td>syringic acid</td>
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</tr>
<tr>
<td>tea and red wine</td>
<td>rutin trihydrate</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>soya</td>
<td>biochanin A</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>soya</td>
<td>daidzein</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>soya</td>
<td>genistein</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>fruits, vegetables and leaves</td>
<td>apigenin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>fruits, vegetables and leaves</td>
<td>myricetin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>fruits, vegetables and leaves</td>
<td>luteolin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>fruits, vegetables and leaves</td>
<td>kaempferol</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>berries</td>
<td>ellagic acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>berries</td>
<td>salicylic acid</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>citrus fruits</td>
<td>hesperetin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>citrus fruits</td>
<td>hesperidin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>citrus fruits</td>
<td>naringin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>citrus fruits</td>
<td>naringenin</td>
<td>$10^{-2} - 10^{-4}$ M</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>red wine</td>
<td>gamay noir</td>
<td>dilutions x100, x1000, x10,000 vol/vol</td>
</tr>
<tr>
<td>red wine</td>
<td>cabernet franc</td>
<td>dilutions x100, x1000, x10,000 vol/vol</td>
</tr>
<tr>
<td>tea</td>
<td>oolong tea</td>
<td>dilutions x10, x100, x1000</td>
</tr>
<tr>
<td>tea</td>
<td>green tea</td>
<td>dilutions x10, x100, x1000</td>
</tr>
<tr>
<td>tea</td>
<td>Earl Grey</td>
<td>dilutions x10, x100, x1000</td>
</tr>
<tr>
<td>fruit extract used in prostate disease</td>
<td>saw palmetto</td>
<td>dilutions x100, x1000, x10,000 vol/vol</td>
</tr>
</tbody>
</table>

Forty-five plant-derived compounds and beverages were tested for agonistic and antagonistic activity on progesterone and androgen receptors. These were picked on the basis of plant-food group in which they are present. The soy isoflavones (Indofine Chemical Co.) and the other pure compounds (Sigma Chemical Co.) were diluted with absolute ethanol on a molar basis. The wines were diluted with ethanol on a vol/vol basis. The teas were steeped for 5 minutes with boiling distilled water, and the water extract was collected. This water extract was diluted with ethanol.
21 compounds were found to have agonistic or antagonistic activity on progesterone or androgen receptors. These compounds have very different structures. Many have hydroxyl groups. Chlorophyll is a very large molecule, with a magnesium core.
3.1.1.1 **Screening for Potential Agonistic Activity**

BT-474 breast cancer cells were grown to confluency as above. For the pure flavonoids, antioxidants, methylxanthines, chlorophylline, and homocysteine, final concentrations of $10^{-5}$, $10^{-6}$, and $10^{-7}$ M were used, with anhydrous ethanol being used for dilutions. The teas were prepared by boiling 300 mL distilled water, adding it to 3.2 g of tea (equivalent to two cups of tea per tea bag) and steeping for 5 minutes. Then dilutions of 10x, 100x, and 1000x were made. For the wines and saw palmetto, dilutions were made of 100x, 1000x, and 10 000x v/v. Cells were incubated 1 μL plant-derived compound in 1 mL media. Alcohol was used as a negative control throughout the study, and norgestrel was used as a positive control. Incubation time was 7 days.

3.1.1.2 **Dose-Response of Agonistic Activity**

BT-474 cells were incubated with 1 μL of plant-derived compound and 1 ml media in the 48-well microtiter plates. The concentrations of compounds used ranged from $10^{-2}$ to $10^{-4}$ M, with a final concentration of $10^{-5}$ to $10^{-7}$ M. All compounds tested were with anhydrous ethyl alcohol. Incubation time was 7 days. The compounds tested are listed in table 3.1.

3.1.1.3 **Assessment of Antagonistic Activity**

T47-D cells were grown to confluency under standard conditions in 48-well microtiter plates. The cells were incubated with plant-derived compound at $10^{-6}$ M final
concentration for one hour. DHT or norgestrel, at final concentration of $10^{-7}$ M was added to form triads of flavonoid alone, flavonoid plus androgen or progestin, and androgen or progestin alone. Alcohol was used as a negative control. Incubation time was 7 days.

Statistics were performed as outlined in chapter 2.

3.2 Results

3.2.1 Screening for Potential Agonistic Activity

Forty-five compounds consisting of flavonoids, antioxidants, vitamins, and beverages were assessed for their agonistic effects on androgen and progesterone receptors in the BT-474 breast cancer cell line (table 3.1). The compounds were preliminarily assessed at $10^{-6}$ and $10^{-8}$ M final concentrations. Compounds that were positive at either concentration were then studied for dose-response at final concentrations of $10^{-5}$ to $10^{-8}$ M.

3.2.2 Dose-Response for Agonistic Activity

After repeated rounds of analyses, the only compound found to have agonistic activity was apigenin. Apigenin had a strong effect at the two highest concentrations, and produced lower, but detectable amounts of PSA at the lowest concentration (fig. 3.2).
Figure 3.2  Dose-Response of Apigenin

Apigenin was the only plant-derived compound found to have agonistic activity on progesterone and androgen receptors. Androgen produced 900 ng/L PSA at $10^{-6}$ M concentration, and dropped precipitously by $10^{-7}$ M. This compound was found to have a linear dose-response.
3.2.3 **Assessment of Antagonistic Activity**

Chlorophylline was found to have the strongest antagonistic effect on both hormone receptors of all the plant-derived compounds (87 ± 7% for PR, 94 ± 3% for AR). This was followed for the progesterone receptor by the antioxidant vitamin β-carotene (82 ± 3%), taxifolin (80 ± 5%), homocysteine (75 ± 15%), and α-tocopherol (65 ± 10%). Other compounds with antiprogesterogenic activity included chlorogenic acid, theobromine, theophylline, saw palmetto, luteolin, rutin trihydrate, and the red wines (table 3.2, fig. 3.3).

After chlorophylline, the greatest antiandrogenic plant-derived compounds included hesperetin (79 ± 11%), syringic acid (72 ± 11), α-tocopherol (65 ± 5%), taxifolin (58 ± 11%), gallic acid (55 ± 7%), and m-coumaric acid (53 ± 11%). Other blockers of AR included caffeine, salicylic acid, pyridoxine, gamay noir, β-carotene, and Earl Grey tea (table 3.2, fig. 3.4).

Plant-derived compounds were divided into 7 food groups: coffee, pigments, antioxidant vitamins, vitamins and related compounds, tea and red wine, soya, and fruits, vegetables and leaves. Differences between groups were assessed. For the androgen receptor, antagonistic activity by pigments (chlorophyll) was significantly greater than blocking by vitamins, coffee, tea and red wine, soya, and fruits, vegetables and leaves (p<0.05). Antioxidant vitamins did not show statistical difference from any other group. The progesterone receptor showed much the same result. Chlorophyll had a greater blocking activity from all other groups (p<0.05) (fig 3.5).
Table 3.2  Antagonistic Activity of Plant-derived Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiprogesterogenic Activity (% block)</th>
<th>Antiandrogenic Activity (% block)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coffee</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>caffeine</td>
<td>22 ± 9</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>caffeic acid</td>
<td>0 ± 0</td>
<td>16 ± 16</td>
</tr>
<tr>
<td>theobromine</td>
<td>38 ± 4</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>theophylline</td>
<td>36 ± 7</td>
<td>30 ± 11</td>
</tr>
<tr>
<td>chlorogenic acid</td>
<td>61 ± 2</td>
<td>24 ± 12</td>
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<tr>
<td><strong>Antioxidant Vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>29 ± 12</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>65 ± 10</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>β-carotene</td>
<td>82 ± 3</td>
<td>48 ± 11</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
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<td></td>
</tr>
<tr>
<td>folic acid</td>
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<td>52 ± 20</td>
</tr>
<tr>
<td>homocysteine</td>
<td>75 ± 15</td>
<td>67 ± 20</td>
</tr>
<tr>
<td>methylcobalamin</td>
<td>24 ± 8</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>pyridoxine</td>
<td>28 ± 10</td>
<td>48 ± 5</td>
</tr>
<tr>
<td><strong>Pigments</strong></td>
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<td></td>
</tr>
<tr>
<td>chlorophylline</td>
<td>87 ± 7</td>
<td>94 ± 3</td>
</tr>
<tr>
<td><strong>Tea and Red Wine</strong></td>
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<td></td>
</tr>
<tr>
<td>oolong</td>
<td>19 ± 8</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>Earl Grey</td>
<td>12 ± 7</td>
<td>33 ± 8</td>
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<td>green tea</td>
<td>18 ± 7</td>
<td>47 ± 20</td>
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<tr>
<td>gamay noir</td>
<td>45 ± 8</td>
<td>50 ± 10</td>
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<tr>
<td>cabernet franc</td>
<td>47 ± 10</td>
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<tr>
<td>rutin</td>
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<tr>
<td>Biochanin A</td>
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<tr>
<td>Genistein</td>
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<td>16 ± 9</td>
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<tr>
<td>Saw Palmetto</td>
<td>25 ± 15</td>
<td>21 ± 11</td>
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1 Mean values ± standard error, N=4.

45 plant-derived compounds were tested for antagonistic activity on PR and AR. These are the percentage blocking of norgestrel and DHT, respectively.
Many plant-derived compounds were found to have antiprogestogenic activity. The greatest effect was seen by chlorophylline, followed by the antioxidant vitamins (α-tocopherol and β-carotene), tea and red wine compounds (taxifolin), and methylxanthines from coffee (theobromine, theophylline, and chlorogenic acid).
Figure 3.4 Antiandrogenic Activity of Plant-derived Compounds

Many plant-derived compounds and beverages were found to have antiandrogenic activity. The greatest effect was seen by chlorophylline, followed by hesperetin, a citrus flavonoid, red wine and tea compounds (syringic acid, taxifolin), and antioxidant vitamins (α-tocopherol, β-carotene).
Figure 3.5  Antagonistic Activity of Plant-derived Compounds by Groups

We compared antagonistic activity of plant-derived compounds by groups. Grey bars represent the progesterone receptor, black bars the androgen receptor. Pigments (chlorophylline) was found to have significantly different activity from all other groups for both receptors ($p<0.05$). No other group was statistically different from another.
Correlations were made between the two receptors. A significant correlation was found between AR and PR for all compounds tested ($r=0.47$, $p=0.001$).
CHAPTER FOUR

GENERAL DISCUSSION

AND

CONCLUSIONS
4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 General Discussion

Our study shows that several plant-derived compounds do indeed interact with progesterone and androgen receptors with agonistic and antagonistic results (fig. 3.3, 3.4). Strong agonistic activity was seen with apigenin, and compounds from many plant sources had significant blocking activity of PR, AR or both.

Flavonoids comprise a field with much scope, but very little in-depth knowledge and literature. Categorizing compounds according to plant-food groups was very difficult for many reasons including ubiquitiveness of flavonoids within the plant kingdom, very little work done in assaying plant samples, and no database being in existence. Therefore, we have separated plant-derived compounds into different groups, according to the Merck Index and articles in Medline. Hopefully in the near future flavonoids will be entered into the database of foods (e.g. United States Department of Agriculture), with concentrations evaluated and specified.

The primary purpose of this study was to assess plant-derived compounds for their activity as agonists and antagonists of the androgen and progesterone receptors, in order to determine their use in prevention and treatment of hormone-dependent cancers. Breast and prostate cancers, as hormone-dependent diseases, have been demonstrated through historical treatment as well as investigations and regiments used today, to be prevented, treated, and manipulated through three sex-hormone systems, singly, or in combination. How this can be done through either dietary manipulation or through utilization of "neutraceuticals" is the ultimate goal we wish to pursue. We
believe that this study has provided a necessary step in this long plan.

In order to examine the usability of our tissue culture system to determine hormone agonism and antagonism, we decided to incorporate compounds for which some blocking studies had been conducted. These synthetic progestin antagonists were made available to us for the purpose of measuring progesterogenic agonistic activity by Southwest Research Foundation for Biomedical Research in San Antonio, Texas. We chose to extend this work to look at the antiprogestin and antiandrogen activity of these compounds as well, and compare our results with those seen by their researchers (table 4.1). As our results generally correlated with theirs, we were able to conclude that our assay system could accurately determine receptor stimulation and inhibition. Positive results were conclusive, with tight standard errors. This allowed us to proceed with the flavonoid study, with the necessary confidence that our results could be interpretable.

The blocking ability of the synthetic antiprogestins varied greatly from 0 to 100% of both the androgen and progesterone receptors. Such variation was seen at both the higher (10^{-7} M) and lower (10^{-9} M) concentrations. By using norgestimate, a pure progesterone agonist (i.e. has no affinity for the androgen receptor) we were able to observe progesterone receptor blockade without androgen receptor cross-activity. The progesterone antagonist activity seen with norgestrel as stimulant corresponded with these results. More interesting to us was the conclusion that three of these synthetic antiprogestins (#2, 5 and #9) were in fact progesterone and androgen agonists as well. The activity demonstrated was quite strong at the higher concentration tested (>100
We received information on various tests the Southwest Foundation for Biomedical Research conducted on the 7 synthetic antiprogestins studied. These included relative binding assay results for the progesterone receptor and the anti-McGinty assay, which determines antiprogestational activity. In general, these results were in accordance with ours.

Table 4.1  Collaborative Results of Antiprogesterogenic Activity

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<td>9</td>
<td>111</td>
<td>-</td>
<td>84, 50</td>
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</table>
ng/L of PSA for 10^{-7} M), and dropped precipitously at lower concentrations (10^{-9} M). This knowledge could be beneficial for determining pharmacological concentrations to use as antagonists, i.e., for only blocking activity, one would use the lower concentration.

Agonistic activity has been observed for other progesterone blockers, namely mifepristone (RU486), but the activity of the synthetic blockers measured here was much greater than the one demonstrated for mifepristone, indicating that in some situations these compounds could be beneficial as partial agonists.

During the initial few months of the plant-derived compound study, we determined the best techniques to use, through much trial and error, encompassing the growing and maintenance of the cells, concentrations of compounds to use, and the number of days to incubate the plates. After several attempts, we found that in order to keep the number of cells consistent, growing them as monolayers was a necessity. This was accomplished by utilizing a syringe with an 18-gauge needle to separate the individual cells immediately before plating them. Concentrations of dry compounds were determined through past protocols of blocking of steroids, where steroids are used at 10^{-7} M final concentration, and potential blockers are at 10-fold higher concentrations than the steroids.

Determining dilutions for the teas and wines took more consideration. After looking at several possibilities, we decided to steep the tea as would be done in a natural situation. It was then injected into the media at full strength, as well as 10x, 100x, and 1000x dilutions. This would allow us to see if any activity would be seen at lower concentrations as well as full strength. The wines and saw palmetto were diluted
on a volume per volume basis at dilutions of 100x, 1000x, and 10 000x. These were equivalent to the dry compound concentrations.

The plant-derived compounds we tested were in their pure forms, i.e. they were not metabolized prior to injecting into cellular media. Hence, we were unsure as to whether or not the molecules would enter the cell, necessary for any potential receptor binding to occur. As other studies have been conducted using these and/or similar compounds, we did not believe this would be a problem. Our results are indicative of the compounds diffusing easily through the cell and nuclear membranes into the nucleus for possible interaction with the nuclear receptors (AR and/or PR). The alternate possibility is that cellular metabolism of these compounds occurs once in the cytoplasm of the cells, conducted by enzymes already present.

The large majority of the plant-derived compounds we tested did not have agonistic activity. The only compound that gave a linear and significant dose-response was apigenin, a flavonol form of quercetin. This compound is found ubiquitously in fruit, vegetables, leaves, and other plant foods. Other compounds with some agonistic activity were naringenin, the non-conjugated form of naringin, derived from citrus fruit, and found in the fruit itself, as well as the peel and seeds.

Several plant-derived compounds were found to have antagonistic activity. These included the methylxanthines from coffee, pigment, antioxidant vitamins, other vitamins and related compounds, tea and red wine flavonoids, and compounds found in fruits, vegetables and berries. The soy isoflavones were not found to have any significant blocking activity on either hormone receptor. Perhaps their estrogenic and/or
antiestrogenic effects accounted for these results. It has been found that estrogens can have blocking actions on androgen and progesterone receptors (Yu et al. 1994). Hence, this may be true for strong phytoestrogens as well.

Many of the compounds found to be either agonists or antagonists of the PR or AR have been documented in the literature as antioxidants. Many of these compounds are known for their ability, as antioxidants, to reduce cardiovascular risks including blood cholesterol and LDL oxidation. The "French paradox", the revelation that although the French eat a diet high in saturated fat do not seem to die from cardiovascular diseases, has been attributed, at least partly, to red wine phenolics. In vitro experiments have concluded that the higher the polyphenolic content of the wine, the greater its antioxidative effect measured as change in lag time in different oxidation systems. Moreover, upon stripping the polyphenols from the beverage, the lag time returns to control levels in isolated LDL (Abu-Amsha et al. 1996). Hot water extracts from green tea, black tea, and various wines, also have been documented to scavenge ascorbyl radicals (Satoh et al. 1996). The relationship between antioxidant activity and hormones, namely estrogen, is an interesting one, though not completely understood. Estrogens have been associated with decreased risk of heart disease, and are used as preventive medicine for post-menopausal women in the form of hormone replacement therapy (HRT). Perhaps the relationship can be further explained by our results; since estrogen and progesterone and androgen seem to have an inverse relationship in some situations, perhaps the antagonistic activity of some plant-derived compounds on PR and AR play a role in this cardioprotection. This may either be through a higher
estrogen:androgen activity ratio, or as a direct result of androgen and progesterone blockade. Such theories must be further investigated.

Chlorophyll, the pigment responsible for the color of all green plants, and the necessary component for photosynthesis, is remarkably unstudied at this point. It seems rather surprising that the molecule found ubiquitously in all green plants and vegetables would not be investigated for antioxidative potential, but this is what the lack of literature seems to indicate. Necessary for photosynthesis, a process based on ultraviolet and other forms of radiation, this compound must be extremely stable, and therefore a supremely good antioxidant. Chlorophyll was found to have exceptional blocking ability for both the PR and AR, significantly greater than all other plant-derived compound groups studied. This compound is an extremely large one (MW ≈ 900), and is not absorbed as such from the gut. However, chlorophyll has a magnesium ion at its core. This trace mineral has gained interest at present, and is being examined for its positive effects on chronic health, especially cancer and heart disease (Singh et al. 1997; Gawaz et al. 1996). We hope this revelation, along with the results of other fruit and vegetables flavonoids presented here, will lead to more investigation in its many roles on chronic health, disease, and aging.

In summary, the data we have obtained indicate that some flavonoids and other organic compounds do function as agonists or antagonists of the androgen and progesterone receptors. The observation that compounds are responsive in such a system, are consistent with the epidemiological and prospective literature demonstrating an association between high vegetable and other plant-food consumption and lower risk
of breast and prostate cancers. How we may manipulate this knowledge to increase health and extend longevity is a possibility that needs further investigation.

4.2 Conclusions

These results convey two major findings. First, the antiprogestin work allows us to deduce that this tissue culture system is a good assay for determining the agonistic and antagonistic activity of compounds on progestin and androgen receptors in at least two breast cancer cell lines. The data are consistent and reproducible, allowing the results to be reliable and interpretable.

Secondly, and more importantly, some purified polyphenolic and related compounds were found to have agonistic and/or antagonistic activity in this breast cancer tissue system. This demonstrates that some of these compounds do enter the cell, and have hormonal or antihormonal consequences on nuclear receptors. Whether the compounds are metabolized by the cell or not is yet to be determined. However, this is a good start in determining the effects of flavonoids on hormonal systems within human breast cancer cell.

Tea and wine polyphenols were found to have weak but significant antagonistic activity in our system. We have also demonstrated that some antioxidant vitamins, other vitamins, and other plant-derived compounds have antiandrogen or antiprogestin activity. These compounds have gained much attention epidemiologically, and our in vitro work confirms data found in the literature. We have potentially determined one mechanism by which they function. However, much more work is needed to what other
mechanisms exist, and how these pathways interact and interrelate.

Finally, this study has focussed our attention on a constituent of plant foods not typically examined: chlorophyll. This extremely important compound may function to decrease cancer initiation and promotion by many mechanisms. At its core is a magnesium ion, which is now being looked at in terms of reducing oxidative damage and CHD. Now we observe a potent anti-hormonal effect of this vital compound. Hopefully this project will lead towards more studies on its beneficial effects on health and chronic disease.

4.2.1 Future Studies

Our preliminary observations of dong quai, a root used in Eastern medicine for treatment of premenstrual and menopausal symptoms indicate that this extract has both strong agonistic and antagonistic activity in both the PR and AR systems. Our observations of yam extract, also used in women's health show a strong blocking ability on these receptors. Further study, with purer and more standardized forms of these compounds is necessary for conclusions to be made.

Experiments are already being conducted to determine the effects of the metabolites produced upon consumption of large amounts of flavonoids and lignans on PR and AR in the T47-D system. Our preliminary results indicate that the metabolites produced after consumption of flaxseed (enterodiol and enterolactone) block PSA production after stimulation with norgestrel or DHT. Once determined which sera have such effects, metabolites may be identified using GC-mass spectrometry (Adlercreutz et
and comparisons may be made of which diets and/or metabolites are most beneficial in yielding such results. Dissecting out compounds from beverages found to have blocking activity (e.g. red wine and tea) will also be helpful to determine what components of these sources produce the effects seen. Resveratrol is a polyphenol which has received a lot of attention regarding its antitumorigenic and antioxidant activities (Soleas et al. 1997; Bonard 1997). This is one compound we intend to study in the future.

Other studies looking into the mechanism of hormone receptor blockade must be undertaken. Furthermore, we hope to investigate effects on the estrogen receptor as well as understand the bioavailability and biotransformation of plant-derived compounds in the human body. Ultimately, the mechanism by which plant foods exert their anti-tumor potential may be elucidated, step by step.

4.2.2 Summary

1. By measuring agonistic and antagonistic activity of synthetic antiprogestins on androgen and progesterone receptors, the PSA assay was found to be useful in determining such activity of the plant-derived compounds chosen.

2. The agonistic activity of plant-derived compounds was determined. The flavonoid apigenin was found to have significant agonistic activity on progesterone and androgen receptors, with a linear dose-response. At $10^{-5}$ M final concentration, 900 ng/L PSA were produced. At $10^{-6}$ M apigenin, 500 ng PSA was produced. This dropped precipitously; by $10^{-7}$ M no PSA was detected.
Naringenin was also found to have some agonistic activity.

3. The antiprogestogenic and antiandrogenic activity of plant-derived compounds was determined. Chlorophyll had the strongest effect (87 ± 7% for PR, 94 ± 3% for AR), followed by the antioxidant vitamins (59 ± 16% and 47 ± 11%, respectively). Methylxanthines from coffee, other vitamins, and fruit and vegetable compounds were also found to be blockers of these hormone receptors. Tea and red wine components were also found to be antagonists both in pure forms and as diluted beverage forms. Soy isoflavones were had no activity on progesterone or androgen receptors in our study.

The implications of this study are that plant-derived compounds could possibly be used in hormonal manipulation necessary in prevention and treatment of hormone-dependent cancers. Whether antiprogestins and agonists would be beneficial in breast cancer treatment, and antiandrogens in prostate cancer treatment, will have to be studied further.
5. REFERENCES


Adlercreutz H. Western diet and western diseases: some hormonal and biochemical mechanisms and associations. Scan J Clin Lab Invest 1990;50:3-23.


Beekman JM, Allan GF, Tsai S-Y, Tsai M-J, O'Malley BW. Transcriptional activation by the estrogen receptor requires a conformational change in the ligand binding domain. Mol Endocrinol 1993;7:1266-74.


Buiatti E, Palli D, Amadori D, Marubini E, Puntoni R, Avellini C, Bianchi S,


Cato ACB, Skroch P, Weinmann J, Butkeraitis P, Ponta H. DNA sequences outside the receptor-binding sites differently modulate the responsiveness of the mouse mammary tumor promoter to various steroid hormones. EMBO J 1988;7:1403-10.


Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240:889-95.


Franceschi S, Favero A, Decarli A, Negri E, La Vecchia C, Ferraroni M, Russo A,


Hunter DJ, Manson JE, Colditz GA, Stampfer MJ, Rosner B, Hennekens CH,


McKeown-Eyssen G, Holloway C, Jazmaji V, Bright-See E, Dion P, Bruce WR. A randomized clinical trial of vitamins C and E in the prevention of recurrence of


Mousavi Y, Adlercreutz H. Genistein is an effective stimulator of sex hormone-binding globulin production in hepatocarcinoma human liver cancer cells and suppresses proliferation of these cells in culture. Steroids 1993;58:301-4.


Sato F, Matsukawa Y, Matsumoto K, Nishino H, Sakai T. Apigenin induces


Thomas DB, Karagas MR. Cancer in first and second generation Americans.


