APIGENIN: CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC POTENTIAL FOR PROGESTIN-DEPENDENT BREAST CANCER

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And hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

I dedicate this dissertation to my parents, Mr Takawira K. Mafuvadze and Mrs Alice Mafuvadze, for all their love, guidance and support since the day I took my first walk to school. Mum and dad your choice to live a modest life while investing in me and my siblings' education has made a huge difference to our lives. Your unparalleled desire to see us succeed academically remains a source of inspiration.

In addition, I dedicate this dissertation to my grandfather, the late Dzingo Mafuvadze, a man who never received any formal education, but, a man who was full of amazing wisdom and knowledge. Sekuru (grandfather) you left a legacy and you continue to inspire us as we chase your long term vision for our family.

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TABLE OF CONTENTS

CHAPTER 5

CHAPTER 6: SUMMARY AND PERSPECTIVES ON FUTURE RESEARCH

LIST OF TABLES

LIST OF ABBREVIATIONS

The following list contains abbreviations that are used commonly throughout this
dissertation:

ACS: American Cancer Society

AICR: American Institute for Cancer Research

ANOVA: Analysis of variance

AP: Apigenin

AR: Androgen Receptor

BCA: Bicinchoninic acid

CK: cytokeratin

DCC: Dextran-coated charcoal

DMBA: 7, 12-Dimethylbenz (a) anthracene

DMSO: Dimethyl sulfoxide

DRSP: Drospirenone

E: Estrogen

EGF   Epidermal growth factor

EGFR: Epidermal growth factor receptor

ELISA: Enzyme linked immunosorbent assay

EPT: Estrogen-Progestin Therapy

ER: Estrogen Receptor

FGF: Fibroblast growth factor

GH: Growth hormone

GR: Glucocorticoid receptor

Her2:   Human epidermal growth factor receptor 2

HIF: Hypoxia-inducible factor

HRT: Hormone Replacement Therapy

IARC: International Agency for Research in Cancer

MHC: Major histocompactibility complex

MNU: N-methyl-N-nitrosourea

MPA: Medroxyprogesterone acetate

mRNA: Messenger Ribonucleic acid

MVD: Microvessel density

NET: Norethindrone

NF-KB: Nuclear factor-Kappa Beta

P: Progesterone

PR: Progesterone Receptor

RANKL: Receptor of activated Nuclear Kappa-beta Ligand

RT-PCR: Reverse transcriptase polymerase chain reaction

TNBC: Triple negative breast cancer

TNF: Tumor necrosis factor

TUNEL: Terminal deoxy-nucleotidyl transferase dUTP nick end labeling

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

WHI: Women's Health Initiative

APIGENIN: CHEMOPREVENTIVE AND CHEMOTHERAPEUTIC POTENTIAL FOR PROGESTIN-DEPENDENT BREAST CANCER

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ABSTRACT

Recently the use of hormone replacement therapy (HRT) consisting of a combination of estrogen and progestin has been linked to increased risk of breast cancer. Commonly available antiprogestins are associated with adverse side-effects and their use is therefore limited in humans. With this in mind we conducted studies aimed at identifying naturally-occurring and synthetic compounds with the ability to prevent and treat progestin-dependent breast disease. In the studies described herein we examined the capacity of the naturally-occurring flavonoid apigenin, which is commonly found in vegetables and fruits, to both prevent the onset, and treat already existing progestin-dependent breast tumors. Using ELISA we assessed the in vitro suppression of progestin-dependent induction of vascular endothelial growth factor (VEGF) by apigenin in human breast cancer cells. Increased production of VEGF, a potent angiogenic factor, is directly associated with growth of tumors. Apigenin suppressed VEGF induction by the endogenous hormone progesterone, medroxyprogesterone acetate (MPA), a commonly used synthetic progestin, as well as a variety of other synthetic progestins which are also of clinical importance. Moreover, using a semi-quantitative RT-PCR assay we found that apigenin inhibited progestin-induced VEGF mRNA synthesis.

In order to determine the therapeutic potential of apigenin we used a progestin-dependent breast cancer model previously described by Liang et al (2007). In this model, the growth of human breast cancer cells in immune-deficient mice is sustained by implanted progestin pellets (Liang et al., 2006; 2010). We assessed the effects of daily administration of apigenin and found that treatment with the flavonoid suppressed the growth of MPA-dependent BT-474 xenograft tumors. Immunohistochemical analysis showed that apigenin dramatically induced apoptosis, suppressed VEGF and Her-2/neu expression, and partially suppressed MPA-induced proliferation of xenograft tumors.

We examined the preventive effects of apigenin using a rat model in which progestins have been shown to accelerate the growth and incidence of dimethylbenz (a) anthracene (DMBA)-induced mammary tumors (Benakanakere et al., 2006) and administered apigenin either intraperitoneally or through dietary supplementation. Intraperitoneal administration of apigenin delayed significantly the occurrence of MPA-accelerated DMBA-induced mammary tumors, as well as decreasing their incidence, in a dose-dependent manner. Immunohistochemical analysis of mammary tissue suggests that apigenin suppressed VEGF. Interestingly, apigenin did not prevent MPA-induced hyperplasia of mammary gland epithelial cells, suggesting that it might specifically target mammary cancer cells at a specific stage of growth. Dietary apigenin, given at a dose of 0.1%, also significantly reduced the overall incidence of mammary tumors, though, perhaps paradoxically, increased tumor multiplicity in those rats that developed tumors.

This study shows that apigenin has chemo-preventive and chemo-therapeutic properties which might be exploited to treat progestin-accelerated mammary tumors, either alone or in combination with other agents. Further studies are required, both in

vivo and in vitro, to determine the mechanism by which apigenin mediates the loss of

tumor cell viability. Additional studies, using animal models, will be necessary to

establish the optimum dose and route of administration of apigenin and clinical trials will

be needed to determine the appropriate inhibitory doses for human use.

STATEMENT OF SIGNFICANCE AND OVERALL HYPOTHESIS

According to the American Cancer Society, breast cancer is the most frequently diagnosed non-skin cancer in American women, with approximately 200,000 new cases reported annually, leading to 40,000 deaths. Numerous studies have shown that postmenopausal women receiving combined hormone replacement therapy (HRT), containing both estrogens and progestins, have an increased risk of breast cancer compared with women taking estrogen alone (Ross et al., 2000; WHI, 2002). However, in spite of overwhelming clinical and epidemiological evidence showing the deleterious effects of combination HRT, an estimated six million women still undergo such therapy in the USA and are therefore more likely to develop breast disease than women consuming only estrogen. Studies, both *in vitro* and *in vivo*, provide evidence that progestins promote breast cancer initiation, progression and metastasis through various mechanisms. For example, progestins have been found to induce the synthesis and release of vascular endothelial growth factor (VEGF) in cultured human breast cancer cells and in DMBA-induced mammary tumors in Sprague Dawley rats (Hyder et al., 1998; Benakanakere et al., 2006; Liang et al., 2007). Consequently, by inducing angiogenesis and creating a microenvironment conducive to tumor growth, progestins accelerate the development of breast tumors.

In recent years a number of studies have sought to arrest progestin-dependent tumor growth by specifically inhibiting VEGF secretion and thereby suppressing angiogenesis. RU-486, a well characterized anti-progestin, has been shown to effectively inhibit progestin-induced VEGF in breast cancer cells (Horwitz, 1992; Hyder et al.,

1998). Unfortunately however, the anti-hormonal effects of RU-486 are not limited to its

binding to progesterone receptors (PR); RU-486 also antagonizes other steroid receptors

such as the glucocorticoid receptor, resulting in potential disruption of critical body

systems (Horwitz, 1992). Since the use of RU-486 is therefore limited in humans there is

an urgent need for alternative compounds that possess anti-progestin activity and can

therefore be used to prevent and treat progestin-dependent breast cancer without serious

side effects. Recently, a number of alternative compounds have been identified that

inhibit progestin-induced VEGF secretion from tumor cells (Hyder and Stancel, 2002;

Carroll et al., 2008). These compounds appear to inhibit signal transduction pathways

that influence PR and, while the mechanisms by which they exert their effects have not

yet been elucidated, it is known that, unlike RU-486, they do not block PR directly by

competing for ligand binding with natural or synthetic progestins. Thus such compounds

likely target alternate pathways and thereby prevent progestin-induced VEGF induction

and tumor growth by yet to be determined mechanisms.

Our laboratory is interested in naturally-occurring compounds that possess

potential anti-progestin and anticancer activity. In recent years, a number of such agents

have been identified, all of which significantly inhibit various types of cancer, while

exhibiting low toxicity (Gopalakrishnan and Kong, 2008). One compound gaining

considerable popularity in this regard is the low molecular weight flavonoid, apigenin, a

substance which is abundant in many fruits and vegetables and one that has been shown

to be anti-mutagenic, while being non-toxic to normal cells (Gupta et al., 2001; Patel et

al., 2007; Chen and Dou, 2008; Meeran and Katiyar, 2008). Acting via several

mechanisms, apigenin blocks the proliferation of a number of tumor cell lines including

those from the breast (Yin et al., 2001), colon (Wang et al., 2004) and prostate (Gupta et al., 2001), as well as inhibiting proliferating leukemia cells (Wang et al., 1999). However, so far the anti-progestin effects of apigenin have not been addressed and hence its possible effectiveness as an anti-proliferative agent for use against progestin-dependent and progestin-accelerated breast cancer remains unexplored. Preliminary studies performed in our laboratory in which a number of naturally-occurring compounds were screened for their anti-cancer properties revealed that apigenin might be used to inhibit progestin-induced VEGF secretion from breast cancer cells. The main objective of this dissertation was to test the hypothesis that *apigenin inhibits progestin-dependent breast cancer.* To test this hypothesis the following specific objectives were addressed;

Specific Aim 1: Determine the effects of apigenin on progestin-dependent VEGF induction in breast cancer cells,

Specific Aim 2: Determine the effects of apigenin on progression of progestin-dependent BT474 human breast xenograft tumors,

Specific Aim 3: Determine the effects of apigenin on progestin-dependent DMBA induced mammary tumors in Sprague Dawley rats.

CHAPTER 1

REVIEW OF LITERATURE

The focus of this dissertation is progestin-dependent breast cancer and the potential chemo-preventive and/or therapeutic effects of apigenin against this deadly disease. Initially I will review the general literature regarding breast cancer, and in particular describe the connection between combined hormone replacement therapy (HRT) and increased breast cancer incidence. Next, I will present an in-depth discussion of synthetic progestins, together with a description of the mechanisms known to be involved in progestin-induced breast cancer. I will also review the different animal models used to study breast cancer, highlighting the advantages, disadvantages, and relevance of each model to progestin-dependent human breast cancer. Because this study focuses on the effects of apigenin, a compound normally found in the diet, a general review of the use of dietary compounds for prevention and treatment of cancer will be given. Finally, I will focus on apigenin and the various mechanisms through which it is reported to exert anti-cancer activity against a variety of tumors.

1.1. Breast cancer epidemiology

Although the incidence of many types of cancer has declined in the last thirty years, the prevalence of breast cancer has been rising worldwide, possibly due to demographic and environmental factors as well as improvements in diagnosis (Dellapasqua et al., 2005). Age-adjusted incidence rates of breast cancer range from 75-100 per 100 000 women in North America, northern Europe, and Australia, to less than 20 per 100 000 in parts of Africa and Asia (IARC, 2007). In the USA and affluent European countries, breast cancer

is the most common cancer affecting women and is the second leading cause of cancer deaths in women (Jemal et al., 2011). It is estimated that 1 in every 8 woman born in the USA has a life-long risk of getting breast cancer (Altekruse et al., 2010). According to the American Cancer Society (ACS), in 2011, about 1.3 million women were expected to be diagnosed with breast cancer worldwide, accounting for nearly 25% of all cancers in women and about 465,000 deaths. The American Cancer Society (ACS) also estimated that approximately 207,090 new cases of invasive breast cancer and 54, 010 cases of carcinoma in situ breast cancer would be diagnosed in the US alone, with an estimated 39,840 deaths.

Historically, breast cancer has generally been a disease associated with high-income nations; however over the last three decades there has been an increasing trend of both breast cancer incidence and mortality in less affluent countries (Porter, 2009). Notable increases in breast cancer have also been observed among pre-menopausal women (Dellapasqua et al., 2005).

1.2.   Classification of breast cancer

Human breast tumors are very heterogeneous with approximately five recognized molecular subtypes (Stingl, 2011). However, molecular genetic profiling categorizes breast cancers into two main subtypes, namely luminal and basal, which comprise 85% and 3-15% of breast cancer cases respectively (Perou et al., 2000). Further, breast cancers are characterized based on expression of or lack of specific receptors. For example, luminal tumor subtypes often express estrogen receptors (ER), progesterone receptor (PR) and cytokeratin 18 (CK18) and are thus denoted as $ER^{+}PR^{+}CK18^{+}$. On the other

hand, the basal subtypes do not express ER, PR and CK18 but express cytokeratin 5 and are consequently denoted as ER$^-$PR$^-$CK18$^-$CK5$^+$ (Horwitz et al., 2008). Because of the presence of steroid receptors breast tumors can therefore be classified based on whether they are steroid hormone dependent or not. Consequently, the majority of human luminal breast cancers are steroid hormone-dependent and respond to a variety of treatment options, including endotherapy, that are based on suppression of steroid-dependent pathways. Triple negative breast cancers (TNBCs) are both ER and PR negative and lack another breast cancer associated protein known as HER2/neu (Lehmann et al., 2011). TNBCs are generally more aggressive and constitute 10%-20% of all breast cancers, frequently affecting young patients and being more prevalent in African-American women (Lehmann et al., 2011). Several subtypes of TNBCs have been identified.

According to Perou et al. (2000), other minor categories of breast cancer include Erb-B2 positive and "normal breast like". A number of additional classifications based on histological criteria and used by physicians and pathologists, are beyond the scope of this discussion and will not be addressed (see Perou et al., 2000; Lehmann et al., 2011). This dissertation will focus primarily on progestin-responsive breast cancer.

1.3.   Risk factors associated with breast cancer

Accumulating epidemiological data suggest that different types of breast cancers have different risk factor profiles which might vary depending on age and environment (Garcia-Closas et al., 2008). A summary of some of the factors that have been linked to increased breast cancer risk is presented in Table 1.1 below. Factors that have been linked to breast cancer risk include; physical inactivity, obesity, smoking, alcohol consumption,

diets high in saturated fat, use of hormone replacement therapy and oral contraceptive

pills, early onset of menarche and late menopause (Porter, 2009). In recent years, the rise

in breast cancer observed in Asia and other parts of the world has in part been attributed

to adoption of western lifestyles (Dellapasqua et al., 2005). Between 4 and 9 percent of

breast cancer cases are hereditary, usually being caused by inherited mutations in either

the BRCA1 or BRCA2 gene. Carriers of BRCA1 mutations are commonly diagnosed

Table 1.1. Factors associated with increased breast cancer risk in women (ACS, 2010).

Risk Factor	Explanation
Gender	Simply being a woman is the main risk factor for developing breast cancer. Although women have many more breast cells than men, the main reason why breast cancer incidence is higher in women than men is the constant exposure to the steroid hormones estrogen and progesterone.
Aging	The risk of developing breast cancer increases with age. About 1 out of 8 invasive breast cancers occur in women younger than 45, while about 2 out of 3 invasive breast cancers arise in women age 55 or older.
Genetic Risk factors	About 5-10% of breast cancer cases are linked to gene mutations. The most common gene changes are those of BRCA1 and BRCA2. Other mutated genes that have are associated with breast cancer include; p53, ATM, CHEK2, PTEN and CDH1.Women with these inherited mutations also have an increased risk for developing other cancers, particularly ovarian cancer.
Family history	Breast cancer risk is higher among women whose close blood relatives have this disease. Having a first-degree relative with breast cancer doubles a woman's risk.
Personal history of breast cancer	Having cancer in one breast results in a 3- to 4-fold increased risk of developing a new cancer in the other breast or in another part of the same breast.
Race and ethnicity	White women are slightly more likely to develop breast cancer than are African-American women. African-American women however tend to develop more aggressive tumors and are more likely to die from breast cancer
Dense breast tissue	Women with denser breast tissue (as seen on a mammogram) have more glandular and less fatty tissue, and have a higher risk of breast cancer.
Certain benign breast conditions	Women diagnosed with certain benign breast conditions such as fibroadenoma, sclerosing adenosis, papillomatosis, radial scar, lobular carcinoma in situ, may have an increased risk of breast cancer.
Menstrual periods	Women who have had more menstrual cycles because they started menstruating at an early age (before age 12) and/or went through menopause at a later age (after age 55) have a slightly higher risk of breast cancer. This may be due to higher lifetime exposure to estrogen and progesterone.
Earlier Radiation exposure	Women who have had chest area radiation treatment earlier in life have a greatly increased risk of developing breast cancer.
Pregnancy	Women who have their first pregnancy after 30 have a slightly higher breast cancer risk. In addition, having many pregnancies and becoming pregnant at a young age reduces breast cancer risk.
Oral contraceptives	Women using oral contraceptives have a slightly greater risk of breast cancer than women who have never used them.
Hormone replacement therapy	The use of combined hormone replacement therapy after menopause increases the risk of developing breast cancer. Metastatic breast cancer is more likely in these subjects, as is breast cancer-related mortality.
Obesity	Obesity has been found to increase breast cancer risk, especially for women after menopause, possibly due to increased estrogen levels.
Alcohol	Consumption of alcohol has been linked to an increased risk of developing breast cancer.

with triple negative breast cancer (Garcia-Closas et al., 2008). Generally, the risk of breast cancer in women increases with age, with most cases diagnosed between 50 and 75 (ACS, 2010). The increased availability of screening facilities in developed countries however has made detection of breast cancer easier and medical intervention more likely to be successful.

1.4.   Breast cancer and hormone replacement therapy (HRT)

There is overwhelming evidence that HRT and oral contraception lead to an increased risk of breast cancer. Women undergo HRT to alleviate the effects of menopause symptoms that include; hot flashes, osteoporosis, insomnia, mood swings, dementia and decreased libido (Narod, 2011). Those HRT regimens which include only estrogen cause endometrial proliferation and increase the risk of uterine cancer; consequently progestins are often co-administered to counter the effects of estrogen on the uterus (Wiehle et al., 2011). The effects of progestins in HRT have come under scrutiny following publication of the Women's Health Initiative (WHI), which showed that combination HRT containing both estrogen and progestin increased the incidence of breast cancer compared with estrogen alone (Chleboskwi et al., 2003), suggesting that progestins might be responsible for cancer development. Tumors develop shortly after the onset of combination HRT, lending support to the notion that progestins promote the development of pre-existing, clinically latent hormone-dependent cancers (Horwitz and Sartorius, 2008). Recent epidemiological studies further support this notion by demonstrating a direct correlation between reduced HRT and declines in ductal carcinoma in situ as well as invasive breast cancer in humans (Clarke et al., 2006; Ereman et al., 2010). For

example, in the USA, the use of systemic HRT fell from 16.3 million in 2001 to 6.1 million in 2009 (Tsai et al., 2011) and a corresponding 13.2% decline in invasive breast cancer was observed between 2001 and 2004 (Hauser et al., 2009).

1.4.1. Progestins

Progestins are synthetic analogues of progesterone that have been developed to overcome the problems of poor oral absorption of progesterone and its rapid first-pass metabolism in the liver (Garefalakis and Hickey, 2008). Naturally-occurring progesterone and synthetic progestins are often collectively referred to as progestogens. Figure 1 below gives a summary of the different classes of synthetic progestins.

As shown in Figure 1.1, progestins differ not only in their structure but in a variety of ways including; potency, clinical effects and endocrine properties (Simons, 2007). Table 1.2 below highlights some of the variations in biological activity of commonly used progestins. As will be discussed in subsequent sections, these differential effects of progestins make it difficult to extrapolate data from one progestin to another.

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                          ┌─────────────────┐
                          │  Progestogens   │
                          └─────────────────┘
                  ┌─────────────────┴─────────────────┐
        ┌───────────────────┐              ┌────────────────────┐
        │ Progesterone (P4) │              │ Synthetic Progestins│
        └───────────────────┘              └────────────────────┘
                        ┌──────────────────────┼──────────────────────┐
        ┌───────────────────┐   ┌──────────────────────┐   ┌──────────────────────┐
        │ 19-nortestosterone│   │ 17α-hydroxyprogesterone│  │ 19-norprogesterone   │
        └───────────────────┘   └──────────────────────┘   └──────────────────────┘
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Estranes

Norethindrone
Norethynodrel
Lynestrenol
Ethynodiol diacetate

Gonanes

Levonorgestrel
Desogestrel
Norgestrel
Gestodene

Medroxyprogesterone
Megestrol acetate
Chlormadinone acetate
Cyproterone acetate

Drosperinone
Trimegestone
Promegestone
Demegestone

Figure 1.1. Classification of synthetic Progestins (modified from Simons, 2007)

Table 1.2.   A comparison of biological activities of progesterone versus commonly used synthetic progestins.

Progestogen		P	E	Anti-E	A	Anti-A	Gluco	Anti-M
Natural	Progesterone	+	-	+	-	±	+	+
Spironolactone	Drospirenone	+	-	+	-	+	-	+
17α-hydroxyprogesterone	MPA	+	-	+	±	-	+	-
	Cyproterone acetate	+	-	+	-	+	+	-
19-nortestosterone	Norethindrone	+	+	+	+	-	-	-
	Levonorgestrel	+	-	+	+	-	-	-

Modified from Garefalakis and Hickey (2008)
Abbreviations: progestagenic (P), estrogenic (E), anti-estrogenic (Anti-E), androgenic (A), anti-androgenic (Anti-A), glucocorticoid (Gluco), anti-mineralocorticoid (Anti-M).

1.4.2. Progesterone Receptor (PR)

Progestins impart their biological action via two major progesterone receptor (PR) isoforms, PRA and PRB. PRA is a truncated form of PRB which, while missing N-terminal amino acids, is otherwise identical to PRB in the DNA- and steroid binding domains (DeMarzo et al., 1991). In human endometrial carcinomas and breast cancer cell lines, the two receptor isoforms are expressed in approximately equimolar amounts (Horwitz, 1992). However, the two PR isoforms have been shown to differ in their ability to activate gene transcription, their interactions with cytoplasmic signaling cascades, and in their requirements for activation by ligand (Kariagina et al., 2010). In mice, PRA has been shown to be involved in mammary gland side branching, while PRB is critical for lobuloalveolar formation during pregnancy (Shyamala et al., 1998; Mulac-Jericevic et al., 2000). Studies in human breast cancer cells have demonstrated that PRA activates transcription of several genes associated with cell transformation and cell motility and is associated with more aggressive tumors (McGowan and Clarke, 1999). On the other hand, PRB seems to mediate cross talk between progesterone and the epidermal growth factor receptor (EGFR) pathway which subsequently leads to sustained phosphorylation of ERK (Faivre and Lange, 2007) and activates several intracellular signaling pathways.

Both PRA and PRB contain ligand- and DNA binding domains (figure 1.3) and, upon binding the progestin ligand, form dimers which recognize palindromic target DNA (DeMarzo et al., 1991). In the absence of the ligand (progesterone or synthetic progestins) PRs are associated with heat shock protein 90 (hsp90) and other proteins, including hsp70 and form an inactivated complex (DeMarzo et al., 1991). Activation of PR therefore involves rapid dissociation of the multiple accessory proteins, and rapid

hormone-dependent phosphorylation of the receptor. According to Horwitz (1992), PR

antagonists can therefore oppose PR activity by blocking any of the multiple stages,

including interfering with the entry of agonists into target cells, binding to receptor,

dimerization of receptors and blocking the transcriptional signal. In this study we

examined the ability of apigenin to suppress the growth of progestin-dependent breast

cancer using *in vitro* and *in vivo* models. In the models that we used, progestin effects are

blocked by the well-characterized PR antagonist RU-486, suggesting that PR is involved

(Hyder et al., 2001).
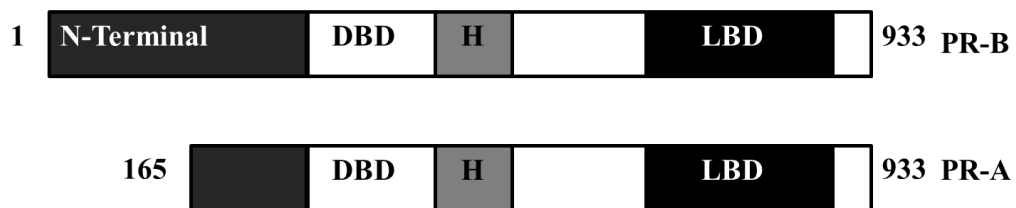
Figure 1.2. Organizational structure of progesterone receptors (PR) A and B showing main functional domains: N-terminal regulating domain, DNA-binding domain (DBD), hinge region (H), ligand-binding domain (LBD).

1.4.3. The influence of progestins on breast cancer incidence

While the role of endogenous progesterone and synthetic progestins in the development and progression of breast cancer has, until quite recently, received little attention, the conventional view that progestins are terminally differentiative in the endometrium and that consequently, by extrapolation, they exert similar effects within breast tissue (Lanari and Molinolo, 2002), must now be questioned. The aforementioned notion that estrogens are primarily to blame for inducing breast cancer, with progestins being anti-proliferative and therefore protective against breast cancer would seem to be flawed, given recent experimental and clinical evidence suggesting that progestins are involved in the induction, progression and maintenance of mammary tumors (Goepfert et al., 2000). Data from the Women Health Initiative study (2002) showed a higher risk of breast cancer in post-menopausal women taking combined estrogen-progestin therapy (EPT), compared with those taking estrogen alone or placebo (Chleboskwi et al., 2003). This suggests that progesterone might be responsible for the effects of EPT on cancer development. In addition to increasing the incidence of breast cancer, EPT also increased benign breast proliferative lesions, further supporting the hypothesis that the combination of estradiol and progestins may promote mammary cancer growth (Wiehle et al., 2011). There is however some evidence which suggests that in some cases and at certain dosages progestins may decrease the risk of breast cancer (Hankinson and Eliassen., 2010). For example, initial studies showed progestins to be anti-proliferative in normal human breast cells (Gompel et al., 1986). More recently, Rajkumar et al (2007) reported that exogenous estrogen and progesterone reduced the incidence of DMBA-induced mammary tumors in genetically engineered mice over-expressing Her2/neu. It is therefore not surprising that

the role played by progestins in breast cancer development has been controversial. Depending on the experimental model system, cell context, specific progestin, dosage and duration of treatment, progestins can elicit either proliferative or anti-proliferative effects on breast epithelial cell growth (Lange et al., 1999). However, cumulative evidence from recent in vivo and in vitro studies support the notion that progestins do in fact stimulate the proliferation of both normal and neoplastic breast cells in a variety of different species (Soderqvist et al., 1997; Raafat et al., 2001; De Lignieres, 2002 and Liang et al., 2007).

Epidemiologic studies show a correlation between HRT containing progestins and an elevated risk of breast cancer in post-menopausal women, while experimental studies conducted in a number of laboratories support this notion by providing mechanistic evidence. Several cross talk mechanisms involving the conventional nuclear PR pathway with different growth factors, neurotransmitters and polypeptide hormones have been described (Lanari and Molinolo, 2002). Many recent studies show that progestins induce growth factors such as vascular endothelial growth factor (VEGF), an essential component of the angiogenesis which is vital for tumor growth (Hyder et al., 1998, 2001; Benakanakere et al., 2010). In addition progestins also regulate several intracellular factors that increase or decrease expression of several proliferation genes (Lange et al., 1999). Overall, progestins appear to create a microenvironment that is conducive to tumor development and progression. Recently, Horwitz and Sartorius (2008) reported that medroxyprogesterone acetate; a progestin commonly used in HRT, increased the stem-like ER-, PR-, CK5+ subpopulation from 2% to more than 20%, suggesting that progestins may play a role in activating and transforming dormant breast cancer stem

cells into intermediate subpopulations with the capacity to differentiate into breast cancer cells.

Schramek et al (2010) and Gonzalez-Suarez et al (2010) reported that progestin-induced RANKL may play a significant role in the development of mammary cancer in mouse. This idea is further supported by the fact that RANKL is only expressed in PRA expressing cells. Kariagina et al (2010) reported that estrogen and progesterone work together in cells expressing ERα, PRA and PRB to induce robust proliferation of hormone-dependent mammary cancers through induction of amphiregulin. Progestins also appear to prime precancerous cells which in turn respond more vigorously to growth factors (Lange et al., 1999). For example, after being primed with MPA, T47D breast cancer cells become highly sensitive to the proliferative effects of epidermal growth factor (EGF) (Groshong et al., 1997). On the other hand, it has been suggested that the cancer risk related to the use of HRT containing estrogen and synthetic androgenic progestins (19-nortestosterone derivatives) could actually be due to non-progesterone-like effects that enhance the proliferative effects of estrogen in sensitive cancer cells (Ferretti et al., 2007). Synthetic progestins have been shown to exert a variety of additional effects on other nuclear receptors such as the glucocorticoid receptor (GR) and androgen receptor (AR) (Koubovec et al., 2005), supporting the notion that progestins could act through these targets. There is therefore overwhelming evidence that progesterone and synthetic progestins play a critical role in the development and progression of breast cancer via a number of different mechanisms.

1.4.4. Targeting progestin-induced angiogenesis for chemoprevention

The role of angiogenesis (the process of new blood vessel formation) in local tumor growth and metastasis to distant sites is now well established as a key component of malignant breast cancers (refer to a review by Schneider and Miller, 2005). The pioneering studies of Folkman generated much of the initial interest in angiogenesis, demonstrating that neovascularization precedes the transformation of hyperplasia to malignancy and that without vascularization tumors cannot grow beyond 1-2mm^3 in size (Folkman, 1990). In recent studies, it has emerged that transfection of tumor cells with angiogenic stimulatory peptides increases tumor growth, invasiveness and metastasis (Miller and Sledge, 2003). Cumulative evidence shows that the initiation or termination of angiogenesis is controlled by a balance between positive and negative regulators. Several angiogenic factors have been described including vascular endothelial factors (VEGF), fibroblast growth factor (FGF) and angiopoietins (Zhai et al., 1999). Preclinical tumor models show that tumors express high levels of pro-angiogenic factors such as VEGF (Hyder et al., 2001), which is over-expressed in many human breast tumors. Indeed, levels of VEGF expression correlate with a number of important clinical features of the disease (Hyder et al., 1998). Furthermore, evidence from cell culture systems also shows that medium conditioned by malignant cells provides a more stable environment for tumor and vessel growth than medium conditioned by nonmalignant cells (Schneider and Miller, 2005). Hyder et al. (1998) first reported the induction of VEGF by progestins in human T47-D breast cancer cells. Further studies have since shown that various pharmacological and endogenous progestins induce VEGF in human breast cancer cells (Hyder et al., 2001), as well as in animal (rat) models (Benakanakere et al., 2010). Using

a murine tumor model Inoue et al (2002) showed that VEGF likely plays an essential and non-replaceable role in tumor angiogenesis, particularly during the early stages of carcinogenesis.

Data from the Women's Health Initiative (2002) and clinical trials (Ross et al., 2000; Chen et al., 2004) suggest that post-menopausal women receiving estrogen plus progestin therapy are at a greater risk for breast cancer than those receiving estrogen alone. Inhibiting progestin-induced VEGF production is therefore a realistic target for preventing breast tumor development in postmenopausal women undergoing HRT. A number of chemotherapeutic agents used routinely to treat breast cancer have known antiangiogenic activity (Sweeney and Sledge, 1999), though generally maximal antiangiogenic activity requires prolonged exposure to low concentrations of the drug (Schneider and Miller, 2005). There are challenges associated with anti-VEGF targeted antiangiogenic therapy such as possible selection for hypoxia resistant tumor cells. For example, Cao (2004) suggested that anti-VEGF reagents might lead to selection of cells producing other angiogenic factors such as FGF-2. Furthermore, single angiogenic factor antagonists may not be particularly effective since tumor cells may switch on angiogenic stimulators and compensate for the inhibited factor by producing an excess of other factors. Consequently inhibitors of angiogenesis which target common angiogenic pathways may be more efficient at disrupting tumor growth than mono-factor targeting agents. Boehm et al (1997) demonstrated that anti-angiogenic drugs such as endostatin, which selectively target vascular endothelial cells, are less likely to induce drug resistance. Recently our laboratory has examined the capacity of dietary compounds to inhibit progestin-induced tumor angiogenesis and we previously reported that curcumin

arrests progestin-dependent tumor growth via mechanisms that involve the suppression of VEGF production (Carroll et al., 2008; 2010). In this study, we investigated the ability of apigenin to inhibit progestin-dependent angiogenesis and tumor growth.

1.5.1. Models used to study breast cancer

Breast cancers are not genetically and phenotypically uniform (Wagner, 2004). Consequently it is not feasible to have a single model that can mimic all aspects of human breast cancer (Gusterson et al., 1999) and so a variety of different *in vitro* and *in vivo* models of the disease has been developed and used in different studies.

In vitro culture of established breast cancer cell lines is probably the most widely used model for preclinical evaluation of breast cancer (Kim et al., 2004). Different human and animal-derived mammary tumor cell lines are used to study the varying aspects of breast cancer. However, *in vitro* models are limited in their applications to the disease situation since they are not three-dimensional; furthermore in cell culture there is an absence of stromal cells which play a significant role in both tumorigenesis and tumor progression (Kim et al., 2004). Such limitations make *in vitro* models less representative of real cancers, and therefore extrapolation of results acquired using cultured cell models to real human cancers is difficult. Animal models in which stroma and structure are present provide better results but in order to be representative of human breast cancer, they would still require genetic and other biomarker abnormalities similar to their human counterparts (Kim et al., 2004).

Rodent models of human breast cancer are commonly used and can be categorized into three main groups: xenograft models, induced models that use chemicals, viruses or ionizing radiation for cancer initiation and genetically engineered

models such as transgenics and knockouts (Wagner, 2004). Models are also generated through a combination of techniques, for example knockout mice treated with a carcinogen (Fernandez-Valdivia et al., 2005). Though the mouse model is widely used, differences in tumor histopathology between mice and women limit the applicability of murine studies, particularly as this suggests different target cell populations for the initiating event (Gusterson et al., 1999; Cardiff, 2001). For example, while more than one-half of human breast cancers are hormone responsive at diagnosis, the vast majority of mouse tumors are hormone-independent (Nandi et al., 1995). The metastatic patterns between humans and mice mammary tumors are also different. While breast cancer in humans reportedly spreads lymphatically, predominantly to bone, brain, adrenal gland, liver and lung, in mice mammary cancers metastasize exclusively to the lung via the haematogeneous route (Kim et al., 2004). Despite these limitations mouse models are still valuable, in particular for defining the molecular pathways involved in mammary epithelial cell transformation and cancer progression and for testing various therapeutic and preventive approaches (Gusterson et al., 1999).

Mammary glands from humans and rats possess a similar ductal-lobular organization. Furthermore, induced mammary cancers in rats are predominantly hormone-dependent, as are most human breast cancers (Kariagina et al., 2010). Moreover the developmental pattern of breast PR isoform expression and co-localization, as well as cell-type specific expression of PR isoforms are similar in human and rat mammary gland (Taylor et al, 2009). In carcinogen-induced rat mammary tumors though, there is a high incidence of ras mutations, which are very rare in human breast cancers (Gusterson et al., 1999).

Canine and feline models have also been used to study breast cancer. As in humans, canine mammary tumors arise spontaneously and constitute almost half of canine cancers (Thuroczy et al., 2007). As will be described in more detail below, canine mammary tumors tend to be hormone-dependent and as such are appropriate models for studying steroid hormone-dependent breast cancer (Queiroga et al., 2005). On the other hand, spontaneously occurring feline mammary tumors tend to be highly aggressive, grow rapidly and metastasize to regional lymph nodes and lungs (MacEwen, 1990). However, most feline mammary tumors are estrogen and progesterone receptor negative but overexpress Her2 (De Maria et al., 2005). The feline mammary cancer model has therefore been identified as a good model for poor prognosis human breast cancer.

Non-human primates such as monkeys make attractive models for the study of human breast cancer because of their genetic, anatomical and physiological similarities to humans. A number of studies have been carried out using monkeys (Tavassoli et al., 1988) which will be described in detail below. The major limitation to the use of monkeys as models for human breast cancer, however, is the low rate at which spontaneous tumors occur, suggesting the effect of genetic differences between humans and monkeys, or the involvement of unidentified environmental factors.

1.5.2. Animal models used to study progestin-dependent breast cancer

As stated previously, human breast cancers are genetically and phenotypically different (Wagner, 2004). Variations in morphology and dependence on endogenous growth factors, differences in gene specific mutations and, most significantly, clinical outcome (Perou et al., 2000) make it infeasible to use a single model to represent all aspects of

human breast cancer (Gusterson et al., 1999). As a result, several models have been used to study the role of steroid hormones in breast carcinogenesis. In this section animal models used for studying progestin-dependent breast cancer will be discussed.

Due to their size and ease of maintenance, rodents are usually employed for studying steroid-dependent breast tumors. The majority of genetically modified murine breast cancer models, as well as most spontaneous, chemically or virally-induced mammary tumors in mice, however, do not express ER and PR and are therefore not appropriate for the study of hormone-dependent human breast cancer (Kordon, 2008). However, while studying the inhibitory effects of medroxyprogesterone acetate (MPA) on artificially induced fibrosarcomas, Lanari et al (1986) observed that MPA induced and increased the incidence of mammary tumors in BALB/c mice. In follow-up studies, the same researchers demonstrated that even naturally-occurring progesterone, when administered at high dose, also induced mammary carcinomas in virgin female BALB/c mice. All ductal mammary tumors arising in this model are ER and PR positive. In this model multiple mammary carcinomas are induced at an average incidence level of 80% (Lanari et al., 2009), making it a good means by which to evaluate the effects of progestins. The major weakness inherent in this rodent model however is the relatively long latency period for tumor development to occur, typically one year. Furthermore, although a few cases of lymph node and lung metastasis were observed, unlike in human breast cancer where tumors arise frequently at sites distant from the breast, metastasis rarely occurs in this model (Lanari et al, 1986).

In addition to the intrinsic evolutionary distance between mice and humans, there are also notable differences in the ductal-lobular organization between mouse mammary

gland and human breast (Uva et al., 2009). The ductal-lobular organization of rat

mammary gland, on the other hand, is similar to that of the human breast (Russo et al.,

1990), in addition to exhibiting similar patterns of expression and co-localization of PRA

and PRB isoforms (Taylor et al., 2009), making it a more appropriate model the study of

human progestin-dependent breast cancer. Moreover, the majority of carcinogen-induced

mammary tumors in rats are reportedly ER and PR positive and hormone-dependent.

They can therefore provide insights into progestin-dependent mechanisms relevant to

human breast cancer (Kariagina et al., 2010). Following studies by Lanari et al (1986)

showing that medroxyprogesterone acetate (MPA) accelerated the growth of DMBA-

induced mammary carcinomas, a number of subsequent studies using the DMBA model

have been carried out to examine the effects of other progestins on tumor development.

We and others have shown that oral administration of a single dose of 20mg DMBA to

45-50 day old Sprague-Dawley rats leads to the development of mammary tumors whose

growth is accelerated by high levels of exogenous progestins administered through

implantation of long term-release pellets (Lanari et al., 1986, Aldaz et al., 1996;

Benakanakere et al., 2006). Several researchers have since adopted this model to evaluate

the capacity of compounds with anti-progestin activity to prevent or treat progestin-

dependent mammary tumors. Commonly used anti-progestins such as RU486 have been

shown to inhibit the growth and progression of progestin-accelerated DMBA-induced

mammary tumors (Horwitz, 1992). Our laboratory uses this model to determine the

ability of various naturally-occurring and synthetic compounds to prevent the

development of progestin-dependent breast tumors and recently showed that curcumin

and apigenin, compounds normally found in the diet, inhibited tumor growth in the

DMBA model (Carroll et al., 2010) (Mafuvadze et al., 2011). The main disadvantage of the DMBA rat model is that the H-ras mutations induced by DMBA, which are responsible for tumor initiation, are very rare in human breast cancer (Chan et al., 2005) which makes some researchers question the relevance of the model to the human situation. However, Finney and Bishop (1993) argued that since more than 90% of rat genes are known to have human homologs, it is possible that H-ras mimics the effects of a more common, as yet unidentified mutated gene in human breast cancer. It is not surprising that Clark et al (1996) observed that aberrant function or over-expression of a ras-related protein, TC21, may be important in human breast cancer development. The effects of progestins on expression of TC21 and other ras-related proteins in human breast cancer remain to be fully investigated.

Another widely used chemically-induced mammary cancer rat model is the N-methyl-N-nitrosourea (MNU)-induced tumor model. Earliest evidence linking estrogen and progesterone to MNU-induced carcinogenesis came from studies performed by Gullino et al (1975) which showed that ovariectomy before or immediately after administration of MNU significantly decreased tumor incidence. Similar to DMBA-induced mammary tumors, MNU-induced tumors have H-ras mutations at codon 12 (Choi et al., 2009), abundantly express ER and PR receptors and are hormone-dependent (Arafah et al., 1980). Using microarray analysis, Chan et al (2003) observed that not only do MNU-induced mammary tumors show significant phenotypic similarities but that they also share molecular signatures with low-to-intermediate grade, ER and PR positive human breast cancer. While MNU-induced mammary tumors exhibit some differences compared with human breast cancer, such as lack of micro calcifications, tumors arising

following MNU induction metastasize to some extent to lymph node, liver, lung, kidney

and spleen (Thompson et al., 2002). This is in contrast to DMBA-induced tumors, which

rarely metastasize. Because of its similarity to human breast cancer, it is not surprising

that the MNU-induced mammary cancer model has been used extensively to study the

effects of progestins on tumor development, though many of the disadvantages inherent

in the DMBA-induced model also apply to this model.

While useful xenotransplantation models in which ER and PR positive human

cancer cell lines are orthotopically inoculated into immune-deficient mice have been

utilized to test various anti-cancer therapies (Bao et al., 1994), it has remained difficult to

establish hormone-dependent tumors without co-inoculation with Matrigel. Van Slooten

et al (1995) were the first to describe a model in which BT-474, a hormone-responsive

cell line, co-inoculated with Matrigel, grew in immune-deficient mice supplemented with

estrogen pellets. However, since Matrigel already contains several growth factors, it

remained difficult to demonstrate progestin-dependent effects with xenograft models. We

recently developed a mouse model for studying the effects of progestins on the growth of

breast cancer xenograft tumors (Liang et al, 2007). In this model, immune-deficient mice

are sequentially implanted with estradiol prior to inoculation with cells (without

incorporation of Matrigel gel) followed by implantation with progestin pellets. We found

that estradiol supports a short burst of tumor cell growth, followed by regression and

tumor cell senescence and/or apoptosis. Supplementation with a progestin rescues tumor

growth and tumors progress further. As shown in Figure 1.3 below, when progestin

pellets are removed, tumor growth is suppressed (Liang et al., 2007), providing further

evidence that this growth is progestin-driven. This model therefore offers a practical

system for evaluating the role of progestins in breast cancer development, as well as for testing different compounds with potential anti-progestin activity. As with most xenograft models, the major shortcoming of this approach is that human cells grow in a mouse environment in which mouse stromal cells interact with cells of human origin. A number of recent studies have highlighted the importance of the interaction between epithelial and stromal cells in tumorigenesis (Potter et al., 2011); however it remains unclear just how the interaction between mouse stromal cells and human breast epithelial cells in this xenograft model influences progestin-dependent effects. Recently developed models use human fibroblasts transplanted together with neoplastic cells, creating a humanized micro- environment (Lanari et al., 2009). Unless specifically manipulated, most xenograft tumors in immunosuppressed mice do not metastasize, though in our model (Liang et al., 2010), in which we use human specific MHC-1 antibodies to identify human tumor cells, we showed that norethindrone significantly increased metastasis of tumor cells to regional inguinal lymph nodes. This model, in addition to being useful for evaluating progestin-dependent tumor growth, can also be used to assess progestin-induced metastasis.
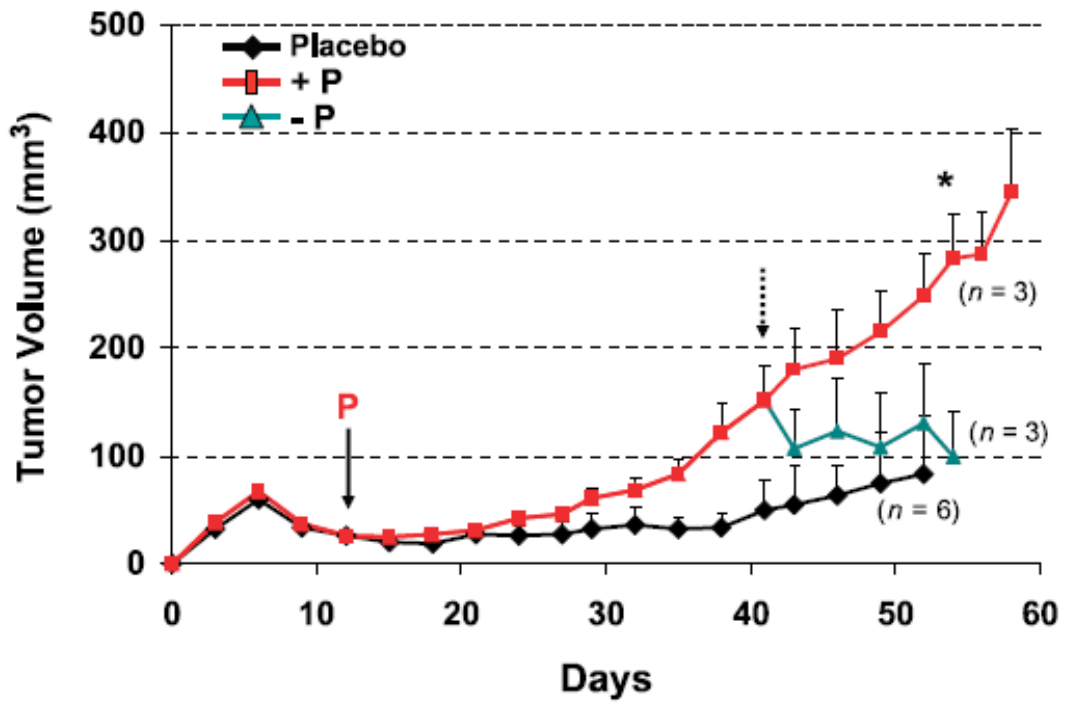
Figure 1.3. **Progesterone promotes growth of human xenograft tumors**. Implanted progesterone pellets (solid arrow) resuscitate and promote BT474 growth following a regression phase. Removal of the progesterone pellets after the initial growth phase (broken arrow) results in tumor regression (Adapted from Liang et al., 2007).

In recent years, companion animals such as dogs and cats, which frequently develop spontaneous tumors, have emerged as suitable models for human cancer, primarily because both animal populations and tumors are genetically heterogeneous (De Maria et al., 2005). Notable clinical and molecular similarities have been described between canine mammary tumors and human breast cancer (Queiroga et al., 2011). Canine mammary tumors are particularly interesting because they occur spontaneously and the majority are ER and PR positive (Queiroga et al., 2005), unlike feline mammary tumors which tend to be ER and PR negative. Furthermore, with the emergence of similarities between the human and canine genomes in recent years (Uva et al., 2009), the dog has emerged as an attractive alternative model for breast cancer. As in human breast cancer, most canine mammary tumors metastasize (Millanta et al., 2005), making the canine model an appropriate and valuable model for studying progestin-dependent breast cancer and metastasis.

Regular administration of exogenous progestins to felines reportedly increases the risk of mammary carcinoma development (Misdorp et al., 1991). Jacobs et al (2010) even reported the development of mammary adenocarcinomas in male cats exposed to MPA. However, since most feline tumors do not express ER and PR, felines are less useful than other animal models for the study of progestin-dependent effects. The felid model can still, however, yield information on transitional mechanisms involved in loss of steroid hormone receptors during tumor progression, which is important for understanding human triple negative breast cancers.

Non-human primates are attractive models for human breast cancer because they share >95% genetic sequence homology with humans, including key genes involved in

breast cancer susceptibility (Archer, 2004). The female *cynomolgus* monkey is one such primate which, in recent years has been used extensively to model human breast cancer. These creatures possess mammary glands that are anatomically similar to humans and also have a 28-day menstrual cycle that is similar to women (Gilardi et al., 1997). A further characteristic feature that makes the cynomolgus monkey an attractive model for progestin-dependent breast cancer is that females display a distinctive menopausal state, which is, however, shorter than humans. A number of studies have examined the effects of combined estrogen and progestin HRT in surgically-created postmenopausal *cynomolgus* monkeys (Cline et al., 1996; Wood et al., 2007). In this model animals are ovariectomized and maintained for a 6-month baseline period to allow for physiologic adjustment to the postmenopausal state prior to progestin administration. A study by Wood et al (2007) shows that synthetic progestins such as MPA, given in conjunction with 17β-estradiol, increased breast epithelial proliferation in female *cynomolgus* monkeys, while little proliferation was seen following the administration of micronized progesterone. This further highlights the difference between naturally-occurring progesterone and synthetic progestins. Despite many similarities with humans, the low rate at which mammary tumors spontaneously occur in *cynomolgus* monkeys and other non-human primates seriously limits their usefulness as models for studying the human disease. The reason why spontaneously occurring breast tumors are rare in *cynomolgus* monkeys is unknown, but could be due to unique genetic factors or as yet unidentified environmental causes (Archer, 2004). It is possible that progestins fail to increase the incidence of breast tumors in this model due to the limited time-frame of the study

compared with much longer periods during which humans are exposed to progestins (Archer, 2004).

1.5.3. Challenges in studying effects of progestins on breast cancer

It is generally accepted that progesterone receptor (PR) is an estrogen-regulated gene whose synthesis in both normal cells and tumor cells requires estrogen and estrogen receptor (ER) (Engman et al., 2008). Consequently it is difficult to study the effects of progestins on progression of breast cancer independent of estrogen. Given that it is now widely accepted that estrogen alone can induce and support the growth of preneoplastic and malignant breast cells (IARC working group,1999; Roy and Singh, 2004), one of the major challenges when studying progestin-dependent effects is to distinguish the effects of progesterone/progestin from those of estrogen (Lange, 2008). Previous studies using PR knockout mice showed that PR is important for DMBA carcinogenicity, indicating a sensitivity that does not seem to require ER (Lydon et al., 1999) in some types of mammary tumor. Combination estrogen/progestin HRT results in a higher incidence of breast cancer compared to treatment with estrogen alone, supporting the notion that progestins are responsible for the harmful effects of this type of therapy (Ross et al., 2000; Chlebowski et al., 2003). It is also possible however, that estrogens and progestins have distinct effects that complement each other, with progestins activating and transforming dormant breast cancer stem cells into an intermediate subpopulation which is then further acted upon and driven to develop into breast tumors by the mitogenic effects of estrogen (Horwitz and Sartorius, 2008). Using the previously described xenograft nude mouse model Liang et al (2007) showed that if progestin pellets were

removed after the initial burst of tumor growth, cancers began to regress even though long-term release estrogen pellets were kept in place (refer to Fig 2). This further suggests that progestins are chiefly responsible for promoting tumor growth though administration of exogenous estradiol in the absence of progestin promotes the growth of several ER positive cancer cells (Pang and Faber, 2001). It is therefore difficult to determine whether the role of estrogen is restricted to inducing PR or whether estrogen exerts synergistic effects with synthetic progestins. It is possible that there are variations in the effects of estrogen and progestins when they are used in combination or alone, or depending on the model employed, doses used or whether progestin is given pre- or post-carcinogen administration (Horwitz, 1992). However, because post-menopausal women still possess estrogen, albeit at low levels produced mainly in adipose tissue and adrenal gland, models that combine the effects of estrogen and progestins are appropriate for studying the effects of progestins used in combination HRT on breast cancer development.

A further confounding factor when studying the effects of progestins is their variable biological actions due to differences in structure, stability, pharmacokinetics and steroid-receptor binding specificity (Schindler et al., 2008), which potentially lead to different proliferative effects in different models. For example, while Neubauer et al (2011) reported that progesterone appears to have no effect on MCF-7 and MCF-7/PGRMC1-3HA cells, MPA, norethindrone (NET) and drospirenone (DRSP) stimulate proliferation of the same cells. Similarly, in the DMBA-induced mammary tumor rat model, Benakanakere et al (2010) reported differential effects of synthetic progestins, with MPA and norethindrone stimulating tumor development, while norgesterol was

protective and suppressed tumor growth. Synthetic progestins also bind to other nuclear receptors such as glucocorticoid receptor (GR) and androgen receptor (AR) (Koubovec et al., 2005); it is therefore plausible that progestins exert biological effects via these targets. For example, in several mutant mouse models GR has been shown to stimulate proliferative activity in the mammary gland (Otto et al., 2010). Given the variation in glucocorticoid, androgenic and anti-mineralocorticoid activity among the different progestins, it is difficult to distinguish between progestin effects involving classical nuclear PR from non-PR receptors. Most of the commonly used PR antagonists such as RU486 are not highly selective for PR, also binding to non-PR receptors such as GR (Horwitz, 1992). However, studies can be performed by selective knockout of mammary PR or non-PR receptors.

Differences in bioavailability and affinity for PR make extrapolation difficult when considering the effects of different progestins. As noted by Otto et al (2010), progesterone and synthetic progestins may be metabolized differently in animals compared with humans. Animal models may therefore prove inappropriate for analyzing the influence of metabolites that occur in humans. Thus, we can only confirm the validity to humans of progestin-dependent results obtained from animal models after further clinical studies with post-menopausal human patients.

1.6.1. Use of Dietary compounds in chemoprevention and therapy

Recent evidence shows that certain dietary factors may reduce the incidence of various cancers (Patel et al., 2007), indicating a possible role in cancer prevention and the potential for cancer therapy. Development of dietary compounds as potential cancer chemopreventive agents is highly desirable, due to their safety, low toxicity and general

acceptance as naturally-occurring agents consumed either as part of a diet or as supplements (Chen and Kong, 2005). Several dietary compounds have been found to exhibit chemo-preventive properties, partially by blocking the main phases of carcinogenesis, namely; initiation, promotion and progression (Nair et al., 2007). Dietary phytochemicals reportedly block cancers by perturbing crucial factors that control cell proliferation, differentiation, senescence or apoptosis. A large body of evidence shows that certain naturally-occurring compounds are able to affect signal transduction pathways, cell proliferation rates and cell differentiation and in addition, many of these compounds have hormone-like, antioxidant and anti-mutagenic activity (Rosenberg et al., 1998). Furthermore, dietary compounds could prevent cancer through several other mechanisms such as modulation of xenobiotic metabolizing enzymes involved in the bio-activation of carcinogens or by induction of apoptosis (Wang et al., 1999; Middleton et al., 2000). Naturally-occurring compounds can further modulate the effects of hormones by interfering with steroid hormone binding proteins in the serum, thereby altering the rate of hormone delivery, metabolism and elimination (Oberdorster et al., 2001). Recent evidence also shows that dietary phytochemicals can prevent cancers through mechanisms that might involve epigenetic processes. For example, according to De Assis and Hilakivi-Clarke (2006) dietary factors can potentially modify hormone-driven signaling and alter later breast cancer risk by inducing epigenetic changes in the genes that regulate mammary cell proliferation, differentiation and survival. Given that the breast is most sensitive to dietary factors at times when it is undergoing extensive growth, *i.e.,* fetal period, puberty and pregnancy, consumption of dietary phytochemicals during these phases may prove to be an effective long-term means of preventing breast cancer.

The anticancer effects of plant-derived nutrients and non-nutritive constituents have been confirmed in a variety of cell culture systems and animal tumor models (Shukla and Gupta, 2004). Widely-used plant-derived anticancer drugs include vincristine and vinblastine (*Catharanthus roseus*), taxotere (yew species), etoposide (*Podophyllum spp*) and camptothecin analogues (*Camptotheca 35rogestog*) (Houghton et al., 2007). Due to their high safety index in normal cells, naturally-occurring compounds are particularly attractive for use as chemoproventive and chemotherapeutic agents (Gopalakrishnan and Kong, 2008). Many studies have demonstrated the ability of phytochemicals to arrest cancer development without affecting normal cells. The exact mechanism through which phytochemicals exert effects that are harmful to cancer cells, while being non-toxic to normal cells is not completely understood but possibly involves differential induction or suppression of specific signaling cascades as well as transcriptional regulation of several defense genes (Gopalakrishnan and Kong, 2008). For example, Nair et al (2007) noted that while prostate cancer cells (PC-3) may require the overexpression of NF-kβ and/ or EGFR for survival, normal cells might not require these molecules to survive. Consequently blockade of NF-kβ would sensitize tumor cells, resulting in their death, while leaving normal cells intact. Gopalakrishnan and Kong (2008) suggest that dietary phytochemicals may induce apoptotic pathways in abnormal cancer cells while at the same time stimulating the production of detoxifying enzymes and thereby rendering them non-toxic to normal cells. An additional advantage to therapies involving phytochemicals is that low dose combinations can be used to achieve therapeutic synergy between individual compounds while reducing individual toxicities (Sporn and Liby, 2005). However, certain natural chemical properties of dietary

compounds might result in poor absorption, extensive metabolism and rapid clearance from the body, rendering them pharmacologically inactive, particularly after oral administration (Chen and Kong, 2005). Once a compound with potential chemotherapeutic benefits has been identified, structural modification can be carried out to improve its pharmacologic characteristics. An example of such an agent being so modified is sulforamate, a synthetic sulforaphane analog (Gerhauser et al., 1997).

It is becoming increasingly apparent that dietary compounds with chemo-preventive properties have great potential in the fight against cancer. Our laboratory is particularly interested in dietary phytochemicals with anti-progestin properties which might be used as chemo-preventive or chemotherapeutic agents against progestin-dependent breast cancers. In this study, we investigated the chemo-preventive/chemotherapeutic potential of apigenin, a flavonoid proven to be anti-proliferative in a number of human cancer cell lines. A brief review of apigenin and its anticancer effects is given in the next section.

1.6.2.   Use of apigenin in cancer prevention and therapy

Apigenin, whose chemical structure is shown in Figure 1.4, is a low molecular weight polyphenolic compound that belongs to a group of ubiquitous compounds called flavonoids and is classified under a subgroup known as flavones (Patel et al., 2007). Apigenin is abundantly present in common fruits, vegetables and beverages (Patel et al., 2007) and several studies have shown it to possess anti-mutagenic properties. It would appear that by virtue of its low intrinsic toxicity and capacity to exert differential effects in normal versus cancer cells, apigenin has great potential as a chemotherapeutic agent

(Gupta et al., 2001). Previous studies have shown that apigenin affects several signaling pathways in cancer cell lines. For example, Shukla and Gupta (2004) reported that apigenin induced apoptosis in androgen-responsive prostate cancer cell lines, possibly by inducing p21 via p53 independent pathways. Recently King et al (2011) suggested that apigenin might exert its effects by activating mutated p53 in human pancreatic cancer cells. Restoration of p53 function in tumor cells is a novel approach to cancer therapy as it targets one of the major differences between normal and cancerous cells (Wang et al., 2003). Given that more than 50% of all breast tumors are known to carry a p53 mutation or have a defective p53 pathway (Bartek et al., 1990), any compound that restores p53 function will likely possess significant potential as a chemopreventive or chemotherapeutic agent.

In addition to its actions described above, apigenin also inhibits the mutated epidermal growth factor receptor (Her2/neu) pathway in human breast cancer cells (Weldon et al., 2005), while in human ovarian cancer cells it has been shown to suppress the expression of VEGF by inhibiting hypoxia inducible factor (HIF1-α) expression via the PI3K/Akt/p70S6K1 pathway (Fang et al., 2005). Evidence exists that apigenin can render cancer cells more responsive to chemotherapy; for example, Horinaka et al (2006) noted that apigenin increased the sensitivity of malignant cancer cells to tumor necrosis factor-α (TNF-α) related apoptosis-inducing ligands. Thus, it is possible that combining apigenin with other chemotherapeutic agents will enhance their activity, facilitating their effectiveness at even lower dosages and thereby reducing their toxicity. We would like to propose that simultaneous consumption of apigenin with HRT might well prevent the development of progestin-accelerated breast cancers. Lamy et al (2008) showed that

apigenin markedly reduced the migratory and invasive properties of smooth muscle cells. Since breast cancer cells commonly metastasize to bones, lungs and liver (Studebaker et al., 2008), it is possible that apigenin may inhibit tumor metastasis by suppressing VEGF and other metastatic and adhesion promoting molecules. Hu et al (2008) reported that apigenin inhibited the expression of focal adhesion kinase which is known to be important for migration and invasion of human ovarian cancer cells.

In vitro studies show that apigenin is bio-transformed into luteolin and conjugated derivatives such as glucurono-, sulfo- and hydroxylated derivatives (Gradolatto et al., 2004). Pharmacokinetics data shows that in rats, apigenin has a long half-life, being detected in plasma nine days after administration of a single dose (Gradolatto et al., 2005). As a result of its slow metabolism and entero-hepatic circulation apigenin remains in the blood for a long time; consequently consumption of a diet rich in apigenin may result in significant accumulation in the body. It is therefore possible that consistent intake might protect against breast cancer.

Thus, by virtue of its ability to inhibit several cancer-signaling pathways, accumulating evidence points to the potential of apigenin as a chemopreventive or chemo-therapeutic agent. To date however, little attention has been paid to apigenin as a compound with anti-progestin activity. In this study, we sought to investigate its potential against progestin-dependent breast cancer, by performing experiments designed to examine the following: can apigenin suppress progestin-dependent breast cancer by inhibiting the expression of VEGF (Hyder et al., 2001) or other progestin-induced factors such as RANKL (Schramek et al., 2010) and does apigenin operate via other pro-apoptotic and anti-proliferative mechanisms.
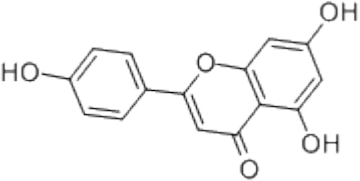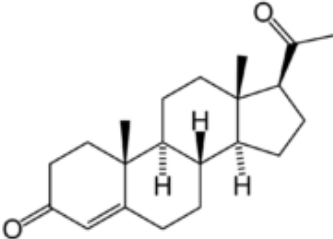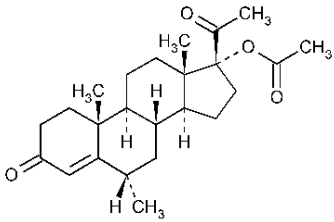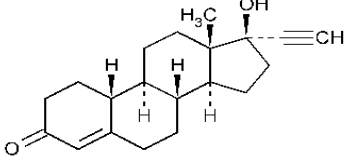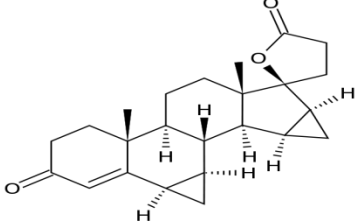
Type (Subtype)	Compound	Structure
Bioflavonoid (flavone)	Apigenin	
Endogenous progestin	Progesterone (P4)	
Synthetic progestin (17α-hydroxyprogesterone)	Medroxyprogesterone acetate (MPA)	
Synthetic progestin (Estrane)	Norethindrone	
Synthetic progestin (19-Norprogesterone)	Drosperinone	

Figure 1.4. Chemical Structure of Apigenin, Progesterone and commonly used synthetic progestins.

Structures of progestins are shown in comparison to that of apigenin.

CHAPTER 2

2.1. Apigenin blocks induction of VEGF mRNA and protein in progestin-treated human breast cancer cells

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Running Title: Apigenin Blocks Induction of VEGF by progestins

Keywords: Apigenin, breast cancer, VEGF, progestin

2.2. ABSTRACT

Results from recent clinical trials indicate that hormone replacement therapy with
estrogen and progestin is associated with higher risk of breast cancer in post-menopausal
women than treatment with estrogen alone or placebo. This observation is consistent with
studies showing that progestins stimulate expression of vascular endothelial growth factor
(VEGF), which in turn stimulates angiogenesis. The objective of this study was to
examine whether apigenin, a natural flavone, inhibits progestin-dependent induction of
VEGF in human breast cancer cells.

T47-D human breast cancer cells were treated with medroxyprogesterone acetate
(MPA) (10 nM) or other synthetic progestins in the presence or absence of anti-progestin
RU-486 and variable doses of apigenin (1-100μM). BT-474 cells were also treated with
MPA $\pm$ 100 μM apigenin. Secreted VEGF was quantified by ELISA and total cellular
VEGF mRNA was quantified by RT-PCR. Expression of VEGF receptor-1 (flt) and -2
(flk), progesterone receptor, and estrogen receptor-alpha was also quantified by Western
blot analysis and/or RT-PCR.

Our results showed that apigenin (50 or 100 μM) prevented progestin-dependent
induction of both VEGF mRNA and protein and reduced progesterone receptor levels in
T47-D cells. Apigenin also blocked MPA-dependent secretion of VEGF from BT-474
cells. Messenger RNA levels of progesterone and estrogen receptor-α were unaffected by
apigenin, which did exert somewhat suppressive, though complex effects on VEGF
receptor expression in MPA-treated T47-D cells.

We thus concluded that apigenin blocks progestin-dependent induction of VEGF
mRNA and protein, and broadly inhibits the ability of progestins to alter expression of

other components of the angiogenesis pathway, including progesterone receptor, vascular endothelial growth factor receptors, in human breast cancer cells. Further studies are however, warranted to explore the potential of apigenin as a chemopreventive agent in post-menopausal women exposed to oral progestins.

2.3.   INTRODUCTION

Clinical trials suggest that post-menopausal women administered hormone replacement therapy (HRT) regimen consisting of both progestin and estrogen are at an increased risk of developing breast cancer compared with women taking estrogen alone or placebo (Ross et al., 2000; Chen et al., 2004). It has been proposed that progestins, which suppress the proliferative effects of estrogen in the uterus, may stimulate the progression of breast cancer cells by inducing vascular endothelial growth factor (VEGF) and increasing angiogenesis (Hyder et al., 1998; Hyder et al., 2001). We and others have recently provided evidence in animal models that both natural and synthetic progestins are capable of accelerating the development of tumors (Aldaz et al., 1996; Benakanakere et al., 2006), including human breast cancer xenografts (Liang et al., 2007). The in vivo results confirm the role of VEGF in the growth of breast cancer cells in vitro (Liang et al., 2006). In view of the established role of progestins in accelerating the development of breast cancer, finding compounds that can block progestin-dependent VEGF production may allow post-menopausal women to continue to benefit from combination estrogen/progestin HRT without increasing the risk of developing breast cancer.

Apigenin is a low molecular weight flavone found in fruits, vegetables, nuts and plant-derived beverages such as tea and wine (Patel et al., 2006). Apigenin is reported to

reduce levels of VEGF and block proliferation of breast (Yin et al., 2001), colon (Wang et al., 2004), leukemia (Wang et al., 1999), skin, thyroid, ovarian and prostate cancer cells (Gupta et al., 2001; Shukla & Gupta, 2004; Hu et al., 2008). Apigenin also down-regulates expression of the endogenous estrogen receptor-alpha (ERα) in mouse uterus (Breinholt et al., 2000). Several mechanisms have been proposed to explain the anti-carcinogenic effects of apigenin. These include suppression of inflammatory responses, free-radical scavenging, proteasome inhibition (Patel et al., 2006; Chen et al., 2007) and suppression of angiogenesis (Fang et al., 2007). Our current concept is that progestins promote tumor progression in the breast by inducing VEGF (Hyder et al., 1998; Hyder et al., 2001; Liang et al., 2005) which then leads to VEGF-stimulated angiogenesis (Liang et al., 2007). Because previous studies suggest that apigenin suppresses VEGF induction, we hypothesized that this naturally-occurring flavone might have the capacity to inhibit progestin-dependent progression of human breast cancer cells.

The goal of this study was to determine whether apigenin blocks induction of VEGF and other angiogenesis-related proteins in progestin-treated human breast cancer cells. To test this hypothesis, T47-D human breast cancer cells were incubated with a commonly used synthetic progestin, medroyprogetserone acetate (MPA) or other synthetic progestins in the presence or absence of the anti-progestin RU-486 and variable doses of apigenin (1-100 μM). Secreted VEGF was quantified by ELISA and total cellular VEGF mRNA was quantified by RT-PCR. Expression of VEGFR-2 (flk), VEGFR-1 (flt), progesterone receptor (PR) and ERα was also quantified by Western blot analysis and/or RT-PCR. To confirm that apigenin exerted its effects on other breast

cancer cells we also determined its ability to influence MPA-dependent secretion of VEGF from BT-474 cells.

The results presented here demonstrate that apigenin inhibits progestin-dependent induction of VEGF mRNA and protein and suppresses expression of PR and ERα protein in T47-D cells. Apigenin also inhibits progestin-dependent VEGF secretion from BT-474 cells. These results suggest that apigenin has significant potential as a chemopreventive agent in post-menopausal women exposed to oral progestins.

2.4. MATERIALS AND METHODS

2.4.1. *Reagents*: Medroxyprogesterone acetate (MPA), progesterone, norethindrone, apigenin and RU-486 were obtained from Sigma–Aldrich (St Louis, MO). Pierce Bicinchoninic acid protein assay reagents were purchased from Fischer Scientific International. Apigenin (100 mM in DMSO) was stored in aliquots at -20°C and thawed immediately prior to use at the indicated final concentration (1-100 μM; ref by Yin et al., 2001; Gupta et al., 2001; Chen et al., 2007).

2.4.2. *Cell culture*: Human T47-D and BT-474 breast cancer cells were grown in a humidified atmosphere of 5% CO_2 at 37°C in 100-mm cell culture plates using phenol red-free Dulbecco's Modified Eagle's Medium (DMEM/F12) supplemented with 10% fetal bovine serum. When cells reached 50–60% confluence, they were washed with PBS and switched into DMEM/F12 supplemented with 5% dextran-coated charcoal (DCC) for 36 hours. Cells were then washed, transferred into fresh DMEM/F12 and treated with MPA (10nM), RU-486 and apigenin, as indicated. Unless otherwise stated, cells were treated with 1 μM RU-486 and 100 μM apigenin for 30 min prior to addition of progestins. Control cells were treated with DMSO.

2.4.3. *VEGF enzyme-linked immunosorbent assay (ELISA)*: Quantikine human VEGF enzyme-linked immunosorbent assay kit (catalog number DVE00) was purchased from R & D Systems, Inc. (Minneapolis, MN). Supernatant was collected from cultured T47-D or BT-474 cells treated with vehicle (DMSO), MPA (10nM) ± either apigenin (100μM) or RU-486 (1μM) and apigenin or RU-486 alone. VEGF concentrations were then

determined using the ELISA kit according to the manufacturer's protocol. Experiments were performed in triplicate, and each sample was analyzed in duplicate. Inter- and intra assay coefficients of variance given by the manufacturer for cell culture supernatant assay are 5-8.5% and 3.5-6.5%, respectively.

2.4.4. *Bicinchoninic acid protein assay*: Cells were harvested and pellets re-suspended in 300 μl lysis buffer (50mM Tris-Hcl pH 8, 150mM NaCl, and 1% NP-40). Protein concentration was determined using the bicinchoninic acid assay (BCA) using bovine serum albumin as standard. Absorbance was measured at 562 nm using a SpecTRA MAX 190 microplate reader (Sunnyvale, CA). All samples were analyzed in duplicate.

2.4.5. *RT-PCR*: RT-PCR was performed using primers for the following genes; VEGF, HIF-1α, VEGFR-2 (flk), VEGFR-1 (flt), ERα, and PR (Table 1). Total cellular RNA was extracted using Trizol reagent (UltraSpec) (Biotecx, Houston, TX), according to the manufacturer's instructions. Two plates from each treatment were pooled for RNA preparation. RNA was further purified using an Ambion DNAse kit and, in order to ascertain RNA quality, aliquots were separated in 1% agarose gels. RT-PCR amplification was carried out using an Invitrogen Superscript III one-step RT-PCR amplification kit in an Applied Biosystems 2700 thermocycler (Foster city, CA) using 1 μg of extracted RNA. RT-PCR conditions were as follows: 60°C for 30 min; 94°C for 2 min; followed by 35 cycles of 94°C for 15s, 55°C for 30s, 68°C for 60s and a final elongation step at 68°C for 5 min. RT-PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. Electrophoresis was carried out in 0.5X

TBE, pH 8.0, at 100V for 1h, after which time gels were photographed under UV light. The human VEGF primer pairs used in this study were obtained from R&D Systems, Inc (Minneapolis, MN) and the other primers; PR, ERα, VEGFR-2, VEGFR-1, hypoxia-inducible factor-1α (HIF-1α), and GAPDH were obtained from Integrated DNA Technologies, Inc (Coralville, Iowa) (Table 2.1).

Table 2.1. Primers used for RT-PCR analysis.

Gene	Forward primer sequence	Reverse primer sequence
GAPDH	5'-TCA ACG GAT TTG GTC GTA TT -3'	5'-CTG TGG TCA TGA GTC CTC CTT CC-3'
PR	5'-AGC CCT AAG CCA GAG ATT-3'	5'-TAG GAT CTC CAT CCT AGA CC-3'
ERα	5'-CCA CCA ACC AGT GCA CCA TT-3'	5'-GGT CTT TTC GTA TCC CAC CTT TC-3'
VEGFR-2 (*flk*)	5'-CAT CAC ATC CAC TGG TAT TGG-3'	5'-GCC AAG CTT GTA CCA TGT GAG-3'
VEGFR-1 (*flt*)	5'-TAC ACA GGG GAA GAA ATC CT-3'	5'-ACA GAG CCC TTC TGG TTG GT-3'
HIF-1α	5'-CCC CAG ATT CAG GAT CAG ACA-3'	5'-CCA TCA TGT TCC ATT TTT CGC-3'

2.4.6. *Western blot analysis*: Total cellular protein extracts were prepared as described

previously (19). Protein aliquots (30μg) were separated by SDS-PAGE, transferred to

polyvinylidene difluoride membrane and blotted with antibodies to PR (1:200 dilution;

Santa Cruz Biotechnology), ERα (1:150 dilution; Santa Cruz Biotechnology) and β-actin

(Sigma Aldrich, St Louis, MO). Protein bands were detected and quantified by blotting

with anti-mouse secondary antibody (1: 2000 dilution; Santa Cruz Biotechnology) and a

chemiluminescent detection system according to manufacturer's instructions (Amersham

Pharmacia Biotech).

2.4.7. *Statistical Analysis of data*: Results shown in figures are presented as mean ±

SEM. Statistical significance was tested by one-way analysis of variance (ANOVA)

using SigmaStat Software version 11 (Sigstat Software Inc., Richmond, CA, USA). For

ANOVA, the assumption of analysis of variance was examined and non-parametric

measure based on ranks was used, as needed. When ANOVA indicated significant effect

(F-ratio, $p<0.05$), the Student-Newman-Keuls multirange test was employed to compare

the means of individual groups. When normality test failed, significance was determined

by Kruskal–Wallis test (one-way ANOVA by ranks) followed by Student-Newman-

Keuls test.

2.5. RESULTS

2.5.1. Effect of apigenin on VEGF protein secretion in MPA-treated cells

Previous studies show that exposure to progesterone, MPA or other synthetic progestins increases the amount of intracellular VEGF mRNA and extracellular VEGF protein secreted by T47-D cells (Hyder et al., 2001; Mirkin et al., 2006). Because this cellular response is blocked by anti-progestin RU-486, it is most likely directly caused by PR/progestin-mediated cellular effects. The goal of this study was to determine whether apigenin can block MPA-dependent induction of VEGF expression and/or secretion. Therefore, T47-D cells were treated with MPA and other progestins in the presence and absence of apigenin. The results show that 100 μM apigenin significantly reduced the amount of VEGF secreted by MPA-treated cells, restoring secreted VEGF to levels close to those measured in untreated cells or cells treated with MPA and RU-486 (Fig. 2.1A; $p<0.05$). Similar results were obtained in T47-D cells treated with progesterone or norethindrone, indicating that apigenin is equally effective in blocking the effects of these three progesterone-related compounds in this experimental system (Fig. 2.1B). These results also suggest that apigenin and RU-486 reduce PR mediated VEGF induction with similar efficacy in this experimental system.
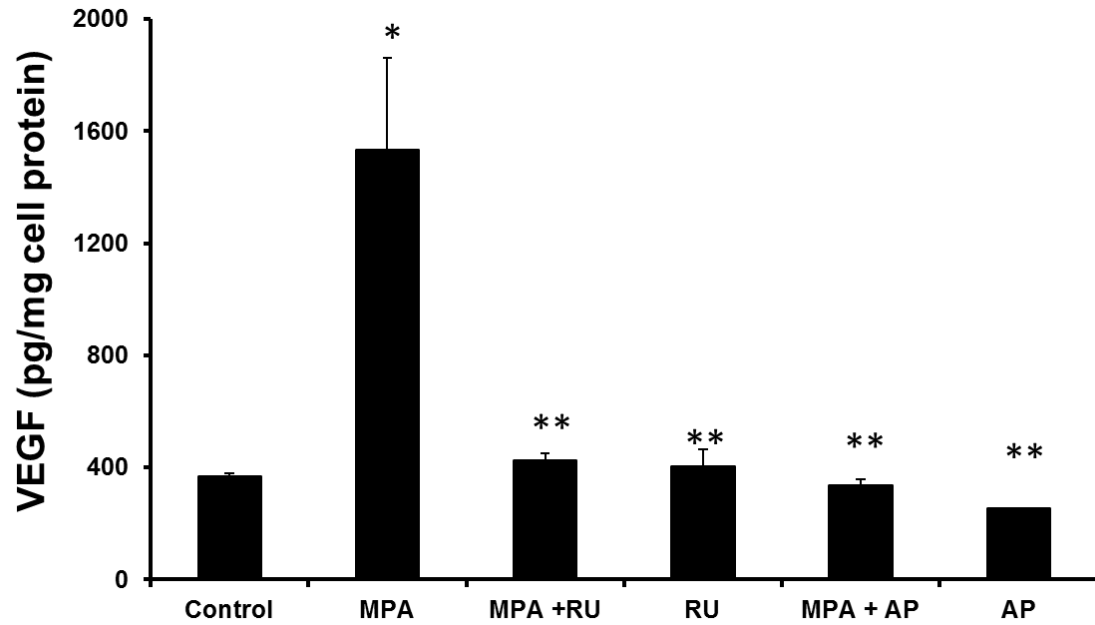
Figure 2.1A. Apigenin inhibits MPA-induced secretion of VEGF from T47-D cells. Cells were treated with vehicle alone (control), 10nM MPA ± 1μM RU-486 (RU) or 100μM apigenin (AP), and with 1μM RU or 100μM AP alone for 18 hours at 37°C. VEGF was measured in the supernatants of cultured cells by enzyme-linked immunosorbent assay as described in the methods. Results are expressed as mean ± SEM (n = 6). * $p < 0.05$, compared with control group. ** $p < 0.05$, compared with MPA group.
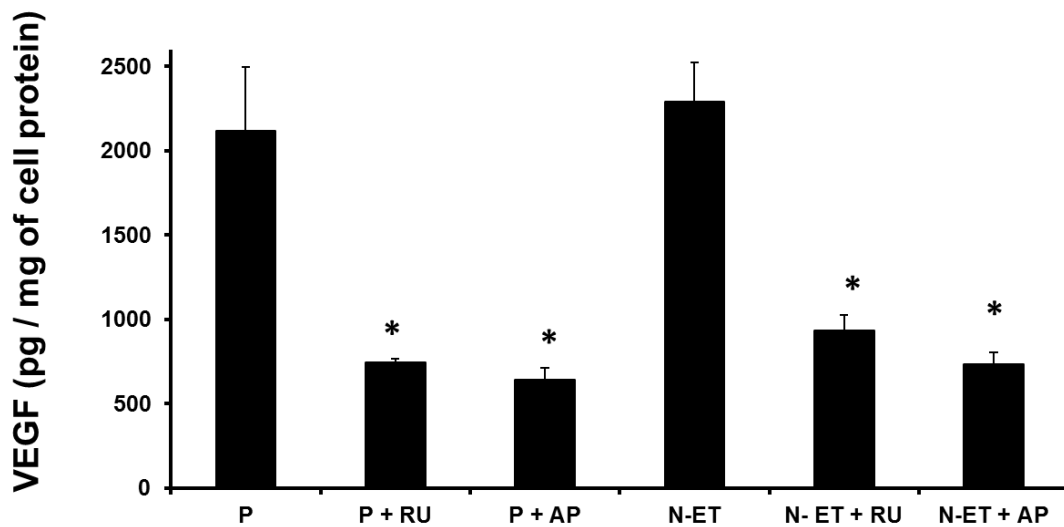
Figure 2.1B. Apigenin inhibits VEGF protein secretion induced by progesterone (P) and norethindrone (NET) in T47-D cells. T47-D cells were treated with 10nM P or N-ET ± either 1μM RU or 100μM AP for 18 hours at 37°C. RU-486 and apigenin were administered 30 minutes before progestins were added to the culture media. VEGF was measured in the supernatants of cultured cells using ELISA as described in methods. Results are expressed as mean ± SEM (n = 6). * $p < 0.05$ as compared to P or NET-treated group.

We also examined the effect of apigenin on MPA-dependent induction of VEGF in BT-474 human breast cancer cells, in which VEGF secretion is also enhanced by progestins. In contrast to our findings with T47-D cells, in BT-474 cells MPA-dependent VEGF secretion was more sensitive to inhibition by 100 μM apigenin than it was to RU-486 inhibition (Fig. 2.1C). Apigenin alone also reduced the basal level of VEGF secretion from BT-474 cells.
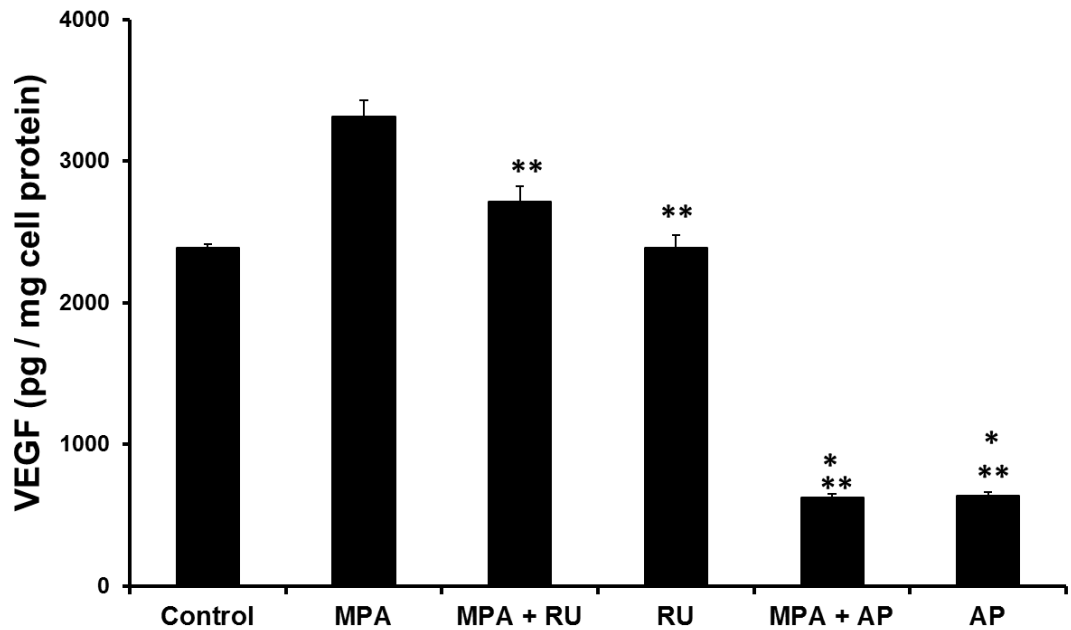
Figure 2.1C. Apigenin inhibits VEGF protein secretion induced by MPA in BT-474 cells.

Procedure as previously described in Fig 3A above. Results are expressed as mean ± SEM (n = 6).

* $p < 0.05$, compared with control group. ** $p < 0.05$, compared with MPA

2.5.2. Effect of apigenin on VEGF mRNA expression in MPA-treated cells

A similar experiment was performed to determine whether apigenin restored a basal level of VEGF mRNA in MPA-treated T47-D cells. For this purpose, total RNA was extracted from T47-D cells cultured in the presence and absence of MPA and apigenin. VEGF mRNA was then estimated using semi-quantitative RT-PCR. As expected, exposure to MPA (10 nM for 6 h; Hyder et al., 2001) significantly increased the level of VEGF mRNA in T47-D cells, but this increase was blocked by 1 μM RU-486 (Fig. 2.2A). Though there was a tendency towards dose-dependent suppression of VEGF mRNA with increasing concentrations of apigenin, inhibition of MPA-dependent induction of VEGF mRNA was not significant at lower concentrations (1-50 μM) and only became so at the highest concentration used (100 μM) (Figs. 2.2A and 2.2B). Neither RU-486 nor apigenin significantly affected the basal level of VEGF mRNA in T47-D cells.

2.5.3. Effect of apigenin on expression of PR

Previous studies demonstrate that progestin-dependent effects on expression of VEGF are mediated by progestin/PR interactions, leading to PR activation and downstream signaling (Liang et al., 2005). Therefore, it was important to determine whether apigenin alters the level of PR protein or mRNA in T47-D cells treated with or without MPA. Our results showed that apigenin has no effect on the level of PR (Fig. 2.3A) or HIF-1α mRNA (data not shown). Previous studies (Hyder et al., 2001) and our results (Fig. 2.3B) also show that MPA and RU-486 down-regulate the levels of PRA and PRB protein (Fig. 2.3B; $p<0.05$), and this study shows a similar effect in cells treated only with apigenin. Interestingly, a detectable amount of PR protein was present in T47-D cells exposed to

MPA, AP or RU-486, at the concentrations and culture conditions used in these experiments.
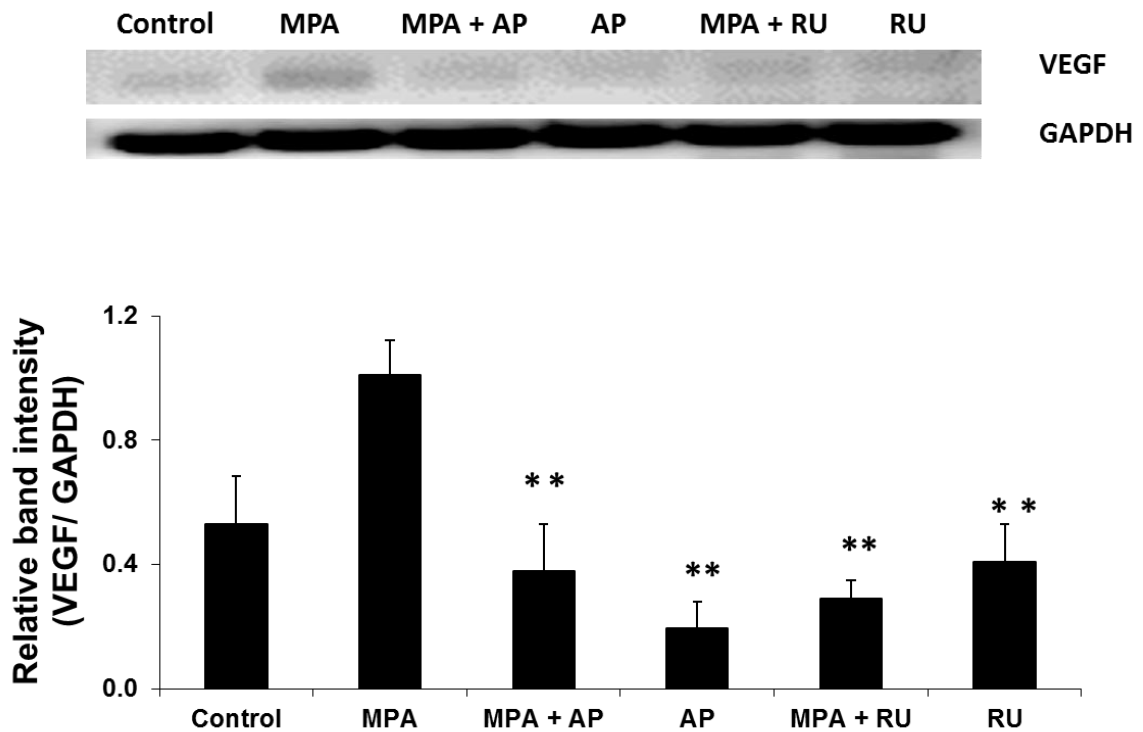
Figure 2.2A. Apigenin (AP) inhibits MPA induced VEGF mRNA expression in T47-D cells.

Cells were treated with vehicle alone (Control) or with MPA (10nM) in the absence of RU (1 μM) or AP (100 μM). Inhibitors were administered 30 minutes prior to exposure to MPA. RNA was collected 6 hours after MPA treatment and analyzed for expression of VEGF message using RT-PCR as described in Methods. Results are expressed relative to controls after normalization with GAPDH and given as mean ± SEM (n=3 for each experimental point taken from three individual experiments). *$p < 0.05$ as compared to control group, ** $p<0.05$ as compared to MPA treated group. ANOVA was used for statistical analysis.
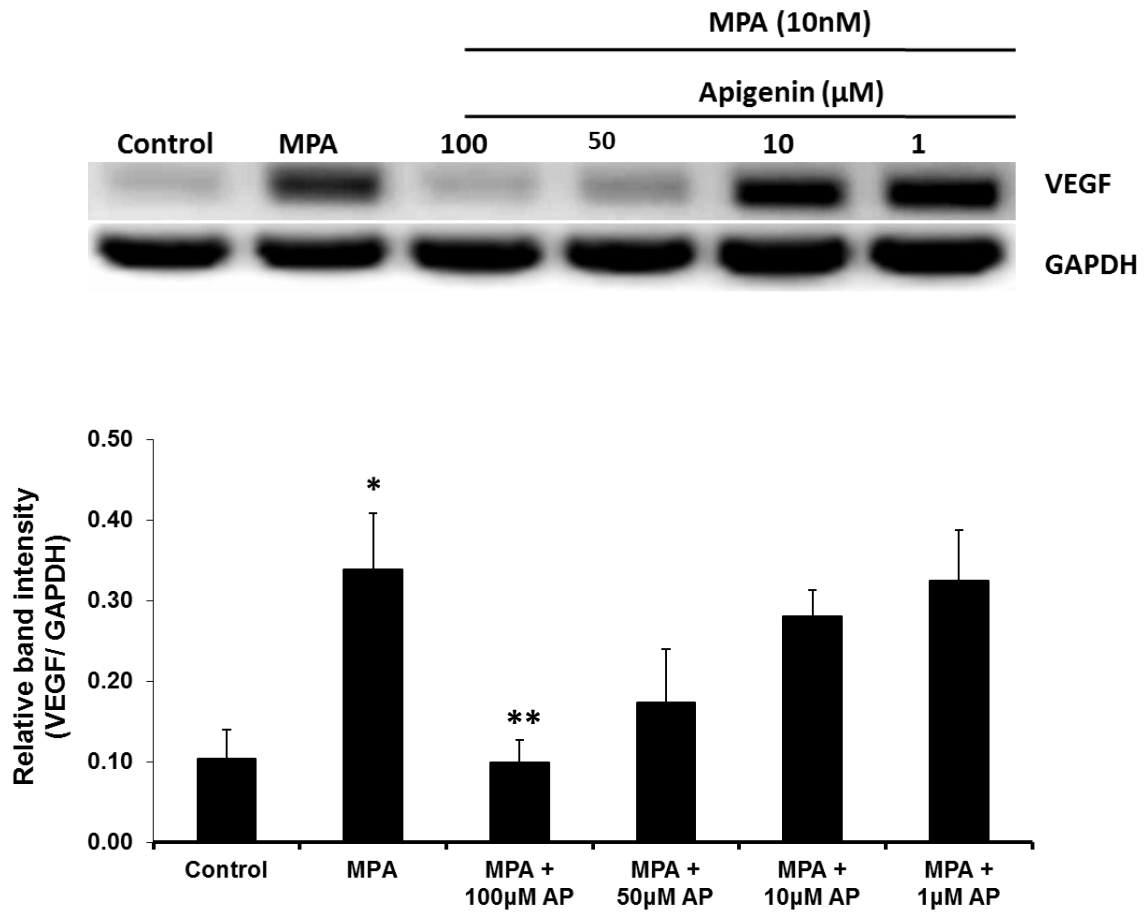
Figure 2.2B. Inhibition of MPA induced VEGF expression in T47-D cells by AP. The procedure was similar to that described in (4A) except that varying doses of AP were used as indicated. Results are expressed relative to controls after normalization with GAPDH and given as mean ± SEM (n=3 for each experimental point taken from three individual experiments). *$p < 0.05$ as compared to control group. ** p<0.05 as compared to MPA group. ANOVA was used for statistical analysis.
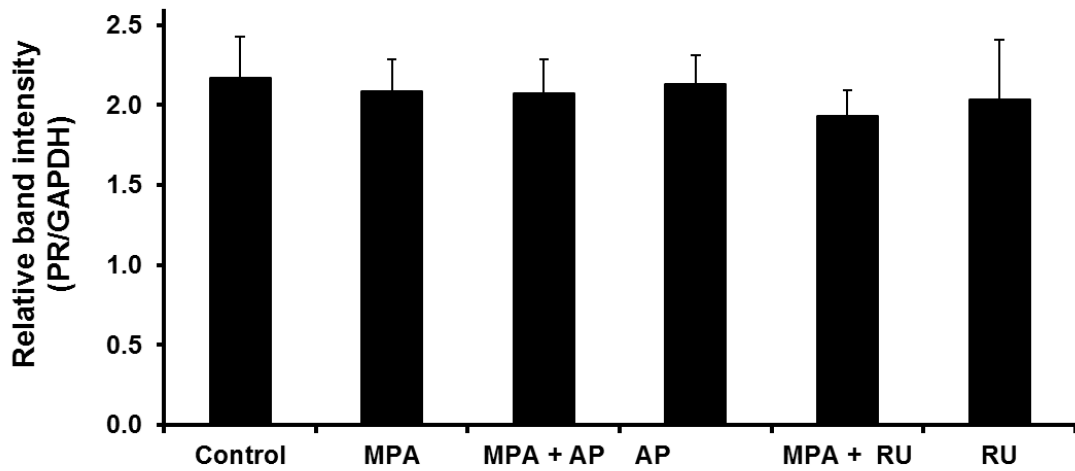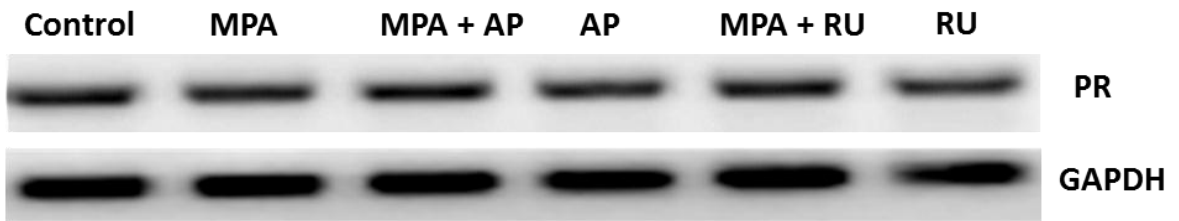
Figure 2.3A. Apigenin does not alter mRNA expression of PR in T47-D cells. T47-D cells were treated with vehicle (Control), with MPA (10nM) ± either apigenin (100μM) or RU-486 (1μM), and with same concentrations of apigenin alone as described in Methods. Total RNA was prepared 6 h following MPA addition as described in Methods and assessed for expression of PR using RT-PCR (n=3)
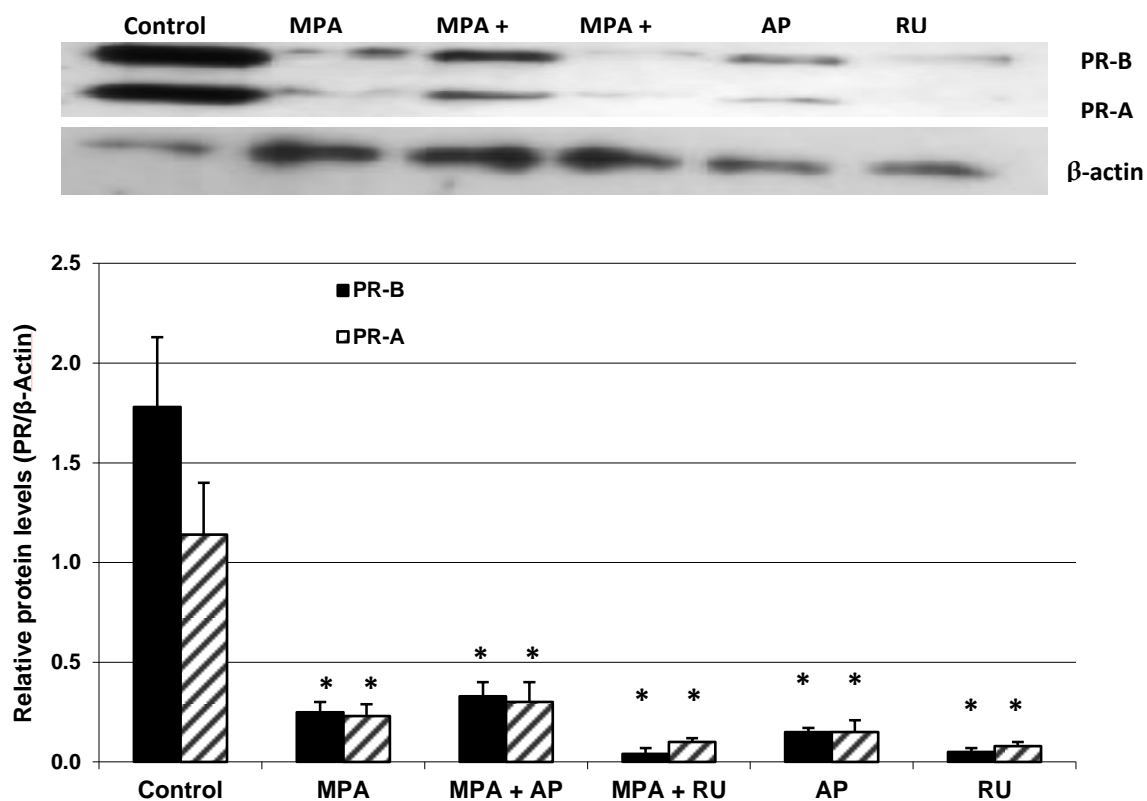
Figure 2.3B. Apigenin inhibits PR protein levels in T47-D cells. Cells were also collected after 6 h and protein preparations were analyzed for PR using Western blot analysis. A representative immunoblot is shown in the upper panel and quantitative data from three experiments is shown in the histogram in the lower panel. The densities of the bands of PRA and PRB were compared with controls after normalization with β-actin. Results are expressed as mean ± SEM (n=3 for each experimental point taken from three individual experiments). * $p < 0.05$ as compared to control group (ANOVA).

2.5.4. Effect of apigenin on expression of ERα and VEGFR in MPA-treated cells.

The effect of apigenin on expression of ERα was also examined in T47-D cells (Fig. 2.4A). ERα protein levels were reduced but not completely eliminated by treatment with MPA, apigenin, MPA + apigenin or MPA + RU-486, though not by RU-486 alone. None of the suppression levels reached statistical significance. Apigenin did not down-regulate ERα mRNA (data not shown).

Given the fact that the downstream effects of VEGF are mediated by VEGFR-2 (flk) and VEGFR-1 (flt), the effect of apigenin on expression of VEGFR mRNA was examined in MPA-treated T47-D cells. MPA down-regulated expression of VEGFR-2 (Fig. 2.4B); however this did not reach statistical significance using the parameters in this manuscript. In the presence of MPA and 100 µM apigenin VEGFR-2 was significantly suppressed in T47-D cells. Surprisingly, the presence of 10 µM apigenin completely prevented the suppression of VEGFR-2, levels of which remained the same as in controls and were significantly different from the suppressed levels of VEGFR-2 mRNA observed with 100 µM apigenin. MPA also down-regulated VEGFR-1 (Figure 2.4C; $p < 0.05$). In contrast to its effects on VEGFR-2 mRNA, apigenin did not significantly alter the MPA-suppressed levels of VEGFR-1 mRNA at any concentration used (1-100 µM), though at its highest concentration (100 µM) apigenin did appear somewhat to compound the effect of MPA in lowering VEGFR-1 expression (Fig. 2.4C). The significance of this observation remains to be determined.
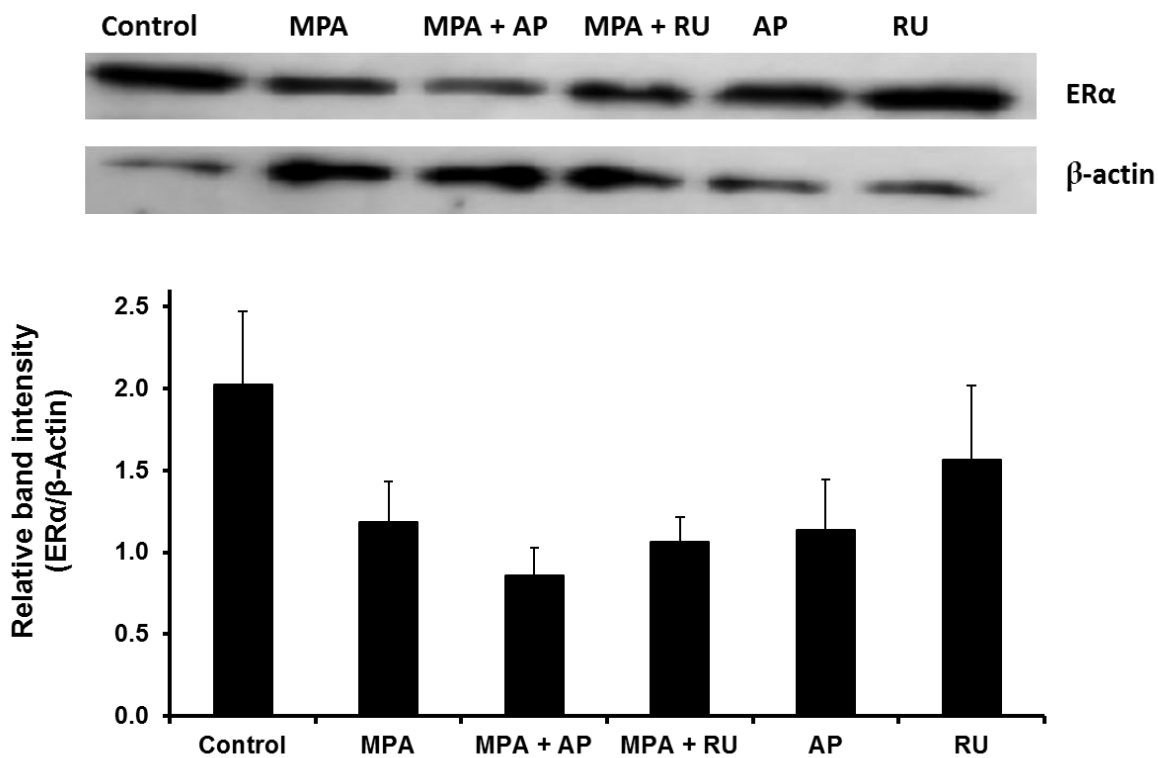
Figure 2.4A. Influence of apigenin on ERα protein levels in T47-D cells. Cells were collected after 6 h and protein preparations were analyzed for ERα using Western blot analysis. A representative immunoblot is shown in the upper panel and quantitative data from three experiments is shown in the histogram in the lower panel. The densities of the bands of ERα were compared with controls after normalization with β-actin. Results are expressed as mean ± SEM (n=3 for each experimental point taken from three individual experiments).
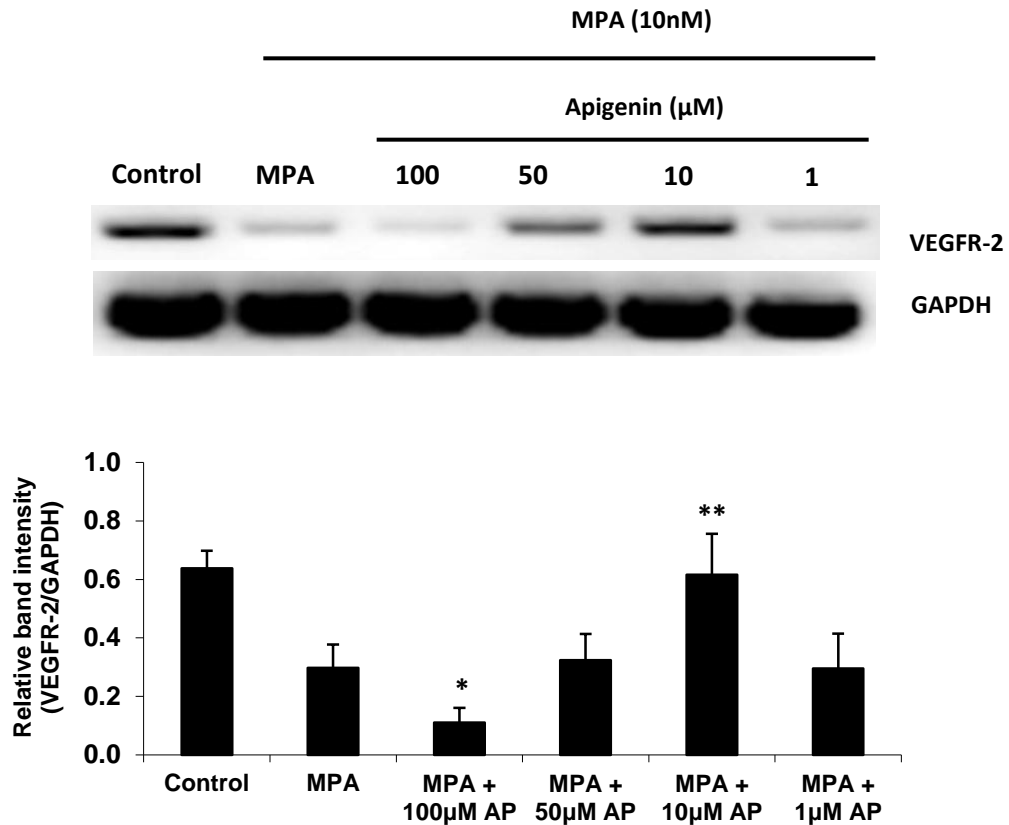
Figure 2.4B. Apigenin inhibits VEGFR-2 mRNA in the presence of MPA. T47-D cells were treated

with either 10nM MPA alone or in the presence of the indicated concentration of AP. Apigenin was

administered 30 minutes prior to exposure to MPA for 6 hours, after which total RNA was prepared and

RT-PCR was performed as described in Methods. Results are expressed relative to controls after

normalization with GAPDH and shown as mean ± SEM (n=3 for each experimental point taken from three

individual experiments).). * $p < 0.05$ as compared to control group; ** $p<0.05$ as compared to MPA + 100

μM apigenin treated group (ANOVA).
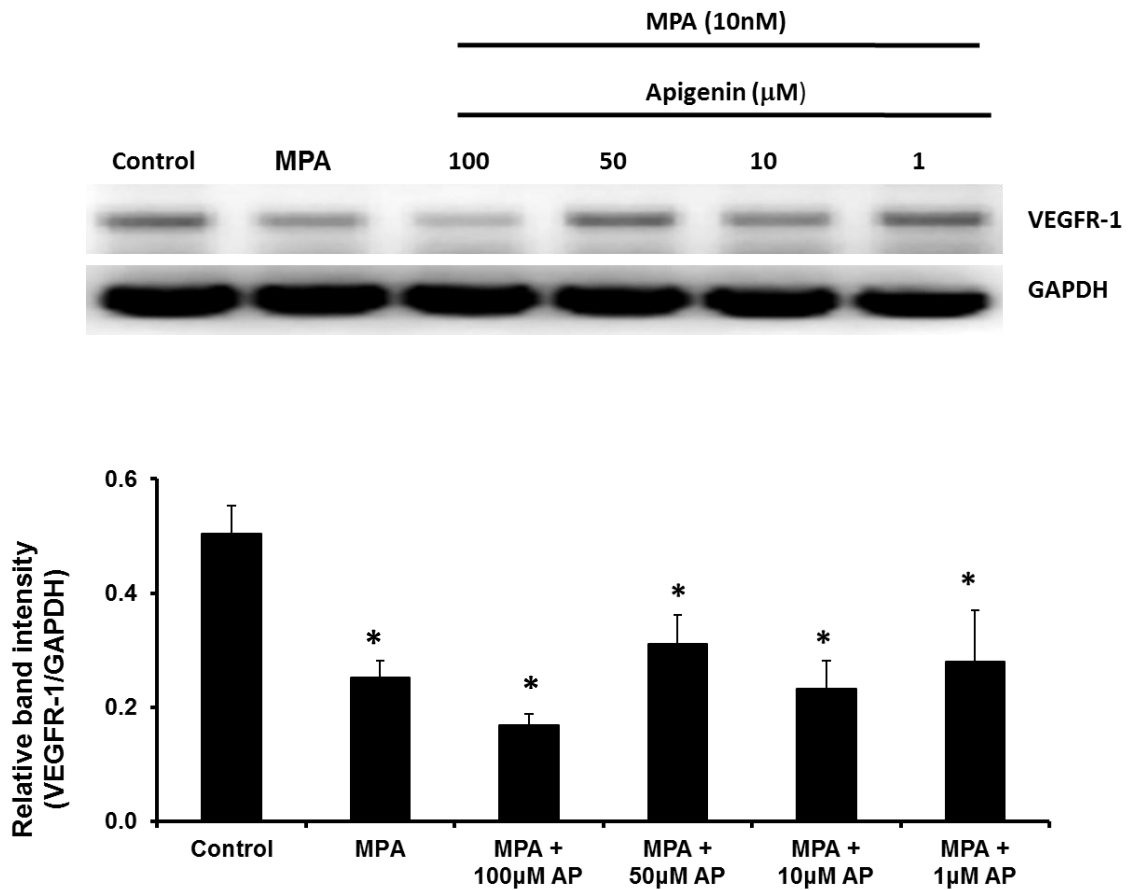
Figure 2.4C. Apigenin does not influence MPA-dependent inhibition of VEGFR-1 mRNA expression in T47-D cells. RNA collected in 6B was also assessed for expression of VEGFR-1 mRNA as described in Methods. Results are expressed relative to controls after normalization with GAPDH and given as mean ± SEM (n=3 for each experimental point taken from three individual experiments). * $p < 0.05$ as compared to control group (ANOVA).

2.6. DISCUSSION

The use of progestins during HT has been linked to increased risk of breast cancer (Ross et al., 2000; Chen et al., 2004). Although the exact role of progestins in the human breast is not known, a number of reports suggest they play a pro-proliferative, anti-apoptotic role in both rodent and human mammary cells (Moore et al., 2004; Mirkin et al., 2006). Due to the rapid rate at which these tumors develop, we and others suggested that progestins most likely increase the proliferation of existing tumor cells in the breast (Hyder et al., 1998, 2001; Chen et al., 2004). Recent xenograft studies from our laboratory showed that progestins do indeed promote the progression of tumor cells (Liang et al., 2007; 2010). In this model, by suppressing progestin-induced elaboration of VEGF with the synthetic anti-progestin RU-486, we were able to bring about tumor regression (Liang et al., 2007). Due to its unfortunate side effects, RU-486 is not suitable for administration to women for extended periods of time. Consequently, we are investigating various compounds which occur naturally and which might be consumed without such undesirable side-effects, with a view to blocking progestin-dependent VEGF induction. Such compounds, we believe, have the potential to both treat and prevent progestin-dependent progression of breast disease.

This study provides evidence that apigenin inhibits progestin-dependent induction of VEGF mRNA and protein and reduces the amount of VEGF secreted by PR-expressing T47-D and BT-474 cells, the latter of which expresses abundant Her-2-neu, which is related to aggressive forms of cancer. Furthermore, apigenin appears to have broad specificity and potency as a PR antagonist, since it blocks VEGF induction by MPA, progesterone and norethindrone in T47-D cells, as well as MPA-dependent

induction of VEGF in human BT-474 cells. Thus, apigenin may be an effective antagonist against many clinically-relevant progestins, as well as having the capacity to act in a variety of breast cancer cell types. Other naturally-occurring compounds such as curcumin inhibit MPA-, but not progesterone-stimulated secretion of VEGF in human breast cancer cells (Carroll et al., 2008). Thus, apigenin may have broader clinical potential than curcumin. In this regard, apigenin could potentially protect against the adverse effects of exposure to environmental progestins, whose exact chemical nature may be unknown. For example, bisphenol A , a compound generally thought to be an endocrine disruptor that binds to estrogen receptors, also has progestational activities (Jung et al., 2005). Other compounds that may bind to progesterone receptors (atrazine and waste water treatment plant effluents) have also been identified. While their effects on breast cancer cells are as yet unknown (Viswanath et al., 2008), we propose that any negative impact of such compounds could potentially be opposed by apigenin and that such a possibility warrants further study.

The mechanism by which apigenin suppresses MPA-stimulated induction of VEGF is not yet known. Three PR response elements in the VEGF promoter play a role in PR-dependent induction of VEGF (Mueller et al., 2003). Therefore, apigenin could block interactions that occur between progestin-activated PR and the PR response elements in the VEGF promoter. Alternatively, apigenin could down-regulate PR sufficiently to globally block activation of PR-responsive genes. Results presented here indicate that apigenin down-regulates PR protein but not mRNA in the absence of MPA. Activated ligand-bound PR protein is normally down-regulated via proteosomal degradation (Ellmann et al., 2009). Thus, PR may be activated but down-regulated in

cells exposed to MPA and apigenin. PR protein was also down-regulated by RU-486, both in the presence and absence of MPA, even though RU-486 blocks progestin-dependent activation of PR. Both apigenin and RU-486 down-regulate PR and suppress induction of VEGF by MPA, suggesting that steps beyond down-regulation of PR are involved in the ability of ligands to suppress PR-mediated effects.

Breinholt et al. (2000) reported that apigenin decreased the level of endogenous ER in mouse uterus. We found that apigenin had a slight, though not statistically significant, effect on the level of ERα protein (Fig. 3C) and ERα mRNA (data not shown). A similar reduction in the level of ERα protein in progestin-treated T47-D cells was reported by Read et al (1989). One interpretation of this result is that ligand-bound PR post-transcriptionally regulates expression of ERα protein. However, we feel it is unlikely that the level of PR protein decreases as a secondary effect of a lower level of ERα in apigenin-treated cells, because PR mRNA is not down-regulated by MPA, apigenin or a combination of MPA plus apigenin. Carroll et al (2009) reported that HIF-1α is essential for progestin-dependent VEGF induction; thus, it is also possible that apigenin could block progestin-dependent induction of VEGF by a mechanism involving HIF-1α. Apigenin did not inhibit expression of HIF-1α mRNA; however it is possible that it down-regulates HIF-1α protein or interferes with downstream functions of HIF-1α. Further studies are required to determine whether apigenin influences the expression and/or activity of HIF-1α in progestin-treated cells.

This study also shows that exposure to MPA moderately down-regulates VEGFR-2 and strongly down-regulates VEGFR-1 mRNA in T47-D cells. This could reflect a protective feedback mechanism in response to the high level of VEGF in MPA-treated

tumor epithelial cells, as previously described for VEGFR-2 in endothelial cells (Duval et al., 2003). We also demonstrate that apigenin exerts complex effects on expression of VEGFR-1 and 2 in MPA-treated T47-D cells. For example, a high concentration of apigenin enhances the suppressive effect of MPA on transcription of VEGFR-1 and 2, while mixed effects on VEGFR-2 mRNA levels were observed when cells were exposed to lower concentrations of apigenin. These results suggest that apigenin may have both agonist and antagonist properties in MPA-treated cells since it can enhance or block progestin effects on VEGFR1 and 2. Previous studies did in fact show that apigenin is a weak PR agonist (Rosenberg et al., 1998). Further studies are needed to determine whether apigenin binds to PR and how it influences expression of VEGFR-1 and 2 in the presence and absence of PR agonists such as MPA. Nevertheless, the low levels of VEGFR-1 and 2 present in cells exposed to apigenin and MPA could enhance its potential anti-angiogenic effects and therefore its clinical utility.

2.7.   CONCLUSION

In summary, in this study, we provide evidence that apigenin inhibits MPA-induced VEGF at both mRNA and protein secretion levels in T47-D human breast cancer cells. Apigenin also reduces PR protein levels in these cells. Since progestin-dependent VEGF induction is crucial for (i) mediating angiogenesis by causing proliferation of endothelial cells, and (ii) proliferation and survival of breast cancer cells, this study provides evidence that apigenin has great potential as a naturally-occurring chemopreventive and/or chemotherapeutic agent against progestin-dependent breast cancer in post-menopausal women. Additional pre-clinical and clinical studies are required to further

investigate the efficacy of apigenin and to determine its suitability as a viable drug

candidate for use against human breast disease.

CHAPTER 3

3.1. Apigenin induces apoptosis and blocks growth of medroxyprogesterone acetate-dependent BT-474 xenograft tumors

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Running title: Apigenin inhibits MPA-dependent BT-474 xenograft tumors

Key words: Apigenin, breast cancer, medroxyprogesterone acetate, BT-474 xenograft tumor, hormone replacement therapy, progesterone receptor, VEGF, RANKL.

3.2.   ABSTRACT

Recent clinical and epidemiological evidence shows that hormone replacement therapy

(HRT) containing estrogen and progestin increases the risk of primary and metastatic

breast cancer in post-menopausal women, while HRT containing only estrogen does not.

We and others previously showed that progestins promote the growth of human breast

cancer cells in vitro and in vivo. In this study, we sought to determine whether apigenin,

a low molecular weight anti-carcinogenic flavonoid, inhibits growth of aggressive

Her2/neu-positive BT-474 xenograft tumors in nude mice exposed to

medroxyprogesterone acetate (MPA), the most commonly used progestin in the USA.

Our data clearly shows that apigenin (50 mg/kg) inhibits progression and development of

these xenograft tumors by inducing apoptosis, inhibiting cell proliferation and reducing

expression of Her2/neu. Moreover, apigenin reduced levels of VEGF without altering

blood vessel density, indicating that continued expression of VEGF may be required to

promote tumor cell survival and maintain blood flow. Although it has previously been

shown that MPA induced expression of RANKL in the rodent mammary gland, MPA

reduced the level of RANKL in human tumor xenografts in a progesterone receptor-

independent manner (i.e., in the presence or absence of the antiprogestin RU-486).

Conversely, apigenin protected against RANKL loss, suggesting that MPA and apigenin

modulate levels of RANKL differently in the rodent mammary gland and in human breast

cancer cells. These data and our previous findings suggest that apigenin has potential for

treating progestin-dependent breast disease.

3.3. INTRODUCTION

Post-menopausal women routinely undergo hormone replacement therapy (HRT) containing estrogen or estrogen and progestin to alleviate the climacteric symptoms of menopause such as hot flashes, osteoporosis, insomnia, mood swings, dementia and decreased libido (Narod, 2011). Progestins have been included in HRT to counteract the increased risk of endometrial cancer associated with post-menopausal estrogen supplementation. However, the Women's Health Initiative (WHI) and subsequent studies of combination HRT showed recently that breast cancer risk is higher in post-menopausal women on estrogen/progestin-based HRT than in post-menopausal women given oral estrogen or placebo (Chlebowski et al., 2003). As a result, use of combination HRT has become controversial (Tsai et al., 2011). Recent epidemiological studies linked the decline in HRT to reduced incidence of ductal carcinoma in situ, particularly hormone-receptor-positive invasive breast cancer (Clarke et al., 2006; Ravdin et al., 2007; Ereman et al., 2010), and progestins increase proliferation of breast epithelial cells in vivo (Haslam, 1988; Clarke and Sutherland, 1990). Due to the rapid onset of tumor growth in postmenopausal women on combination HRT, we and others have suggested that progestin-containing HRT promotes progression of latent tumor cells or stimulates proliferation of cancer stem cells (Hyder et al., 2001; Aupperlee et al., 2006; Joshi et al 2010).

A number of mechanisms have been proposed to explain progestin-stimulated breast cancer in post-menopausal women (Lanari and Molinolo, 2002). Several studies showed that progestins induce expression of vascular endothelial growth factor (VEGF),

a potent pro-angiogenic factor, in human and rodent breast cancer cells (Hyder et al., 1998; 2001; Benakanakere et al., 2010). Another study showed that progestins activate several genes that promote cell transformation, increase cell motility and increase the rate of cancer metastasis (McGowan and Clarke, 1999). Recently, Schramek et al. (2010) and Gonzalez-Suarez et al. (2010) reported that progestin induces Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL), and that higher RANKL may correlate with higher rate of mammary cancer in mice. Kariagina et al. (2010) also reported that in cells co-expressing estrogen and progesterone receptors, estrogen and progesterone increase expression of amphiregulin, and that the two hormones co-operate to stimulate robust proliferation of hormone-dependent mammary cancer. Thus, there is an urgent need to identify compounds which can be used in a clinical context to counteract the tumor-promoting effects of progestins in breast cells.

Apigenin is a low molecular weight flavonoid, common in fruits, vegetables, nuts and plant-derived beverages that inhibit growth of human cancer cells in vitro and in vivo (see Review by Patel et al., 2007). We previously showed that apigenin prevents and/or delays the appearance of medroxyprogesterone acetate (MPA)-dependent DMBA-induced tumors in vivo (Mafuvadze et al., 2011). In the present study, we demonstrate that apigenin inhibits progression and development of MPA-dependent BT-474 xenograft tumors in nude mice by a mechanism involving apoptosis of proliferating human breast cancer cells.

3.4. MATERIALS AND METHODS

3.4.1. *Animals***:** Female athymic *nu/nu* nude mice, 5 to 6 weeks old (20-22g) were purchased from Harlan Sprague-Dawley, Inc. Mice were housed in a laminar air-flow cabinet under specific pathogen-free conditions. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current federal regulations and standards. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri (Columbia, MO) and were in accordance with procedures outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 85-23).

3.4.2. *Experimental protocol*: We followed the protocol previously described by Liang et al. (2007; 2010), proceeding as shown in Figure 3.1. Briefly, nude mice were implanted with 17-β-estradiol pellets (1.7mg/pellet, 60-day release, Innovative Research of America, Sarasota, FL) 48 hours prior to subcutaneous inoculation with BT-474 tumor cells into both flanks (Liang et al. 2007; 2010). Tumors were serially measured every 3 days using a digital caliper and tumor volume calculated using the formula (length x width x height) x $\pi/6$ (El Etreby and Liang, 1998). The mean $\pm$ SE was then calculated for each experimental group for each time point. As previously reported by Liang et al. (2007), in this model tumors regress following an initial burst of growth. Once the majority of tumors began to regress, animals were split into 2 groups [MPA (n=6) and MPA + apigenin (n=6)], both of which were implanted with pellets containing MPA (10mg/pellet, 60-day release) on day 8 following inoculation with tumor cells. A third group (n=5) was implanted with placebo pellets.

71

Apigenin was dissolved in 50% DMSO, 15% ethinyl alcohol and 35% phosphate buffered saline. Starting on day 25 after inoculation with tumor cells, apigenin (50 mg/kg) or vehicle were administered daily by intra-peritoneal injection for a total of 21 injections. Animals were sacrificed and tumors were collected 3 h after the last injection. Figure 3.1 summarizes the entire experimental protocol for this study.
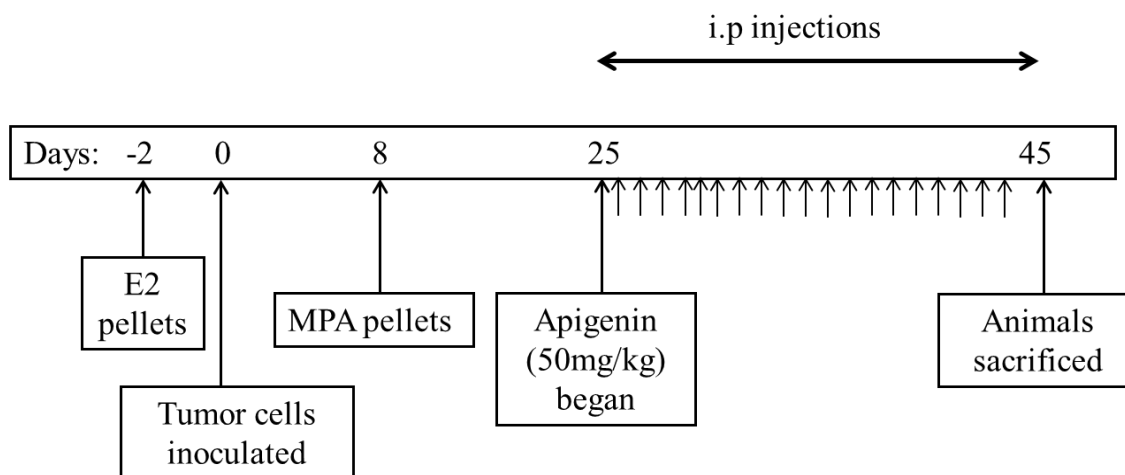
Figure 3.1. Experimental Protocol and Timeline. Nude mice were inoculated with BT-474 cells two days

after subcutaneous implantation of pellets containing 17ß-estradiol (1.7 mg/60-day release pellet). On day 8

post inoculation, animals were implanted with either MPA pellets (25 mg/60-day release pellet) or placebo

pellets. On day 25 animals given MPA pellets were split into two groups, which received daily i.p.

injections of either apigenin (50mg/kg) or vehicle, until day 45 when the experiment was terminated.

Animals were observed daily for toxicity, and tumor volume was measured every 3 days.

3.4.3. *Immunohistochemistry*: Tumors were collected immediately after sacrifice and placed in 4% paraformaldehyde for immunohistochemical (IHC) staining or frozen in liquid nitrogen for future analysis. Collected tissues were then processed for IHC staining using standard procedures previously described by Liang et al. (2007). Stained sections were assessed and analyzed for expression of the following proteins; VEGF, VEGFR-1, VEGFR-2, PR, ERα, ERβ, Ki-67, CD31 and RANKL. Antibodies and dilutions were as follows: anti-progesterone receptor (PR) antibody (1:50 dilution [A0098], DAKO, Carpinteria, CA), anti-estrogen receptor-α (ER)α (1:300 [sc-542], Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ERß (1:25 [MCA1974S], AbD Serotec), anti-VEGF antibody (1:100 [sc-152], Santa Cruz Biotechnology, Inc.), anti-VEGFR-1 antibody (1:50 [Flt-1, H225, sc-9029], Santa Cruz Biotechnology, Inc.), anti-VEGFR-2 antibody (1:50 [Flk-1, ab2349], Abcam, Inc., Cambridge, MA), anti-RANKL antibody (1:100 [sc-7628], Santa Cruz Biotechnology, Inc), anti-CD31 antibody (1:100 [ab28364], Abcam, Inc., Cambridge, MA), anti-Ki-67 antibody (1:600 [RB1510-P], Fisher Scientific), anti-HER2/neu antibody (1:250 [sc-284], Santa Cruz Biotechnology, Inc).

Tumor samples were stained immunohistochemically and relative staining intensity was quantified using morphometric software (Fovea Pro 3.0, Reindeer Graphics, Asheville, NC). Four images were recorded at 20x magnification per given tumor, and threshold image intensity was adjusted for measurement in pixels. To determine microvessel density (MVD), 4 representative photographs of CD31-labelled sections were taken at 20x magnification for at least 5 tumor samples per treatment group. The total number of vessels per field was counted. A vessel was defined as an open lumen

with adjacent CD31-positive cells. MVD was then expressed as mean microvessel number per field ± SEM.

3.4.4. *Proliferation index*: Ki-67 staining was quantified as previously described by Burcombe et al. (2006). A minimum of 5 randomly-selected 40X high-power fields were examined, each containing representative sections of the tumor with at least 1500 total cells. Epithelial cells with nuclear Ki-67 immunoreactivity were counted and the proliferation index determined (i.e., number of Ki-67 positive cells divided by total cells).

3.4.5. *TUNEL staining*: Tumor tissues were stained by terminal deoxy-nucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), (*in situ* cell death detection kit, cat no: 11684817910, Roche) and counterstained with haematoxylin and eosin (H&E). The procedure was performed following instructions provided by the manufacturer and in reference to previous studies (Sun et al., 2003). A minimum of 3 randomly-selected high-power fields containing representative sections of each tumor were imaged. At least 5 tumors were examined per treatment group. TUNEL-positive cells and total cells were counted and percent apoptotic cells calculated.

3.4.6. *sRANKL Assay:* Human sRANKL levels were measured in supernatant collected from cultured BT-474 cells using an enzyme-linked immunoassay kit (RD193004200R, BioVendor, Czech Republic) with a sensitivity of 0.4pmol/L, an intra-assay coefficient of variation (CV) of 7% and an inter-assay CV of 11%. Supernatant was collected from cultured BT-474 cells treated with vehicle (DMSO), MPA (10nM) ± either apigenin

(100μM) or RU-486 (1μM) and apigenin or RU-486 alone. All experiments were performed in triplicate following the manufacturer's recommended protocol, and each sample was analyzed in duplicate.

3.4.7. *Statistical analysis*: Statistical significance was tested with one-way ANOVA using SigmaStat Software version 3.5 (Sigmastat Software Inc., Richmond, CA). The assumption of ANOVA was examined and a nonparametric measure based on ranks was used as needed. Values are reported as mean ± SEM. When ANOVA indicated a significant effect ($p<0.05$), Student-Newman-Keuls posthoc-test was used to compare the means of individual groups. When normality test failed, significance was determined by the Kruskal-Wallis test (one-way ANOVA by ranks) followed by the Dunn test as a posthoc test.

3.5.   RESULTS

3.5.1.   Apigenin prevents progression of MPA-dependent BT-474 xenograft tumors in nude mice

We previously developed a mouse model for studying estrogen- and MPA-dependent xenograft tumors in mice (Liang et al., 2007; 2010). Here, we examine the effect of apigenin on estrogen and MPA-implanted mice, which develop spontaneous xenograft tumors after subcutaneous inoculation with human BT-474 cells, an aggressive progestin-dependent HER2-positive and p53-defective human breast cancer cell line (Liang et al., 2007; 2010). The dose regimen and experimental protocol used for this study is shown in Figure 3.1. Tumor-bearing animals were injected daily with apigenin (50mg/kg, i.p.) or vehicle on days 25 to 45 after tumor cell inoculation. As shown in Figure 3.2A, tumor size reached a stable plateau rapidly after apigenin dosing began, while tumors grew at a fairly constant rate until animal sacrifice at day 45 in animals dosed with vehicle. The mean tumor size difference reached statistical significance after 12 injections of apigenin. At sacrifice (i.e., day 45), mean tumor size was 208 ± 50 mm^3 in animals that were injected with vehicle, 70 ± 10 mm^3 in animals injected with apigenin, and 32 ± 7 mm^3 in control animals (inoculated with tumor cells but not implanted with an MPA pellet) (Figure 3.2A). Representative photographs at sacrifice of animals in the three treatment groups are shown in Figure 3.2B. Average animal body weight was similar for all three treatment groups (Figure 3.2C) and no changes in behavior, eating habits or mobility were observed in animals treated with apigenin (data not shown).

As previously noted in the rat model for DMBA-induced MPA-accelerated mammary gland tumors (Mafuvadze et al. 2011), apigenin did not block MPA-induced hyperplasia in the tumor-free areas of mammary glands of nude mice (Figure 3.3), suggesting that apigenin specifically inhibits proliferation of tumor cells.

A.

B.

Control MPA MPA + AP
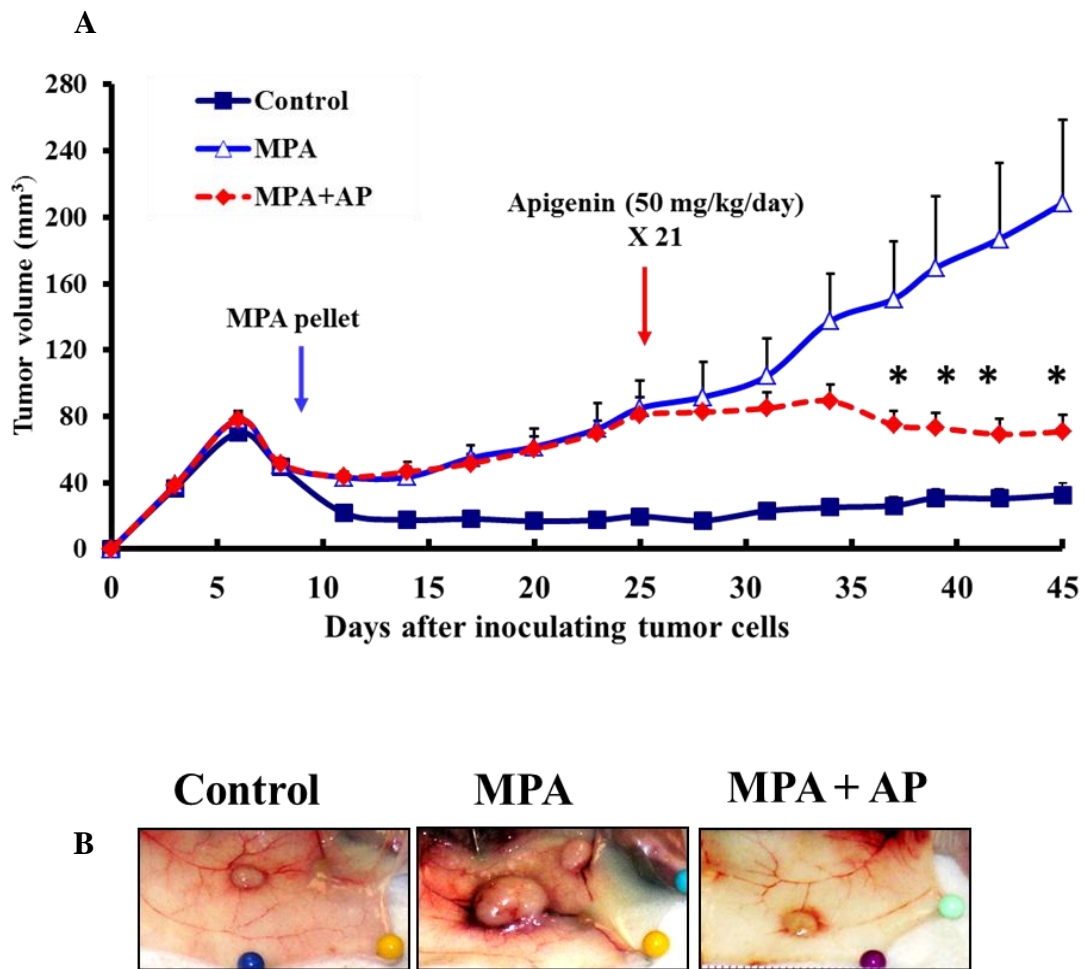
Figure 3.2. A. Apigenin treatment inhibited MPA-dependent growth of BT-474 xenograft tumors.

B. Representative tumors for the different treatment groups at the termination of study.
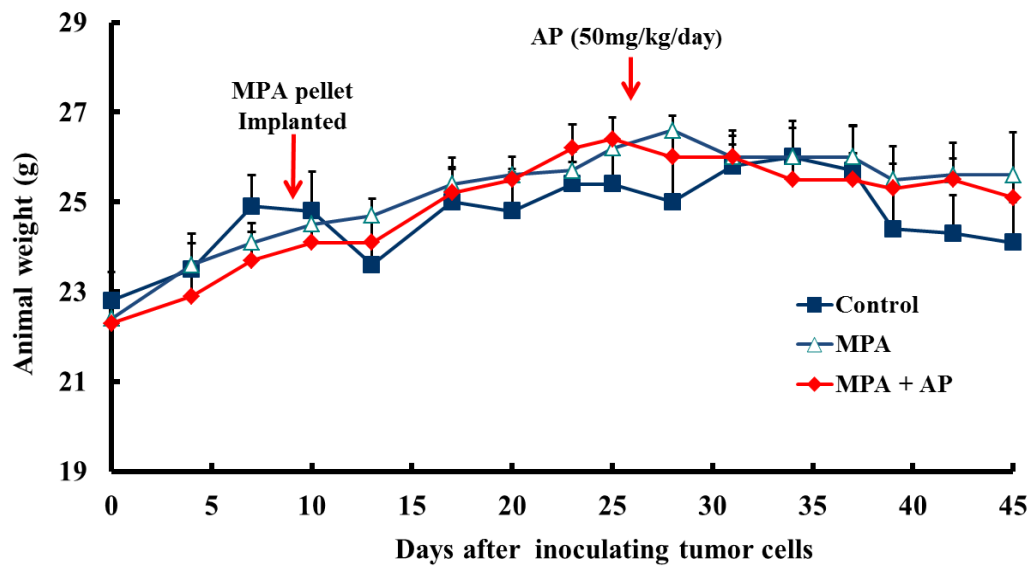
Figure 3.2C. Animals weight among treatment groups throughout the study timeline. There were no significant weight differences among the three treatment groups

MPA   **MPA+AP**   **A**
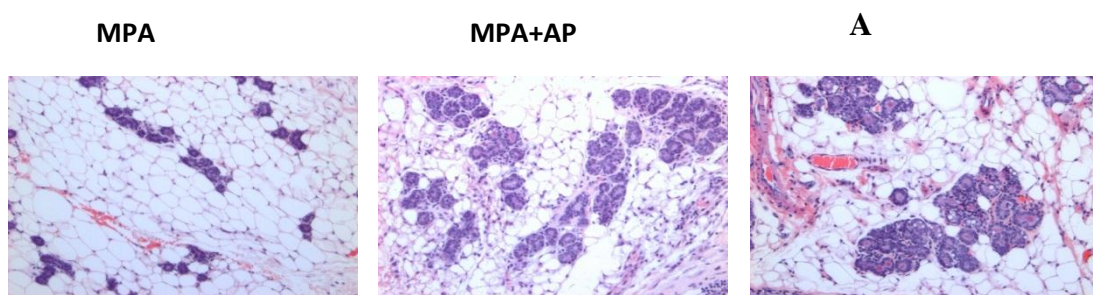
Figure 3.3. Apigenin did not block MPA-induced mammary epithelial hyperplasia in host-mice. H & E staining demonstrating the presence of epithelial hyperplasia in animals given MPA alone and MPA + apigenin. Hyperplasia was absent in control animals. Images taken at 10x magnification.

3.5.2. Immunohistochemical analysis for Her2/neu

BT-474 cells express a high level of Her2/neu, resulting in aberrant growth factor signaling and rapid cell proliferation (Saal et al., 2005). Because previous studies showed that apigenin reduces the level of Her2/neu in breast cancer cell lines overexpressing it (Way et al., 2004), the effect of apigenin on Her2/neu expression was examined in BT-474 xenograft tumors. As shown in Figure 3.4A, apigenin significantly reduced the level of Her2/neu in xenograft tumor cells, suggesting that it reduces growth factor signaling via Her2/neu in these cells.

3.5.3. Histological and immunohistochemical analysis of proliferation and apoptotic markers

Apigenin could reduce the rate at which a tumor's size increases by increasing the rate of tumor cell death and/or by decreasing the rate of tumor cell proliferation. To determine which mechanism is relevant to the effect of apigenin on MPA-dependent BT-474 xenograft tumors in nude mice, the fraction of cells undergoing apoptosis was measured in tumors from apigenin-treated and untreated mice by TUNEL assay. The results show that the fraction of apoptotic cells was significantly higher ($P<0.05$) in tumors from apigenin-treated mice ($34 \pm 3\%$) than in tumors from untreated mice ($7 \pm 1\%$) or from control mice ($14 \pm 2\%$) (Figure 3.4B). Conversely, apigenin slightly lowered immunoreactivity for Ki-67, a marker for cell proliferation, to $47 \pm 2\%$ from $53 \pm 1\%$, with 45 ± 3 % control cells staining positively for Ki67 (Figure 3.4C). These data suggest that the anti-tumor effects of apigenin primarily reflect its ability to increase the

fraction of tumor cells that undergo apoptosis, while it has a smaller effect on tumor cell proliferation.
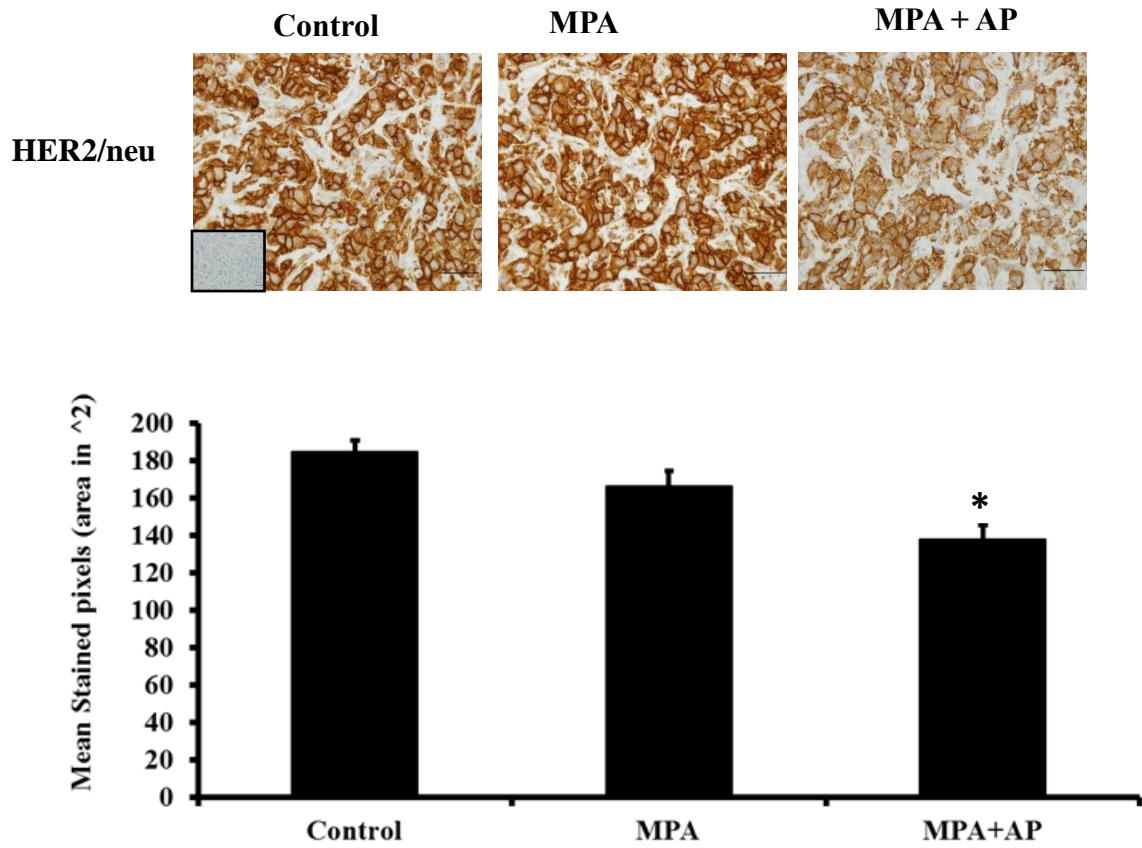
Figure 3.4A. Immunohistochemical analysis of apigenin-treated and vehicle-treated MPA-induced tumor samples. Representative sections showing Her2/neu at 40x magnification. Positive staining was quantified (see Materials and Methods) and results are shown as bar graphs. Error bars show standard deviation. Asterisk (*) indicates significantly different from other treatment groups, $p < 0.05$.
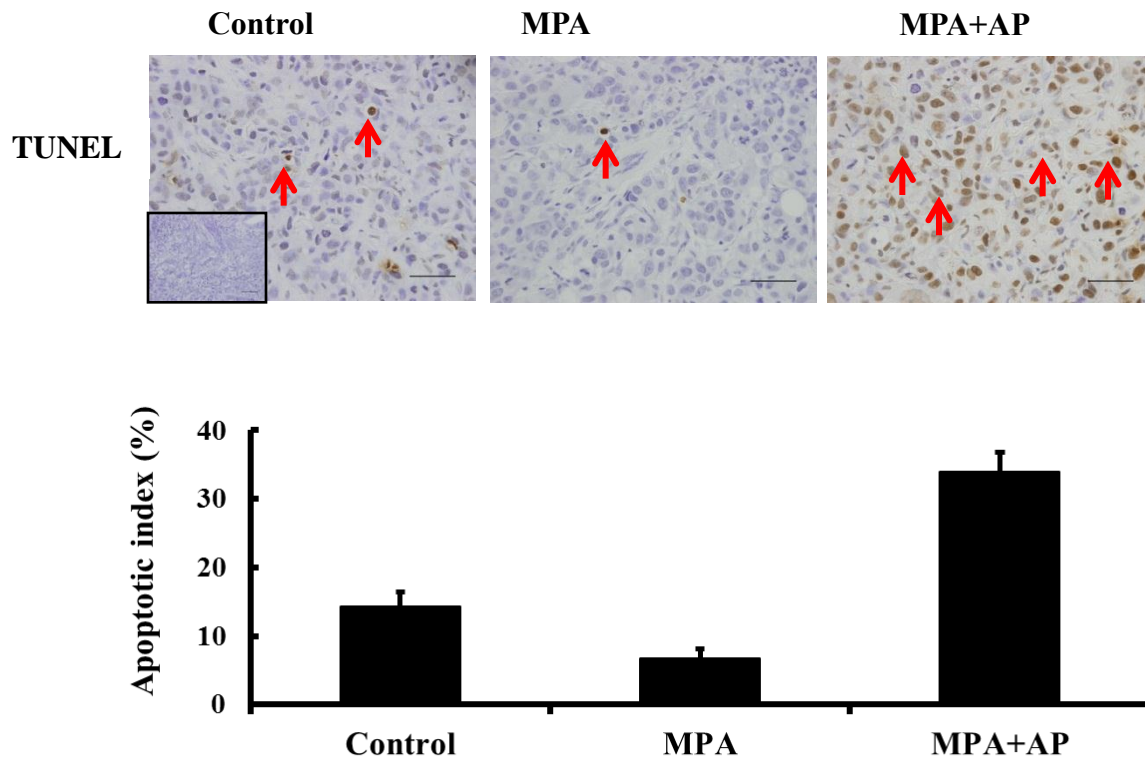
Figure 3.4B. Top panel. Representative sections showing apigenin-induced apoptosis in BT-474 xenograft tumors. Apoptotic cells manifested as brownish staining in the nuclei (red arrow, MPA image). Bottom panel shows apoptotic index. Number of TUNEL positive cells were counted in a field and expressed as a percentage of total cells. * Significantly different from the other treatment groups, $p<0.05$
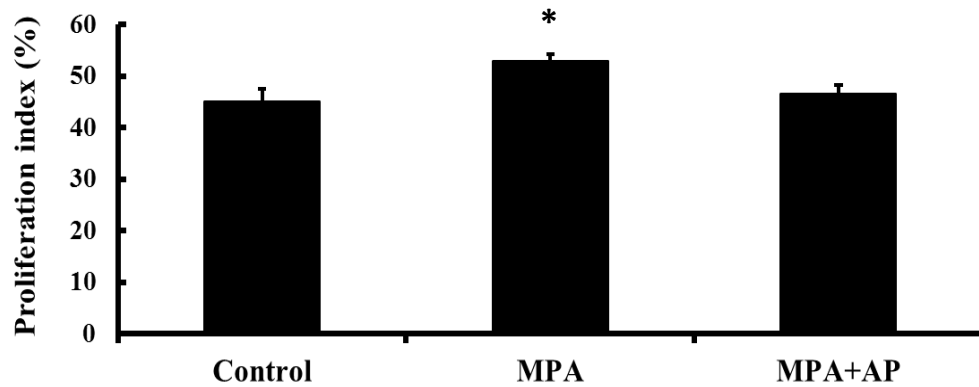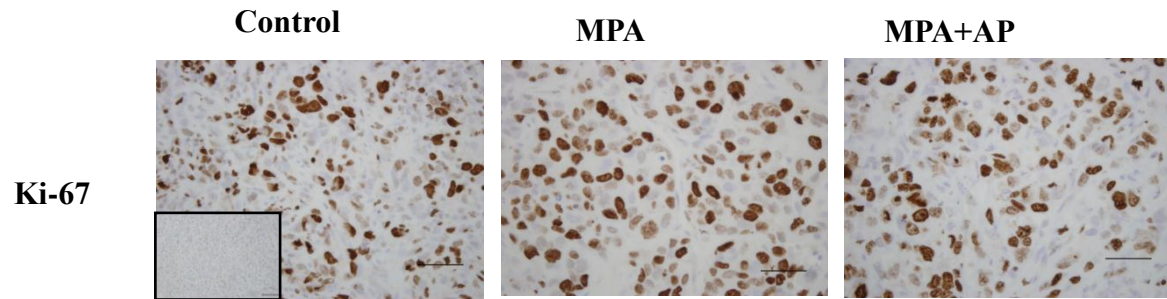
Figure 3.4C. Representative Ki-67 stained slides. The proliferation index as determined by number of Ki-67 positive cells expressed as a percentage of total number of cells in a field. * Significantly different from the other treatment groups, p<0.05.

3.5.4. Immunohistochemical analysis for PR

Our previous studies showed that RU-486, an anti-progestin, blocks the progression and growth of progestin-dependent BT-474 xenograft tumors, suggesting that PR activity is essential (Liang et al., 2007). To rule out the possibility that apigenin treatment leads to complete loss of PR as a mechanism for explaining apigenin mediated tumor regression we assessed levels of PR in tumor sections. Our findings concur with those of previous studies (Dennis et al., 2005; Benakanakere et al., 2010), showing that the number of PR-positive cells is significantly lower in mice treated with both MPA, or MPA and apigenin ($p<0.05$) than in controls (Figure 3.5). Such a partial loss of PR has been associated with functional PR activity (Ellmann et al., 2009) and suggests that apigenin is unable to block partial loss of PR, and also that apigenin treatment does not lead to complete loss of PR that can explain the ability of apigenin to inhibit tumor growth in this model.

3.5.5. Immunohistochemical analysis of angiogenic factors/blood vessel density

In contrast to previous studies from our laboratory (Liang et al, 2010), the amount of immunoreactive VEGF was similar in tumors from control animals and MPA-treated animals (Figure 3.6A). However, the amount of immunoreactive VEGF was significantly lower in xenograft tumors from apigenin-treated animals. Additional experiments confirmed that VEGF receptor 1 (VEGFR-1 or flt) and VEGF receptor 2 (VEGFR-2 or flk) were expressed at a similar level in animals treated with or without apigenin (data not shown), and, based on CD31 immunoreactivity, maintained a similar blood vessel density in the tumor (Figure 3.6B), with average microvessels per field in the range 19 to 22 for all three treatment groups (i.e., control (22 ± 2), MPA (20 ± 2) and MPA+AP (19 ± 1)).

However, the lumen size [internal diameter] of tumor blood vessels was smaller in controls and apigenin-treated animals, suggesting that apigenin might reduce blood flow in BT-474 xenograft tumors.
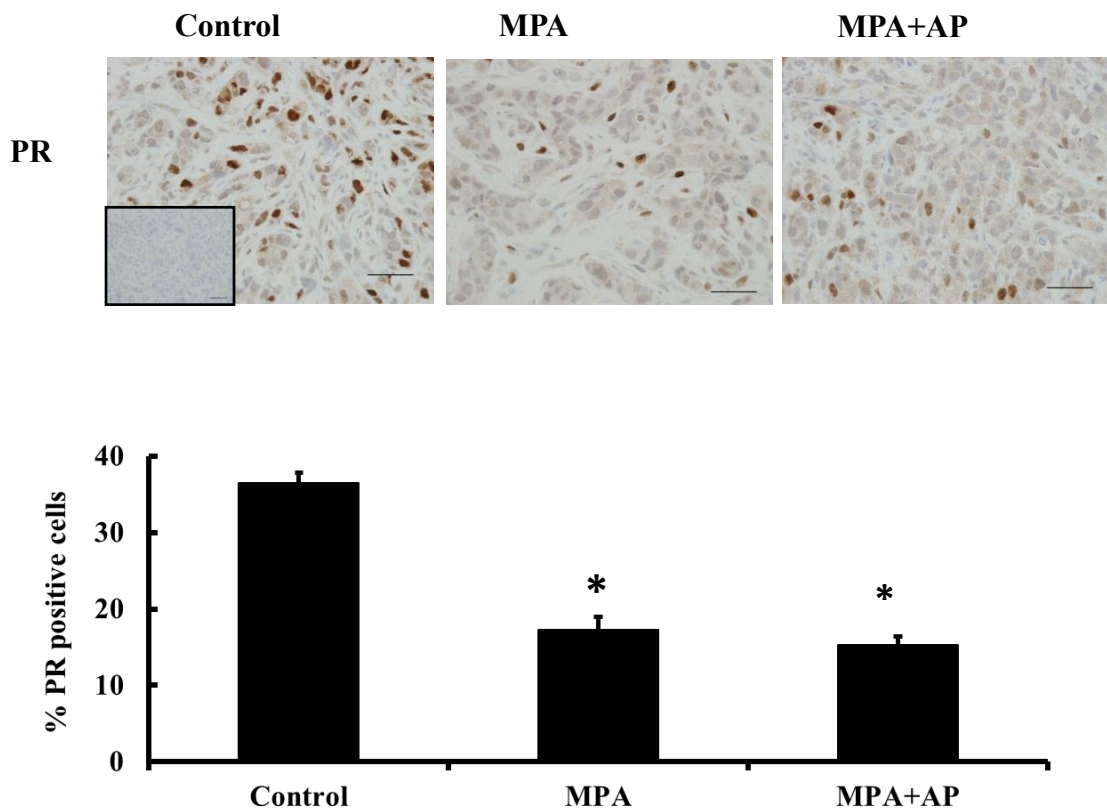
Figure 3.5. Apigenin does not affect MPA-dependent loss of PR in BT-474 tumor xenografts. Upper panel: Immunohistochemical staining for PR in representative tumor sections from control and apigenin-treated mice. Lower panel, Positive staining was quantified (see Materials and Methods) and data are shown as a bar graph. Error bars show standard deviation. Asterisk (*) indicates significantly different from control ($p < 0.05$).
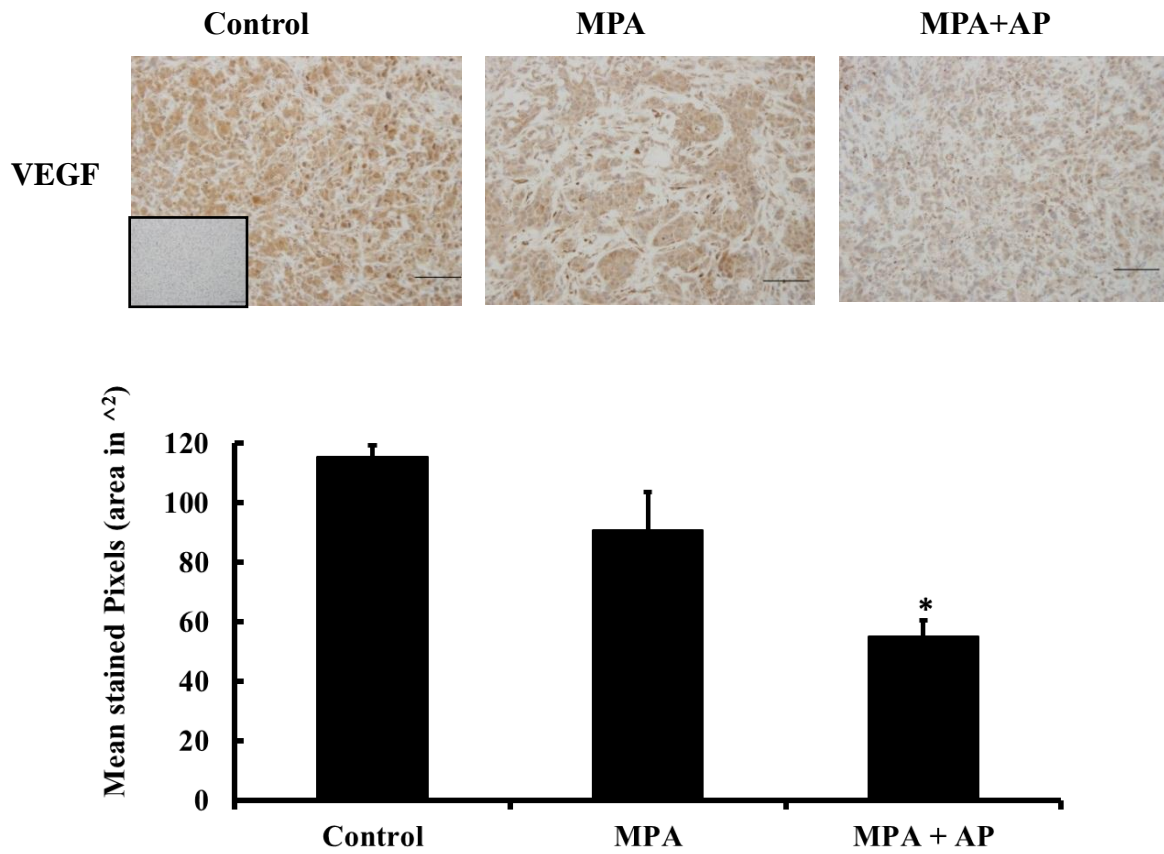
**Figure 3.6A. Immunohistochemical analysis of VEGF in BT-474 xenografts treated with apigenin.** Representative tumor samples were analyzed for VEGF. Positive staining was quantified (see Materials and Methods) and data is shown as a bar graph. Error bars show standard deviation. Asterisk (*) indicates significantly different from control (p<0.05).
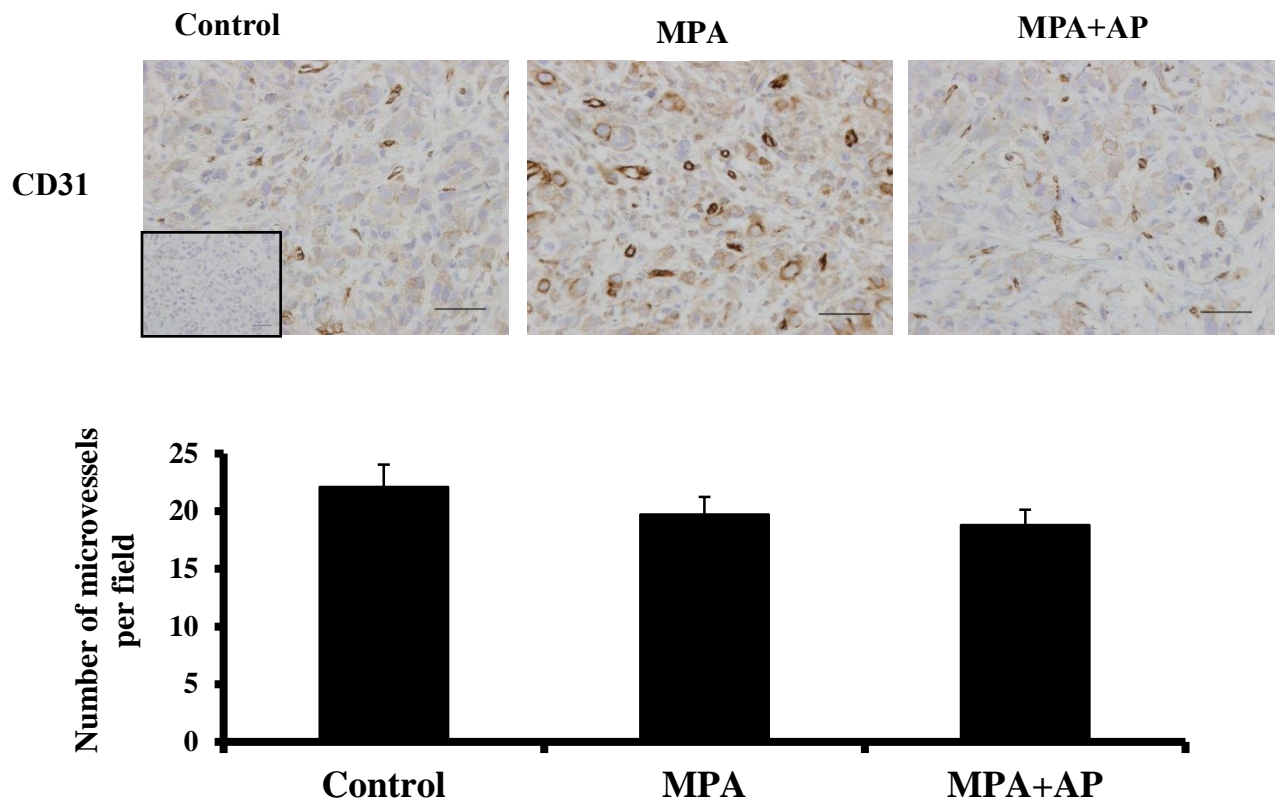
Figure 3.6B. Immunohistochemical analysis of CD-31 in BT-474 xenografts treated with

apigenin. Representative tumor samples were analyzed for CD-31. Microvessels were counted per

field (see Materials and Methods) and data is shown as a bar graph. Error bars show standard error

mean.

3.5.6. Immunohistochemical and ELISA analysis of RANKL

Recent studies in rodents showed that progestin induces expression of RANKL, and that RANKL may play a significant role in mammary tumor development (Schramek et al 2010; Gonzalez-Suarez et al., 2010). Therefore, the effect of apigenin on expression of RANKL in xenograft tumor-bearing mice was examined using an immunohistochemical approach. In contrast to previous studies in mice, we found that MPA significantly inhibits the production of RANKL in human BT-474 xenograft tumors ($p<0.05$) and this effect was independent of co-treatment with apigenin (Fig 3.7A). Similarly, when total soluble human RANKL (sRANKL) was evaluated in cultured BT-474 cells, we observed that MPA lowered the level of sRANKL, although this effect was not blocked by RU486, but was blocked by 100 μM apigenin in cultured cells (Figure 3.7B).
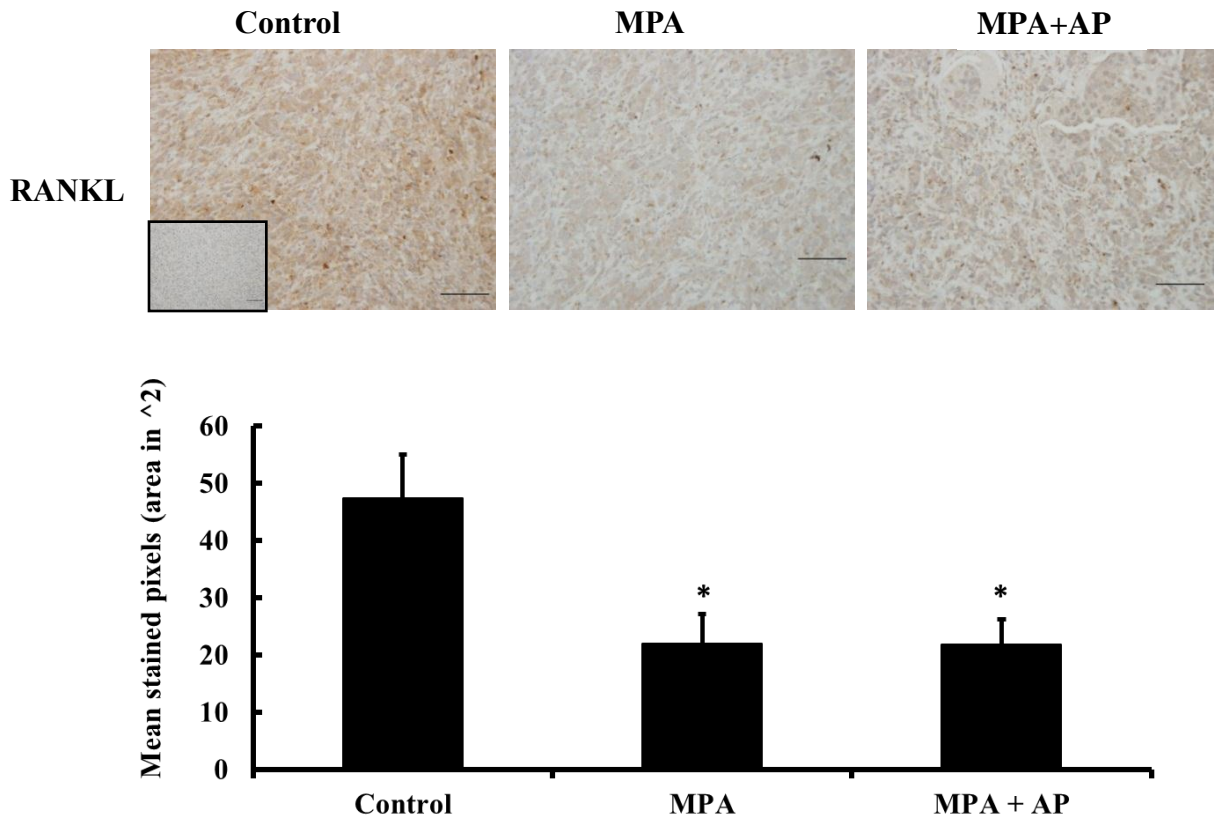
Figure 3.7A. **Quantification of RANKL expression in BT-474 xenograft tumors.** RANKL expression was measured in xenograft tumors from mice treated with or without apigenin and or in control mice. Data is shown as a bar graph. Asterisk (*) indicates significantly different from control, $p < 0.05$.

Total sRANKL in BT-474
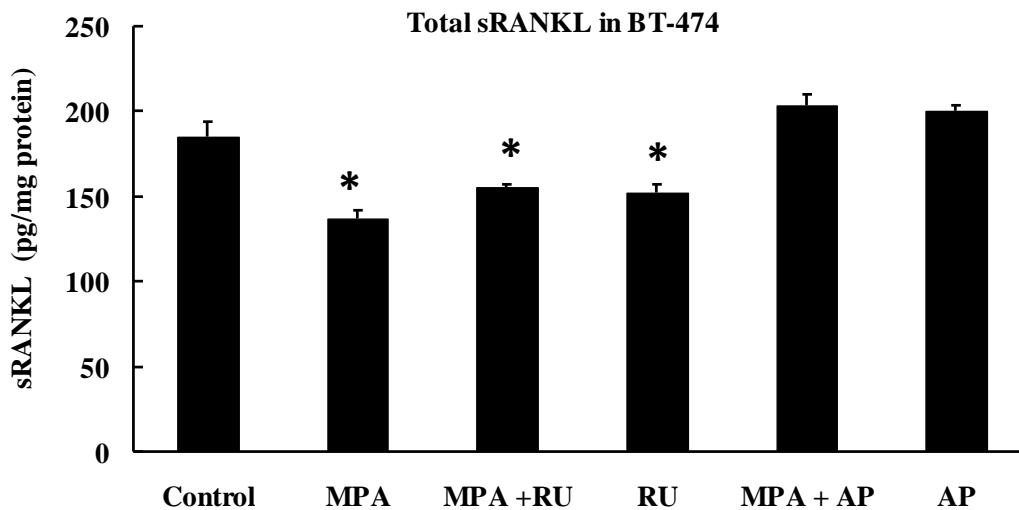
Figure 3.7B. **Quantification of RANKL expression in BT-474 cultured cells.** BT-474 cells were treated with 10 nM MPA ± 1μM RU or 100μM AP for 18 hours at 37°C. RANKL was quantified in media from BT-474 cells grown in liquid culture. RU-486 and apigenin were administered 30 min prior to addition of MPA. Soluble RANKL was measured in the supernatant of cultured cells using ELISA (see Materials and Methods). Results are expressed as mean ± SEM (n = 6). Asterisk (*) indicates significantly different from control, $p < 0.05$.

3.6. DISCUSSION

Our previous study demonstrated that apigenin reduced the frequency of DMBA-induced, progestin-accelerated mammary tumors in the rat (Mafuvadze et al., 2011). The present study examines the effect of apigenin on growth and progression of progestin-dependent BT-474 xenograft tumors in nude mice. BT-474 cells are an aggressive progestin-responsive highly metastatic human breast cancer cell (Liang et al., 2007; 2010). Under the dose regimen used here (21 daily 50 mg/kg subcutaneous injections of apigenin), apigenin significantly inhibited growth of BT-474 xenograft tumors. The dose selected was from a previous study (Chen et al., 2007) though higher levels of apigenin might prove more potent against tumor growth, though this remains to be established. TUNEL assays showed that apigenin induces tumor cell apoptosis, but only modestly inhibits tumor cell proliferation. Wang et al (2011) reported similar findings in colorectal cancer xenografts. Apoptosis is also induced by other tumor growth-inhibiting phytochemicals (Zhao et al., 2002) and anticancer drugs such as 5-flurouracil (Hsueh et al., 1998) and cisplatin (Henkels and Turchi, 1999). Previous studies demonstrated that apigenin induces apoptosis, and suggest the following mechanisms that may be involved: induction of caspase3 (Vargo et al., 2006), induction/activation of p53 (Zheng et al., 2005), up-regulation of FADD (Wang et al., 2011), and, in MDA-MB-435 cells, stimulation of proteasome-dependent degradation of HER2/neu (Way et al., 2004). Apigenin also was associated with reduced tumor cell Her-2-neu immunoreactivity in the present study. Although it is possible that apigenin stimulates proteasome-dependent degradation of Her2/neu in BT474 xenograft tumors, there are other possible mechanisms that might explain reduced immunoreactivity and its consequences. These include

reduced Her2/neu autophosphorylation, binding of apigenin to Her2/neu, or interactions that block events downstream of Akt or PI3-kinase. The effect of reduced Her2/neu on tumor cell growth may relate to its potential role in maintaining human breast cancer stem cells (Korkaya et al., 2008).

As expected, MPA stimulates proliferation of BT-474 xenograft tumor cells, although the proliferation index was modest. This supports the hypothesis that progestins may promote the proliferation of latent breast tumor cells, which may contribute to the increased breast cancer risk associated with progestin-containing HRT (Hyder et al., 2001; Liang et al., 2007; Joshi et al., 2010). Apigenin blocked MPA-induced proliferation, reducing levels of tumor cell proliferation to those observed in controls. This is consistent with previous studies, and a number of mechanisms by which apigenin could inhibit proliferation have been proposed (Ujiki et al., 2006; Patel et al., 2007; Zhao et al., 2011)

In accord with previous studies (Dennis et al., 2005; Benakanakere et al., 2010), we observed that MPA inhibits expression of PR in human breast cancer cells. In this study, this effect of MPA is observed in BT-474 xenograft tumors in apigenin-treated and untreated animals. As described previously, exogenous progestins stimulate proteasomal degradation of PR (Ellmann et al., 2009) which is associated with functional PR activity. Apigenin does not appear to modulate the effects of MPA on PR in tumor cells and thus it is unlikely that it modulates functionality of PR in tumor cells though this remains to be firmly established.

Contrary to our earlier findings (Liang et al., 2007; 2010), MPA did not stimulate expression of VEGF in BT-474 xenograft tumors in this study, possibly indicating that

the level of VEGF is differentially affected by MPA at different stages of tumor growth. However, in this study, basal VEGF immunoreactivity was lower in tumors from apigenin-treated animals. Since VEGF has been shown to be involved in survival and proliferation of mammary tumor cells (Lee et al., 2007; Su et al., 2007), it is possible that loss of VEGF leads to loss of tumor cells. Furthermore, although blood vessel density was similar in tumors from all animal groups, apigenin-treatment correlated with smaller blood vessel lumen size. It is therefore conceivable that apigenin restricts tumor blood supply, although the mechanism for this possible effect is not yet known.

Recent studies show that MPA induces RANKL in DMBA-induced mammary tumors, which is consistent with a role for RANKL in the growth of progestin-dependent breast cancer (Schramek et al 2010; Gonzalez-Suarez et al., 2010). Because MPA stimulates proliferation of BT-474 xenograft tumor cells, we examined whether RANKL might also be induced by MPA in BT-474 cells. However, in contrast to findings in rodents, RANKL expression was lower in BT-474 xenograft tumor cells from animals treated with MPA (with or without apigenin) than in controls (i.e., not exposed to MPA). A similar effect was seen in vitro; namely, exogenous MPA inhibits expression of RANKL in cultured BT-474 cells, and this effect was not blocked by RU-486 , but it was blocked by apigenin. The significance of this observation remains to be determined. A previous study indicated that membrane-associated PR is insensitive to the negative regulatory effect of antiprogestins (Bottino et al., 2011), thus it is possible that membrane-associated PR mediates MPA-dependent down-regulation of RANKL. Moreover, the effect of progestin-dependent RANKL on human breast cancer progression is controversial (Petrie and Hovey 2011), and it is possible that progestin

regulates expression of RANKL via different mechanisms in murine and human

mammary cells. Earlier studies reported that serum levels of RANKL are unaffected by

progestin-containing HRT in postmenopausal women (Di Carlo et al., 2007). However, it

is possible that different tumors (or different cells within a single tumor) might

differentially induce or suppress expression of RANKL in response to MPA. Further

studies are needed; in particular, the effect of progestin on expression of RANKL should

be evaluated in other human breast cancer cells. It is worth noting that progesterone has

strain-specific effects on RANKL expression in mice (Aupperlee et al., 2009).

Although this study shows that apigenin blocks breast cancer xenograft tumor

growth by inducing apoptosis, apigenin does not affect proliferation and hyperplasia of

ducts and lobular epithelial cells in the normal mammary gland of nude mice. Similar

observations were made by Mafuvadze et al. (2011) in the context of the DMBA-induced

progestin-accelerated rat mammary tumors and by Gupta et al. (2001), who suggested

that apigenin may exert different effects on prostate cancer cells than on normal cells. By

virtue of its ability to selectively induce apoptosis in rapidly growing breast cancer cells,

while having no effect on normal mammary cells, apigenin would seem to be an

extremely promising chemotherapeutic agent.

In conclusion, we show that apigenin inhibits the growth of MPA-dependent BT-

474 xenograft tumors, both by inducing apoptosis and by inhibiting proliferation of tumor

epithelial cells. Apigenin may also reduce the production by tumor cells of pro-

angiogenic VEGF, which is essential for growth and maintenance of blood vessels in the

tumor microenvironment. Although the pharmacokinetics and effectiveness of apigenin

in humans need to be evaluated, combination therapy including apigenin and other

chemotherapeutic agents may also warrant further study. For example, apigenin and

tamoxifen or aromatase inhibitors should be explored, because apigenin does not

influence the expression of ER in breast cancer cells in vivo as was demonstrated in the

present study.

The study discussed above demonstrated the therapeutic potential of apigenin in

progestin-dependent human breast cancer. A number of reports in the literature have also

suggested that apigenin may also have preventive potential against tumorigenesis. In this

respect we explored whether apigenin has potential to prevent progestin-dependent breast

cancer using the DMBA-induced and progestin-accelerated rat mammary gland model.

The next chapter describes the results for the preventive study with the rat model.

CHAPTER 4

4.1. Apigenin prevents development of medroxyprogesterone acetate-accelerated 7,12-dimethylbenz(a)anthracene-induced mammary tumors in Sprague-Dawley rats

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Running title: Apigenin delays MPA-accelerated DMBA-induced mammary tumors

Key words: Apigenin, breast cancer, medroxyprogesterone acetate, chemoprevention

4.2.   ABSTRACT

The use of progestins as a component of hormone replacement therapy has been linked to

an increase in breast cancer risk in postmenopausal women. We have previously shown

that medroxyprogesterone acetate (MPA), a commonly administered synthetic progestin,

increases production of the potent angiogenic factor vascular endothelial growth factor

(VEGF) by tumor cells, leading to the development of new blood vessels and tumor

growth. We sought to identify nontoxic chemicals that would inhibit progestin-induced

tumorigenesis, preventing the *in vivo* formation of MPA-induced tumors. We used a

recently developed progestin-dependent mammary cancer model in which tumors are

induced in Sprague-Dawley rats by 7, 12-dimethylbenz (a) anthracene (DMBA)

treatment. The flavonoid apigenin, which we previously found to inhibit progestin-

dependent VEGF synthesis in human breast cancer cells *in vitro*, significantly delayed the

development of, and decreased the incidence and multiplicity of, MPA-accelerated

DMBA-induced mammary tumors in this animal model. Whereas apigenin decreased the

occurrence of such tumors, it did not block MPA-induced mammary tissue hyperplasia.

Apigenin blocked MPA-dependent increases in VEGF in hyperplastic lesions, and

suppressed VEGF receptor-2 (VEGFR-2) but not VEGFR-1 in these lesions. No

differences were observed in estrogen or progesterone receptor levels, or the number of

estrogen receptor-positive cells, within the mammary gland of MPA-treated animals

administered apigenin, MPA-treated animals, and placebo treated animals. However, the

number of progesterone receptor-positive cells was reduced in animals treated with MPA

or MPA and apigenin compared with those treated with placebo. These findings suggest

that apigenin has important chemo-preventive properties for those breast cancers that develop in response to progestins.

4.3. INTRODUCTION

Recent clinical trials and studies demonstrate an increased risk of breast cancer in postmenopausal women undergoing hormone replacement therapy (HRT) that consists of both estrogen and progestin (E+P) compared with women taking estrogen alone or placebo (Ross et al., 2000; Rossouew et al., 2002; Chen et al., 2004). Furthermore, women on a regimen of E+P are more at risk of metastasis and death than those taking estrogen alone (Chlebowski et al., 2010). Progestins are prescribed in HRT regimens to counter the unopposed proliferative effects of estrogen on the uterus that could lead to uterine cancer; however, overwhelming evidence shows that progestins stimulate proliferation of normal and neoplastic breast cells in various species, including humans (Soderqvist et al., 1997; Raafat et al., 2001; Liang et al., 2007; 2010).

A number of mechanisms have been implicated in the progestin-induced increase in breast cancer risk observed in women undergoing HRT, including induction of angiogenic growth factors such as vascular endothelial growth factor (VEGF) (Hyder et al., 1998; Liang and Hyder, 2005), which renders the microenvironment conducive to tumor development and progression. VEGF is considered one of the most important growth factors and its effects have been reported to be vital to the angiogenesis that is integral to the growth and metastasis of tumors (Ferra, 2002), including those that arise in response to progestins (Liang et al., 2007). Recently, other mechanisms have also been proposed to explain progestin-dependent breast tumor development and progression. For

example, in animal models, RANK-L has also been associated with progestin-dependent increased proliferation of breast cancer cells (Gonzalez-Suarez et al., 2010; Schramek et al., 2010), while it has also been reported that medroxyprogesterone acetate (MPA), a progestin commonly used in HRT, increased the stem-like cell subpopulation in mammary glands (Horwitz and Sartorius, 2008). Collectively, these observations suggest that progestins promote the growth of latent breast tumor cells by increasing their proliferative potential and/or promote tumor development through activation and transformation of dormant breast cancer stem cells into intermediate subpopulations, which then differentiate into breast cancer cells.

We have previously shown that progestins accelerate the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in Sprague-Dawley rats (Benakanakere et al., 2006; 2010; Carroll et al., 2010). Whereas the molecular basis of this phenomenon has not been fully established, it is clear that progestins accelerate the tumor development process by increasing VEGF and subsequent angiogenesis in this model (Benakanakere et al., 2006; 2010; Carroll et al., 2010). Thus, it appears that once tumor initiation has occurred, progestins have the capacity to increase tumor cell proliferation, producing palpable tumors sooner. The progestin-accelerated DMBA-induced mammary tumor model is therefore a useful model in which to identify natural "neutraceuticals" that can prevent the development of progestin-accelerated breast cancers. Because HRT is used widely to alleviate the severe symptoms of menopause, the inclusion of neutraceuticals in a drug regimen could well prove beneficial, as these compounds would selectively limit the proliferative effects of progestins in the breast, protecting against the development of frank tumors.

Previous studies have shown that apigenin inhibits the growth of several human cancers (Patel et al., 2007). Apigenin is a low molecular weight flavonoid commonly found in fruits, vegetables, nuts, and plant-derived beverages (Birt et al., 2001). Apigenin exhibits low intrinsic toxicity and appears to have little effect on normal cells (Gupta et al., 2001; Chiang et al., 2006), making it an attractive candidate for chemoprevention. While apigenin has been shown to be effective against several types of tumors (Patel et al., 2007), its ability to act as an antiprogestin as a possible mechanism by which it exerts its antitumor action has not been well-studied. However, we recently demonstrated that apigenin inhibits progestin-dependent VEGF induction in human breast cancer cells *in vitro* (Mafuvadze et al., 2010). These findings provided a strong rationale for us to extend our studies to an *in vivo* model and examine apigenin's ability to inhibit progestin-accelerated DMBA-induced mammary tumors. Herein we provide evidence that apigenin significantly delays the appearance of and decreases the incidence and multiplicity of MPA-accelerated, DMBA-induced mammary tumors, though it is unable to prevent the formation of MPA-induced hyperplastic mammary lesions. We also show that at least one of the mechanisms by which apigenin exerts its preventive effects is through suppression of progestin-induced VEGF and VEGFR-2 expression, both of which are vital components of the angiogenic pathway and VEGF has been shown to be under progestin control (Hyder et al., 2001; Ferra, 2002).

4.4. MATERIALS AND METHODS

4.4.1. *In Vivo Studies***:** Intact 40- to 45-day-old female Sprague-Dawley rats (Harlan Breeders, Indianapolis, IN) were used in this study. The rats were maintained according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International under conditions of 12-hour light/dark cycles and *ad libitum* access to food and water. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri (Columbia, MO) and were in accordance with procedures outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 85-23). Each experiment was performed at least twice.

4.4.2. *Apigenin***:** Apigenin (Indofine Chemical Company, Hillsborough, NJ) was dissolved in a dimethyl sulfoxide (DMSO)/PBS mixture (1:4) to produce the required concentration. Fresh apigenin was prepared every two days, with the remaining apigenin solution stored at -20°C for use the following day.

4.4.3. *Experimental Design***:** We followed a protocol previously established by our laboratory for progestin-acceleration of DMBA-induced mammary tumors in rats (15-17; Figure 4.1). Briefly, 45- to 50-day-old Sprague-Dawley rats were given a single dose of 20 mg DMBA (Sigma, St. Louis, MO) in vegetable oil by gavage (on day 0). On day 28 post-DMBA administration, animals were anesthetized and implanted with a single 25 mg-60 day release MPA or placebo pellet (Innovative Research, Sarasota, FL) subcutaneously on the dorsal part of the neck. The dose of apigenin used initially (50

103

mg/kg) was based on a previous report (Chen et al., 2007), and was administered daily via intraperitoneal injection, beginning 21 days after the initial dose of DMBA and continuing for 10-13 days. This was done in order to allow the DMBA-induced tumor initiation process to occur. Control animals from the MPA implanted group and those implanted with placebo pellets only were treated with DMSO/PBS vehicle on the same schedule. Following pellet implantation, animals were palpated every 2 days to detect tumors. The time preceding the appearance of the first tumor (latency) and tumor size, number, and location were recorded. Tumor length (L) and width (W) were measured with a micrometer caliper, and tumor size was calculated using the formula L/2 X W/2 X π (Benakanakere et al., 2006). All animals were sacrificed and the experiment terminated on day 60 and the abdominal mammary tissue lacking tumors were collected.

In order to determine potential mechanisms by which apigenin could exert anticancer effects against MPA-accelerated DMBA tumors, we hypothesized that differences in morphology and protein expression in mammary tissue between apigenin- and nonapigenin-treated animals would best be distinguished if tissues were collected immediately after the last apigenin injection. Therefore, during the course of our experiment as described above, we collected abdominal mammary tissues, devoid of tumors, from randomly selected animals on day 33, which was 5 days after implantation of MPA (or placebo) pellets and 6 hours after the last apigenin injection. We selected 5 days after implantation of MPA pellets as the tissue collection time point because we normally first detect tumors at around this time in this model, and therefore expected sufficient hyperplasia to be present within the mammary glands at this point. To further

confirm the effects of MPA and apigenin, we monitored the remaining animals for tumor development as described above until the study was terminated on day 60.
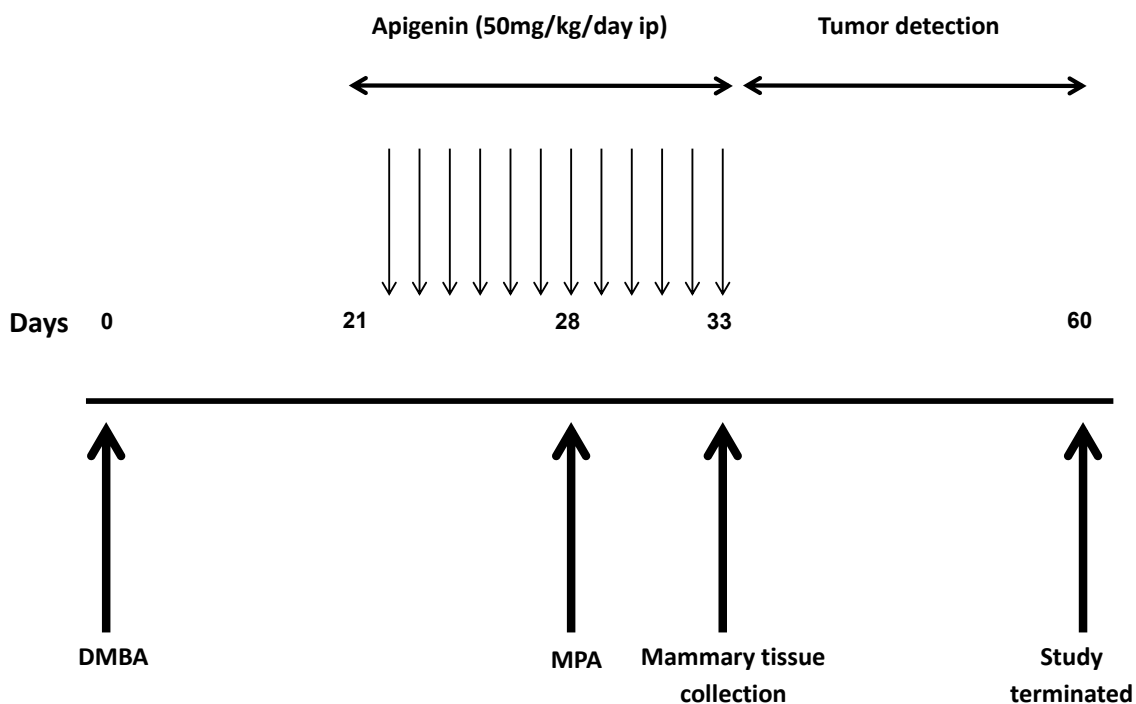
Figure 4.1. Treatment protocol followed in the DMBA-induced MPA accelerated mammary tumor study. Sprague-Dawley rats were treated with DMBA and subsequently implanted with a 25 mg/60-day release MPA (or placebo) pellet on day 28 as described in the Materials and Methods. Apigenin (AP; 50 mg/kg) or vehicle injections were given once daily between days 21 and 31 following DMBA treatment. Following pellet implantation, animals were palpated for tumors every other day through day 60.

4.4.4. *Histology and Immunohistochemical Analysis*: Immunohistochemical staining of mammary tissue was carried out according to a previously described method (Carroll et al., 2010; Benakanakere et al., 2010). The following polyclonal antibodies were used: anti-progesterone receptor (PR) antibody (1:50 dilution [A0098], DAKO, Carpinteria, CA), anti-estrogen receptor (ER) α (1:300 dilution [sc-542], Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ERβ (1:25 dilution [MCA1974S], AbD Serotec) , anti-VEGF antibody (1:100 dilution [sc-152], Santa Cruz Biotechnology, Inc.), anti-VEGFR-1 antibody (1:50 dilution [Flt-1, H225, sc-9029], Santa Cruz Biotechnology, Inc.), anti-VEGFR-2 antibody (1:50 dilution [Flk-1, ab2349], Abcam, Inc., Cambridge, MA). Histologic staining was then quantified using morphometric software (Fovea Pro 3.0, Reindeer Graphics, Asheville, NC). Images were recorded at 20X magnification, and threshold image intensity was adjusted for measurement in pixels. Two sections were prepared for each animal (right and left abdominal mammary glands), and 4 different images per section were obtained, for a total of 8 images per animal. Section images were collected from at least 3 animals in each treatment group. Staining area was recorded and the means determined. For further quantitation of PR and ER, PR-, ERβ-, and ERα-positive cells were counted and results expressed as a percentage of total cells. At least 150-200 cells were counted per section prepared as described above.

4.4.5. *Statistical Analysis***:** Time-to-event data (latency) was analyzed using the LIFETEST procedure in SAS. We estimated survival functions for each group using the Kaplan-Meier method and made comparisons across groups using the Wilcoxon log-rank test. Those animals that had not developed a tumor by the end of the study were censored.

Tumor incidence among groups was compared using a General Linear Mixed Model procedure in SAS (PROC GLIMMIX), where the link function was a logit and the distribution binomial and differences were determined using the LS means statement. Multiplicity and immunohistochemical staining data were analyzed using Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Tukey's procedure as a posthoc test. For all comparisons, $P<0.05$ was regarded as statistically significant. Values are reported as mean ± standard error of the mean (SEM).

4.5. RESULTS

4.5.1. Apigenin inhibits MPA-accelerated DMBA-induced mammary tumors

Using our well-established rodent model in which MPA reduces the latency of DMBA-induced mammary tumors (Benakanakere et al., 2006) we examined the ability of apigenin to prevent the MPA-induced acceleration of tumor development. In brief, 21 days after a single dose of DMBA was given, apigenin was administered daily at a dose of 50 mg/kg body weight as described previously (Chen et al., 2007) for one week. A single pellet containing MPA was then implanted, and treatment with apigenin was continued for an additional 3 days, after which animals were palpated for tumors up to day 60. In those DMBA-treated animals given MPA alone, mean tumor latency was 41.0 ± 1.7 days (mean ± SEM), compared with 58 ± 1.6 days in DMBA-treated animals with only a placebo pellet implanted. However, when DMBA-treated animals were administered both MPA and apigenin, the mean latency period increased considerably, to 55.8 ± 0.3 days (p<0.05), suggesting that apigenin significantly delays the development of MPA-induced mammary tumors. Furthermore, by the time the first tumor was detected in the MPA + apigenin group (day 54), tumor incidence in the group given MPA alone had already reached a peak of 66.7% (Fig. 4.2A). In two different experiments, tumor incidence in MPA + apigenin-treated animals varied from 20-44%, compared with 66.7-87.5% in animals given MPA alone at day 60. The apigenin dose used in these experiments (50 mg/kg/day for 10 days i.p.) was deemed nontoxic, as the animals maintained their body weight throughout the study (data not shown).

When tumor multiplicity was examined, apigenin was found to significantly reduce the number of tumors per tumor-bearing animal (Fig 4.2B). In those animals receiving MPA alone, the average number of tumors was 1.57 ± 0.4, compared with 1.25 ± 0.3 for the placebo group, and 1.0 in animals administered MPA + apigenin.
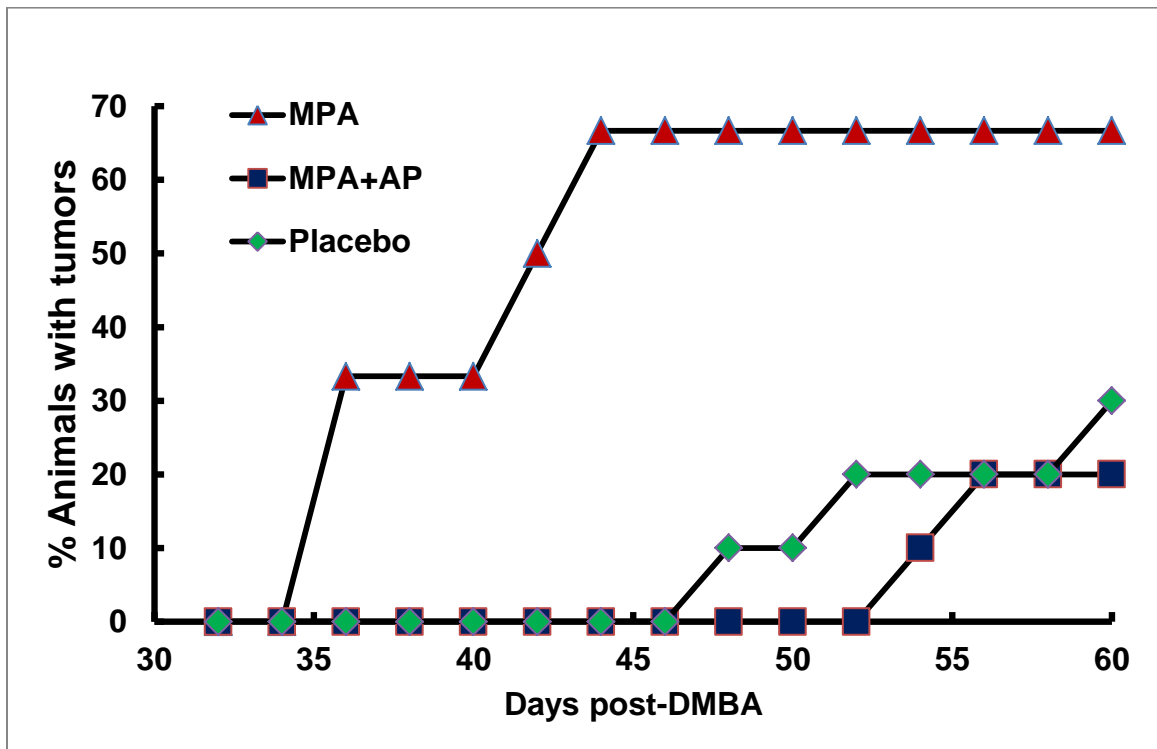
Figure 4.2A. Preventive effect of apigenin on MPA-accelerated DMBA-induced mammary tumors.
The percent of animals with tumors in each experimental group at each time point is presented (n=6-10 animals/group). Tumor latency was assessed using LIFETEST procedure in SAS as described in the Materials and Methods; * indicates p<0.05 compared with tumor latency in the MPA-treated group. Tumor incidence was determined using the General Linear Mixed Model procedure in SAS as described in the Materials and Methods; ** indicates P<0.05 compared with tumor incidence at day 60 in the MPA-treated group.
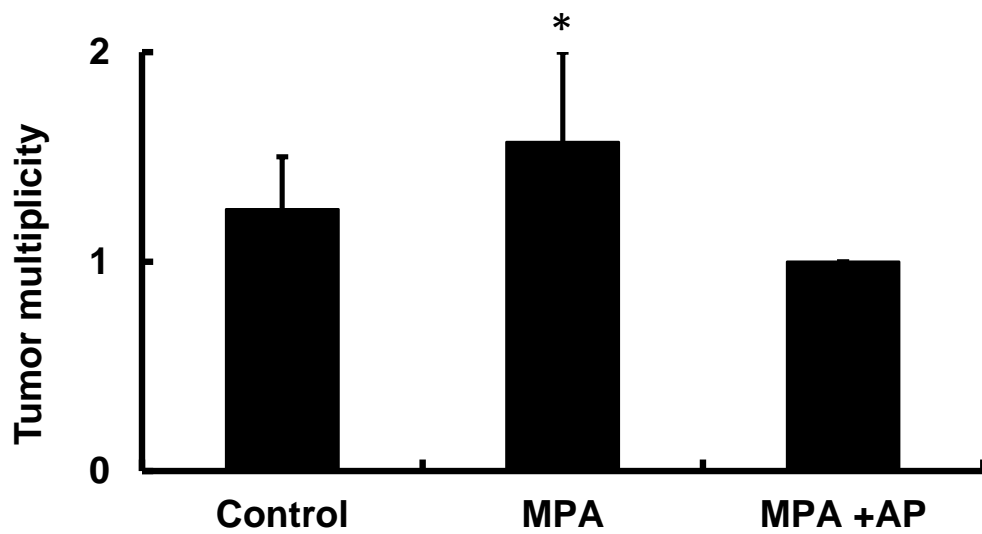
Figure 4.2B. Effect of apigenin on tumor multiplicity. The mean number of tumors per tumor-

bearing animal at the conclusion of the study in 2A (60 days after DMBA treatment) is presented.

* indicates p<0.05 compared with the other two groups; error bars represent SEM.

4.5.2. Prevention of MPA-accelerated tumors by apigenin is dose-dependent

To determine whether apigenin suppresses MPA-accelerated tumor development in a dose-dependent manner, we treated animals with 10, 25, and 50 mg/kg apigenin daily as described above. Each dose significantly delayed the first appearance of progestin-accelerated tumors compared with animals treated with MPA alone (Fig. 3.2C). However, at the lowest dose of 10 mg/kg apigenin, tumor incidence at termination of the study was not statistically different from that seen in animals treated with MPA alone; indicating that at a lower dose apigenin was less effective at preventing tumor formation.
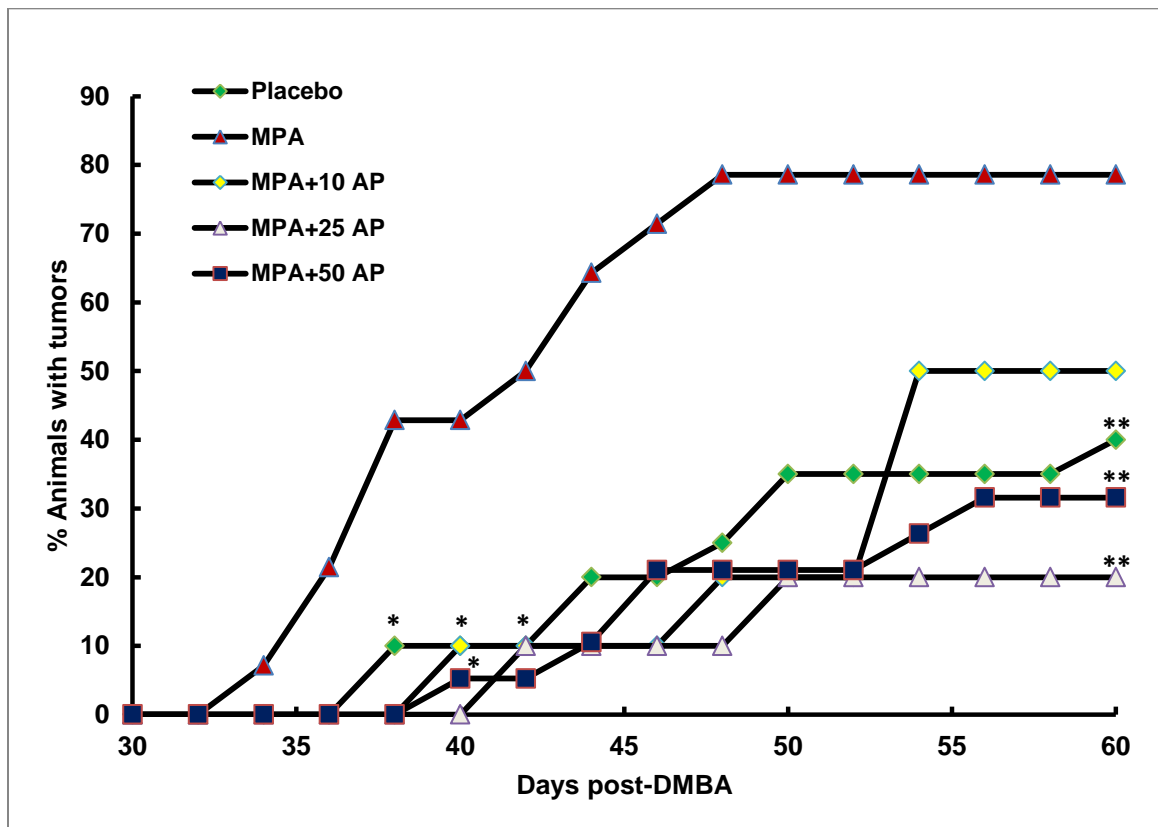
Figure 4.2C. Dose-response effects of apigenin on MPA-accelerated DMBA-induced mammary tumors. Experimental details are as described above except that varying amounts of apigenin were used as indicated in the figure. 10AP, 10 mg/kg dose of AP; 25AP, 25 mg/kg dose of AP, and 50AP, 50 mg/kg dose of AP. * indicates p<0.05 compared with MPA-treated group (latency) and ** indicates p<0.05 compared with MPA-treated group at day 60 (incidence). Combined results from two different experiments are shown (n=10-20 animals/group).

4.5.3. Apigenin does not prevent MPA-induced morphological changes in rodent mammary gland

Previous studies have shown that mammary tissue from animals exposed to progestins exhibited extensive proliferation of the mammary epithelium, resulting in formation of hyperplastic alveolar nodules and intraductal hyperplasia (Benakanakere et al, 2006; 2010; Carroll et al., 2010). In order to determine whether apigenin prevents MPA-induced hyperplastic lesions, we collected abdominal mammary tissue 5 days after implantation of MPA pellets and 6 hours after the last apigenin injection from DMBA-treated animals as described in the Materials and Methods. A second group of treated animals from the same experiment was maintained up to day 60 to ensure that the effects of MPA and apigenin were observed. Our observations concurred with previous reports in that extensive hyperplastic lesions occurred within the mammary glands of animals treated with MPA for 3-5 days (Aldaz et al., 1996; Benakanakere et al, 2006; 2010; Carroll et al., 2010), whereas minor lesions were observed at the same tine in animals given placebo pellets alone. Apigenin failed to block the formation of hyperplastic lesions that developed in response to MPA (Fig. 4.3A). However, even though apigenin was unable to prevent hyperplasia in MPA-treated animals, by the end of the experiment it had reduced overall tumor incidence (Fig. 4.2B). This suggests that apigenin arrests the development of MPA-induced tumors at a stage beyond the formation of hyperplastic lesions.

Placebo   MPA   MPA + AP
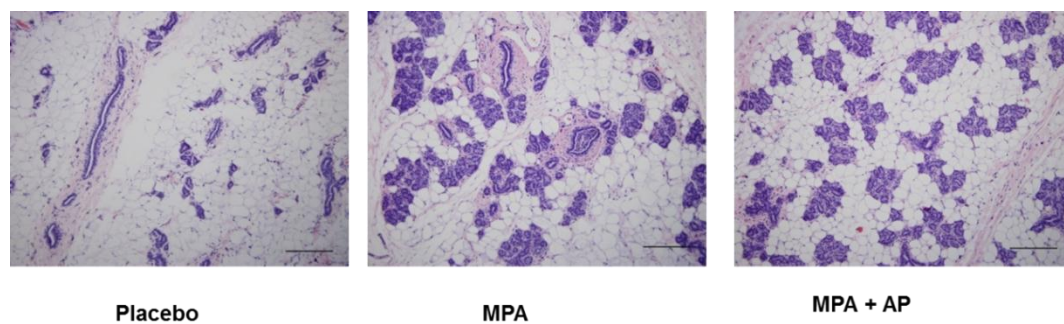
Figure 4.3. Apigenin does not block MPA-induced morphological changes in mammary gland tissues. Sprague-Dawley rats were treated with DMBA and subsequently implanted with a 25 mg/60-day release MPA pellet on day 28 as described in the Materials and Methods. Apigenin (AP; 50 mg/kg) or vehicle injections were given once daily between days 21 and 33 following DMBA treatment. **(A)** Abdominal mammary tissues were collected from randomly selected animals sacrificed on day 33, 6 hours after the last apigenin injection and processed as described in the Materials and Methods. One representative H&E stained section is shown for each experimental group. Images were taken at 20X magnification; scale bar, 100μm.

4.5.4. Immunohistochemical analysis

In previous studies, we showed that MPA-induced elevation in tumor cell VEGF was an important mechanism by which the progestin accelerated development of mammary tumors (Benakanakere et al, 2006; 2010; Carroll et al., 2010). In the present study, we sought to determine whether the antitumor effects of apigenin might arise as a consequence of its ability to suppress VEGF production within hyperplastic lesions of MPA-treated animals. We examined VEGF staining in sections of mammary tissues from animals in our model treated with MPA, MPA + apigenin, or placebo pellets. We found that VEGF staining in hyperplastic lesions of mammary tissues from animals in the MPA + apigenin group was significantly lower than that observed in animals receiving MPA alone or in control animals receiving placebo ($p<0.05$; Fig. 3.4A &3.4B). These findings led us to propose that the ability of apigenin to prevent MPA-accelerated DMBA-induced tumor development is at least in part due to its effect on VEGF expression.

VEGF signaling is mediated largely by the VEGF receptors, VEGFR-1 (flt) and VEGFR-2 (flk). Consequently, we conducted studies to determine whether apigenin influences VEGFR-1 and VEGFR-2, which are essential mediators of the proliferative and survival effects of VEGF (26). In mammary tissues of the animals in our experimental model, we found that VEGFR-2 (flk) expression in the hyperplastic lesions was significantly decreased in animals in the MPA + apigenin group compared with animals receiving MPA alone or placebo ($p<0.05$; Fig. 3.4C). In contrast, apigenin had no effect on levels of VEGFR-1 expression in the hyperplastic lesions of MPA-exposed mammary glands, and no significant difference was seen among VEGFR-1 expression levels in MPA, MPA + apigenin, and placebo-treated animals (Fig. 3.4D).
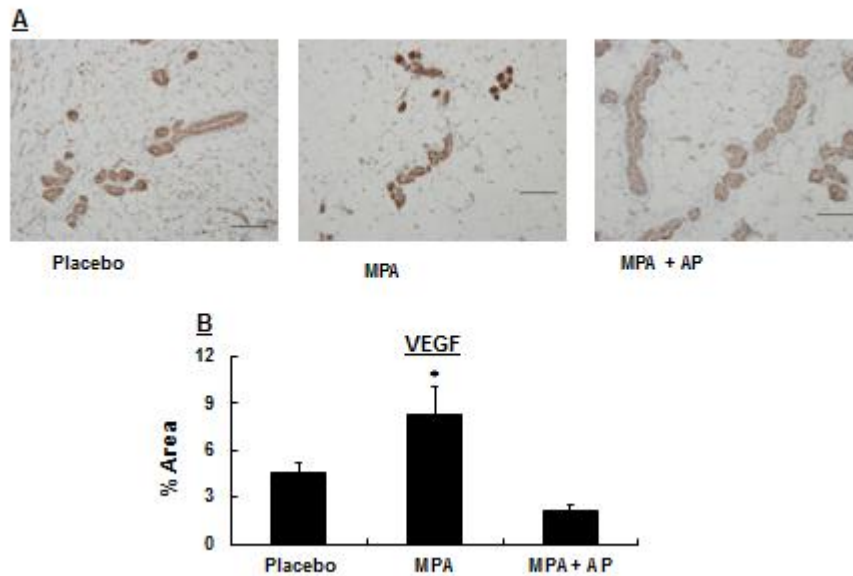
Figure 4.4. Apigenin suppresses expression of VEGF in DMBA-induced MPA-treated mammary glands. Sprague-Dawley rats were treated with DMBA and subsequently implanted with a 25 mg/60-day release MPA pellet on day 28 as described in the Materials and Methods. Apigenin (AP; 50 mg/kg) or vehicle injections were given once daily between days 21 and 33 following DMBA treatment. **(A)** Mammary glands were collected from randomly selected animals sacrificed on day 33, 6 hours after the last apigenin injection and processed and stained for VEGF as described in the Materials and Methods. One representative section is shown for each group. Images were taken at 20X; scale bar, 100 μm. Quantitative analysis of VEGF **(B)** expression in mammary tissues described in A using Fovea Pro software as described in the Materials and Methods. Data represent the mean percent area positive for staining; error bars represent SEM.* indicates $p < 0.05$ compared with the rest of the groups by Kruskal-Wallis one-way ANOVA on ranks and all pair-wise multiple comparisons by Tukey's procedure.
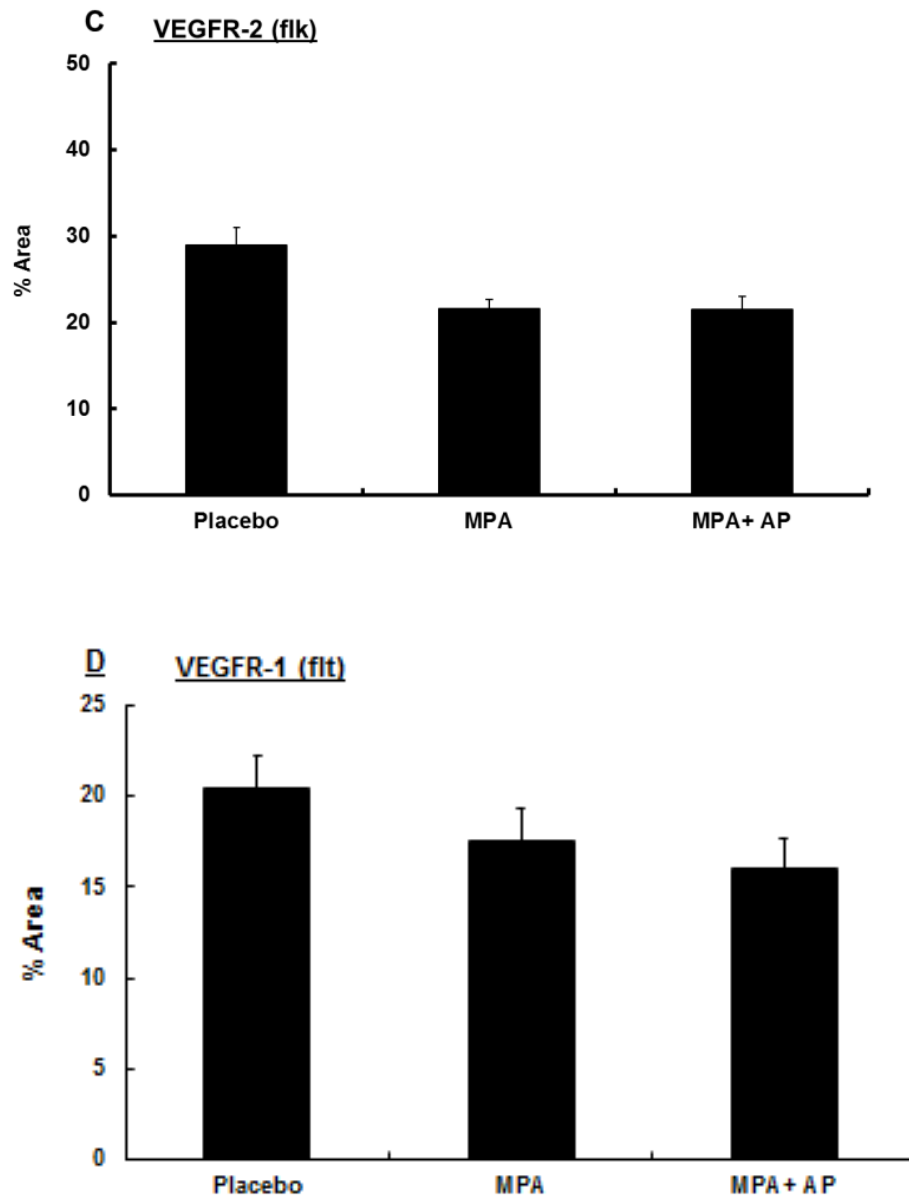
Figure 4.4 C and D. **Quantitative analysis of VEGFR-2 (*flk*) (C), and VEGFR-1 (*flt*)**

(D) expression in mammary tissues described in 10A above using Fovea Pro software as

described in the Materials and Methods. Data represent the mean percent area positive for

staining; error bars represent SEM.

Previous studies have shown that signaling through ERs is critical for PR expression, and that PR activity is essential for VEGF induction (Horwitz et al., 1978; Narduli et al., 1988). We therefore carried out studies aimed at determining whether apigenin blocks MPA-dependent effects by suppressing expression of ER and/or PR. We found that, while levels of PR expression in mammary tissue were similar among all three treatment groups (MPA, MPA + apigenin, and placebo; data not shown), the proportion of cells staining positive for PR was significantly lower in animals in the MPA and MPA+ apigenin groups compared with that in animals in the placebo group (Fig. 3.5A). This decline in PR could be a result of increased PR phosphorylation and rapid receptor turnover leading to a lack of detection by immunohistochemistry as previously reported (Dennis et al., 2005; Benakanakere et al., 2010). This is further evidence that apigenin does not block all MPA-dependent effects. Similarly, we found that expression of both ERα (Fig 4.5B) and ERβ (data not shown) was similar in the mammary tissues of MPA-treated animals, whether or not animals were administered apigenin. The proportion of cells in mammary tissue that stained positive for ERα was similar for animals in the MPA and MPA + apigenin groups, and was not significantly different from that observed in the placebo group (Fig. 4.5B), while almost all epithelial cells were ERβ positive in all the groups mentioned above (data not shown).
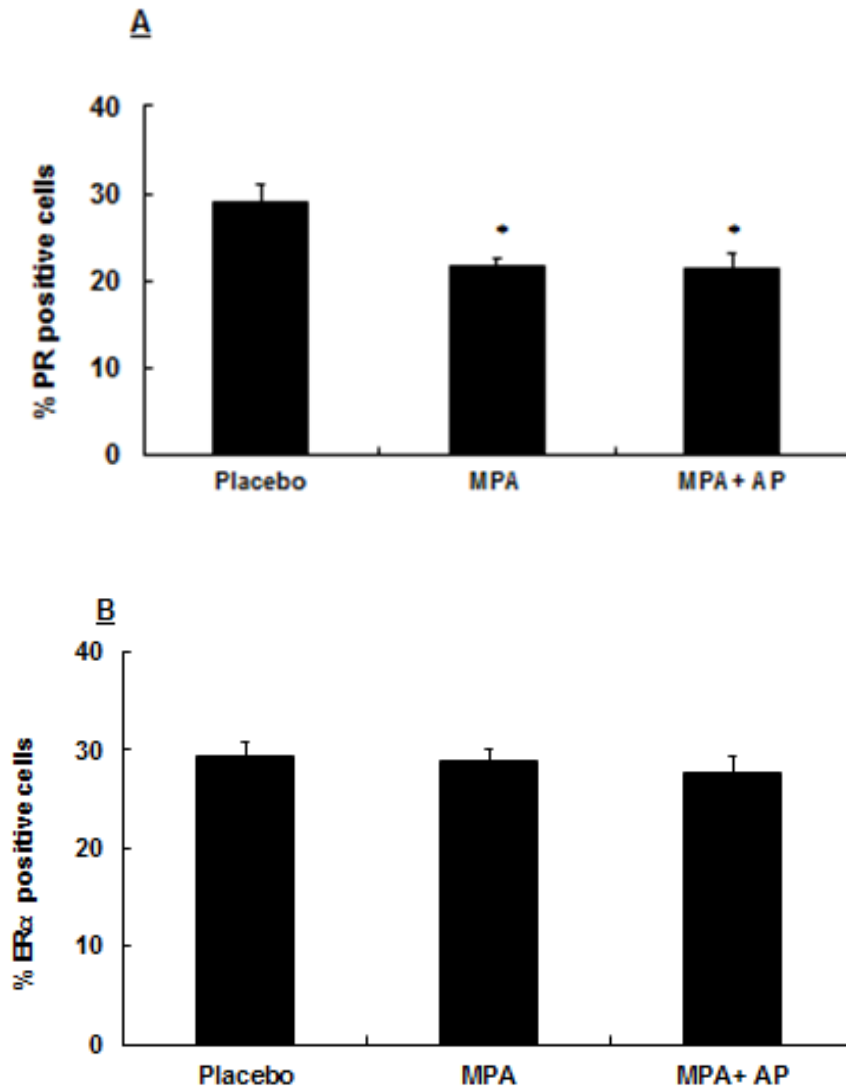
Figure 4.5. Immunohistochemical analysis of ER and PR in the mammary glands of DMBA-induced MPA accelerated tumors in rats with and without apigenin treatment. Mammary glands were collected from randomly selected animals sacrificed on day 33, 6 hours after the last apigenin injection and processed and stained for PR and ER as described in the Materials and Methods. Images were taken at 40X, and at least 150 cells counted per section as described in the Materials and Methods. Data represent mean percentages of PR- **(A)** and ERα-positive **(B)** stained cells in DMBA-induced MPA-treated mammary glands of animals treated with or without apigenin; error bars represent SEM. * indicates $p<0.05$ compared with placebo group by Kruskal-Wallis one-way ANOVA on ranks and all pair-wise multiple comparisons by Tukey's procedure.

4.6. DISCUSSION

In this study, we provide evidence that apigenin significantly delays the appearance of progestin-accelerated breast tumors in a DMBA-induced tumor model, reduces tumor multiplicity, and reduces tumor incidence. While several mechanisms have been proposed for the anticancer effects of apigenin, to our knowledge this is the first report indicating that this naturally occurring flavonoid might inhibit *in vivo* tumor development by opposing the effects of progestins. Using cell culture, we previously showed that apigenin inhibits progestin-dependent induction of VEGF in human breast cancer cells (Mafuvadze et al., 2010). With this in mind, we conducted a series of *in vivo* studies aimed at determining whether apigenin might be used to prevent the emergence of mammary tumors, especially in women exposed to synthetic progestins as a component of E+P HRT. Our studies were focused on examining the effects of apigenin on MPA-accelerated mammary tumors in an animal model, because MPA is the most widely prescribed progestin and its use is associated with increased risk of breast cancer and mortality (Ross et al., 2000; Rossouew et al., 2002; Chen et al., 2004; Chlebowski et al., 2010).

Following the administration of apigenin to DMBA-treated animals that were also given MPA, tumor latency was significantly increased. Furthermore, tumor incidence decreased at least 50% in these animals, compared with animals that were treated with vehicle alone. Importantly, apigenin also reduced tumor multiplicity to levels below those normally observed following progestin treatment. It was interesting to note that, even though apigenin suppressed tumor development, it did not prevent the MPA-induced morphological changes and hyperplasia in the mammary gland of DMBA-treated

animals, a finding in direct contrast to the effects of curcumin (Carroll et al., 2010), a natural plant derived curcuminoid with anti-cancer properties (Jaiswal et al., 2002). Surprisingly though, with respect to its ability to increase tumor latency and reduce the incidence of MPA-accelerated tumors in this model, apigenin was a more effective preventive compound than curcumin.

Apigenin has been reported to exert its anticancer effects via a variety of mechanisms, including induction of cell cycle arrest and apoptosis, through both tumor necrosis factor-α induced NFκB-mediated and intrinsic apoptosis pathways (Horinaka et al., 2006) and attenuation of the phosphorylation of epidermal growth factor receptor and MAP kinase (Yin et al., 2001, Patel et al., 2007). Compelling evidence from a variety of model systems suggests that VEGF is essential for tumor development and progression (Ferra, 2002; Hyder, 2006), and it has been postulated that, unless tumors develop blood vessels, they will not grow beyond 2 mm^3 (Folkman, 1995). Our earlier *in vitro* studies using human breast cancer cell lines (Mafuvadze et al., 2010) have demonstrated that apigenin significantly reduces the expression of both VEGF and its receptor (VEGFR-2), an essential mediator of VEGF-dependent tumor cell proliferation (Hyder, 2006). It is therefore likely that, by suppressing VEGF expression, apigenin renders the mammary tissue microenvironment less conducive to tumor development.

Accumulating evidence has shown that many types of cancer, including breast cancer, are initiated by a small population of cancer stem cells (Li et al., 2010). Due to the rapid rate at which breast tumors develop in postmenopausal women taking E+P HRT, we and others have suggested that progestins most likely increase the proliferation of existing tumor cells in the breast (Hyder et al., 1998; 2001; Joshi et al., 2010).

Recently, Horwitz and Sartorius (2008) suggested that progestins may promote tumor development through activation and transformation of dormant breast cancer stem cells into intermediate subpopulations, which then differentiate into breast cancer cells. Because we found that apigenin did not prevent MPA-induced hyperplasia, but did inhibit the emergence of tumors and reduce the incidence and multiplicity of tumors normally associated with MPA treatment, apigenin may target a subset of cells essential for tumor development (cancer stem cells) in this model system. Recent studies showing that sulphorane, a component of broccoli, prevents tumor development by targeting and killing critical cancer stem cells (Li et al., 2010) support this idea. Furthermore, a number of dietary compounds such as curcumin (Jaiswal et al., 2002), quercetin, and epigallocatechin-gallate (Pahlke et al., 2006) have long been recognized as agents capable of suppressing cancer stem cell proliferation. Further studies are necessary to confirm whether apigenin might prevent the development of progestin-accelerated DMBA-induced mammary tumors via a mechanism that targets breast cancer stem cells.

Breinholt et al. (2000) reported that apigenin reduced levels of endogenous ER in mouse uterus. With this in mind, we sought to determine whether apigenin might have a similar effect in mammary gland. ER plays a vital role in controlling mammary levels of PR (Narduli et al., 1988), a fact which could explain the lack of MPA effects, because the presence of adequate levels of PR is essential if MPA is to exert effects such as induction of VEGF (Hyder et al., 1998; Hyder, 2006). However, we did not detect any differences in the expression levels of either ER or PR in the mammary gland of MPA-, MPA + apigenin-, or vehicle-treated animals. Apigenin has been shown to function in both an ERα-dependent and independent manner (Long et al., 2008) and thus it is a possibility

that in our study apigenin is mediating its effect in an ERα independent manner, although this remains to be tested. Interestingly, we observed that while the percentage of cells expressing ERα was similar in all treatment groups, the percentage of PR-positive cells was significantly lower in MPA-treated animals (both those treated with and without apigenin) compared with the placebo group. However, insufficient PR expression is unlikely to explain the antiprogestin effects of apigenin, and we cannot rule out the possibility that it blocks PR-mediated functions by modifying its activity, either through suppressing phosphorylation of the PR protein or through an unidentified mechanism. These scenarios remain to be examined.

Although data on the bioavailability of dietary apigenin in humans is extremely limited, Meyer et al. (2006) reported that, following ingestion of parsley, which is rich in apigenin, plasma levels of the flavonoid increased to a concentration of 0.34 µmol/l. Consequently, it appears that concentrations of apigenin sufficient to be biologically effective can be obtained orally through the diet. Furthermore, it has been reported that apigenin's slow pharmacokinetics allow it to remain in the circulation for a prolonged period (Chen et al., 2007), suggesting that it may accumulate within tissues at sufficient levels to exert chemopreventive effects. Consumption of apigenin-rich foods may be chemopreventive, particularly with respect to progestin-accelerated tumors. Furthermore, regular intake of foods rich in the flavonoid may decrease the cancer risk in postmenopausal women undergoing HRT with a progestin component, as well as in those who have already been exposed to combined HRT. Fortunately, apigenin appears to be nontoxic, since it appears to have different effects on normal versus cancerous cells (Gupta et al., 2001; Chiang et al., 2006), suggesting that the intake of high doses will

likely prove benign. Indeed, we administered apigenin repeatedly to animals at levels up to 50 mg/kg for 10-13 days and observed no signs of toxicity.

In this study, our main focus was to determine the effects of short-term apigenin administration (10 days) on preventing the development of progestin-accelerated tumors in a model of DMBA-induced mammary carcinogenesis. In the future, we will perform long-term studies using apigenin given either as ip injections or incorporated in the diet with a view to determining whether continuous treatment with apigenin can further prevent the appearance of tumors in animals while remaining nontoxic. Such studies will be valuable in helping to determine whether the reduction in tumor incidence (approximately 50%) seen following short-term treatment with apigenin (10 days) can be further improved in a long-term preventive strategy. The present study is also limited in that we have yet to determine the maximum tolerated dose that might be used to prevent the emergence of MPA-accelerated tumors in DMBA-treated animals. In the future, we will also look into whether administration of apigenin before the carcinogenic insult protects against the initial formation of DMBA-induced mammary tumors, as well as opposing the effects of MPA on tumor development. Additional preclinical and clinical studies are required to further investigate the efficacy, pharmacokinetics, and suitability of apigenin as a chemopreventive candidate for progestin-accelerated breast cancer and other hormone-dependent cancers in humans.

In the next chapter we describe studies aimed at determining whether apigenin incorporated in diet and taken orally will be able to prevent MPA-accelerated DMBA-induced mammary tumors.

CHAPTER 5

5.1.   Effects of feeding apigenin-containing diets on MPA-accelerated DMBA-induced mammary tumors in rats: a preliminary study.

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Key words: Breast cancer, diet, apigenin, progestin.

Manuscript: In Preparation

5.2.   ABSTRACT

Based on our earlier studies which showed that intraperitoneal administration of apigenin prevents progestin-dependent DMBA-induced mammary tumors in rats, we conducted preliminary studies aimed at investigating the capacity of dietary apigenin to prevent the development of the same type of tumors. Animals were fed either an apigenin supplemented or control diet and implanted with either placebo or MPA pellets. Animals were then palpated every two days to detect the presence of tumors.

We observed that rats implanted with MPA pellets and given a diet supplemented with 0.1% apigenin had a significantly reduced incidence of mammary tumors compared with animals exposed to MPA and fed a control diet. However, mean tumor multiplicity was significantly higher in rats fed dietary apigenin than those fed a control diet. Histological characterization showed no morphological differences between tumors from animals given MPA irrespective of whether they were fed apigenin or a control diet.

5.3.   INTRODUCTION

In recent years naturally-occurring dietary compounds have received increasing attention for their cancer chemopreventive properties (Aggarwal and Shishodia, 2006). The consumption of substances with anti-cancer properties is convenient and would be preferred by most people, particularly as a means of long-term disease prevention (Patel et al., 2007). The effectiveness of natural compounds as chemotherapeutic and chemopreventive agents varies however, depending on method of administration (Houghton et al., 2007). For example, Bansal et al (2011) reported that intraperitoneal administration of curcumin effectively prevented the development of mammary tumors.

However, when given orally at even higher doses, curcumin was ineffective at preventing breast tumor growth. A number of mechanisms have been proposed to explain the lack of effectiveness when certain naturally-occurring compounds are administered orally. For example, metabolic breakdown of active compounds, lack of absorption in the gastrointestinal tract and first-pass metabolism in the liver might significantly reduce the systemic concentration of a particular agent (Laupattarakasem et al., 2003). In addition, it is possible that absorption and metabolism of orally administered compounds might also be affected by other compounds present in the diet.

In previous studies (Mafuvadze et al., 2011), we showed that short-term intra-peritoneal administration of apigenin prevented the development of MPA-accelerated DMBA-induced mammary tumors in rats. Herein we examined the ability of long-term consumption of dietary apigenin to prevent the progression of such tumors. Leonardi et al (2010) reported that 0.1% dietary apigenin decreased significantly the incidence of azoxymethane-induced high multiplicity aberrant crypt foci which are known to correlate with future colon tumors in rats. Au et al (2006) reported that 0.025% and 0.1% dietary apigenin modestly reduced colonic tumors compared with controls, though in a non-dose response manner. In the present study, we also used a diet containing 0.1% apigenin to determine how an apigenin-supplemented diet might affect the growth and development of MPA-accelerated DMBA-induced mammary tumors. We found that 0.1% dietary apigenin significantly reduced overall tumor incidence in Sprague Dawley rats implanted with MPA compared with those fed a control diet. However, animals fed an apigenin-supplemented diet exhibited significantly higher tumor multiplicity (number of tumors per animal) compared with controls. Detailed results are described below.

5.4. MATERIALS AND METHODS

5.4.1. *Animals*: Intact 40- to 45-day-old female Sprague-Dawley rats (Harlan Breeders, Indianapolis, IN) were used in this study. The rats were maintained according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International under conditions of 12-hour light/dark cycles and *ad libitum* access to food and water. All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri (Columbia, MO) and were in accordance with procedures outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 85-23).

5.4.2. *Experimental protocol*: We followed a protocol previously described by Benakanakere et al (2006; 2010). In brief, all animals were given 20mg of DMBA dissolved in vegetable oil by oral gavage. Two weeks post-DMBA administration animals were divided into 3 groups; control (n=14) fed control diet (2016S), MPA (n=12) fed control diet and MPA + AP (n=12) fed 2016S supplemented with 0.1% apigenin (Harlan-Teklad). Four weeks post-DMBA, animals were either implanted with 25mg-60day releasing MPA pellets (MPA, MPA+AP groups) or placebo pellets (control group). Animals were then examined for mammary tumors by palpation once every two days. The endpoints for data analysis were presented as follows; latency to tumor appearance, tumor incidence, and the number of tumors per animal (tumor multiplicity). All animals were sacrificed 70 days post-DMBA administered which corresponded to 6 weeks of feeding apigenin-supplemented diets. Tumor tissues were collected and

preserved in 4% paraformaldehyde for histological and immunohistochemical analysis.

A summary of the experimental protocol that we followed is given below.
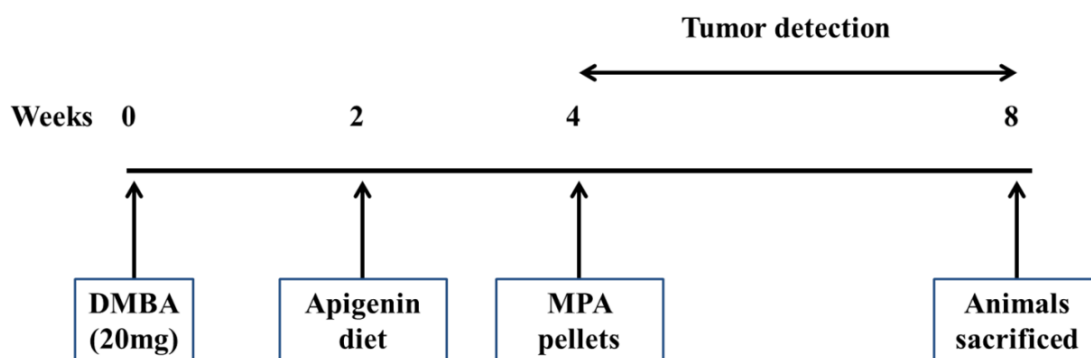
Figure 5.1. Treatment protocol followed in the DMBA-induced MPA accelerated mammary tumor study. Sprague-Dawley rats were treated with DMBA and subsequently implanted with a 25 mg/60-day release MPA (or placebo) pellet on day 28 as described in the Materials and Methods. Beginning two weeks post-DMBA administration animals were either fed a control diet or a diet supplemented with 0.1% Apigenin. Following implantation of MPA pellets animals were palpated for tumors every two days.

5.4.3. *Immunohistochemical analysis*: Tumor tissues were collected in 4%

paraformaldehyde and processed as previously described above (ref to section. 4.4.4).

Fixed tissues were then stained for the following molecular markers; VEGF, PR, Ki-67

and caspase-3. We quantified VEGF expression using the fovea pro software as

previously described above (ref to section 4.4.4). PR staining was assessed by a

pathologist who was blinded with respect to treatment groups and scored on a 0-3+ grade

based on percentage of cells that were positive and on staining intensity, as previously

described by Mohsin et al (2004). The overall score assigned was based on the following;

A + B =C, where A is the percentage of positive cells (0-25=1, 26-50=2, 51-75=3, 76-

100=4), B is the determined intensity (0= no staining, 1= weak, 2= moderately strong, 3=

Very strong staining), C is sum of A and B (if C≤2: -, C=3-4: +, C=5-6: ++, C=7: +++).

Ki-67 and caspase-3 were assessed by determining the percentage of positive cells.

5.4.4. *Statistical Analysis***:** Time-to-event data (latency) was analyzed using using the

Kaplan-Meier method and comparisons across groups made using the Wilcoxon log-rank

test. Those animals that had not developed a tumor by the end of the study were censored.

Multiplicity and immunohistochemical staining data were analyzed using Kruskal-Wallis

one-way analysis of variance (ANOVA) followed by Tukey's or Dunn' procedure as

posthoc tests. For all comparisons, P<0.05 was regarded as statistically significant.

Values are reported as mean ± standard error of the mean (SEM) unless otherwise stated.

5.5. RESULTS

5.5.1. *Tumor Incidence and Multiplicity*

As previously reported in this model (Benakanakere 2006; 2010; Mafuvadze et al., 2011), implantation of MPA pellets significantly increased overall tumor incidence ($P<0.05$) and reduced tumor latency ($P<0.05$) compared with control animals implanted with placebo pellets (Figure 5.2). By feeding MPA-treated rats a diet supplemented with 0.1% apigenin we were able to significantly lower the incidence of tumors ($p<0.05$) by the end of the study (Figure 5.2), though as shown in figure 5.3 apigenin-fed rats demonstrated a significantly higher tumor multiplicity compared with rats implanted with pellets containing MPA or placebo and fed the control diet ($P<0.05$).
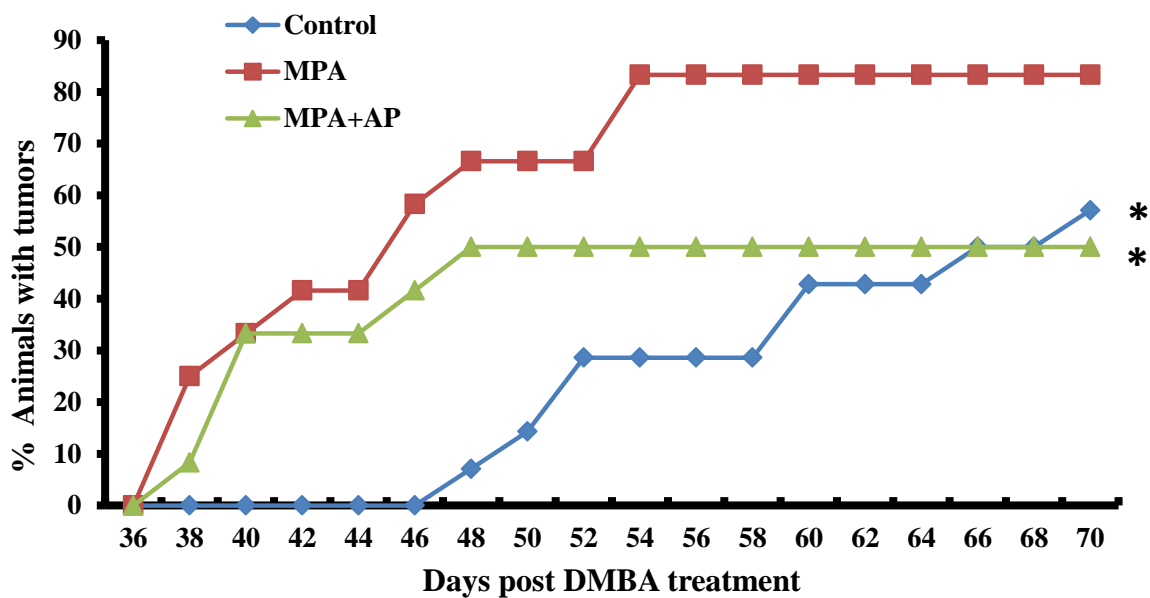
Figure 5.2. **Preventive effect of 0.1% apigenin (AP)-supplemented diet on MPA-accelerated DMBA-induced mammary tumors.** Feeding a diet supplemented with 0.1% apigenin decreased the incidence of MPA-dependent DMBA-induced tumors significantly (p<0.05) as compared to MPA alone groups. * Significantly different from MPA group.
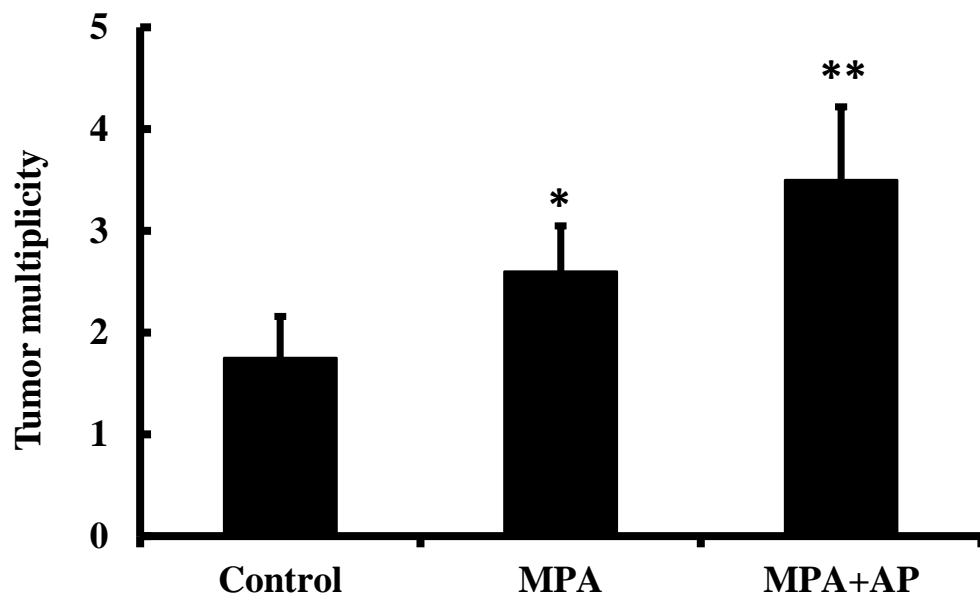
Figure 5.3. Tumor multiplicity. Animals fed a 0.1% apigenin (AP)-supplemented diet that developed tumors had a significantly higher number of tumors per animal (tumor multiplicity). ** Significantly different from MPA and control groups, $p < 0.05$, * significantly different from control.

5.5.2. *Body weight and weight gain*

We assessed whether feeding a diet supplemented with 0.1% apigenin could have adverse effects on the health of the animals by monitoring the weight of all animals throughout the study. At the termination of the study, mean body weights of animals from the three treatment groups were as follows; control (261.2 ± 4.67g), MPA (264.6 ± 4.81g), MPA+AP (275.7 ± 5.54g). Since body weights did not differ appreciably between the groups (Figure 5.4) we concluded that dietary apigenin did not adversely affect animal health.
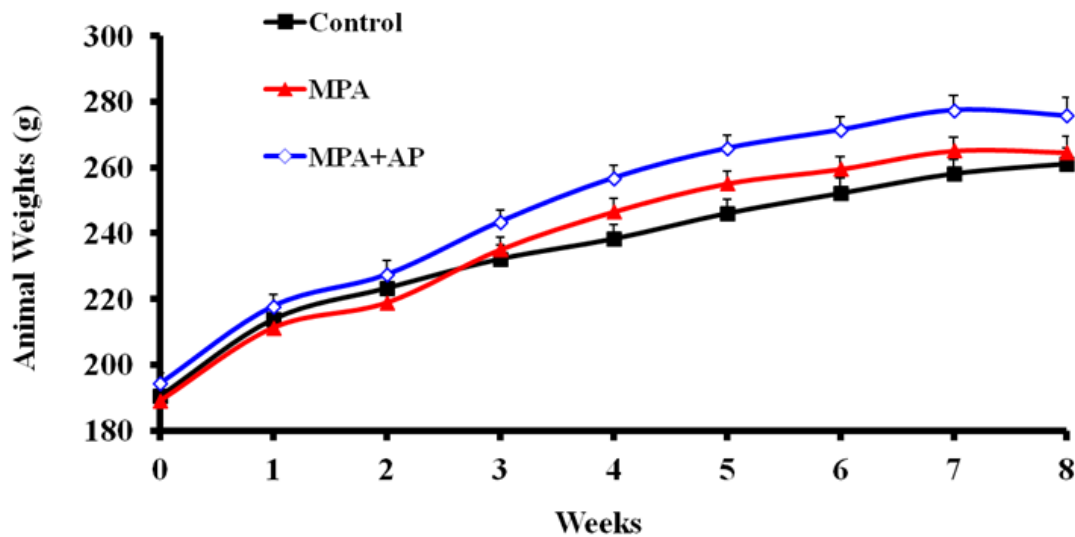
Figure 5.4. Weekly mean weight for animals fed either a control diet or a diet supplemented with 0.1% apigenin (AP) for the entire experimental period. Repeated ANOVA showed no difference (p>0.05) among the 3 treatment groups.

5.5.3. *Histological and Immunohistochemical analysis*

As stated previously animals that were given both MPA and fed a diet supplemented with 0.1% apigenin demonstrated significantly higher tumor multiplicity compared with those given a control diet. We hypothesized that tumors arising in animals receiving dietary apigenin could be less malignant and morphologically different compared with tumors from animals implanted with MPA pellets and fed a control diet. In order to examine this possibility we characterized haematoxylin and eosin stained sections of fixed tumors based on their degree of differentiation and organization (e.g whether encapsulated or not), the appearance of gland-like structures and the ability to invade surrounding stromal or adipose tissue.

Tumors from rats implanted with placebo pellets and fed a control diet were generally well differentiated and encapsulated with fibrous tissue and had a tendency to show gland-like structures (Figure 5.5A). On the other hand, tumors collected from rats implanted with MPA pellets and fed a control diet tended to be poorly differentiated and had an increased tendency to invade into surrounding stromal (Figure 5.5B) and adipose tissue (Figure 5.5C). Tumors obtained from rats implanted with MPA and fed a diet supplemented with 0.1% apigenin were morphologically similar to tumors from rats given MPA and a control diet, suggesting that apigenin at the administered dose had no significant effect on MPA-induced morphological changes.
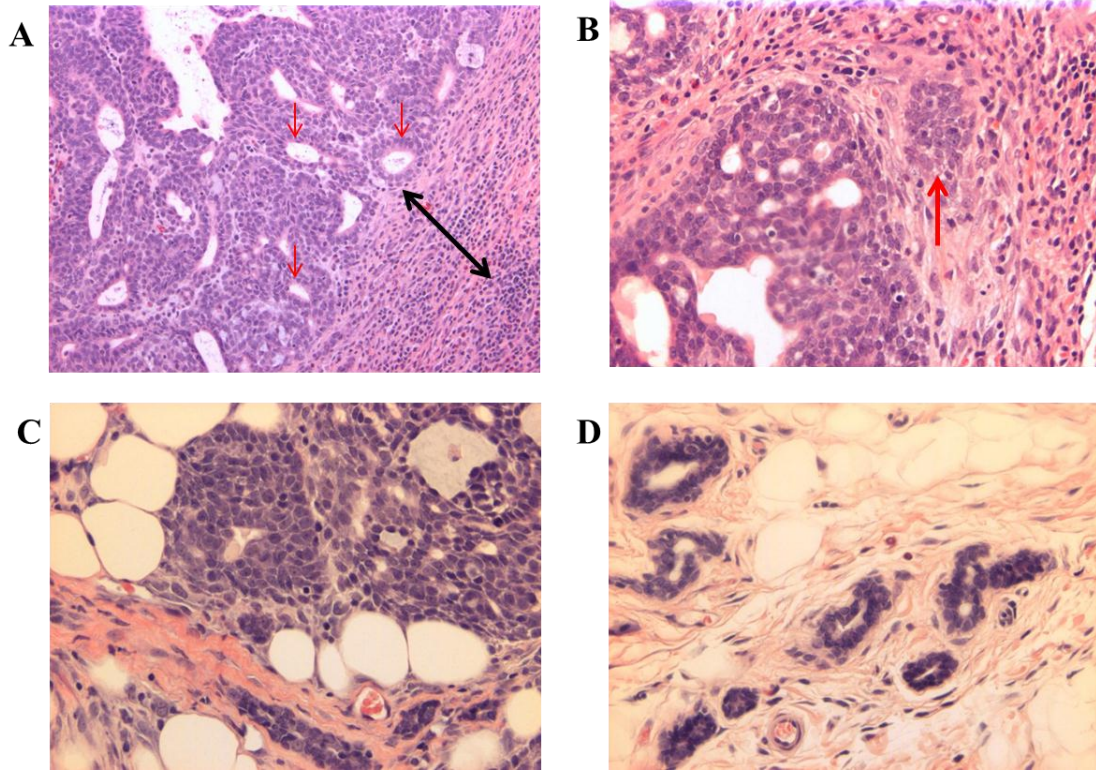
Figure 5.5. Morphological characterization of mammary tumors (H&E stained). A. Well differentiated and encapsulated (black arrow) mammary tumors with gland-like structures (narrow red arrow). 10X magnification. B. Mammary tumor invading into surrounding stromal tissue (thick red arrow). C. Mammary tumor invading into surrounding adipose tissue. D. Normal mammary glands (see typical two layered glands surrounded by adipose tissue). B, C, D images taken at 20X magnification.

Using immunohistochemical staining we determined the expression of specific molecular markers known to be involved in tumor angiogenesis (VEGF), proliferation (Ki67), apoptosis (caspase-3) and progestin responsiveness (PR). In contrast to our previous study in which intraperitoneal administration of apigenin suppressed VEGF expression, no significant differences in expression of VEGF were observed between the three experimental groups. Percentage areas of VEGF expression were as follows; control (18.2 ± 2.37), MPA (11.3 ± 2.52 and MPA+AP (13.0 ± 3.13) respectively. Similarly, PR expression was roughly the same in the three experimental groups, with the following mean values; control (1.80 ± 0.20), MPA (1.78 ± 0.22) and MPA + AP (1.83 ± 0.41). While not reaching the level of statistical significance, Ki-67 expression was higher in tumors from the MPA group compared with those obtained from controls or animals given apigenin (Figure 5.6A). Interestingly, there was a tendency towards increased Ki-67 positive staining in stromal cells from tumors in the MPA group (Figure 5.6B) compared with controls or the MPA + AP group (Figure 5.6C). Immunohistochemical staining for caspase-3 showed no evidence of apoptosis in the majority of tumors from all three treatment groups, suggesting that most tumors were proliferating.

A Control MPA MPA + AP

B

C

Control MPA
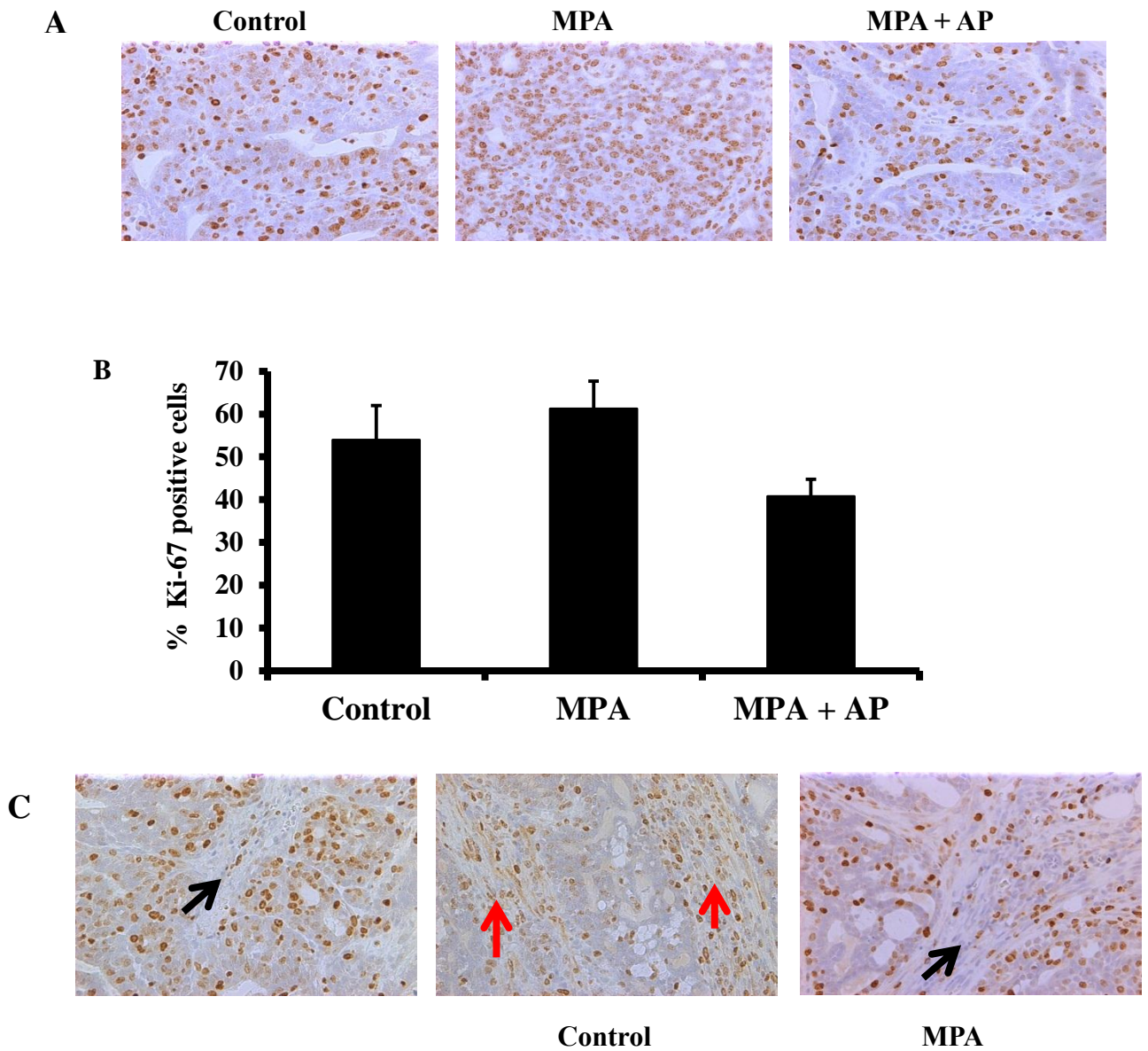
Figure 5.6. A. Ki-67 positive epithelial tumor cells. **B**. Apigenin treatment tended to decrease the number of positive epithelial tumor cells. **C.** Increased Ki-67 staining in cells the stroma of tumors from animals implanted with MPA pellets and fed a control diet (red arrow) and decreased Ki-67 staining in cells in stroma of tumor collected from control animals and apigenin treated animals (black arrow). All images are taken at 20X magnification.

5.6.   DISCUSSION

In recent years the anticancer effects of a number of naturally-occurring compounds have been reported, based on epidemiological evidence and experimental data obtained from both *in vitro* and *in vivo* studies (Patel et al., 2007). Previously we reported that intraperitoneal administration of apigenin prevented the growth of MPA-accelerated DMBA-induced mammary tumors in rats (Mafuvadze et al., 2011). In this preliminary study, our focus was to examine the effectiveness of dietary apigenin in preventing the development of such tumors, using the DMBA rat model previously described by Benakanakere (2006; 2010) and a regimen of 0.1% apigenin incorporated into the diet. A dietary dose of 0.1% apigenin was used since this level has previously been shown to effectively prevent the growth and development of colonic tumors in rats (Au et al., 2006; Leornardi et al., 2010).

We found that consumption of a diet containing 0.1% apigenin significantly reduced the overall incidence of MPA-accelerated DMBA-induced mammary tumors. Unlike in our previous study, in which intra-peritoneal administration of apigenin significantly reduced tumor incidence and increased tumor latency (Mafuvadze et al., 2011), dietary apigenin at the administered level did not appear to have a significant effect on tumor latency. It is possible that when apigenin is given intra-peritoneally, higher levels of the compound are able to accumulate in the systemic circulation, thereby reaching concentrations that are inhibitory to DMBA-induced mammary tumors at an earlier stage of their development. On the other hand, dietary apigenin, at least at the dose used in this study, might take longer to accumulate to levels sufficient to affect tumor latency.

Interestingly, we found that rats treated with MPA and fed a 0.1% apigenin-supplemented diet demonstrated higher tumor multiplicity compared with similar MPA-treated rats consuming a control diet. To our knowledge, this is the first time apigenin has been shown to actually promote the proliferative effects of a progestin *in vivo*, resulting in an increased number of mammary tumors. Apigenin and flavonoids in general have been reported to exhibit different properties *in vitro* depending on the dose and the type of cells (Xu et al., 2011). For example, Long et al (2008) reported that lower concentrations of apigenin (1µM) stimulated MCF7 cell growth, whereas higher concentrations (≥10µM) inhibited the growth of MCF7 cells. It is therefore likely that the effect of apigenin on breast cancer cells depends on the systemic concentration of the drug. It is possible that when rats consume apigenin as a part of their diet, the systemic concentration of the flavonoid is at a level sufficient to stimulate breast cancer cell growth, a notion proposed by Long et al (2008). However, as systemic concentrations of apigenin, and levels within mammary tissue increase with chronic dietary intake, a level is reached that inhibits tumor growth. Such a scenario would account for the observed initial increase in MPA driven tumors and the overall reduction in tumor incidence. It is however also possible that orally ingested apigenin is metabolized by microflora in the gastrointestinal tract into substrates that might increase the tumorigenic effects of MPA on DMBA-induced mammary tumors. A further possibility is that at the concentration used, apigenin might selectively affect a subtype of cells that are more responsive to the effects of MPA. A rebound effect on tumor growth has previously been observed with other antiangiogenic drugs, possibly as a result of killing sensitive cells while sparing

resistant mutant cells that are less sensitive to hypoxia and which consequently proliferate (Blagosklonny, 2004).

These preliminary studies demonstrate that apigenin possesses both potentially beneficial and possibly deleterious effects in terms of breast cancer development. Given the observed effects of dietary apigenin on tumor multiplicity, it is possible that oral supplements of the compound might increase breast cancer risk in people who are genetically predisposed to the disease, such as those with mutant BRCA genes. Consequently, if we are to develop safe and effective doses of apigenin for dietary supplementation and chemoprevention, further studies on the mechanisms involved in increased tumor multiplicity are required. One area of future study might involve microarray analysis to identify those genes that are activated and over-expressed, and those that are suppressed as a result of apigenin administration. It is possible that the observed effects of apigenin on tumor multiplicity could be reduced by increasing dietary levels of the flavonoid; consequently we will perform studies using higher levels of apigenin. It is also possible that dietary apigenin may prevent mammary tumor growth if administered prior to DMBA administration.

We initially hypothesized that there could be morphological differences between tumors obtained from animals given apigenin and those fed control diets lacking apigenin. As previously reported by Benakanakere et al (2006), tumors obtained from rats treated with MPA had a tendency to be more invasive and poorly differentiated (Figure 5.5B&C) compared with tumors from control rats, which tended to be well differentiated and well encapsulated (Figure 5.5A). Dietary apigenin at the 0.1% level used in these studies appeared to have no significant effect on tumor morphology, most tumors being

morphologically similar to those from rats given MPA and a control diet. This suggests that tumors arising in these animals are progestin-dependent and that apigenin at the level administered is ineffective at producing anti-proliferative effects.

In earlier studies we used a xenograft model to demonstrate extensive induction of apoptosis following intra-peritoneal administration of apigenin. A previous report also showed that apigenin induces apoptosis in several different tumor types (Patel et al., 2007). However, in this study we observed limited caspase-3 staining in tumors from apigenin treated animals, supporting our observation that once the process of tumorigenesis was initiated, apigenin at the administered level did not have a significant effect on tumor growth.

 In the present study we observed increased Ki-67 staining within stromal cells of tumors collected from animals implanted with MPA pellets and fed a control diet, though the significance of this apparent proliferation and expansion of stromal cells during progestin-induced mammary tumor growth remains to be explored. Recent studies have however shown an increased role of stromal tissue in breast cancer initiation and progression (Khamis et al., 2011) and it is possible that changes within stroma might reflect a passive or active reaction to the malignant transformation induced by the progestin.

Previous studies using this model show increased VEGF expression in tumors collected from animals given MPA (Benakanakere et al., 2006; 2010). We did not however see any differences in VEGF expression in tumors collected from animals in the treatment groups employed in this study, possibly because they were collected 42 days after implantation of MPA pellets. VEGF expression is elevated during the hyperplastic

to neoplastic transition (Woessner et al., 1998). Hence, it is possible that we might have seen differential expression of VEGF if we had collected tumors at an earlier stage of development. In contrast to a previous study (Mafuvadze et al., 2011) in which intra-peritoneal administration of apigenin suppressed VEGF expression in mammary gland tissue, in this study 0.1% dietary apigenin had no significant effect on the expression of tumor VEGF. It is possible that higher levels of dietary apigenin might be more effective at suppressing VEGF expression but this remains to be determined.

These preliminary studies suggest that the consumption of 0.1% apigenin in the diet might be both protective, as evidenced by an overall reduction in tumor incidence, and potentially harmful, as shown by increased tumor multiplicity. Consequently, we intend to pursue further studies aimed at determining the effects of higher levels of dietary apigenin on progestin-dependent breast cancer. Studies will also be performed to ascertain the mechanisms involved in apigenin-mediated increases in tumor multiplicity, as reported in this study.

CHAPTER 6: SUMMARY AND PERSPECTIVES ON FUTURE RESEARCH

6.1. SUMMARY

Breast cancer continues to be one of the leading causes of cancer death in women worldwide. In the USA and other developed countries the use of hormone replacement therapies (HRT) containing both estrogen and progestin is known to increase the risk of breast cancer in postmenopausal women (Chlebowski et al., 2010). Cumulative evidence suggests that the progestin component of combination HRT may be responsible for this increased risk and it is not surprising that there has been a worldwide decline in HRT use in recent years. Commonly available anti-progestins such as RU486 have adverse effects in humans and are therefore of limited use. With this in mind our laboratory focuses on screening and identifying both naturally-occurring and synthetic compounds which possess anti-progestin properties and might therefore be used as chemo-preventive and chemotherapeutic agents against progestin-dependent breast cancer. In this dissertation my main focus was to examine the chemo-preventive and therapeutic potential of apigenin against progestin-dependent breast cancer.

Apigenin is a flavonoid which is abundant in fruits and vegetable and which has been shown to suppress the growth of a number of cancer cell lines. Due to its wide availability and high safety index, apigenin would seem to be an ideal candidate for preventing and treating progestin-dependent breast cancer (Patel et al., 2007). In this study we focused in particular on the ability of apigenin to oppose the *in vivo* proliferative effects of medroxyprogesterone acetate (MPA), a synthetic progestin widely used clinically.

Our initial *in vitro* studies sought to determine the ability of apigenin to inhibit progestin-dependent VEGF induction. Previous studies from our laboratory have shown that progestins induce VEGF in human breast cancer cell lines that are progesterone receptor positive and contain mutant p53 (Liang et al., 2007; 2009). Secreted VEGF is directly related to the proliferative response of progestins *in vivo*. Using ELISA kits we measured levels of secreted VEGF in cultured human cells treated with MPA and incubated with different concentrations of apigenin and found that the flavonoid inhibited MPA-induced VEGF induction in BT-474 and T47D human breast cancer cells in a dose-dependent manner (Mafuvadze et al., 2010). We also showed that apigenin suppressed VEGF induction by the natural hormone progesterone, as well as a number of other synthetic progestins. Furthermore, using semi-quantitative RT-PCR we demonstrated that apigenin inhibited VEGF mRNA synthesis in a dose-dependent manner (Mafuvadze et al., 2010).

Having demonstrated that apigenin inhibits the production of VEGF by human breast cancer cells we conducted studies using an animal model previously described by Liang et al (2006) to examine its therapeutic potential against progestin-dependent breast cancer. In this model, human breast cancer cells are injected into nude mice in the absence of Matrigel. Inoculated cells initially grow into tumors, which begin to regress after 8-10 days. Implantation of progestin pellets arrests tumor regression, and tumors begin to grow even more aggressively. Tumor growth is blocked by anti-progestins, demonstrating progestin-dependence. In this study we inoculated nude-mice with BT-474 human breast cancer cells; after cells had resumed progestin-dependent growth, daily intra-peritoneal injections of 50mg/kg apigenin were given. We found that apigenin

induced apoptosis and suppressed the growth of MPA-dependent BT-474 xenograft tumors. Immunohistochemical analysis of xenograft tumors showed that apigenin treatment significantly decreased the expression of Her2/neu, a protein highly expressed in BT-474 cells and known to be important for proliferation and survivability of cancer cells (Korkaya et al., 2008). Furthermore, we observed that apigenin suppressed VEGF expression. Recent studies suggest that RANKL might play an important role in mediating the effects of progestins on proliferation and tumor growth (Schramek et al., 2010; Gonzalez-Suarez et al., 2010). In contrast to previous findings however, our study showed a decrease in levels of RANKL in MPA-treated animals, an observation we believe could be explained by species differences. Further studies will be necessary to determine if MPA has a similar effect on RANKL in other human cancer cell lines.

We previously showed that MPA accelerates the development and increases the incidence of DMBA-induced mammary tumors in Sprague Dawley rats (Benakanakere et al., 2006; 2010). Using the DMBA model to examine the ability of apigenin to prevent the development and growth of breast tumors, and initially administering apigenin intra-peritoneally at doses varying from 10-50mg/kg, we found that the flavonoid significantly reduced tumor incidence while increasing the latency of MPA-accelerated DMBA-induced mammary tumors. In addition, apigenin significantly blocked MPA-stimulated tumor multiplicity. Histological and immunohistochemical analysis of mammary tissues collected 5 days after MPA pellet inoculation showed that apigenin did not prevent MPA-induced hyperplasia of normal mammary gland epithelial cells, suggesting that it might act differently on normal mammary gland compared with its effects on mammary tumor cells. Such a differential effect has been reported in prostate cancer (Gupta, 2001).

Further immunohistochemical analysis showed that apigenin blocked MPA-dependent increases in VEGF and suppressed VEGF receptor 2 (VEGFR-2). However, no apigenin-related differences were observed in levels of either estrogen or progesterone receptors, suggesting that co-administration of an anti-estrogen drug such as tamoxifen, which is antiproliferative in many types of cancer, might be beneficial.

In our previous study we used the DMBA model of carcinogenesis to examine the effects of dietary apigenin on the growth and development of mammary tumors. While dietary ingestion of apigenin would seem to be the ideal means by which to take in the compound for long-term cancer prevention, earlier studies show that the effectiveness of phytochemicals might be reduced when they are taken orally (Houghton et al., 2007). In order to investigate the effectiveness of dietary apigenin we formulated diets that incorporated the flavonoid at a level of 0.1% and observed its effects on MPA-accelerated DMBA-induced mammary tumors, following protocols previously described by Benakanakere et al (2006). A diet containing 0.1% apigenin significantly reduced the incidence of MPA-accelerated DMBA-induced mammary tumors compared with animals given MPA and fed a control diet. Unexpectedly, those animals fed an apigenin-supplemented diet which went on to develop tumors exhibited significantly higher tumor multiplicity. This suggests that dietary apigenin might be both proliferative and anti-proliferative with respect to tumor development, effects which could depend on its dosage and/or metabolism by gastrointestinal microflora into as yet unidentified compounds that might enhance the proliferative effects of MPA in a subset of mammary tumor cells.

Overall our studies show that apigenin exhibits great potential as a chemopreventive and chemotherapeutic agent against progestin-dependent breast cancer, especially when administered intra-peritoneally. Our findings however, also highlight the potentially harmful effects of long-term intake of dietary apigenin. Further studies are clearly essential in order for us to better understand the complex mechanisms involved in proliferation vs anti-proliferation following apigenin consumption. In the following section we detail those issues needing to be addressed.

6.2.   PERSPECTIVES ON FUTURE STUDIES

This study suggests that apigenin has potentially beneficial chemopreventive and therapeutic properties against human progestin-dependent breast cancer. However, due to fundamental limitations of models employed in this study (cell cultures and rodent models), the feasibility of achieving comparable results in humans is not guaranteed. Previous studies have shown that certain naturally-occurring compounds exhibit distinct pharmacokinetics in humans, resulting in poor absorption and rapid clearance from the body and consequently rendering them pharmacologically inactive (Chen and Kong, 2005). Although some studies have been carried out to determine the pharmacokinetics of apigenin in rats, there is limited corresponding information pertaining to humans (Meyer et al, 2006). It is possible that the intra-peritoneal doses used in our animal studies might be higher than levels attainable in humans through consumption of dietary apigenin. Consequently if we are to use apigenin for long-term prevention of breast cancer in post-menopausal women, it is important that human clinical trials be done in order to fully characterize its pharmacokinetics. It is also important that we explore alternative

methods of long-term apigenin delivery for sustained pharmacological effectiveness. Given our observation of increased tumor multiplicity in animals ingesting apigenin, it is possible that consumption of the flavonoid might increase breast cancer risk in humans with a genetic predisposition to the disease, such as those with mutant BRCA genes. It is essential therefore that further studies be carried out in order to understand the specific mechanisms responsible for this troubling effect of dietary apigenin. As previously described *in vitro*, apigenin might exert proliferative effects at low levels, while being anti-proliferative at higher doses; consequently it is important that we determine supplementation levels appropriate for cancer prevention. Future studies will employ higher levels of dietary apigenin to determine whether elevating the amount of consumed apigenin abolishes adverse effects on tumor multiplicity.

As a therapeutic agent, apigenin has great potential if used in combination with other commonly used anticancer drugs. Combination therapy is now advocated by most experts in cancer research because of its potential to reduce adverse side effects commonly seen with clinically used chemotherapeutic drugs. In our study apigenin differentially induced apoptosis in xenograft tumors without affecting the growth of normal mammary epithelial cells. Consequently we contend that it might be safely used to increase the sensitivity of cells to other chemotherapeutic drugs, thereby making them effective at lower dosages. For example, apigenin appeared to have no effect on ERα expression, implying that a combination of apigenin and a selective ER modulator such as tamoxifen might be especially effective against hormone-responsive breast cancer. In addition, based on earlier studies by McVean et al. (2000) suggesting that apigenin might act to some extent by increasing the stability of wild type p53, it would be interesting to

examine its effectiveness when used in combination with other compounds known to reactivate and/ or stabilize p53 protein, such as PRIMA-1 (Liang et al., 2007; 2009) and RITA (Spinnler et al., 2011).

In this study, apigenin was administered to Sprague Dawley rats at a stage equivalent to puberty. Recent studies however suggest that dietary compounds might affect cancer risk by inducing epigenetic changes (De Assis and Hilakivi-Clarke, 2006); consequently we propose to expose fetuses *in utero* to apigenin in order to determine whether early exposure to the flavonoid might prevent later development of progestin-accelerated DMBA-induced mammary tumors. The mammary gland undergoes extensive growth during the fetal period; thus if apigenin is able to induce epigenetic changes at this early stage of development, it may well be beneficial for long term breast cancer prevention. It is also important that we perform studies to determine whether administration of apigenin prior to the carcinogenic insult protects against the initial formation of DMBA-induced mammary tumors, as well as opposing the effects of MPA on tumor development.

Although this study examined some of the possible molecular mechanisms through which apigenin might exert its effects on progestin-dependent mammary tumors, more studies are needed. The possibility that apigenin induces epigenetic changes is an exciting area for future studies. Since apigenin did not prevent MPA-induced hyperplasia of normal mammary epithelial cells in both the DMBA and xenograft models, further studies are required to explain the differential effects observed between normal mammary gland stem cells and cancer stem cells. A more comprehensive dose study is also

necessary if we are to better understand the effects at the molecular level of low and high

dose apigenin on breast cancer stem cells.

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APPENDIX

<u>Collaborative Projects</u>

Differential expression of FGF family members in a progestin-dependent BT-474 human breast cancer xenograft model.

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Running Title: FGF expression in BT-474 xenograft tumors

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ABSTRACT

Members of the fibroblast growth factor (FGF) family have been associated with tumor progression and angiogenesis, though the mechanism through which they affect the progression of breast cancer remains elusive. We recently showed that progestins increase the production of the potent angiogenic factor VEGF in an in vivo BT-474 human breast cancer cell-derived xenograft model. In this study we sought to determine the effect of progesterone (P) on regulation of specific FGF family members (FGF-2, FGF-4 and FGF-8), in the same model. Using immunohistochemistry we found that treatment with P significantly reduced FGF-2 and FGF-8 levels, while modestly increasing the levels of FGF-4 in tumors collected at the termination of the study or soon after P treatment began. The in vivo observations with FGF-2 were confirmed in cultured BT-474 cells, though the P-mediated reduction in FGF-2 was not blocked by the anti-progestin RU-486, suggesting that classical progesterone receptors (PR) are not involved in FGF-2 down-regulation. Also, P did not affect levels of FGF-2 mRNA in BT-474 cells, indicating that P exerts its effects on FGF-2 post-transcriptionally. Our observations suggest that the in vivo stimulation of BT-474 cell growth by P is associated with down-regulation of FGF-2 and FGF-8. Furthermore, since FGF-4 levels increased during P-treatment, FGF-4 may be required for tumor growth and maintenance and might therefore be a potential therapeutic target through which to suppress P-dependent tumor growth.

Breast Cancer Knowledge and Awareness among High School and College Students in Mid-Western USA.

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ABSTRACT

Early detection of breast cancer and its subsequent treatment lead to better patient prognosis and survival rates. However, despite advanced screening and detection techniques, the disease all too frequently remains undetected until it has reached an advanced stage. Despite a number of means of disseminating breast cancer awareness, such as public health programs, public and private media and the internet, breast cancer remains a major health problem which exerts a tremendous economic burden on the health care system. Consequently, promoting early awareness of the disease to students at both high school and college level might potentially lead to improved health habits later in life. This study investigated breast cancer awareness among college and high school students. A self-administered questionnaire was given to 355 high school students and 132 college students in a Mid-Western area of the USA to determine their perceptions of breast cancer, risk factors and the need for breast self-examination. Despite many annual efforts to disseminate breast cancer awareness, and the availability of information about the disease, our study showed that both college and high school students have a poor understanding of breast cancer. Most participants, however, were receptive to the provision of more information at school. Based on our findings, we recommend more breast cancer awareness programs targeting high school and college students. Breast cancer awareness could be included as a component of the high school curriculum, as well as via internet, and public health programs.

VITA

Benford Mafuvadze was born in Gweru, Zimbabwe on May 20, 1977. He grew up in a remote rural area called Matsai. Benford did his advanced level studies at Mashoko High School before enrolling for his Bachelor of Veterinary Science degree at the University of Zimbabwe in March 1997. After completing his Bachelor's degree in 2002, Benford began and successfully completed a Masters degree in Veterinary Physiology in 2004. He then accepted a teaching position in the Department of Preclinical Veterinary studies where he taught Physiology to Veterinary Science students for 3 years. While teaching at the University of Zimbabwe, Benford worked as a part-time small animal veterinarian at the Society of Prevention to Animal Cruelty between 2002 and 2006. In 2007, he was appointed chair of the Department of Preclinical Veterinary Studies, a position he held until he left to join the Department of Biomedical Sciences at the University of Missouri for his doctoral studies at the beginning of 2008. At University of Missouri, Benford joined Dr. Salman Hyder's laboratory where he successfully completed his doctoral studies looking at the chemo-preventive and therapeutic potential of apigenin on progestin-dependent breast cancer. Benford is married and has two children aged 14 and 6 year.