INHIBITION OF PROGESTIN-INDUCED VEGF IN MAMMARY CANCER BY CURCUMIN AND 2-METHOXY ESTRADIOL AND THEIR POTENTIAL ROLE AS ANTI-ANGIOGENIC & CHEMOPREVENTATIVE COMPOUNDS

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DEDICATION PAGE

I would like to dedicate this dissertation and my PhD to my parents, Dr. Jerome A Carroll and Mrs. E Loretta McCune Carroll. They are the inspiration of my life and the reason why education has been made a priority in my life. My parents were my first teachers—of those things small and large, but most of all, they taught me love, respect, and family are the most important things in life. With that, I share the honor of this degree with my siblings, Sandi D Carroll and Kenneth J Carroll, along with my immediate, extended family Mrs Tiffany Williams Carroll, and Ms Jayden Arie Carroll. I pray that this is the first of many honors I am able to share with you.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
1. HRT	Hormone Replacement Therapy
2. VEGF	Vascular Endothelial Growth Factor
3. 2ME2	2-methoxyestradiol
4. HIF-1α	Hypoxia-Inducible Factor 1-{alpha}
5. MPA	Medroxyprogesterone Acetate
6. PR	Progesterone Receptor
7. ER	Estrogen Receptor
8. VPF	Vascular Permeability Factor
9. ERE	Estrogen Response Element
10. PRE	Progesterone Response Element
12. wt-p53	Wild Type p53
13. mt-p53	Mutant p53
14. ARNT	Aryl hydrocarbon Receptor Nuclear Translocator
15. pVHL	Protein von Hippel-Lindau
16. HyRE	Hypoxia Response Element
17. DMBA	7,12-Dimethlybenzy[a]anthracene
18. E_2	Estrogen
19. DCC	Dextran-Coated Charcol

20. ELISA	Enzyme-Linked Immunosorbant Assay
21. BCA	Bicinchoninic Acid
22. SRB	Sulforhodamine B
23. AR	Androgen Receptor
24. H&E	Hemotoxylin and Eosin
25. FIH	Factor Inhibiting HIF
26. MVD	Micro Vessel Density
27. IHC	Immunohistochemistry

ABSTRACT

Hormone replacement therapy (HRT) is commonly prescribed to alleviate the symptoms of menopause in post-menopausal women. HRT supplements increase estrogen levels; however estrogen replacement therapy increases the risk for uterine cancer. In order to reduce the likelihood of uterine cancer, progestins, mainly medroxyprogesterone acetate (MPA), are prescribed alongside estrogen to prevent proliferation of uterine cells. Interestingly, studies have revealed that progestin + estrogen treatment increases the risk of breast cancer compared to women who received estrogen alone or placebos, indicating that the progestin component associated with HRT increases the risk of breast cancer. It has been reported that progestins cause breast cancer cells to up-regulate the expression of vascular endothelial growth factor (VEGF) and to secrete high levels of this potent angiogenic growth factor. VEGF plays a critical role in angiogenesis, the formation of new blood vessels from existing ones, making it an ideal target for progestin-dependent angiogenesis. RU-486 (Mifepristone), a known anti-progestin, has been shown to block progestin-induced VEGF secretion from human beast cancer cells; however RU-486 is not specific for the progesterone receptor (PR) and interferes with other hormone receptors that are essential for normal physiological functions. Consequently, there is a need for compounds that will inhibit progestin-induced VEGF secretion from breast cancer cells without undesirable side effects. The present study screens several natural compounds to investigate their ability to inhibit progestin-induced VEGF secretion from T47D human breast cancer cells. Two compounds, curcumin and 2-methoxyestradiol (2ME2) showed the most promise as anti-angiogenic agents and were investigated in

detail in experiments reported in this thesis. Curcumin, a derivative of the turmeric root inhibited MPA-induced VEGF secretion specifically from T47D breast cancer cells, without reducing the expression of PR. Though no mechanism became apparent from the present study, curcumin delayed progestin-accelerated DMBA-induced tumorigenesis in Sprague-Dawley rats and decreased the chance of tumor formation. The results from the curcumin studies showed that curcumin may prove beneficial to post-menopausal women who are prescribed combined HRT. 2ME2, an estrogen metabolite with no affinity for the estrogen receptor (ER), also showed significant promise as an anti-angiogenic compound. 2ME2 targeted hypoxia inducible factor -1{alpha}, (HIF-1α), which was discovered to be an essential transcription factor for progestin-induced VEGF transcription. Mechanistic studies using YC-1, a specific HIF-1α inhibitor confirmed the involvement of HIF-1α, since inhibition of HIF-1α activity with YC-1 or 2ME2 blocked progestin-induced VEGF secretion from T47D cells without decreasing HIF-1α or PR protein levels. YC-1 blocked MPA-induced up-regulation of VEGF mRNA transcript levels. Although there was no binding of PR to the VEGF promoter, increased binding of HIF-1α to the VEGF promoter in response to MPA treatment did occur. Such binding was blocked by treatment with either YC-1 or 2ME2 treatment, suggesting that PR binds indirectly to the VEGF promoter through HIF-1α. This was confirmed via IP assay showing a protein-protein interaction between HIF-1α and PR. Progestin-dependent *in vivo* studies showed that blocking HIF-1α with YC-1 brought about tumor regression in both DMBA-induced tumors in Sprague-Dawley rats and T47D and BT474 human breast cancer xenografts in nude mice. Decreased VEGF expression levels within tumor tissues were observed in both rat and mouse models. Although there was no statistically

significant reduction in blood vessel numbers, DMBA studies revealed that the blood vessels present were non-functional in that there was little or no blood perfusion within the tumors. It may be concluded from these data that HIF-1α is required for progestin-induced VEGF transcription and that PR interacts with the VEGF promoter indirectly through HIF-1α, which binds directly to the VEGF promoter. In conclusion, both curcumin and 2ME2 show promise as chemotherapeutic agents and HIF-1α may prove to be a beneficial target for hormone-dependent mammary cancer.

CHAPTER 1

INTRODUCTION

1.1 Description of Chapters

The following is a brief description of each chapter and its content, to be found herein. Chapter one provides an in depth discussion on the relationship existing between the progestin component of hormone replacement therapy (HRT), breast cancer, vascular endothelial growth factor (VEGF), and hypoxia. Also, the rationalization of why natural compounds were preferred for chemoprevention studies is also discussed in chapter 1. Chapter 2 describes the methods and results of how two compounds, curcumin and 2-methoxyestradiol (2ME2) were chosen for more in depth and detailed studies. Chapter 3, published in part in Menopause Journal, reports the ability of curcumin, an Indian spice, to specifically and dose-dependently inhibit MPA-induced VEGF secretion from T47-D human breast cancer cells. Also reported in chapter 3, are the protective *in vivo* effects of curcumin on the MPA-exposed mammary gland in Sprague-Dawley rats, as well as its effects on delaying in MPA-driven tumor development. Hypoxia-inducible factor 1-{alpha} (HIF-1α) is the focus of chapter 4, which reports significant findings with 2ME2 and YC-1, HIF-1α inhibitors. Chapter 4 describes studies undertaken to investigate the ability of 2ME2 and YC-1 to inhibit HIF-1α activity, providing evidence that HIF-1α is required for progestin-dependent VEGF induction in human breast cancer cells. YC-1 was used to investigate HIF-1α inhibitory mechanisms due to its ability to specifically target HIF-1α, as opposed to 2ME2, which is a more broad-spectrum compound. YC-1 exhibits similar inhibitory effects as 2ME2, confirming a significant role of HIF-1α in progestin-induced VEGF induction. YC-1 was also administered to animals in studies to

determine the effectiveness of YC-1 as a chemotherapeutic agent *in vivo*; the results provide strong evidence that HIF-1α could be a molecular target in the fight against progestin-dependent breast cancer (a novel finding). Chapter 5 concludes the chapters herein and discusses suggestions for future directions and investigations for this project.

1.2 Breast Cancer and Hormone Replacement Therapy

Breast cancer is the second highest cause of cancer death in the United States among women, with approximately 200,000 new cases reported annually, leading to 40,000 deaths [1]. The risk of developing breast cancer is increased in post-menopausal women compared to pre-menopausal women [1, 2]. Although the cause of this increased risk has not yet been elucidated, according to the American Cancer Society, there is an increased risk of breast cancer associated with the prolonged use of combined hormone replacement therapy (HRT) estrogen in combination with progestin, as compared with estrogen alone or placebo [2, 3]. In post-menopausal women, estrogens are usually prescribed to women to combat the symptoms of menopause such as hot flashes, dementia, loss of bone density, etc. Unfortunately, the estrogens prescribed in these treatments cause proliferation of cells in the endometrium and uterus—resulting in an increased risk of uterine cancer [4]. As a result, progestins are prescribed alongside estrogens to block the proliferative effects of estrogen [5, 6]. Subsequently, it was reported that the combination therapy increased the risk of breast cancer by 26% in these women compared to women receiving only estrogen alone [2]. In recent years, the natural and synthetic hormones, progesterone and medroxyprogesterone acetate (MPA) found in oral contraceptives and HRT, have been shown to induce secretion of vascular endothelial growth factor (VEGF) in human breast cancer cells (T47-D and BT-474),

both of which express progesterone receptors (PR) and estrogen receptors (ER) [7, 8]. Thus, it is tempting to speculate that progestins play an agonistic role in the proliferation of breast cancer cells.

Progestins have been reported to elicit both proliferative and anti-proliferative effects in breast tissue [9, 10], resulting in a controversy over the role of progestins in breast cancer. It is known that higher doses of progestins are indeed anti-proliferative and used to treat hormone responsive cancers [10, 11]. However, there are numerous reports that illustrate that progestins cause mammary cells to proliferate [12-16]—thus, providing more evidence that progestins increase the risk of breast cancer.

Moore et al. have reported that progestins play a protective role, and inhibit cell death in breast cancer cells; specifically, T47-D, MCF-7, and MDA-MB-231 breast cancer cells. [17]. Moore et al. speculate that this phenomenon, although controversial, explains why combined HRT containing both estrogen and progestin, is more problematic than the use of estrogen alone. They reason that progestins are protecting breast cancer cells from undergoing apoptosis, while estrogens lack this protective property. Therefore, estrogens have a lower risk of breast cancer in postmenopausal women, compared to women receiving combined HRT—as reported in numerous HRT studies [2, 18].

1.3 VEGF and Angiogenesis under Steroid Hormone Regulation

Vascular endothelial growth factor, or VEGF, has been generally defined as a potent angiogenic growth factor that plays a critical role in the regulation of angiogenesis [19, 20]. Also referred to as the vascular permeability factor (VPF), VEGF increases the permeability of blood vessels [21] that ultimately increase the nourishment of tissues,

including tumors—thus promoting tumor growth. VPF/VEGF was reported to be secreted from a variety of human and rodent tumor cell lines [22]. Soon after this report, it was proposed and proven that solid tumors were angiogenesis-dependent [23]; and evidence later showed that endothelial cells contain VEGF receptors, Flk/Flt, during embryogenesis [24], vasculogenesis, and angiogenesis [25, 26]. As a result, it has been concluded that tumors release growth factors, including VEGF, which signal blood vessels to grow, or bud off, in the direction of the tumors to increase blood flow to the tumor (Figure 1.1).

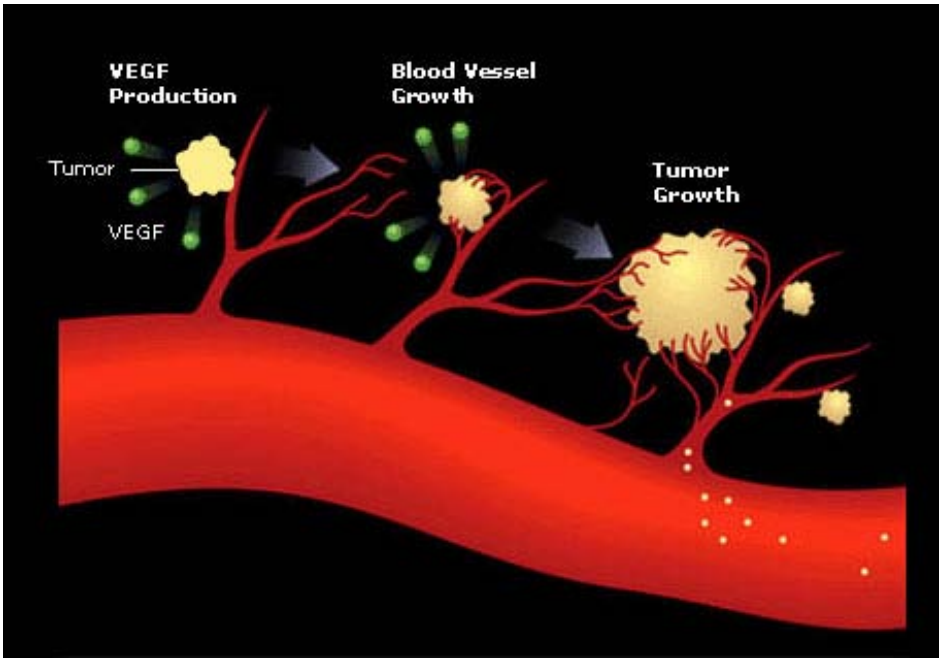

Figure 1.1: Tumors release growth factors, including VEGF, signaling endothelial cells to bud off in the direction of the angiogenic signal, resulting in angiogenesis [27].

There is also evidence that VEGF functions in an autocrine and paracrine manner with tumor cells, thus promoting permeability of nutrients within a tumor environment, which in turn promotes tumor cell survival [28] and proliferation of tumor cells [29]. These studies have provided strong evidence that VEGF is an ideal target for anti-cancer therapies [23, 30]. However, like many anti-cancer therapies, specifically targeting

VEGF in tumor cells becomes extremely problematic because VEGF is critical in other biological functions including wound healing and neovascularization for growth and development [31].

Because angiogenesis is required for endometrial vascularization—a target tissue for sex steroids, it was hypothesized that hormones play a role in angiogenesis [32]. Consequently, it was shown that VEGF is under steroid hormone regulation [7, 33, 34]—thus the presence of hormones has a marked effect on VEGF expression. The VEGF promoter region has been shown to contain non-consensus binding sites for estrogen and progesterone steroid hormone receptors [34-36]; anti-hormones have been shown to block hormone-induced VEGF induction in breast cancer, both *in vitro* and *in vivo*, thus confirming the role of estrogen and progesterone receptors in VEGF regulation [3, 28, 29].

1.3.1 Estrogen Receptors

Estrogen receptors (ER) are ligand-activated transcription factors [37]. Both ERα (66kDa) and ERβ (56kDa) contain ligand- and DNA-binding domains, and upon binding the estradiol ligand in the nucleus, the ER forms a homodimer, or a heterodimer with other transcription factors, and binds DNA to influence transcription of estrogen-mediated genes [38]. Originally considered to be sequestered to the nucleus, there is growing evidence that supports the existence of extranuclear steroid receptors [39]. It is reported that these extranuclear receptors have specific functions including activating specific kinase pathways resulting in non-genomic cellular changes [39]. Although no consensus estrogen response element (ERE) has been reported on the VEGF promoter, based on ERE homology, two putative EREs have been reported to be present within the

rat VEGF 5' and 3'-untranslated region [32]. However, this region contains an ERE sequence that deviates from the consensus ERE sequence, which may explain tissue-type specificity; also, an increase in VEGF expression is only mediated in the reverse orientation of the 5' untranslated region [32]. Other groups report that ER, and other steroid hormone receptors, interact with the VEGF promoter by interacting with other transcription factors including Sp1 and Sp3, which have been shown to bind directly to the VEGF promoter [40-42]. Even though much controversy surrounds a possible ERE on the VEGF promoter, it is widely accepted that ER does plays a major role in the expression of the VEGF protein in the uterus. Evidence that ER mediates VEGF induction in breast cancer cells remains controversial [7, 43, 44].

1.3.2 Progesterone Receptors

The progesterone receptor (PR) is a steroid hormone receptor with two isoforms, PR-A and PR-B [12]. PR-B is considered the dominant isoform in PR-mediated gene regulation, including VEGF expression [8]. Like the ER, both PR isoforms contain a ligand- and DNA binding domain. PR, forming a homodimer upon binding the progestin ligand, binds to a progesterone response element (PRE) on the DNA of progestin-mediated genes, thus influencing transcription [12] as shown in **Figure 1.2**.

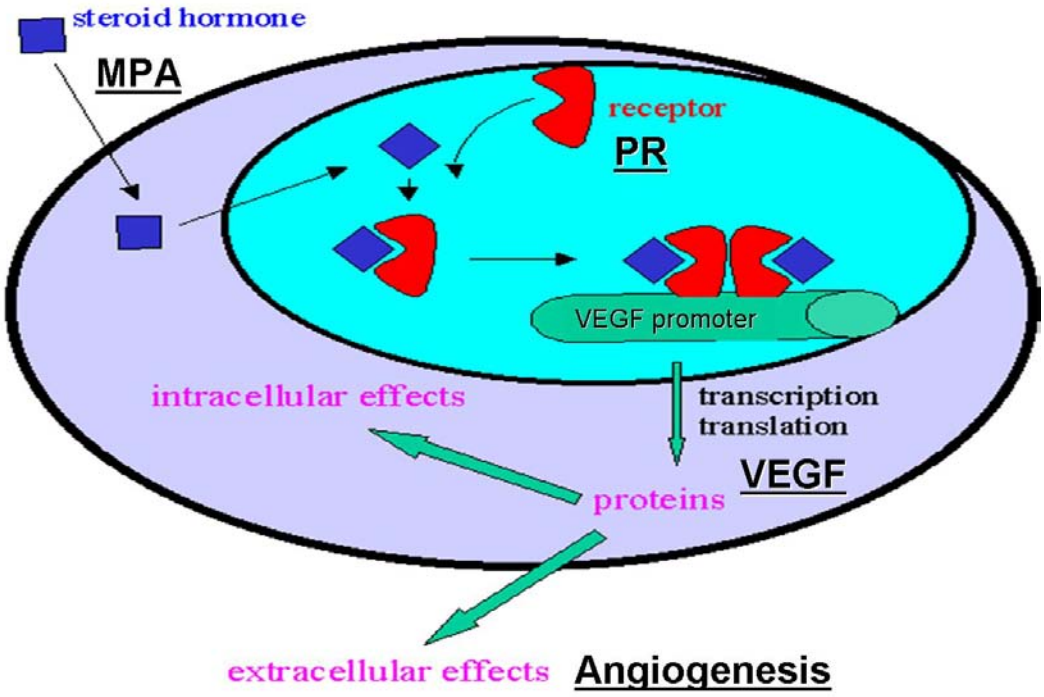

Figure 1.2: The ligand, MPA, binds to the progesterone receptor. Two ligand-bound receptors dimerize and bind to the promoter of the target gene (VEGF), influences transcription. The VEGF promoter results in the release of angiogenic factors. [45]

PR is also well-known for its presence within the nucleus, however evidence is growing to support the hypothesis that extra-nuclear and membrane progesterone receptors exist [39]. The VEGF gene is also regulated by progestins [7, 35], the VEGF promoter contains a complex progestin response element (PRE), although no consensus binding site has been elucidated [35].

1.4 Mutant p53, VEGF, and Breast Cancer

The tumor suppressor gene, *p53*, plays a critical role in cancer prevention [46, 47]. Normal, or wild-type, p53 (wt-p53), accumulates in the nucleus and becomes activated upon cellular stress which leads to growth arrest or apoptosis [47]. This allows for selection against cells with damaged DNA, thus preventing the development of tumors. However, it has been reported that more than half of all cancer cases have a

mutation in the *p53* gene [48]. These mutations usually occur in the DNA-binding domain of the protein, resulting in its inability to bind DNA and influence transcription [48]. As a result, p53-dependent tumor suppression by the mutated p53 does not occur. Interestingly, it has been shown that a mutation in the *p53* gene is required for the progestin-dependent response of breast cancer cells to increase VEGF secretion [49]. It has recently been shown that reactivation of the p53 protein to a wild-type p53-functioning protein in breast cancer cells results in a decreased, or complete, loss in the ability of cells to secrete VEGF in response to progestin treatment [50].

Wild-type p53 has been shown to bind to Sp1, a transcription factor, thus inhibiting binding to the VEGF promoter and preventing transcription of VEGF [51]. The mutant form of the p53 protein is unable to bind DNA, thus resulting in an increased transcription of the VEGF gene [52]. Other studies report that loss of function of wt-p53, via mutation or deletion, causes an increase in levels of the α subunit of the complex hypoxia-inducible factor 1 (HIF-1) [53], which is discussed in detail in the next section *(1.5)*. This increase in HIF-1α subsequently leads to an increased expression of HIF-1 target genes, including VEGF.

1.5 Tumor Environment and Hypoxia

The tumor microenvironment is described as hypoxic, which means the growing tumor(s) lack adequate oxygen and nutrients due to a decreased blood supply [54]. This dynamic leads to angiogenesis to overcome this hypoxic environment. As a result, hypoxia inducible factor 1 (HIF-1), specifically the alpha (α) subunit, is becoming a more popular target for anti-angiogenic and anti-cancer therapies [55, 56] because HIF-1α regulates a number genes, including VEGF, that promote tumor cell growth [57, 58].

HIF-1 is a heterodimer that consists of an alpha (α) and beta (β) subunit—also referred to as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1α is a transcription factor that accumulates in cells under hypoxic and stressful environments [59]. HIF-1α plays a specific role in cellular homeostasis in response to hypoxia. Under normoxic conditions, the HIF-1α subunit is hydroxylated at two proline residues (402 and 577), ubiquitin-tagged for degradation by the von Hippel-Lindau (pVHL) E3 ubiquitin ligase complex [54]. The tagged protein is then quickly degraded by the 26S proteosome [54]. However, under hypoxic conditions, HIF-1α is accumulates and binds to the constitutively expressed HIF-1β subunit. The HIF-1 complex is then translocated to the nucleus, and binds to the hypoxia response element (HyRE) on target DNA, along with other transcription factors, to influence transcription of hypoxia-regulated genes, which as previously described can potentiate tumor development. Thus, one method to combat cancer may include blocking the formation of the HIF-1 complex and inhibiting HIF-1 DNA-binding [54, 55, 58].

Although the idea of targeting HIF-1 in cancer therapy seems to be gaining merit, few efforts have been directed at utilizing HIF-1α to curtail the growth of endocrine related cancers, especially breast cancer. One study, however, has shown that estrogen induces the activation of HIF-1α and increases edema and VEGF in the rat uterus [33]. This provides strong evidence that HIF-1α activity can be mediated by the presence or absence of hormones.

1.6 Anti-Progestins

The angiogenic effects of progestins can be inhibited by RU-486 also referred to as mifepristone [7]. RU-486 is an anti-progestin that binds to the PR and blocks ligand

binding [60]. As a result, progestin-mediated genes are not influenced by the presence of progestins. The anti-progestin has shown inhibitory effects towards 7,12-dimethylbenz[a]anthracene (DMBA)-induced, progestin-accelerated mammary tumors in rats [61] as well as in progestin-driven human xenografts in mice [62]. Unfortunately, RU-486 also cross reacts with other steroid receptors, including glucocorticoid receptors, which is detrimental to general health [60]. However, this positive inhibitory effect does show promise and supports the idea that the progesterone receptor is a plausible target for combating progestin-driven breast cancer. Nonetheless, due to the lack of specificity of RU-486 to the breast tissue, it is not ideal for breast cancer treatment due to its ability to disrupt other critical body systems [60].

Interestingly, the anti-estrogen, ICI, 872,780 inhibits progesterone induced VEGF secretion from human breast cancer cells [63]. However, the anti-estrogen failed to inhibit MPA-induced VEGF secretion [49] . This suggests that natural and synthetic progestins may be inducing VEGF secretion from breast cancer cells through different mechanisms. This shows promise that a compound may have the potential to target synthetic progestins, without blocking the effect of the natural hormone. This may be achieved by targeting specific pathways unique to the synthetic hormone.

Although not categorized as an anti-progestin, the novel drug PRIMA-1 has been shown to exhibit cell-specific, anti-progestin effects. PRIMA-1 targets mt-p53, causing it to function like wt-p53. As previously stated, mt-p53 is essential for progestin-induced VEGF transcription. PRIMA-1 does not affect the activity of PR, thus providing evidence that some progestin-dependent regulatory pathways can be blocked by means other than the progesterone receptor—suggesting other targets may possibly be used to

combat progestin-dependent breast cancer, perhaps with more specificity than the aforementioned treatments.

1.7 Natural Anti-angiogenic/Chemopreventative Compounds

Many treatments for disease are derived from plants, or naturally occurring compounds. Plant-derived, natural compounds are becoming more popular in the medical field as we search for new cures. Current cancer treatments already utilize natural plants and compounds such as vinblastine, from the Madagascar periwinkle plant [64] , paclitaxel (formally known as taxol) and docetaxel from the bark of the pacific yew tree [65]. These plant derivatives serve as a model for numerous synthetic analogues that are used in most chemotherapies today. Because these plants and their derivatives are naturally occurring and are currently included in the daily diet, they were thought to have few, if any, toxic effects in mammals. Although these derivatives have been proven to be cytotoxic to cells and prevent DNA replication [66], cancer cells are not specifically targeted in these treatments. While the original theory of utilizing these compounds was simply to specifically target cancer cells and prevent DNA replication, the necessary level of specificity has yet to be achieved.

Over the years, more findings have been reported regarding various levels of hormone-like activity of many plant extract components. Specifically, phytoestrogens and their associated risk of breast cancer have become well documented [67, 68]. Phytoestrogens are defined as plant-derived, diphenolic compounds that display estrogen-like, as well as anti-estrogen-like activity, depending on the dose used [69]. Studies have provided strong evidence that many plant derivatives do, in fact, display estrogen-like activity, including soy isoflavones, genistein, daidzein, and clycitein [69]. Based on this

evidence, the question arises, “do phytoprogestins exist?” In other words, are these plants, or plant derivatives, capable of functioning as progestins or anti-progestins? Is it possible that a natural progestin can prove less harmful than synthesized progestin, and thus reduce the increased risk of breast cancer among post-menopausal women? Or, does there exist a natural and safe anti-progestin, which can be taken alongside HRT to reduce breast cancer risk? These questions are what led me to screen a number of naturally occurring compounds, and a few synthetic compounds, that were defined as anti-angiogenic and/or anti-cancer. Compounds were tested via VEGF ELISA for their ability and efficacy in inhibiting progestin-induced VEGF secretion from human breast cancer cells. Upon concluding my initial screenings, I found two compounds, both naturally occurring—one a plant derivative, the other an endogenous estrogen metabolite, that showed significant promise in blocking harmful effects of progestin-induced activity in human breast cancer cells. Two compounds, curcumin and 2-methoxyestradiol (2ME2), were chosen for the studies described herein. They are both are currently in clinical trials for treatments of various types of cancer other than hormone driven breast cancer. This dissertation discusses the in depth studies I undertook to investigate the potential of these two compounds to be used as effective treatment strategies for progestin-dependent breast cancer.

CHAPTER 2

INITIAL SCREENING OF COMPOUNDS FOR INHIBITION OF MPA-INDUCED VEGF INDUCTION IN T47-D HUMAN BREAST CANCER CELLS

2.1 Introduction

Clinical trials show an increased risk, incidence, and recurrence of breast cancer among post-menopausal women who receive both estrogen and progestins in combined hormone replacement therapy (HRT), compared to women who receive estrogen alone [2]. This and other more recent studies suggest that progestins play a major role in the progression and initiation of mammary tumorigenesis [12, 61, 62]. Earlier reports show that human breast cancer cells, T47-D, BT-474, and HCC-1428, secrete vascular endothelial growth factor (VEGF), in response to progestin treatment [7]. VEGF is a potent angiogenic growth factor that is critical for tumor progression and survival [23]; increased levels of VEGF are associated with increased tumor size and volume, as well as accelerated tumor development [29, 61]. This finding has led researchers to target VEGF in the fight against cancer. Induction of VEGF by progestins is blocked by the anti-progestin RU-486 [7], also known as mifepristone. RU-486, however, is not an ideal anti-progestin due to its lack of specificity to the progesterone receptor [60]. As a result, a safe and specific compound that displays anti-progestin-like activities and curtails the production of progestin-dependent angiogenic growth factors from breast cancer cells remains to be discovered. Such compounds may prove effective against progestin-dependent breast cancer.

Presently, most cancer treatments utilize natural plants and plant derivatives such as vinblastine, from the Madagascar periwinkle plant [64] , paclitaxel (formally known as

taxol) and docetaxel from the bark of the pacific yew tree [65]. These plant derivatives serve as models for numerous synthetic analogues that are used in most chemotherapies today. Because these plants and their derivatives are naturally occurring and are currently consumed in daily diets, it was assumed that they would prove non-toxic to mammals. Although these derivatives have proven to be cytotoxic to tumor cells and prevent DNA replication [66], they are also cytotoxic to normal cells. Thus, the necessary level of specificity for cytotoxic agents has yet to be achieved.

Many plant compounds that have been shown to exhibit hormone-like (especially estrogenic) activities [70, 71] have been termed phytoestrogens. Phytoestrogens are defined as plant-derived, diphenolic compounds that display estrogen- or anti-estrogen-like activity [69]. The risk of breast cancer associated with phytoestrogens, including soy isoflavones, genistein, daidzein, and clycitein [69], have been well studied in recent years [67, 68]. Interestingly, it has been reported that specific foods and plant derivatives display both agonist and antagonist bioactivity of ER and PR [70] and also have the ability to regulate growth rates in breast cancer cell lines [70]. This study provides strong evidence that naturally occurring compounds, including plant derivatives, may have the ability to play in inhibitory or preventive role in hormone-dependent breast cancer.

In the present chapter, several naturally occurring compounds, which have been defined as anti-angiogenic and/or anti-cancer, were screened to determine their potential to block progestin-induced VEGF secretion in T47-D human breast cancer cells. Based on these initial screenings, two of the following 14 compounds, curcumin and 2-methoxyestradiol, were selected for further investigation. Their detailed effectiveness

and limitations as chemotherapeutic and chemopreventive agents are explored in later chapters.

Alliin

Alliin is an extract of garlic that has been reported to display anticarcinogenic [72] and antiangiogenic properties [73]. One study reports that alliin inhibits VEGF-induced angiogenesis in the chick chorioallantoic membrane (CAM) model, as well as bFGF-induced human endothelial cell tube formation [73]. Another report demonstrated that garlic powder, with various levels of alliin, fed to rats reduced the appearance and size of hepatic glutathione S-transferase (GST) loci, with the largest reduction seen in rats with the highest alliin content [72]. These anticancer activities of alliin led to its addition in these initial screenings.

Apigenin and Naringenin

Both apigenin and naringenin are termed phytochemicals [74], displaying both estrogenic and progestational activity [71]. Apigenin and naringenin are dietary isoflavonoids, with apigenin found mainly in fruits and vegetables [75], and naringenin in citrus fruits [74]. Both compounds have been shown to exhibit steroid hormone-like activity [71]. Interestingly, it has been reported that apigenin is antiangiogenic by virtue of its ability to reduce HIF-1α and VEGF expression [76]. Both compounds gained interest through reports of steroid hormone-like activity as well as the potential to inhibit angiogenesis.

Curcumin

Curcumin is an Indian spice derived from the turmeric root, known mostly for its rich, orange color [77]. Found in many Indian dishes, including curry, curcumin has been

utilized historically for both its antibacterial and anti-inflammatory properties [77]. Recent studies have reported many other potential effects of curcumin on multiple diseases including Alzheimer's and cancer [77-79]. There are a growing number of reports that suggest curcumin is a chemopreventive agent in multiple neoplastic diseases [77]. Turmeric has been reported to bind with low affinity to both the estrogen and progesterone receptors [70], suggesting curcumin may be used to target PR and/or ER in an effort to combat progestin-induce VEGF induction, and eventually progestin-dependent breast cancer.

Green Tea Catechins

Catechins, including epicatechin (Ec), epicatichen gallate (EcG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG), are found in green tea, which has recently been shown to possess anticancer activity and was reported to inhibit mammary tumorigenesis in a C3(1)/SV40 mouse model [80]. EGCG is the most abundant of the green tea catechins [81] is able to inhibit VEGF induction in breast cancer cells [82] and EGCG has been shown to block angiogenic VEGF signaling by disrupting the receptor complex formation [81]. These catechins showed promise as anticancer and antiangiogenic agents, hence their inclusion in the initial screening.

Indole-3-Carbinol

Termed a phytochemical [83], indole-3-carbinol is found in cruciferous vegetables, including cabbage, broccoli, radishes, etc., [84]. Indole-3-carbinol and its derivatives have many molecular targets [84] thus increasing its potential as an anticancer agent. It has been reported to inhibit the activation of SP1, ER, the androgen receptor (AR), and nuclear factor-E_2-related factor 2 (Nrf2) [84]. Having exhibited inhibition of

estrogen-induced ER-α activity in human breast cancer cells and inhibition of estrogen-dependent tumors [84], indole-3-carbinol may have the potential to inhibit the cross-talk between ER and PR, thus inhibiting progestin-induced VEGF secretion from human breast cancer cells.

Lycopene

Most commonly known for its presence in tomatoes, lycopene is a carotenoid. Eighty percent of the U.S. source of lycopene is from tomatoes [85], however, lycopene is found in other plant foods including apricot, pink grapefruit, watermelon, and papaya [85]. Although lycopene is well studied in the prevention of prostate cancer, until recently, few studies focused on lycopene ingestion and its effects on breast cancer risk. As the number of studies regarding this correlation increases, the overall conclusion remains controversial. One study reports that there is no correlation between higher dietary or plasma levels of lycopene and a decreased risk of breast cancer [85], while another study conducted with Sweden women, reports a decreased risk of beast cancer with lycopene ingestion, especially in menopausal women [86]. An *in vitro* study supports the notion of lycopene being effective in breast cancer treatment, reporting that it inhibits estrogen-dependent and independent cell proliferation of breast cancer cells [87]. The role that lycopene may play in progestin-dependent proliferation of breast cancer cells, as well as its capacity to inhibit or block progestin-dependent VEGF secretion from breast cancer cells remains to be determined.

Resveratrol

Resveratrol is a naturally occurring phenol that is most commonly found in grape peel [88], thought it also occurs in peanuts and other fruits [89]. Resveratrol induces

apoptosis in colon cancer cells [90], and inhibits HIF-1α accumulation and VEGF expression in human tongue squamous cell carcinomas and hepatoma cells [91]. These studies strongly suggest that resveratrol, due to its apoptotic and anti-VEGF properties, may be an effective agent against cancer. Resveratrol has been reported to show little to no activation of the PI3-K and MAPK pathways in MC-7 cells [92], providing a rationale for its anti-proliferative activity. Activated PI3-K and MAPK pathways have been reported to play an agonistic role in progestin-induced secretion of VEGF in breast cancer cells [93], thus providing evidence that resveratrol may be effective against progestin-dependent VEGF induction.

Sulindac Sulfide

A synthetic compound, sulindac sulfide is a non-steroidal anti-inflammatory drug (NSAID), originally used to treat inflammatory diseases [94]. Sulindac sulfide was recently reported to decrease ER and PR expression in human breast cancer cells [94] and inhibits cell growth by arresting cells in G_1 phase [94]. As a result, though not naturally occurring, sulindac sulfide was added to this short list of compounds to be tested. Interestingly, it was reported that sulindac sulfide decreases the number and size of colorectal polyps [95] as well as preventing polyp recurrence [95]. Sulindac sulfide derivatives were also reported to inhibit growth and promote apoptosis in prostate cancer cell lines [96]. Though originally designed as an NSAID, this drug has shown promise as an antitumor agent, and may be used to target PR and ER expression.

Thalidomide

Originally used as a sedative and anti-nausea drug, thalidomide was removed from the market when it was found to be a strong teratogen [97], resulting in extremely

abnormal fetal development [98]. Later found to inhibit angiogenesis [99], thalidomide is now being considered as an advance treatment for tumors including ovarian and breast tumors [100]. A phase II clinical trial reports that thalidomide is well-tolerated at an oral dose of 100 mg daily; however, only 16% of patients with renal cancer showed a decrease in tumor size while another 16% experienced no further progression of their disease for up to 6 months [100]. Nonetheless, the anti-angiogenic properties and high dose-tolerance of thalidomide resulted in its inclusion in this initial study.

2-Methoxyestradiol

2-methoxyestradiol (2ME2) is an endogenous estrogen metabolite that is known to be anti-angiogenic as well as anti-mitotic [101-104]. Although 2ME2 is an estrogen (E_2) derivative, it has little-to-no affinity for ER [105] and whilst it is anti-angiogenic, 2ME2 may operate via multiple mechanisms [106], including blocking hypoxia-induced VEGF secretion from breast cancer and prostate cancer cells [101]. The anti-angiogenic and anti-proliferative properties displayed by 2ME2, as well as a high dose-tolerance [107], probably due to its endogenous presence [108], suggests that 2ME2 has the potential to combat progestin-dependent breast cancer by inhibiting progestin-induced VEGF upregulation.

2.2 Materials & Methods

Reagents. Alliin, apegenin, epicatechin (Ec), epicatechin gallate (EcG), epigallocatechin (Egc), lycopene, and thalidomide were purchased from LKT Laboratories, Inc. (St. Paul, MN). Resveratrol, epigallocatechin gallate (EGCG), and curcumin were obtained from Cayman Chemical Co. (Ann Arbor, MI), and 2ME2 was purchased from Toronto Research Chemicals, Inc (Ontario, Canada). We purchased

naringenin, indole-3-carbinol, sulindac sulfide, medroxyprogesterone acetate (MPA) and RU-486 were from Sigma-Aldrich (St. Louis, MO). Pierce BCA protein assay reagents were from Fischer Scientific International.

Cell Culture. T47-D cells were cultured in 100 mm cell culture plates in DMEM/F12 cell culture media containing 10% Fetal Bovine Serum (FBS). At 50-60% confluence, media was changed to DMEM/F12 media containing 5% dextran-coated charcoal (DCC)-treated serum for 24 h. After washing with PBS, DMEM/F12 media containing 5% DCC-treated serum was replaced and cells were treated for 16-18 h with MPA (10 nM) and/or the designated anti-angiogenic compound at 1 or 100 μM, based on literature. Control cells were treated with the solvent of the reagent (ethanol or DMSO). Each treatment was performed in triplicate.

VEGF ELISA. Quantikine Human VEGF ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Supernatant was collected from cells treated with progestins and/or test compounds and analyzed using the VEGF ELISA kit according to the manufacturer's protocol. Experiments were performed in triplicate and each sample was analyzed in duplicate. According to the manufacturer, the minimum detectable concentration of VEGF is less than 5 pg/ml, and the intra-assay precision has a coefficient variance (CV%) between 3.5-6.5%, while the CV% of the inter-assay ranges from 5.0-8.5%.

BCA Protein Assay. Cells were harvested and cell pellets were re-suspended in 300 μl lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, and 1% NP-40) and incubated on ice for 30 min. Samples were centrifuged and protein concentration of supernatant determined by BCA assay using BSA as a standard. Absorbance was measured at 562 nm

using a SpecTRA MAX 190 microplate reader (Sunnyvale, CA). All samples were analyzed in duplicate.

Statistical Analysis. The average value for VEGF levels (pg/mg) in the untreated (control) samples was calculated from the number of determinants in that group and used to calculate VEGF levels in untreated and treated groups as a percentage value. Within each group, results are plotted as bar graph representing mean $\pm$ SEM. Data were analyzed by one-way ANOVA and all groups compared with controls. Data sets with inhibitors were compared statistically with VEGF values in progestin treated samples and differences considered significant when p values were <0.05. All data was checked for normality and homogeneity of variance. Since a number of analyses did not satisfy these assumptions, data was ranked and ANOVA performed as outlined by Conover and Iman [109]. Fisher's protected least significant difference (LSD) was performed to determine treatment differences, as suggested by Chew [110]. All significance was based on the ranked transformations; however treatment values are presented as actual values. Sigma Stat software was used for data analysis. SRB assay was performed in multiples of six. In each figure, all data sets were compared to controls for significant difference following treatment.

2.3 Results

T47-D human breast cancer cells were pre-treated for 30 minutes with potential inhibitory compound and treated with 10nM Medroxyprogesterone acetate (MPA) for 16-18 hours as described in the methods and materials section. VEGF ELISA kits were used to determine VEGF levels in cultured media. Apigenin and curcumin showed significant inhibition at the lower concentration of 1 μM (Figure 2.1), while some compounds

including 2ME2 and indole-3-carbinol showed slight inhibition; however, this inhibition was not statistically significant. Nonetheless, this trend suggested that a higher concentration of the treatment compound may result in a statistically significant value. As a result, compounds were screened for inhibition using 100 μM of treatment compound (Figure 2.2). This screening resulted in multiple compounds that blocked MPA-induced VEGF secretion from T47-D breast cancer cells at a higher concentration. Of these inhibitory compounds, 2ME2 showed the most inhibition and was used in further chemopreventive studies discussed in later chapters.

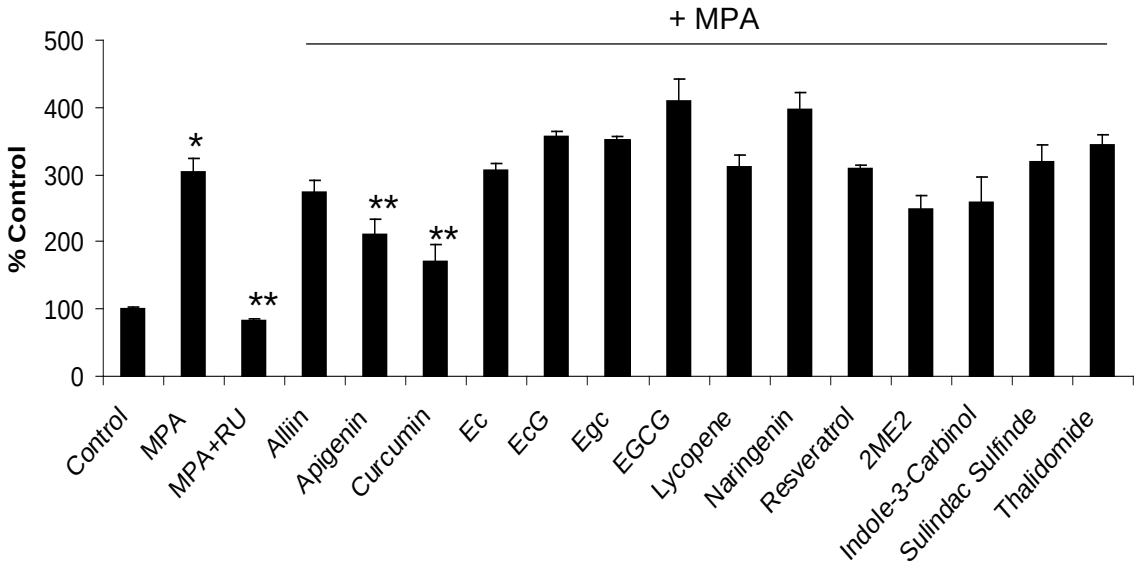

Figure 2.1: VEGF ELISA: Initial screening of compounds at 1 μM. T47-D cells were treated with 10 nM MPA as described in methods and materials. Control = 365 ± 22 . * represents significance compared to control. ** represents significant inhibition compared to MPA. One way ANOVA, Fisher's least significant difference (LSD) was performed to determine significance (p = <0.001), n = 3-36. Error bars represent standard error of means (SEM).

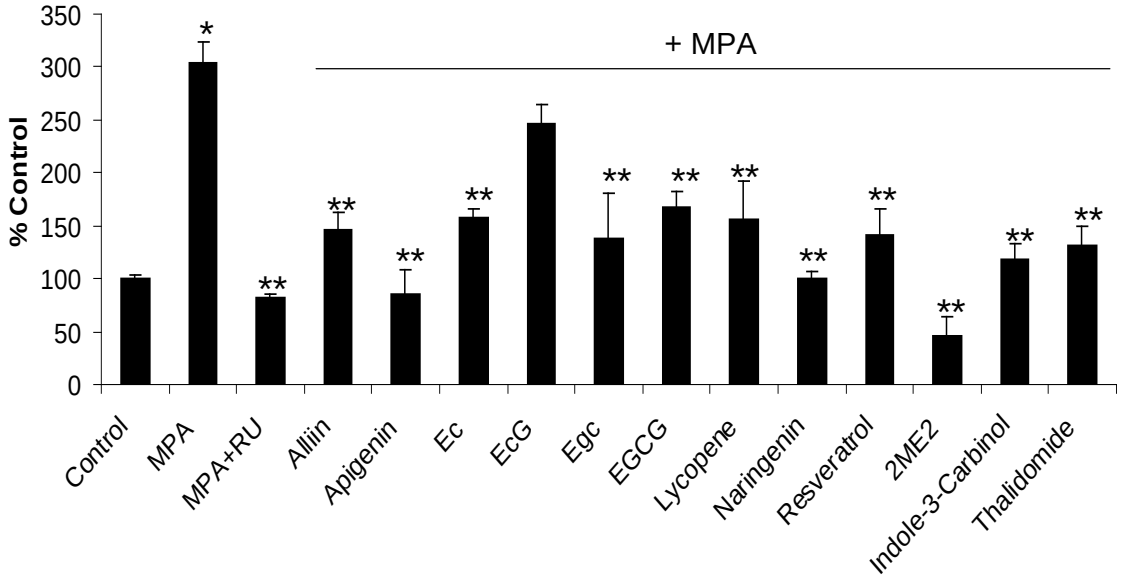

Figure 2.2: VEGF ELISA: Initial screening of compounds at 100 μM. T47-D cells were treated with 10 nM MPA as described in methods and materials. Control = 365 ± 22. * Represents significant induction compared to control. ** Represents significant inhibition compared to MPA group. One-way ANOVA, Fisher's LSD was performed to determine significance (p = <0.001), n =3-36. Error bars represent SEM.

Interestingly, curcumin showed inhibition at 1 μM and was toxic to T47-D cells at 100 μM. As a result, a dose curve was performed to determine at what concentration curcumin becomes toxic to these cells. Figure 2.3 shows an initial dose curve that was performed with 1, 10, 50, 75, and 100 μM of curcumin. In this preliminary experiment, 10 μM curcumin appeared to elicit an increase in VEGF secretion from T47D cells; however, more detailed studies (as shown in Chapter 3) reveal this result to be uncommon. Unexpectedly, only 1 and 10 μM curcumin treatment produce reportable data because cells could not tolerate concentrations higher than 10 μM (Figure 2.4).

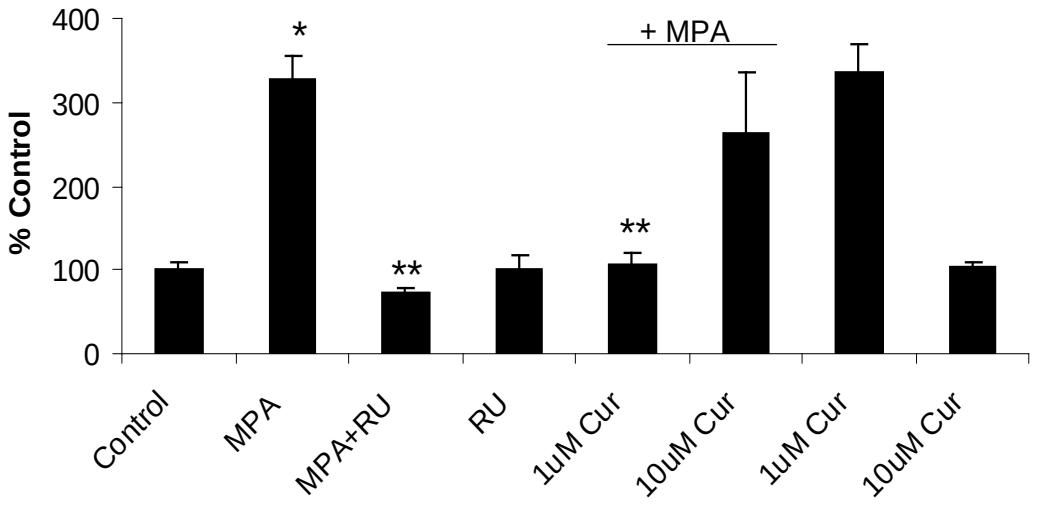

Figure 2.3: VEGF ELISA: Initial curcumin dose curve. T47-D cells were pre-treated as described in methods and materials with 1, 10, 50, 75, and 100 μM curcumin. Curcumin proved toxic at concentrations higher than 10 μM. Control = 404 ± 36. * Represents significant induction compared to control. ** Represents significant inhibition compared to MPA. One-way ANOVA, Fisher's LSD was performed to determine significance (p = <0.001), n = 3-9. Error bars represent SEM.

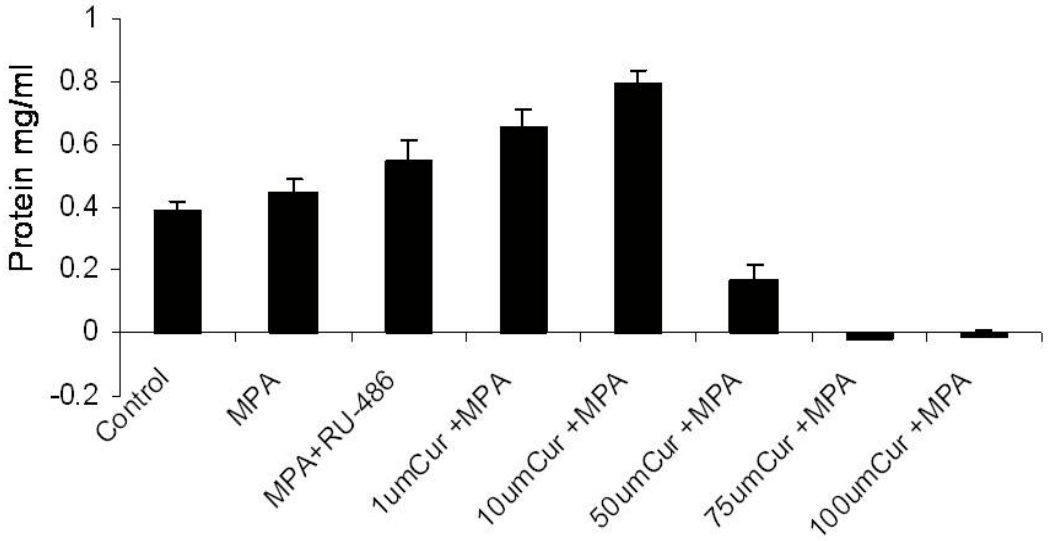

Figure 2.4: BCA Protein Assay: Average protein content of T47-D cells per cell culture dish treated with 1, 10, 50, 75, and 100 μM curcumin for 16-18 hours. Control = 0.39 ± 0.029. Error bars represent SEM. n=6

BCA protein assay shows a marked decrease in protein per cell culture dish at 50 μM curcumin, while 75 μM and 100 μM curcumin showed complete loss of cells. Because curcumin showed inhibition at low concentrations, it was chosen for further studies as a chemopreventive agent. These studies are described in detail in the following chapter (Chapter 3).

Because lycopene is known to have anti-cancer effects in prostate cancer which is also hormone-repsonsive and 2ME2 showed the most inhibition (Figure 2.2), a dose curve with lycopene and 2ME2 was performed to further investigate the inhibitory trend of these compounds. Both lycopene and 2ME2 had a dose-dependent, inhibitory effect on MPA-induced VEGF secretion from T47-D cells (Figure 2.5). This inhibition was more pronounced with 2ME2, compared with lycopene.

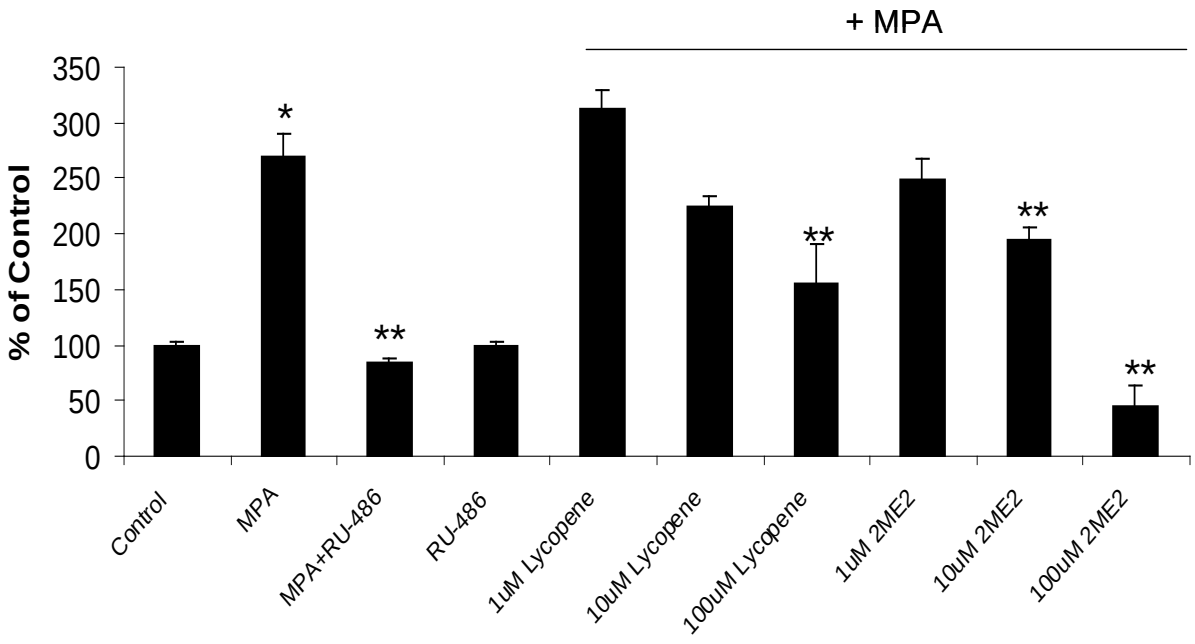

Figure 2.5: VEGF ELISA: Initial dose curve of lycopene and 2ME2 at 1, 10, and 100 μM. T47-D cells were treated with 10 nM MPA as described in methods and materials. Control = 366 ± 20. * Represents significant induction compared to control. ** Represents significant inhibition compared to MPA. One-way ANOVA, Fisher's LSD was performed to determine significance, (p = <0.001), n=3-15. Error bars represent SEM.

2.4 Discussion & Conclusion

Based on the results described in the previous section, curcumin and 2ME2 were selected for further investigation as potential anti-cancer agents against progestin-dependent breast cancer. While curcumin and apigenin, both with the ability to bind PR and ER [70, 71], showed significant inhibition at low concentrations, curcumin proved to be cytotoxic to T47-D human breast cancer cells at higher concentrations (Figure 2.4). It was reported that curcumin induces apoptosis in mammary epithelial cell carcinomas at the G_2 phase of the cell cycle [111], but interestingly, curcumin does not induce apoptosis of normal mammary epithelial cells by preventing cell cycle progression into the G_2 phase [111], suggesting that it may specifically target cancer cells. As a result, further investigation of curcumin as a potential chemopreventive agent was conducted and is discussed in more detail in the following chapter.

Other compounds that showed promise based on these initial studies were 2ME2 and lycopene. Although lycopene and 2ME2 were able to inhibit MPA-induced VEGF secretion at higher concentrations (100μM), 2ME2 proved most effective (Figure 2.2). Previous reports provide evidence that lycopene is a promising chemopreventive agent for prostate cancer [112]; however, its role in breast caner, remains controversial. In vitro data suggests lycopene inhibits estrogen-dependent as well as estrogen-independent breast cancer [87], while two cohort studies report contrasting findings; one study suggests that there is no relationship between increased lycopene ingestion and increased plasma levels or decreased breast cancer risk [85], while another study, conducted with Swedish women, reports that lycopene reduces breast cancer risk in menopausal women [86]. The results from this initial study suggest that lycopene may, in fact, prove

beneficial in the prevention of progestin-dependent breast cancer, a hypothesis that warrants further investigation.

2ME2 did not show inhibition at lower concentrations (1 μM), but there was a marked inhibition of MPA-induced VEGF secretion to below control basal levels at higher concentrations (Figure 2.2). The inability of 2ME2 to inhibit VEGF secretion at lower concentrations may be due to its being present endogenously at low levels [108]; a higher concentration must therefore be achieved in order to elicit an inhibitory response. 2ME2 was reported to display anti-cancer activities including, microtubule disruption [103, 104], cell cycle arrest [113], and HIF-1α inhibition [101, 104, 114], strongly suggesting that it may be effective in inhibiting progestin-induced angiogenesis. Also, because 2ME2 does not bind to ER or PR [115], the estrogen metabolite would appear to be ideal in combating breast cancers that are hormone dependent, since it will not elicit an agonistic effect. 2ME2 was studied in depth to determine its effectiveness as a chemotherapeutic agent. These investigations eventually led to the hypothesis that ***HIF-1α is required for progestin-dependent VEGF induction in breast cancer***, a novel hypothesis which is discussed in detail in the following chapters.

CHAPTER 3

INHIBITORY EFFECTS OF CURCUMIN ON MPA-INDUCED VEGF SECRETION FROM T47-D BREAST CANCER CELLS AND ON MPA-EXPOSED RAT MAMMARY GLANDS

3.1 Introduction

Breast cancer is the second most common cause of cancer death in the United States among women, with 200,000 new cases and 40,000 deaths reported annually [1]. According to the American Cancer Society, prolonged use of hormone replacement therapy (HRT) containing estrogen and progestin is associated with increased risk of breast cancer [1], compared to estrogen alone [1, 2, 18]. The role of progestins in breast cancer is controversial, as they have been implicated in promoting cell cycle arrest [116], as well as an anti-apoptotic and proliferative hormones [12, 17]. Nonetheless, most evidence suggests that progestins specifically increase the risk of breast cancer in post-menopausal women receiving combined HRT.

Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor that is under steroid hormone regulation and plays a critical role in tumor growth and expansion [36]. Benakanakere et al. [61] showed that MPA and progesterone stimulate angiogenesis and tumor growth in the 7, 12-dimethylbenz[a]anthracene (DMBA)-induced rat model of breast cancer, possibly via a mechanism involving the up-regulation of VEGF in tumor tissue and increased tumor vascularization. Other reports confirm that natural and synthetic progestins stimulate expression of VEGF mRNA and protein in breast cancer cells [28, 117] and that up-regulation of VEGF can be blocked by the anti-progestin RU-486 (mifepristone) [117]. Because RU-486 interacts with the progesterone

receptor (PR) as well as androgen and glucocorticoid receptors [60], it is not an ideal drug of choice for modulating PR-specific cellular effects. Thus, additional compounds are needed for the purpose of blocking PR-mediated effects of progestins on breast cancer cells during HRT.

Curcumin, a phytoestrogen derived from turmeric root, is an Indian spice used in cooking and to color food. Historically, curcumin has been used as an antibacterial or anti-inflammatory agent and to treat skin wounds and some tumors [118]. As described in the previous chapter, curcumin binds with low affinity to the estrogen receptor (ER) and PR [70], and blocks estrogen-induced cell proliferation [119]. Recent studies indicate that curcumin has chemopreventive and anti-angiogenic properties and has potential as a therapeutic agent for several neoplasms, including breast cancer [77]. As shown in chapter 2 during the initial screening of compounds, curcumin inhibited VEGF secretion from T47D cells in response to MPA-exposure. However, it is not known whether such an inhibitory effect occurs *in vivo*. Nonetheless, Singletary et al., [120] reported that curcumin (50-200 mg/kg i.p.) inhibits DMBA-induced mammary tumorigenesis and formation of DMBA-DNA adducts in Sprague-Dawley rats. Since curcumin has been shown to exhibit anti-angiogenic properties and to modulate VEGF levels in breast cancer [121], the study reported in this chapter examines the effects of curcumin on progestin-induced secretion of VEGF in human breast cancer cells, with a view to determining its efficacy as a chemopreventive agent against progestin-dependent breast cancer.

3.2 Methods and Materials

Reagents. Curcumin was purchased from LKT Laboratories, Inc. (St. Paul, MN). MPA, progesterone, norgestrel, norethindrone, RU-486, and DMBA were purchased from Sigma-Aldrich (St. Louis, MO). Pierce BCA protein assay reagents were from Fischer Scientific International.

Cell Culture. T47-D cells were cultured in 100 mm cell culture plates in DMEM/F12 cell culture media containing 10% Fetal Bovine Serum (FBS). At 50% confluence, media was changed to DMEM/F12 media containing 5% dextran-coated charcoal (DCC)-treated serum for 24 hours. After washing with PBS, DMEM/F12 media containing 5% DCC-treated serum was replaced and cells were treated for 16-18 hours with progestins and/or curcumin. When present, the progestin concentration was 10nM. Control cells were treated with ethanol (vehicle). Each treatment was performed in triplicate.

Animal Studies. Intact virgin 40-45 day old female Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care under conditions of 12-hour light/dark cycles and *ad libitum* access to food and water. All surgical and experimental procedures were in accordance with procedures approved by the institutional Animal Care and Use Committee and outlined in the "Guide for Care and Use of Laboratory Animals (NIH publication 85-23).

Animals were given a single dose of 20 mg/rat of DMBA in peanut oil via gavage on day 0. On day 26, daily i.p. injections of curcumin (200 mg/kg/day) [120] were given; control animals were given peanut oil. On day 30, animals were anesthetized and MPA pellets implanted subcutaneously on the dorsal surface. Daily injections of curcumin

were continued through day 50. Animals were palpated and tumors were measured 2-3 times weekly. Non-tumor mammary tissues were collected for immunohistochemical (IHC) analysis from days 51-61.

VEGF ELISA. Quantikine Human VEGF ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Supernatant was collected from cells treated with progestins and/or test compounds and analyzed using the VEGF ELISA kit according to the manufacturer's protocol. Experiments were performed in triplicate and each sample was analyzed in duplicate. According to the manufacturer, the minimum detectable concentration of VEGF is less than 5 pg/ml, and the intra-assay precision has a coefficient variance (CV%) between 3.5-6.5%, while the CV% of the inter-assay ranges from 5.0-8.5%.

BCA Protein Assay. Cells were harvested and cell pellets were re-suspended in 300 µl lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, and 1% NP-40) and incubated on ice for 30 minutes. Samples were centrifuged and protein concentration determined by BCA assay using BSA as a standard. Absorbance was measured at 562 nm using a SpecTRA MAX 190 microplate reader (Sunnyvale, CA). All samples were analyzed in duplicate.

Cell Viability Assay. Cell viability was estimated using Sulforhodamine B (SRB) as previously described [28]. Briefly, 100 µl cell culture (3×10^4 cells/ml) was incubated overnight in a 96 well microtiter plate at 37 ℃ in DMEM/F12+10% FBS. Fresh DME/F12 media containing 5% DCC serum was added and incubation continued for 18–24 hours. Media was replaced a second time and incubation continued for 24 to 48 hours in the presence of 10 nM progestin or 10 µM curcumin. Media was removed from each

well, and surviving or adherent cells were fixed *in situ* in 100 µl PBS and 100 µl 50% cold trichloroacetic acid for 1 hour at 4 °C. Cells were washed with ice-water, dried and incubated for 8 minutes in 50 µl SRB. Cells were washed five times with cold 1% acetic acid and dried at room temperature. Adherent stain was solubilized with 150 µl 10 mM Tris and absorbance at 520 nm was measured with a SpecTRA MAX 190 microplate reader (Sunnyvale, CA).

Preparation of Nuclear Extract. Nuclear extract was collected from treated cells using the ActiveMotif Nuclear Extract Kit (Carlsbad, CA). Cells were washed with PBS/Phosphatase inhibitors, scraped from cell culture dishes, and collected in a conical centrifuge tube and centrifuged for 6 minutes at 1000 rpm. Supernatant was removed, pellets were re-suspended in 500 µl 1X hypotonic buffer and incubated for 15 minutes on ice. Twenty-five ml of detergent was added to samples which were vortexed for 10 seconds and centrifuged for 1 minute at 13,000 rpm. Supernatant was removed and the nuclear pellet re-suspended in complete lysis buffer, vortexed for 10 seconds, and incubated on ice for 30 minutes. Samples were vortexed for 30 seconds and centrifuged for 12 minutes at 13,000 rpm. Supernatant (nuclear fraction) was collected. Nuclear extract is 80% pure, according to the manufacturer's data sheet.

Western Blotting. Protein, 10 µg per lane, was separated on a 7.5% Tris-HCl Criteron pre-cast gel from Bio-Rad Laboratories (Hercules, CA). Separated proteins were transferred to a PVDF membrane from Bio-Rad Laboratories (Hercules, CA). The blots were blocked for 1 hour at room temperature (or overnight at 4°C) in 5% non-fat dry milk in TBS containing 1% Tween-20 (TBS-T). Blots were incubated with primary antibody at the specified dilution for 2 hours at room temperature (or overnight at 4°C):

anti-PR (1:200 dilution of AB-52 from Santa Cruz, Biotechnology), anti-ERα (1:100 dilution of D-12 from Santa Cruz, Biotechnology). Blots were washed 3 times for 5 minutes each in TBS-T, and then incubated with secondary anti-body for 1 hour at room temperature. Blots were washed again, 3 times for 5 minutes each in TBS-T, and immunoreactive bands were visualized following 5-minute incubation with the ECL plus detection kit from Amersham Pharmacia Biotech (Arlington Heights, IL). Membranes were stripped and re-blotted for β-actin (Sigma Aldrich, St. Louis, MO). Bands were normalized to the β-actin signal in the same sample.

NF-κB Transcription Factor ELISA. Nuclear fraction was extracted to perform the NF-κB transcription factor ELISA from Active Motif (Carlsbad, CA). BCA protein assay was conducted to determine the protein concentration of samples. Briefly, 40 µl binding buffer (prepared according to manufacturer's protocol) was added to each well in the sample plate. 10 µl positive control was added to positive control wells (negative contained binding buffer). 10 µl of nuclear extract was added to sample wells. The plate was incubated 30 minutes at room temperature. The assay plate (different from sample plate) was washed three times with 200 µl wash buffer. 45 µl aliquots from the sample plate were added to the assay plate and incubated for 1 hour at room temperature. Contents of wells were emptied, the plate was washed three times with wash buffer, and 100 µl primary antibody (1:200) was added to each well. Incubation was for 1 hour at room temperature. Contents of wells were discarded and wells were washed with 3 times with wash buffer. Secondary antibody (100 µl, 1:200) was added to each well and incubated at room temperature for 1 hour. Contents of wells were emptied and wells were washed three times with wash buffer. Substrate solution (100 µl) was added to each

well and incubated for 15 minutes in the dark. Stop solution (100 μl) was added to each well, and plates were read on a SpecTRA MAX 190 microplate reader (Sunnyvale, CA) at 450 nm. Cells were treated in triplicate and samples were run in duplicate.

Immunohistochemistry. The effect of curcumin on mammary tumorigenesis was assessed by measuring expression levels of VEGF, ER-α, ER-β, and PR in the non-tumor mammary tissues. Both auxiliary and abdominal mammary glands were used for analysis.

Tissues were fixed overnight in 4% paraformaldehyde for immunohistochemistry. Tissues were processed for paraffin infiltration and embedding. Sections (5 μm) were mounted onto ProbeOn Plus microscope slides (Fischer Scientific, Inc., Pittsburgh, PA). Light microscopic examination of serial H&E stained sections representative of a given tissue was performed for classification using previously published methods [122]. Before immunohistochemisty, sections were dewaxed in xylene, rehydrated through graded concentrations of ethanol, then rinsed in distilled water, and stored in PBS at 4°C until use. Sections were heated to induce epitope retrieval in 10 mmol/L citrate buffer (pH 6.0) for PR, ER-α, and VEGF immunolabeling. Slides were treated with 3% H_2O_2 in absolute methanol (to inactivate endogenous peroxidase activity) before being washed 3 times in PBS and then immersed in 10% bovine serum albumin for 20 minutes. Sections were incubated for 60 minutes at room temperature with each of the following polyclonal antibodies: anti-PR antibody [1:50 dilution of rabbit anti-human PR polyclonal antibody (A0098) that reacts with the DNA binding domain (amino acids 533-547); DAKO Carpinteria, CA], anti-ER-α [1:300 dilution of a rabbit anti-ER-α polyclonal antibody (sc-542) raised against an ER-α peptide of mouse origin; Santa Cruz Biotechnology, Inc.,

Santa Cruz, CA], anti-VEGF antibody [1:100 dilution of a rabbit anti-VEGF polyclonal antibody (sc-152); Santa Cruz, Biotechnology]. Sections were then washed and sequentially incubated with a secondary antibody [biotinylated swine anti-rabbit IgG (DAKO) and a streptavidin-linked horseradish peroxidase product (BD PharMingen, San Diego, CA)] for 30 minutes at room temperature. Alternatively, some sections were incubated with EnVision+, a horseradish peroxidase-labeled polymer conjugated with anti-rabbit antibodies (DAKO). Bound antibodies were visualized following incubation with 3,3'-diaminobenzidine (0.05% with 0.015% H_2O_2 in PBS) solution (Zymed Corp., San Francisco, CA) for 3-5 minutes. Sections were counter stained with Meyer's hematoxylin, dehydrated, cleared, and cover-slipped for microscopic examination.

Statistical Analysis. The mean average value for VEGF levels (pg/mg) in the untreated (control) samples was calculated from the number of determinants in that group and used to calculate VEGF levels in untreated and treated groups as a percentage value. Within each group, results were plotted as a bar graph representing mean $\pm$ SEM. Data were analyzed by one-way ANOVA and all groups compared with controls. Data sets with inhibitors were compared with VEGF values in progestin treated samples and differences considered significant when p values were <0.05. SRB cell viability assay was performed in multiples of six. In each figure, all data sets were compared to controls for significant difference following treatment. All data was checked for normality and homogeneity of variance. Since a number of analyses did not satisfy these assumptions, data was ranked and ANOVA performed as outlined by Conover and Iman [109]. Fisher's protected least significant difference (LSD) was performed to determine treatment differences, as suggested by Chew [110]. All significance was based on the

ranked transformations; however treatment values were presented as actual values. For animal studies, groups were compared with respect to tumor latency and multiplicity at the conclusion of the study. Latency period differences were compared using a general linear model (PROC GENMOD in SAS) in which the link function was logit and the distribution was binomial. Multiplicity data was analyzed using Kruskal-Wallis One Way ANOVA. FoveaoPro 3.0 ® software analysis was used to determine positive staining by area in immunohistochemical studies. One-Way ANOVA was used to statistically compare VEGF staining differences among experimental groups. For all statistical comparisons, $p<0.05$ was regarded as statistically significant.

3.3 Results

The effects of curcumin on progestin-induced secretion of VEGF were examined by incubating human T47-D breast cancer cells with MPA and variable amounts of curcumin. The results showed that curcumin significantly reduced MPA-induced secretion of VEGF in a dose-dependent manner and at concentrations as low as 0.001μM (Figure 3.1). Half-maximal inhibition was observed at a concentration of approximately 0.1 μM curcumin. VEGF levels in cells incubated with MPA + Curcumin were significantly lower than VEGF levels in cells incubated with MPA alone. Curcumin alone did not alter VEGF from cells compared to untreated controls except at 0.001 and 0.01 μM concentrations. At these two concentrations, curcumin decreased the control levels of VEGF by approximately 40%. RU-486 was used as a positive anti-progestin control (Figure 3.1). RU-486 (1 μM) totally abolished MPA-induced secretion of VEGF, as observed previously [28], and reduced the basal level by 50% compared to control, as shown in Figure 3.1.

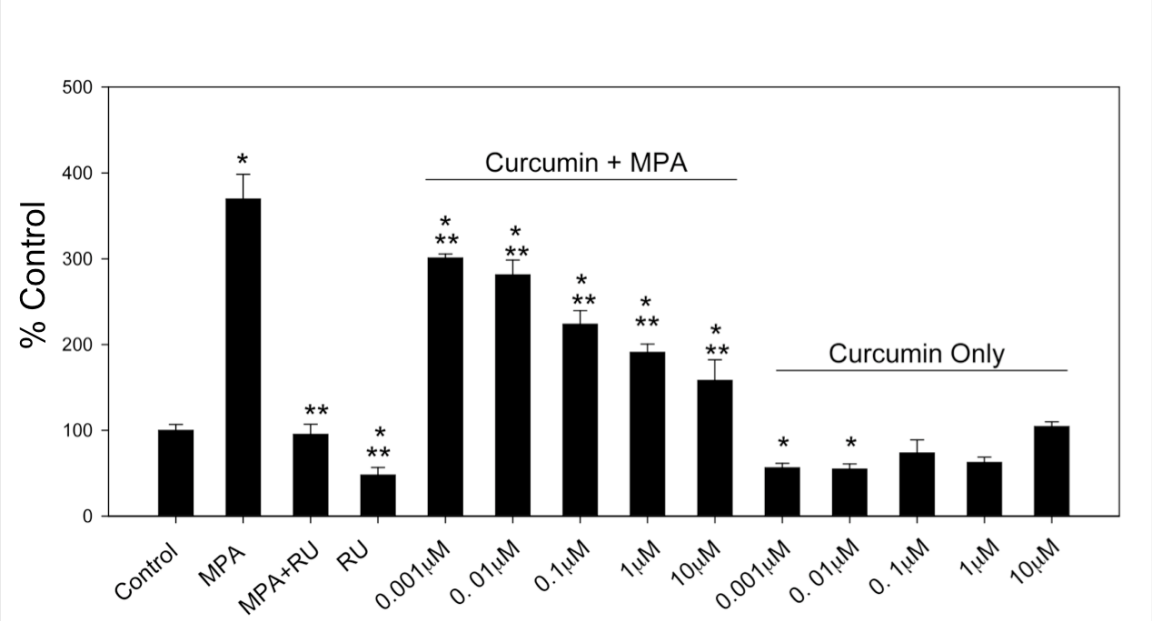

Figure 3.1: VEGF ELISA: Effect of curcumin on MPA-induced secretion of VEGF. T47-D cells were treated with vehicle (control), with MPA (10 nM) ± either 1 μM RU-486 or 0.001-10 μM curcumin and with curcumin alone. Following incubation of cells for 18 h at 37°C, VEGF levels were measured in the supernatants of cultured cells by ELISA as described in Materials and Methods. The output of the assay is VEGF concentration in pg/mg cellular protein. Error bars show SEM (n=3-9). The 100% control value is 438 ± 62 pg/mg. One-way ANOVA was used to test for statistical significance; Fischer's protected least significant difference test was used for ranking. An asterisk (*) indicates values that differ significantly from control (no MPA, no curcumin). A double asterisk (**) indicates values that are significantly different from MPA-treated control in MPA treated samples that also include inhibitors ($p < 0.05$). RU=RU-486

SRB assay results showed that cell viability was not reduced during a 24-hour incubation with curcumin at concentrations lower than 50 μM (Fig. 3.2), indicating that curcumin was not cytotoxic at low to moderate concentrations. Thus, it is possible that the ability of curcumin to inhibit growth of MPA-treated T47D cells is related to its ability to inhibit secretion of VEGF.

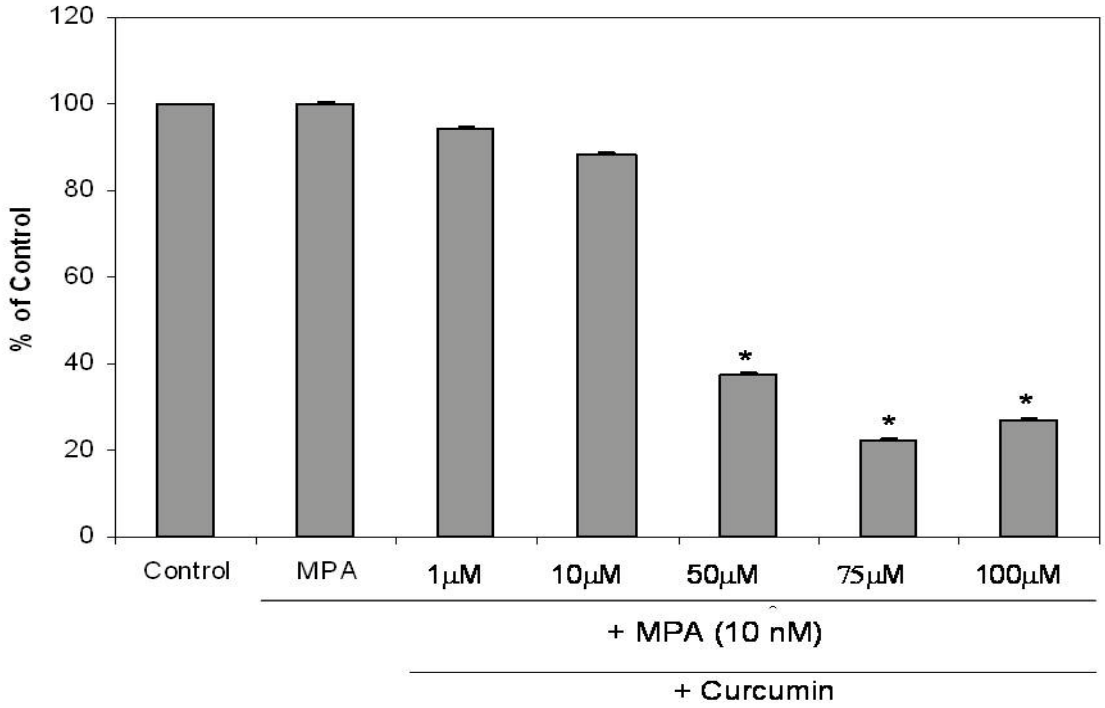

Figure 3.2: SRB Assay: Cells were treated with vehicle or with 10 nM MPA with and without 1-100 μM curcumin for 18 hours at 37°C. Cell viability was measured using SRB Assay as described in the Materials and Methods. One-way ANOVA was used to test for statistical significance; Fischer's protected least significant difference was used for ranking. SEM was <0.02 and error bars are not clearly seen. An asterisk (*) indicates values that were significantly different than control ($p<0.05$).

Because curcumin has been reported to have a low affinity for both PR and ER [70], there is a possibility that it could be down-regulating these receptors, thus blocking the efficacy of MPA to induce VEGF secretion from T47D cells . With this in mind, a western blot was performed to assess the protein expression of PR and ER, shown in Figure 3.3. PR and ER levels were not decreased with 10 μM curcumin treatment (Figure 3.3).

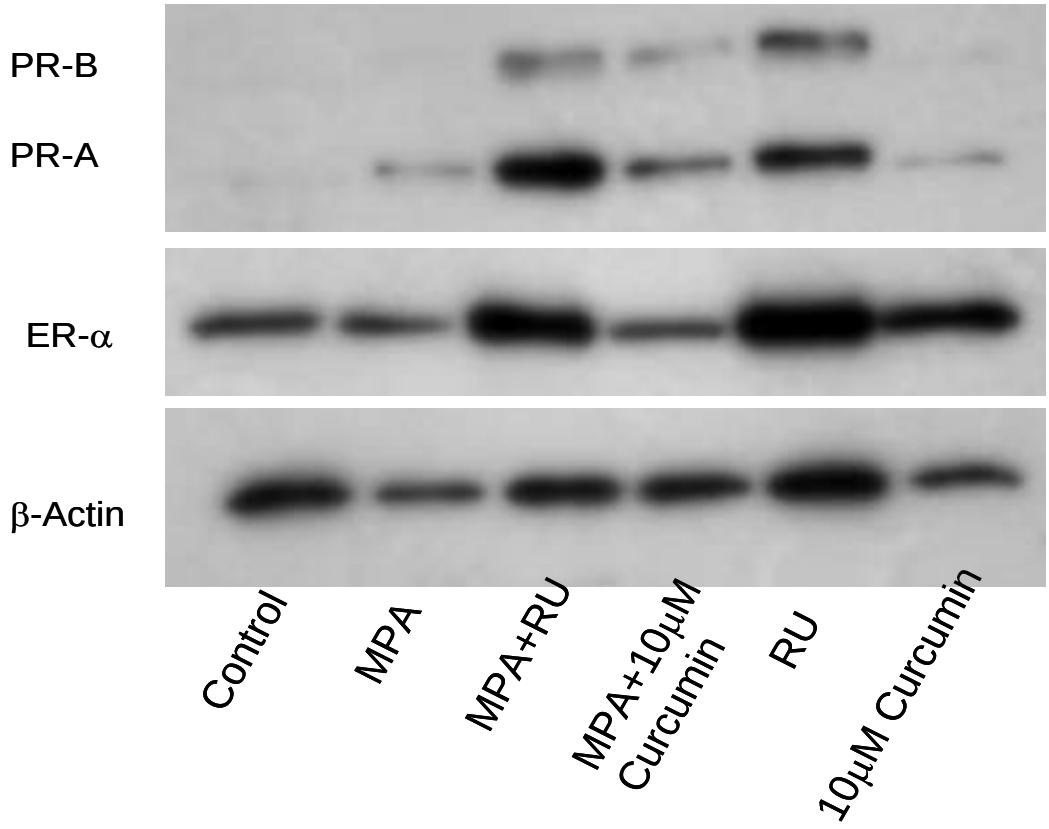

Figure 3.3: Western Blot Analysis. Nuclear Extracts from T47D cells were analyzed for PR and ER-α protein expression. 15μg of protein was loaded.

Because there was no marked decrease in ER or PR protein expression after curcumin treatment, the ability of curcumin to inhibit secretion of VEGF induced by other progestins was examined in T47-D cells. Interestingly, curcumin did not inhibit secretion of VEGF from cells treated with progesterone, norgestrel or norethindrone (Fig. 3.4 and 3.5) at the tested concentration. However, as expected, RU-486 did inhibit progesterone-induced secretion of VEGF (Figure 3.4).

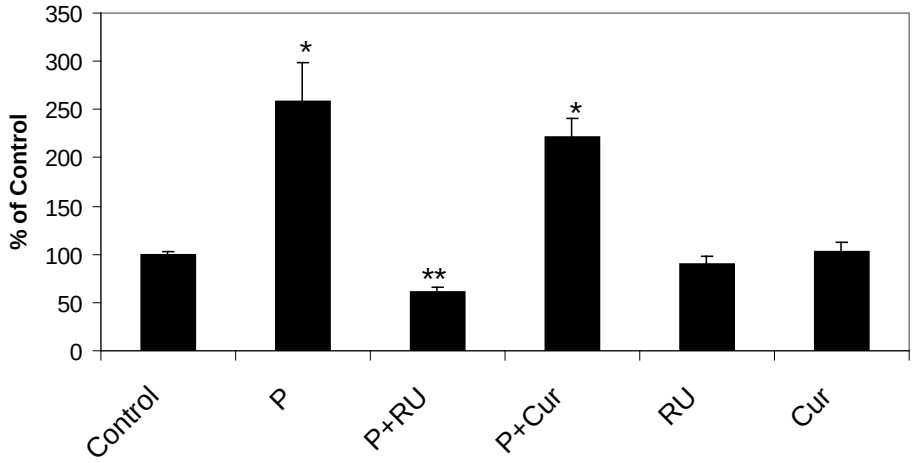

Figure 3.4: VEGF ELISA: Effects of curcumin on progestin-induced secretion of VEGF secretion. Cells were treated with vehicle alone or with 10 nM progesterone ± either 1 μM RU-486 or 10 μM curcumin, and with the same concentrations of RU-486 and curcumin alone for 18 h at 37°C. VEGF levels in the supernatants of cultured cells were measured via ELISA as described in Materials and Methods. The output of the assay is VEGF concentration in pg/mg cellular protein. Error bars show SEM (n=6). The 100% control value is 558 ± 52 pg/mg. One-way ANOVA was used to test for statistical significance; Fischer's protected least significant difference was used for ranking. An asterisk (*) indicates values that were significantly different than control (no MPA, no curcumin). A double asterisk (**) indicates values that were significantly different from P-treated control (p < 0.05). P=Progesterone, RU=RU-486, Cur=Curcumin

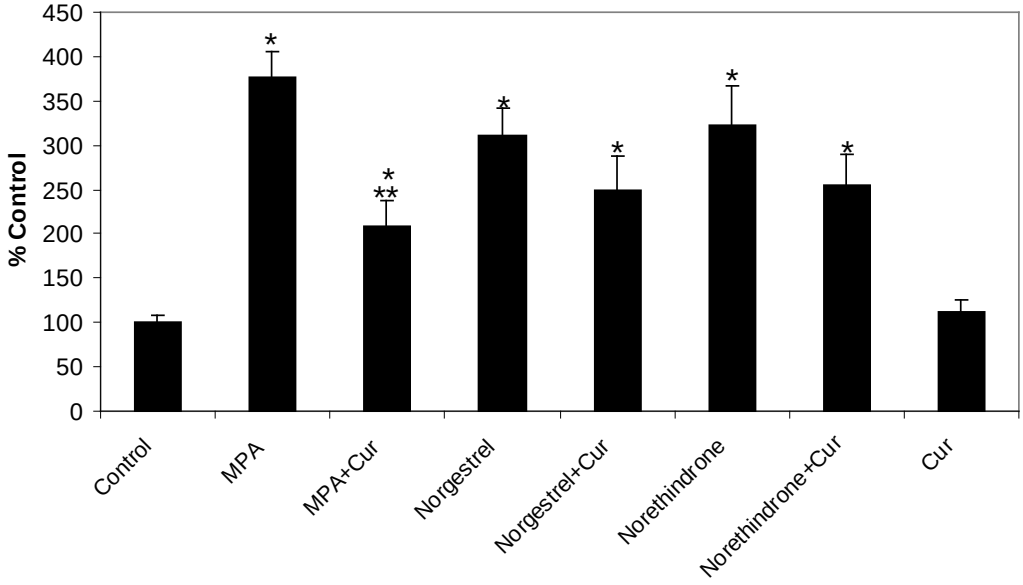

Figure 3.5: VEGF ELISA: Curcumin specifically inhibits MPA-induced VEGF induction. Cells were treated with 10 nM MPA, norgestrel, or norethindrone in the presence and absence of 10 μM curcumin. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. The 100% value is 475 ± 38 pg/mg. An asterisk (*) indicates values that were significantly different than control (no progestin, no curcumin). A double asterisk (**) indicates values that were significantly different from the respective progestin treated group (p < 0.05).

To determine if the inhibitory property displayed by curcumin was cell-type specific, its ability to inhibit progestin-induced VEGF secretion from a second cell line, BT474 was investigated. Interestingly, curcumin displayed no inhibitory properties on this cell line (Figure 3.6). Previous reports show that progestins induce VEGF secretion in T47D and BT474 cells via distinct pathways [93]. It is possible that curcumin inhibits the pathway specific for MPA-induction in T47D cells, and does not affect VEGF induction in BT474 cells.

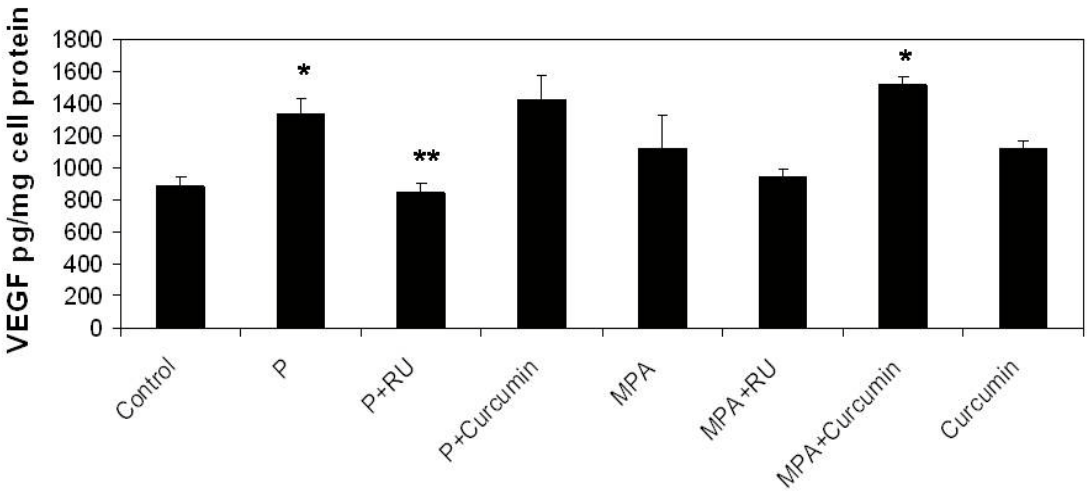

Figure 3.6: VEGF ELISA (BT474): Curcumin does not inhibit progestin-induced VEGF secretion in BT474 Cells. As described in Methods and Materials, cells were pre-treated for 30 minutes with 10 μM curcumin or 1 μM RU-486. Cells were then treated with 10 nM MPA or progesterone for 16-18 hours. Cell culture supernatant was collected and analyzed for the presence of VEGF as described in Methods and Materials. Secreted VEGF levels were given in pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyzed data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$.

Because curcumin specifically inhibited MPA-induced VEGF secretion, an MPA-specific activation pathway was briefly investigated. MPA has an affinity for the androgen receptor (AR) and disruption of AR signaling has been associated with an increased risk of breast cancer [123]. Thus, it seemed possible that the MPA-induced

secretion of VEGF may be mediated in part via the AR. However, this possibility was not supported by cell culture experiments showing that flutamide, a specific inhibitor of AR, did not inhibit MPA-induced secretion of VEGF (Fig 3.7).

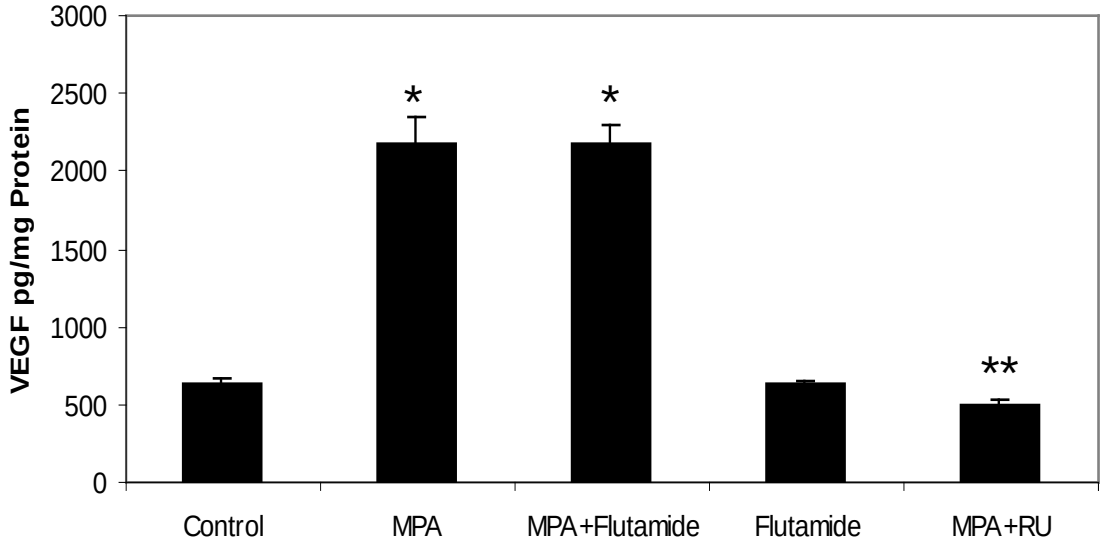

Figure 3.7: VEGF ELISA: 0.1 μM flutamide was tested as an inhibitor of MPA induced VEGF via VEGF ELISA analysis as described in Methods and Materials. An asterisk (*) indicates values that were significantly different than control (no MPA, no fultamide). A double asterisk (**) indicates values that are differ significantly from MPA-treated group ($p \leq 0.05$), n=3.

This result was consistent with the hypothesis that the effects of MPA on T47-D cells were primarily mediated by PR, and was not mediated by AR. However, additional experiments were needed to support this hypothesis. As a result, an animal experiment was conducted in an effort to block MPA-accelerated, DMBA-induced mammary tumors with flutamide. A previously established DMBA-induced, MPA-accelerated mammary tumor model in female Sprague Dawley rats [61] was utilized to investigate this possibility. Figure 3.8 depicts the protocol used in this experiment.

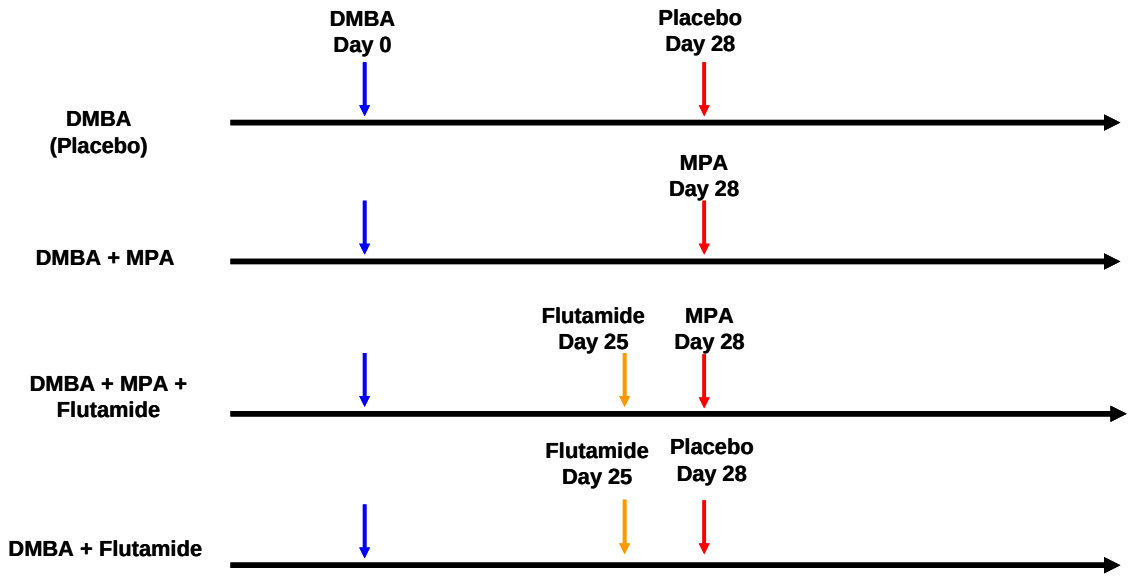

Figure 3.8: Protocol utilized in DMBA-induced, MPA-accelerated mammary tumor model with female Sprague-Dawley rats. 20 mg/rat of DMBA was given via gavage on Day 0. Flutamide pellets (25mg) were implanted 3 days prior to the placement of MPA (25mg) or placebo pellets. Animals were palpated and tumors measured 2-3 times weekly, (n=8-10)

Female Sprague Dawley rats were given 20 mg of DMBA via gavage on day 0. As described in Methods and Materials, 25 mg flutamide pellets were placed on day 25, and 25 mg MPA pellets were placed on day 28. Animals were palpated and tumors measured 2-3 times weekly throughout the study.

The results showed a short delay in the appearance of the first tumor in the flutamide-treated MPA group (Figure 3.9); however, this was only seen in one animal. The appearance of tumors in the flutamide-treated MPA groups and MPA-treated animals did not differ statistically and resulted in the same number of tumor-bearing animals. This result coincided with *in vitro* studies that show flutamide does not inhibit VEGF secretion. As a result, we did not observe a significant affect in tumor formation in DMBA-induced, MPA-accelerated animals, suggesting that MPA does not increase VEGF production via the AR.

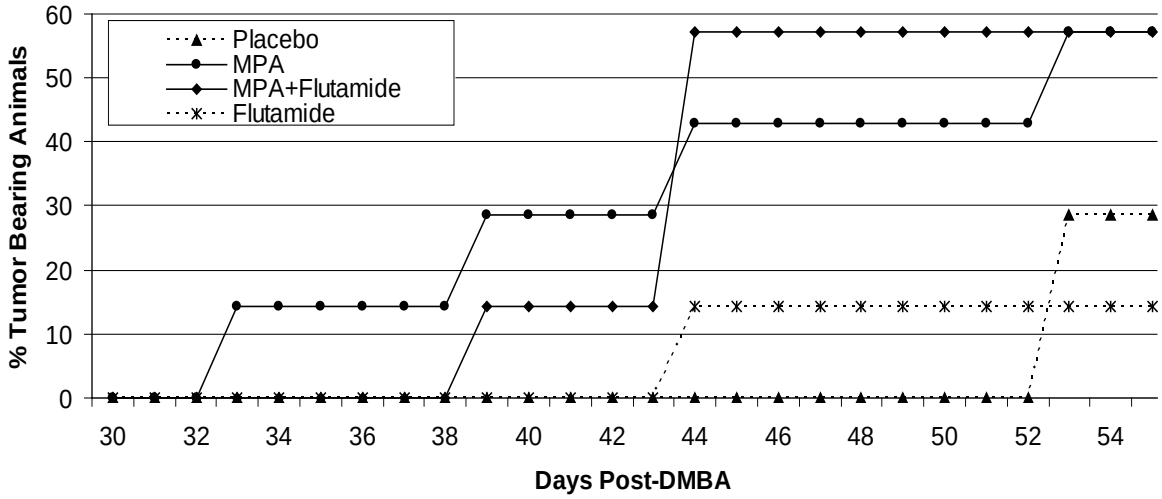

Figure 3.9: Flutamide does not block or delay the appearance of DMBA-induced, MPA-accelerated mammary tumors at the utilized concentration, n=8-10.

Flutamide also showed no inhibition of tumor multiplicity (Figure 3.10). These results further suggested that MPA does not bind to AR during the induction of VEGF secretion of breast cancer cells.

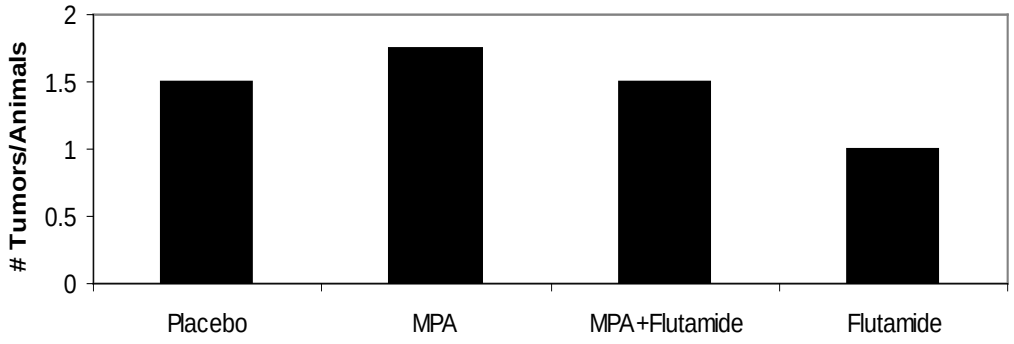

Figure 3.10: Tumor multiplicity in tumor bearing animals.

Efforts were made to elucidate the inhibitory mechanism of curcumin. It has been reported that curcumin down-regulates and blocks the activation of the transcription factor, NF-κB, in breast cancer cells [124]. Curcumin inhibits the phosphorylation/activation of the inhibitory kappa B (IκB), resulting in a decrease in the

nuclear translocation of NFκB [125]. Thus there was a decrease in NF-κB binding to DNA. The role of NF-κB in the progestin-dependent model is unknown and not well studied. As a result, NFκ-B was explored as a possible molecular target for curcumin in the MPA-induced model. An NF-κB transcription factor, DNA-binding assay was utilized to determine whether curcumin alters the DNA-binding abilities of NF-κB after MPA treatment, without affecting DNA-binding after progesterone treatment, due to the fact that curcumin specifically inhibits MPA-induced VEGF secretion. The results from the DNA binding assay (Figure 3.11) suggests that curcumin does not affect NF-κB binding activities after MPA or progesterone treatment.

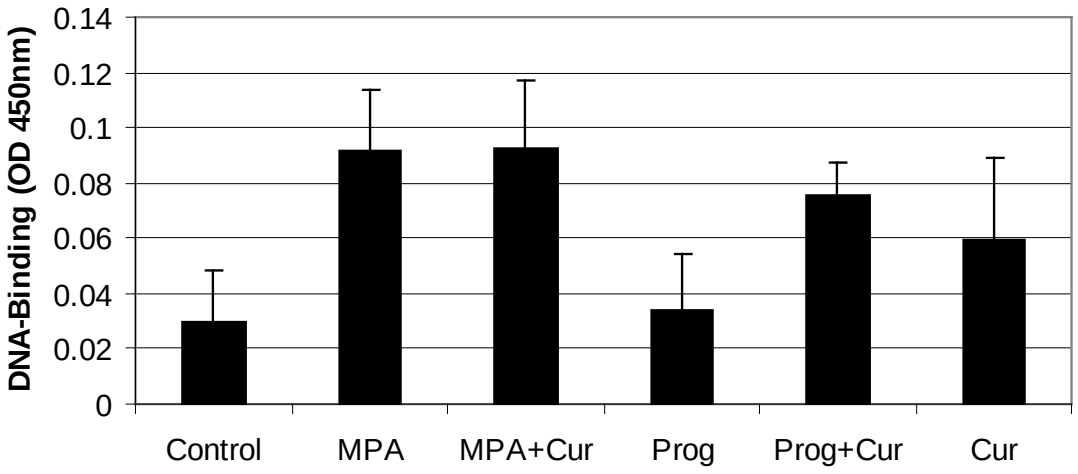

Figure 3.11: NF-κB Transcription Factor DNA-Binding ELISA. T47D cells were pre-treated with 10μM Curcumin for 30 minutes. Cells were then treated with 10 nM MPA or progesterone for 3 hours. Nuclear Extract was collected as described in Methods and Materials. NF-κB transcription factor ELISA was performed according to the manufacturer's protocol. ANOVA Fisher's LSD showed no significance at $p \leq 0.05$. Error Bars represent SEM. n=3.

In Vivo Studies

The inhibitory actions of curcumin as a chemopreventive agent against progestin-driven breast cancer were investigated *in vivo*, utilizing the previously established

DMBA-induced, MPA-accelerated mammary tumor model in Sprague Dawley rats [61]. The specific protocol utilized to determine the chemopreventive effectiveness of curcumin is depicted in Figure 3.12.

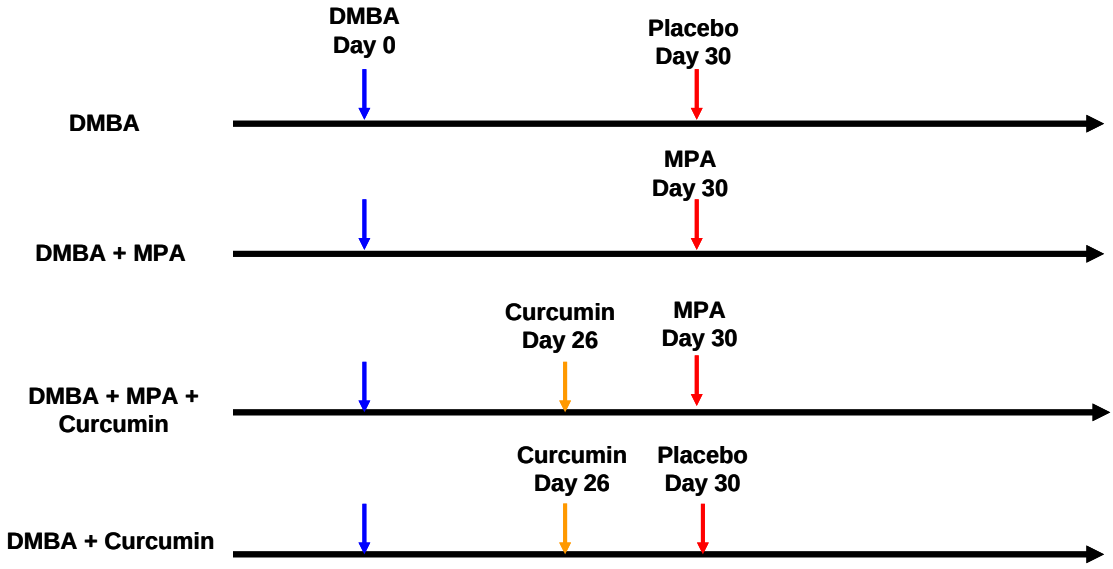

Figure 3.12: Protocol utilized in DMBA-induced, MPA-accelerated mammary tumor model with female Sprague-Dawley rats. 20 mg/rat of DMBA was given via gavage on Day 0. Curcumin was administered daily via i.p. injections at 200 mg/kg beginning on day 26. Placebo or MPA (25 mg) pellets were placed on day 30. Fifteen (15) animals were used in each group; however 19 animals were used in the MPA group. Animals were palpated and tumors measured 2-3 times weekly.

As described in Methods and Materials, 20mg of DMBA was administered to female Sprague Dawley rats via gavage on day 0. Daily curcumin injections (200 mg/kg, i.p.) began on day 26, and 25 mg MPA pellets were placed on day 30. Daily injections continued through day 50. This treatment protocol was selected based on previous studies in which curcumin was administered i.p. for 2 weeks with no negative outwardly effects based on animal weight [120]. The current protocol continued treatment with curcumin for a total of 24 days while monitoring animal weight for signs of negative

effects. On days 51-61, animals were sacrificed and non-tumor mammary glands were collected for immunohisotchemical analysis.

The results show that MPA + curcumin treatment resulted in a 7-day delay in the appearance of the first tumor, compared to that of MPA alone. Comparison of latency data from treatments of MPA and MPA + curcumin did not show a significant difference when analyzed using χ^2 test. However, when compared using a general linear model (PROC GENMOD in SAS) in which the link function was logit and the distribution was binomial, according to the calculated odds ratio, the odds of cancer were 2.2 times greater with MPA than with MPA + curcumin and was 3.05 and 4.4 times greater than the curcumin alone or placebo group, respectively. Curcumin was unable to delay the natural tumors that developed in response to DMBA (Figure 3.13). Thus, curcumin delayed DMBA-induced tumors in which development was accelerated by MPA. Also, it was observed that there were fewer tumor bearing animals when treated with MPA + curcumin (35%), compared to MPA only (55%). No delay or significant difference in tumor incidence was observed in animals given curcumin alone compared to DMBA, placebo animals. (Figure 3.13)

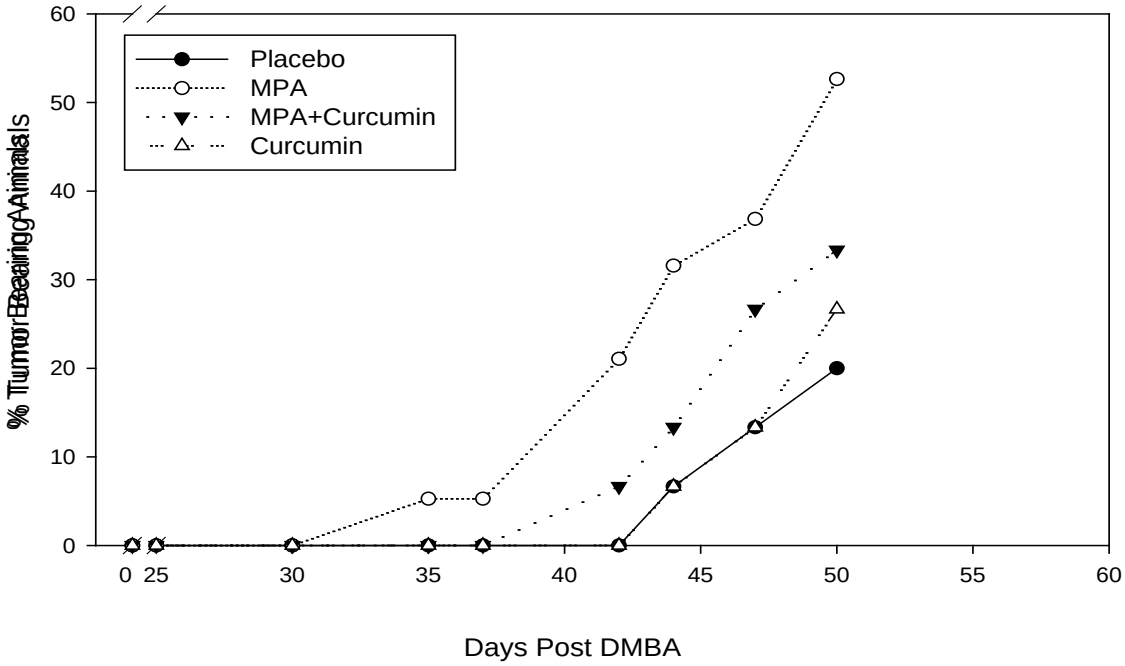

Figure 3.13: Curcumin delays MPA-accelerated tumorigenesis. Animals were palpated 2-3 times each week and tumors were measured. Latency period differences were compared using a general linear model (PROC GENMOD in SAS) in which the link function was logit and the distribution was binomial. MPA treated DMBA-induced tumors were 2.2 times more likely to develop tumors than DMBA-induced tumors alone and 4.4 times more likely than the placebo or curcumin alone group. (n= 15-19/group).

Importantly, curcumin treatment (MPA + curcumin) reduced tumor multiplicity, compared to MPA alone (Figure 3.14). While tumor bearing animals treated with MPA + curcumin, placebo, and curcumin alone experienced an average tumor burden of only one (1 tumor), tumor bearing animals treated with MPA alone experienced an average tumor multiplicity of about two tumors (1.9 tumors) at the conclusion of the study.

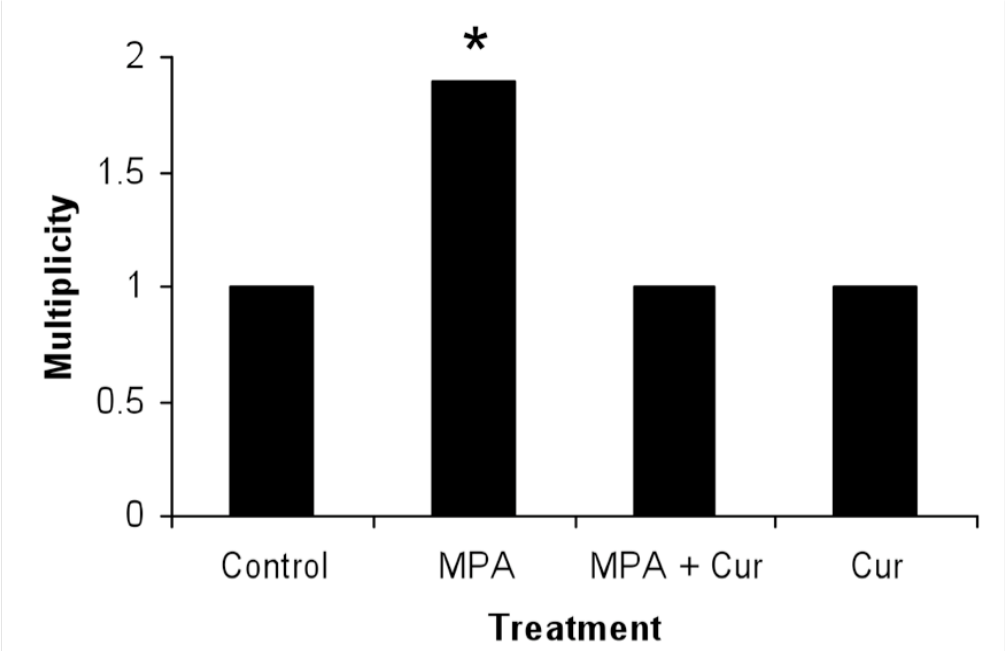

Figure 3.14: Tumor Multiplicity. The average number of tumors per tumor bearing animal at the conclusion of the study was 1.9 for MPA treated animals and 1 for Control, MPA + Curcumin and Curcumin alone. *Significantly different from the rest of the groups (ANOVA, $p<0.05$), Errors bars represent SEM. (Cur=Curcumin)

Curcumin inhibits MPA-induced morphological changes in mammary glands

At the conclusion of the study, animals were sacrificed and non-tumor mammary gland tissue was collected for immunohistochemical analysis. Microscopic examination of gross morphology showed a marked difference in mammary gland histology in MPA + curcumin-treated animals, compared to MPA-treated animals. Placebo animals displayed 'normal' mammary tissue morphology, showing few, if any, pre-neoplastic lesions. Ductal and lobular structures were distinct and distinguishable (Figure 3.15). MPA-treated animals displayed numerous pre-neoplastic lesions and few ductal structures were observed, however numerous lobular structures appeared to be extremely proliferative. Animals that received a combination of MPA + curcumin, or curcumin alone, displayed fewer, if any, pre-neoplastic lesions and were more comparable to the placebo

morphology in that ductal and lobular structures were more distinct and distinguishable, compared to animals treated with MPA alone. (Figure 3.15)

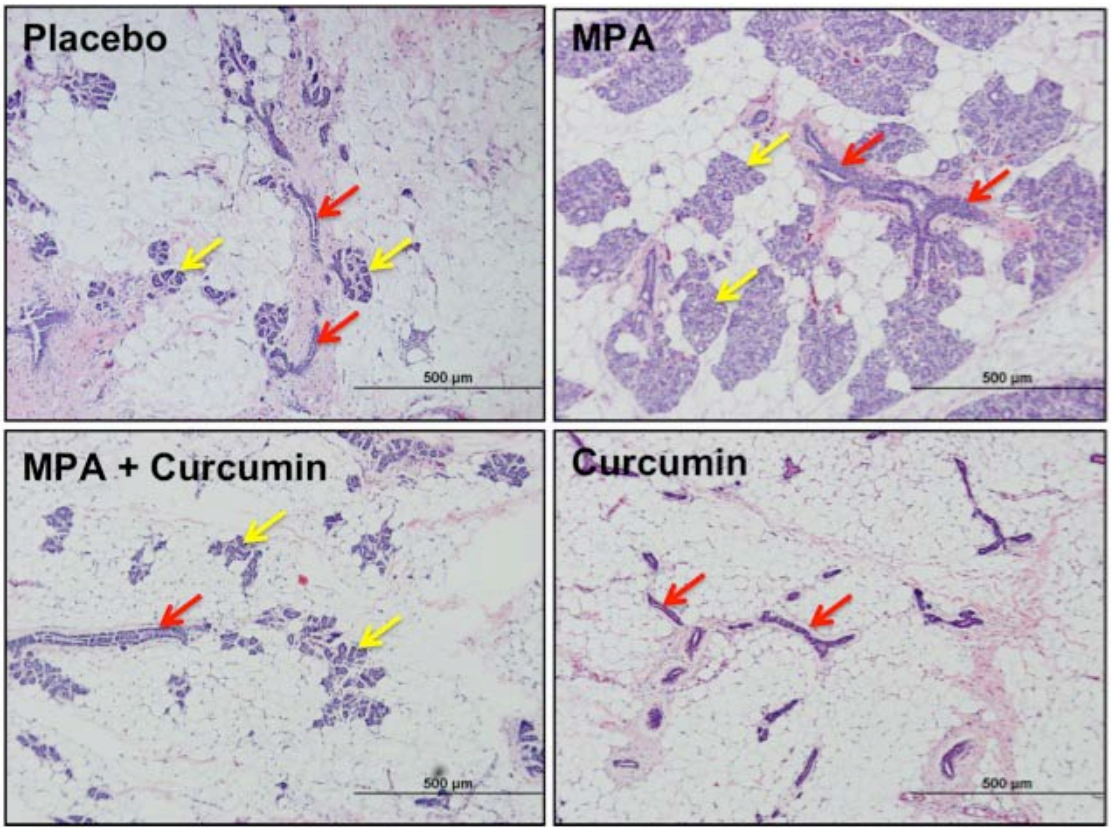

Figure 3.15: H&E Staining of non-tumor mammary gland. Curcumin prevents MPA-induced morphological changes in the mammary gland. Mammary gland tissue was collected at the conclusion of the study on day 52, sectioned, and stained with H&E. One representative section is shown for each group. (scale bars= 500 mm). Red arrows show ductal structures and yellow arrows show lobular structures.

Immunohistochemical staining was performed to determine the effects of curcumin on the expression of the estrogen receptors α and β, as well as PR and VEGF protein expression. Signaling through ERs is critical for PR expression [126, 127] and PR activity is essential for VEGF induction [7]. Because curcumin does bind to PR and ER, though with low affinity [70], ER and PR levels in the mammary glands of treated and untreated rats play a role in determining whether curcumin might up- or down-regulate the ER and/or PR as a result of treatment. Curcumin did not eliminate the

expression of steroid receptors ER α and β, or PR. However, there was an inhibition of VEGF staining, particularly in the ductal epithelial cells, with curcumin treatment, compared to MPA-treated mammary tissues (Figure 3.16).

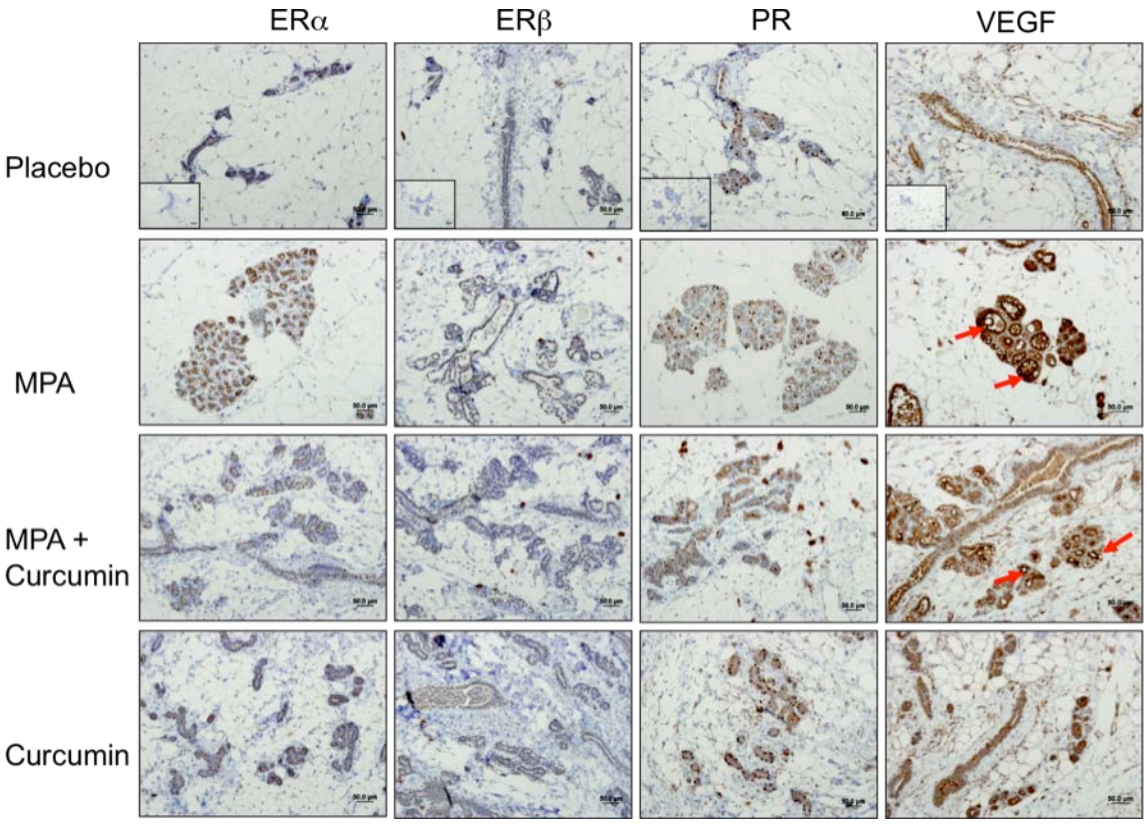

Figure 3.16: ER and PR expression are unaffected by curcumin and VEGF levels are decreased by curcumin in MPA +curcumin treated DMBA-induced mammary tumors. Mammary gland tissues were collected at the conclusion of the study on day 52, sectioned, and immunostained for ER-a, ER-b, PR and VEGF as described in the Methods section. No significant differences were observed in the intensity of the staining among the treatment groups for ER-α, ER-b, and PR, however, curcumin blocked MPA-driven increases in VEGF levels in hyperplastic lesions (red arrows). Insets represent negative controls with no primary antibody staining for each antibody.

The inhibition of VEGF expression in the ductal epithelial cells was quantified using FoveaPro ® Software. The area of positively stained pixels is summed and averaged among the six analyzed slides (Figure 3.17). As expected, the MPA-treated animals had increased expression of VEGF in the mammary gland compared to placebo animals. The VEGF staining within the dutcal epithelial cells of the MPA + curcumin-

treated mammary glands were reduced though not statistically reduced compared to that of the MPA-treated animals.

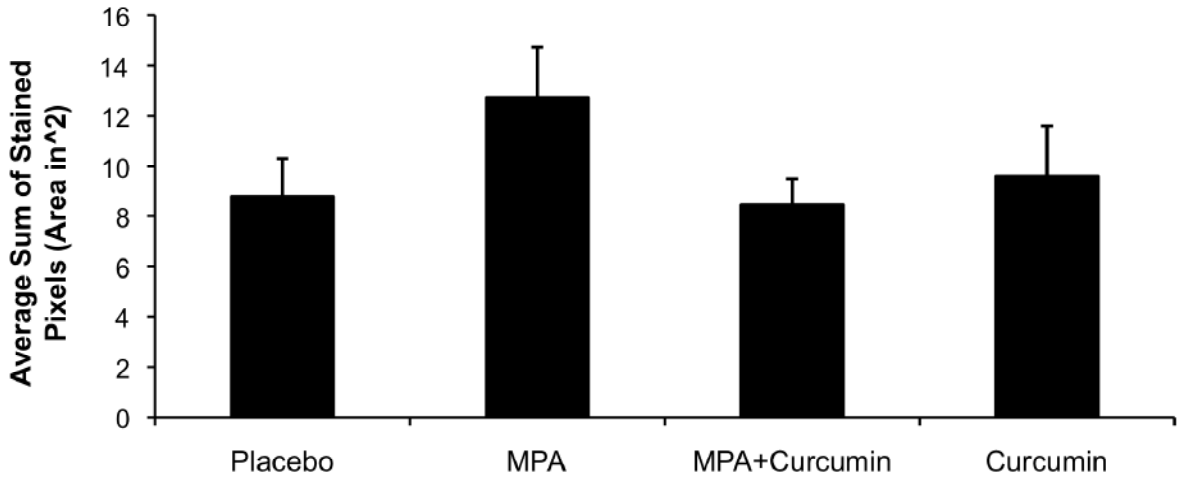

Figure 3.17: Quantification of the area of positively stained pixels for VEGF of the ductal epithelial cells. Six slides were analyzed, the area of positively stained pixels is summed from each slide and the graph depicts the average of the sum. Photographs of slides were analyzed using FoveaoPro 3.0 analysis software. Positive VEGF staining was quantified as the number of VEGF-positive pixels in three different fields. Though not statistically different, the amount of VEGF was reduced in the group treated with MPA + Curcumin compared to the group treated with MPA alone. No significance was determined when analyzed using ANOVA.

3.4 Discussion & Conclusion

Previous studies show that progestins stimulate the secretion of VEGF from human breast cancer cells expressing PR and mutant p53 protein [28]. VEGF in turn stimulates proliferation of PR-positive and PR-negative breast cancer cells that express receptors for VEGF [28]. VEGF also suppresses apoptosis and stimulates survival of endothelial and breast tumor cells [29]. In DMBA-treated rats, progestins accelerate PR- and VEGF-dependent development of mammary gland tumors [61]. Thus, inhibition of progestin-dependent secretion of VEGF may be an effective strategy by which to control the growth and spread of progestin-dependent breast cancer.

The studies described in the current chapter show that curcumin reduces MPA-induced secretion of VEGF from T47-D cells. Surprisingly, although curcumin interfered with the cellular effects of MPA, it did not interfere with the cellular effects of progesterone and other progestins. This suggests that PR may not be a direct target of curcumin, and is consistent with earlier observations suggesting that progesterone and MPA induce secretion of VEGF by distinct pathways [93]. Although a speculative idea, it is possible that curcumin interacts with or interferes with extra-nuclear pathways that are specific to MPA and not to progesterone [128]. It is also possible that curcumin activates an inhibitory pathway that is selective in its suppression of MPA-induced VEGF but does not interfere with VEGF induction by other progestins. Also, because curcumin failed to inhibit MPA-induced VEGF secretion in BT474 cells, it is possible due to the different pathways that regulate MPA-induced VEFG secretion in different cell lines [93], that the inhibitory mechanism of curcumin blocks the specific pathway in T47D cells and not BT474 cells. In this context it is interesting that recent studies have identified membrane progesterone receptors [129, 130], which may also be responsible for some of the differential curcumin effects observed in the present *in vitro* studies. Nevertheless, these data imply that curcumin may be useful as a therapeutic agent to minimize the cancer risk associated with MPA-containing HRT. Indeed, curcumin is already under investigation as an anti-tumor agent in a Phase II clinical trial [131]. Curcumin appears to be well tolerated, lacking dose-limiting effects. Such studies should encourage future clinical trials designed to evaluate the efficacy of curcumin in lowering breast cancer risk in women exposed to MPA as a component of HRT.

We previously reported that curcumin specifically inhibits MPA-induced VEGF secretion from T47-D human breast cancer cells [132]. MPA is not a specific agonist of the PR, as it also functions as an agonist for the androgen receptor [123]. Because studies have reported curcumin to be a potent inhibitor of the androgen receptor [133-135], the possibility that MPA induces VEGF secretion via the androgen receptor was explored. However, it was found that co-treatment with flutamide (0.1 mM), a specific androgen receptor inhibitor, was not able to block the MPA induction of VEGF *in vitro* [132] and was unable to inhibit MPA-accelerated, DMBA-induced tumor development in vivo, thus, AR levels were not investigated. However, ER and PR levels in the mammary glands were investigated after curcumin treatment. As a phytoestrogen, studies have reported that curcumin has a low affinity for ER and PR [70], however, a change in ER or PR levels was not observed. The current chapter reports that curcumin had no effect on ER/PR expression levels in the mammary gland of Sprague-Dawley rats exposed to DMBA and MPA. This provides evidence that curcumin may exert anti-cancer properties on the mammary gland with little to no effect on ovarian hormone receptor expression. Although no significant changes were observed in ER and PR levels, VEGF staining revealed a decrease in VEGF levels in the ductal epithelial cells of the mammary gland from animals treated with MPA + curcumin. These findings may play a critical role in elucidating the inhibitory mechanism of curcumin on DMBA-induced, MPA-accelerated tumor formation in the rat.

The present chapter does however show that curcumin delays MPA-accelerated, DMBA-induced tumor formation by approximately one week, and markedly inhibits morphological changes in MPA-exposed mammary glands. As expected, curcumin

blocked the increase in VEGF levels caused by MPA (Figure 3.16). This supports the anti-angiogenic properties of curcumin, and provides some insight towards determining the mechanism by which curcumin inhibits angiogenesis. It was hypothesized from previous *in vitro* studies, [132] that curcumin would delay MPA accelerated tumors via inhibition of VEGF expression. An earlier report showed that curcumin delays the onset of DMBA tumorigenesis [120], however, no studies utilized curcumin as a chemopreventive agent against progestin-dependent mammary tumors. To our knowledge, this is the first study that utilizes an animal model to provide evidence that curcumin has the ability to exert chemopreventive effects on progestin-dependent breast cancer. A phase I clinical trial with curcumin reports that no toxicity was observed when patients were given up to 8000 mg/day [136]. The dose administered in the current study show little to no negative effects on the animals, based on animal weight, and it is reported in the current chapter that curcumin exerts protective effects on MPA-induced morphological changes in the mammary gland in the process of tumorigenesis. There were increased proliferating lobules in the MPA-treated group, while curcumin-treated animals shared more histological similarities with placebo, suggesting that curcumin blocks changes caused by MPA. This could prove beneficial to women receiving combined HRT.

Though not discussed in detail, a brief pilot study was conducted to determine the effectiveness of curcumin as a therapeutic agent. In this study, three animals with established DMBA-induced, MPA-accelerated tumors were treated with injections of curcumin (1 injection every other day) at the previously determined dose (200 mg/kg, i.p.), for approximately 2 weeks (controls received vehicle). After no significant changes

were observed, this study was terminated and it was suggested that curcumin may not be useful as a therapeutic agent, but evidence suggests that it could prove effective as a chemopreventive agent.

The specific mechanism by which curcumin inhibits angiogenesis is unknown; however, based on the results described in the current chapter, the inhibition of VEGF may play a critical role. Nonetheless, there are studies that suggest cyclin D [111] and p21 [137] are involved in the anti-cancer effects of curcumin, however, no studies provide information on how curcumin interacts with progestin-dependent mammary cancers. Some studies also report that NF-κB is a target for curcumin [125, 138, 139]. Curcumin was reported to inhibit NF-κB activation, thus decreasing VEGF mRNA expression in breast cancer cells and ovarian cancer cells, reducing the expression in matrix metalloproteinases (MMPs), and transcriptional down-regulation of AP-1 [140]. Although it is possible that curcumin inhibits NF-κB activity in the DMBA study described herein, it was also reported that curcumin specifically induces apoptosis in cancer cells and causes cell cycle arrest in the G2 phase [111], which may also be a possible mechanism for curcumin inhibition. Nonetheless, literature reports a vast number of molecular targets affected by curcumin that may result in suppression of tumor growth or progression [141], as depicted in Figure 3.18. Due to the numerous possible targets of curcumin, the detailed inhibitory mechanism through which it acts in this model remains unknown.

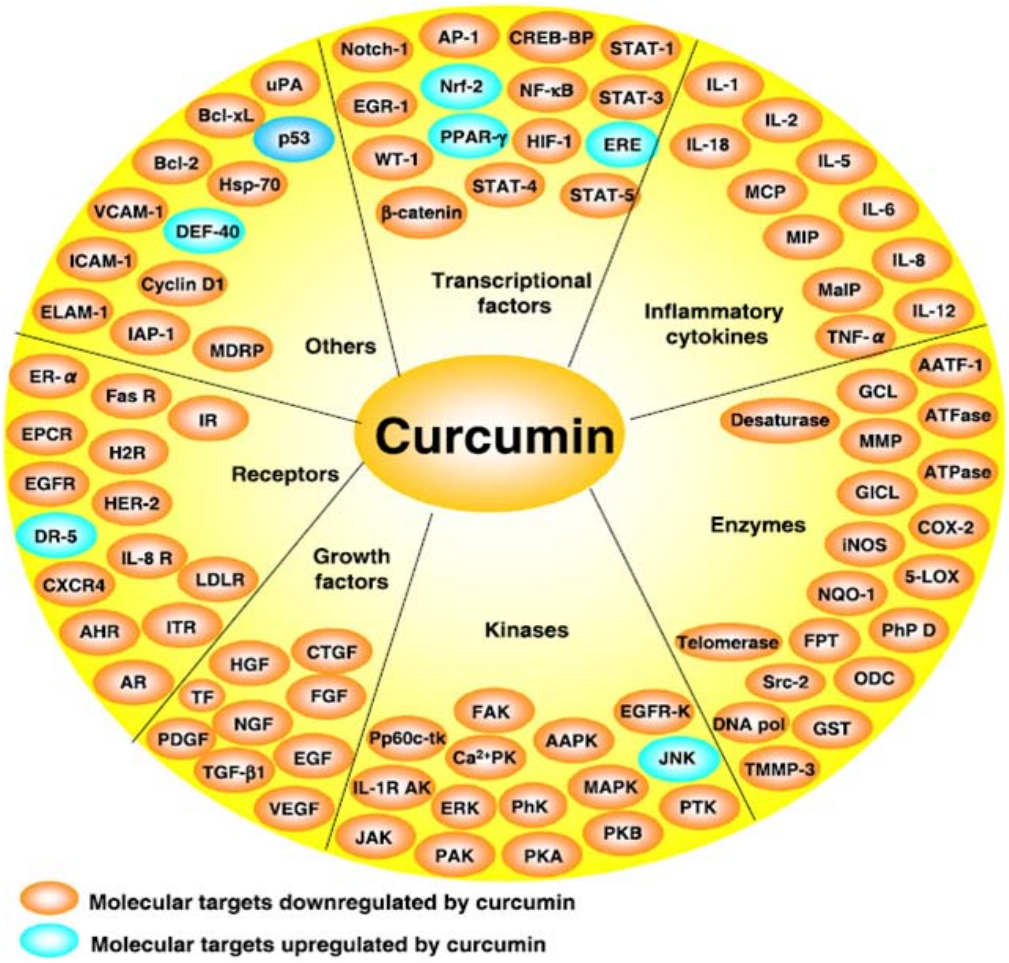

Figure 3.18: Multiple Targets of Curcumin. [141]

In conclusion, the current pre-clinical study reports that curcumin has inhibitory properties on MPA-driven mammary tumorigenesis. It reduces the latency period of progestin-accelerated, DMBA-induced mammary tumors in rats and importantly, reduces tumor multiplicity. The specific mechanism by which curcumin elicits these inhibitory properties is not elucidated in this study; however, it is shown that AR, PR, and ER are not involved in the inhibitory mechanism. Lastly, it is shown that curcumin blocks the morphological changes in the mammary gland caused by exposure to MPA in the process of tumorigenesis and inhibits MPA-induced VEGF expression in the mammary gland exposed to DMBA, suggesting that the turmeric root derivative may decrease the risk of

breast cancer associated with HRT. This study provides strong evidence that curcumin may be an excellent candidate as a chemopreventive agent for clinical trials for postmenopausal women who are prescribed combined hormone replacement therapy that includes both estrogens and MPA as a progestin.

CHAPTER 4

HIF-1α IS REQUIRED FOR PROGESTIN-INDUCED VEGF-INDUCTION AND MPA-ACCELERATED MAMMARY TUMOR PROGRESSION IN THE RAT AND MOUSE

4.1 Introduction

Millions of women in the U.S. undergo hormone replacement therapy (HRT) for postmenopausal symptoms [2]. Usually, estrogen is prescribed with a progestin component such as medroxyprogesterone acetate (MPA) to counteract the proliferative effects of estrogens in the uterus. Recent clinical trials suggest that ingestion of progestins among postmenopausal women is associated with increased incidence and recurrence of breast cancer [2]. The specific mechanism by which progestins increase this incidence, however, is unknown. We have shown that progestins increase levels of vascular endothelial growth factor (VEGF) in a subset of human breast cancer cells, which express mutant p53 protein [7]. VEGF is a potent angiogenic growth factor that plays a critical role in tumor development. We have also demonstrated that progestins increase VEGF levels in vivo resulting in accelerated tumor development and growth [61, 62]. In these studies, we have shown that blocking progestins with known anti-progestin, RU-486, inhibits VEGF secretion from breast cancer cells and blocks tumor growth *in vivo* [61, 62]. As previously established, RU-486 is not an ideal candidate for chemoprevention due its lack of specificity of the progesterone receptor (PR) [60]. As a result, there remains an urgency to find compounds that effectively combat progestin-dependent breast cancer.

It is well known that growing tumors have a hypoxic environment. Due to this fact, hypoxia-inducible factor-1α, (HIF-1α), has become a potential therapeutic target for cancer treatment and prevention [142]. HIF-1α is a subunit of the HIF molecule, a basic helix-loop-helix transcription factor that controls many hypoxia-regulated genes, including VEGF. Under normoxic conditions, the HIF-1α subunit is degraded by proteosome. However, under hypoxic conditions, as in the tumor micro-environment, HIF-1α is accumulated, dimerizes with the HIF-1β subunit, and binds to the HIF-1 response element (HyRE) of HIF-1-dependent genes, thus regulating gene expression. Interestingly, there is little evidence that shows that HIF-1α is a good molecular target for hormone-dependent cancers. There is evidence, however, that HIF-1α is involved in estrogen-dependent cancer [33]. To our knowledge, there is no data that suggests that progestin-dependent mammary tumors are HIF-1α dependent. The present study explores the idea that HIF-1α may be required for progestin-dependent breast cancer.

2-methoxyestradiol, (2ME2), is a known anti-angiogenic compound that has been reported to display effective anti-tumor properties [106]. 2ME2 functions in multiple capacities including as a microtubule disrupter [104], cell cycle inhibitor [103, 113, 143], inhibitor of angiogenesis [101, 144], as well as a HIF-1α inhibitor [101]. 2ME2 has also been tested in clinical trials for solid tumors [107, 145]. Mabjeesh et al [101] has thoroughly demonstrated the anti-angiogenic and HIF-1α inhibitory properties of 2ME2, by reporting that 2ME2-treated breast cancer cells decrease HIF-1α protein expression as well as VEGF secretion into cultured medium. Numerous studies have shown that 2ME2 is an effective anti-angiogenic compound *in vivo*. Klauber et al [104] demonstrated that 2ME2 blocked corneal neo-vascularization of growth factor induced angiogenesis. It has

also been shown that MVD in mammary tumors of 2ME2-treated animals is significantly less than in control animals [101]. HIF-1α was shown to be inhibited from nuclear accumulation, *in vitro*, with 2ME2 treatment of breast cancer cells as well as prostate cancer cells [101]. The present study provides strong evidence that 2ME2 may be extremely effective in combating progestin-dependent breast cancer, specifically targeting HIF-1α.

Because 2ME2 is not specific for HIF-1α, we used 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole), YC-1, to specifically target HIF-1α. YC-1 is a compound that was first described by Ko et al, in 1994 [146]. YC-1 was initially designed to treat thrombosis and hypertension, as a NOS-independent activator of soluble guanylyl cyclase (sGC) [147]. Interestingly, YC-1 has been shown to inhibit HIF-1 binding to the DNA of HIF-1 regulated genes [142]. Recent studies, however, have shown that YC-1, specifically targets HIF-1α, and may have potential to effectively battle cancers [147]. It has been reported that YC-1 is effective in preventing tumor development in multiple cell types in nude mice [147], as well as in treating established tumors [147]. In the current study, we have used YC-1 to confirm that HIF-1α is the target used in 2ME inhibition. Also, due to its specificity, we used YC-1 for mechanistic studies, as well as determining the role of HIF-1α in progestin-dependent breast cancers. Based on the effectiveness of HIF-1α as a molecular target in other cancer models [147], including the estrogen-dependent breast cancer model, **we hypothesize that HIF-1α is required for progestin-dependent VEGF induction of breast cancer cells and HIF-1α may be a useful target for the inhibition progestin-dependent mammary tumor growth.**

4.2 Methods & Materials

Materials. Human breast cancer cell lines T47-D and BT-474 were from ATCC (Manassas, VA). 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole) [YC-1], was purchased from Biomol International, LP (Plymouth Meeting, PA). 2-Methoxyestradiol was purchased from Toronto Research Chemical, Inc. (North York, Ontatio). Phenol red-free DMEM/F12 medium, phosphate-buffered saline, 0.05% trypsin-EDTA, and SOC Medium were purchased from Invitrogen Corporation & Life Technologies (Grand Island, NY) and fetal bovine serum (FBS) was purchased from JRH Biosciences (Lenexa, KS). G418 antibody was purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture. T47-D and BT-474 cells were grown separately on 100 mm cell culture plates to 60-70% confluence. Cells were washed in PBS and media was changed to 5% DCC media for 24 hours before treatment of cells. Cells were treated for 16-18 hours, unless otherwise stated and assayed accordingly.

Animal Studies Intact virgin female Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) that were 40-45 days old were housed according to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care under conditions of 12-hour light/dark cycles and *ad libitum* access to food and water. All surgical and experimental procedures were in accordance with procedures outlined in the "Guide for Care and Use of Laboratory Animals (NIH publication 85-23).

DMBA Animal Study: Animals were given a single dose of 20 mg/rat of DMBA in peanut oil via gavage on day 0. On day 30, animals were anesthetized and MPA pellets were implanted subcutaneously on the dorsal side [61]. On Day 68, YC-1 (3.75 mg/day), or vehicle, was administered to animals via tail vein injection for 5 days.

Animals were palpated and tumors were measured 2-3 times weekly. Twenty-four (24) days after the initial YC-1 treatment, animals were administered 0.5 mg Texas red-tomato lectin conjugate 10 minutes prior to sacrificing. Mammary tumor tissues were collected at the time of sacrifice for IHC analysis.

Animal Xenograft Tumor Study: Female athymic nu/nu mice, 5-6 weeks of age, purchased from Harlan Sprague Dawley, Inc (Indianapolis, IN), were housed in a laminar airflow cabinet under specific pathogen-free conditions. Nude mice were inoculated with 17-β-estradiol pellets (60-day timed released, 1.7 mg) 24-48 hours before implantation of BT474 or T47D cells. Cell were harvested via trypsinization and washed two times with DMEM/F12 media. Cell pellets were re-suspended (1×10^7 cells in 0.15 μl) in DMEM/F12 media and injected subcutaneously into the left and right flanks of each mouse. Tumor volume was measured every 3 days using a digital caliper and calculated using the formula $(L \times W \times H) \times \pi/6$. Tumors began to regress after reaching 60 to 100 mm^3 in size (6-10 days), when tumor volume had decreased by ~50%, mice were inoculated with MPA pellets (60-day timed release, 10mg).

In the T47D-initiated tumors: On day 59, 3 mice were treated with YC-1 (600 μg/mouse, i.p.) for 10 days; 2 mice were treated at the same time with vehicle (DMSO). On day 81, 2 mice were treated with YC-1 (600 μg/mouse, i.p.) for 10 days; 3 mice were treated at the same time with vehicle. Tumor volume and animal weights were measured every 2-3 days. Four animals were used as no-MPA controls, receiving only estrogen (E_2) throughout the study. Animals were sacrificed and tumors collected 2 hours following the tenth and final treatment. At the conclusion of the study, data were merged

based on treatment day (1-10) and average tumor volume was calculated and statistically analyzed via One-Way ANOVA.

In the BT474-initiated tumors: On day 50, mice were treated with YC-1 (600 μg/mouse, i.p.) or vehicle (DMSO), for 7 days. Tumor volume and animal weights were measured every 2-3 days through day 71, when animals were sacrificed. Tumors were collected for IHC analysis at the conclusion of the study.

Histology and Immunohistochemisty. For both rat and mouse tumors, tissues were fixed overnight in 4% paraformaldehyde and processed for paraffin infiltration and embedding. Sections (5 μm) were mounted on ProbeOn Plus microscope slides (Fischer Scientific, Inc., Pittsburgh, PA) and routinely stained with hematoxylin and eosin (H&E) or prepared for immunohistochemical labeling. Prior to immunohistochemistry, sections were dewaxed in xylene, rehydrated through graded concentrations of ethanol, rinsed (wash buffer, DAKO Carpinteria, CA) prior to immersion and heated in 10 mmol/L citrate buffer (pH 6.0) for 20 minutes for heat-induced epitope retrieval. This tissue treatment was performed for PR, ER-α, ER-β, CD34 and VEGF immunolabeling. Slides were cooled for 20 min, treated with 3% H2O2 (to inactivate endogenous peroxidase activity) and rinsed prior to incubation with 5% bovine serum albumin for 20 minutes. Sections were then incubated for 60 minutes at room temperature with each of the following antibodies: anti-PR antibody (1:50 dilution of a rabbit anti-human PR polyclonal antibody [A0098], DAKO), anti-ER-α (1:300 dilution of a rabbit anti-ER-α polyclonal antibody [sc-542], Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-ER-β (1:50 dilution of a mouse anti-ER-β monoclonal antibody [MCA1974s] AbD Serotec, Raleigh, NC), anti-CD34 (1:100 dilution of a goat anti-CD34 polyclonal antibody, and an

anti-VEGF antibody (1:100 dilution of a rabbit anti-VEGF polyclonal antibody [sc-152]; Santa Cruz Biotechnology, Inc.). Sections were then washed, incubated for 30 minutes with a biotinylated secondary antibody (rabbit anti-mouse IgG [DAKO] for anti-ER-β labeled sections and a rabbit anti-goat IgG [DAKO] for the anti-CD34 probed sections) and then for 30 minutes with a streptavidin-linked horseradish peroxidase product (DAKO). Sections for PR, ER-α and VEGF were incubated with EnVision, a horseradish peroxidase–labeled polymer conjugated to anti-rabbit antibodies (DAKO). Bound antibodies were visualized following incubation with 3,3'-diaminobenzidine solution (0.05% with 0.015% H_2O_2 in PBS; DAKO) for 3-5 minutes. Sections were counterstained with Meyer's hematoxylin, dehydrated, and cover-slipped for microscopic examination.

Texas red conjugated-tomato lectin: Sectioned frozen tumors at 8 microns on cryostat. After rinsing, sections were incubated with 4% paraformaldehyde, rinsed again, and mounted with DAPI (Vectashield Hardset with DAPI, Vector Lab, Burlingame, CA) and cover slipped. In order to visualize Texas red-labeled tomato lectin staining, samples were imaged with a 590 nm bandpass filter at 1/2.0 second exposure times.

Transformation of E. coli with shRNA plasmid for HIF-1α. T47-D cells were stably transfected with four shRNA sequences specific for HIF-1α (SuperArray Bioscience Corporation [Frederick, MD]) and one non-specific shRNA (control), selected for neomycin resistance and tested for HIF-1α protein expression. Stably transfected T47-D cells were selected for significant HIF-1α protein expression. Selected cells were maintained in 10% FBS DMEM/F12 medium with 200 μg/ml of G418 antibody. Competent E. coli cells (100 μl, JM109, $>10^{-7}$ cfu/μg [Promega]) were transformed with

1 of 5 plasmids containing shRNA sequences (SuperArray) by thawing cells on ice. The appropriate shRNA (2 μl) was added to the cells and incubated on ice for 10 minutes. Mixture was heat shocked (42°C) for 1 minute, and then incubated on ice for 2 minutes. SOC medium (900 μl) was added to each transformed mixture and incubated for 1 hour at 37°C. Transformed mixtures of 100 μl (1:10, 1:100, and undiluted) were plated on agar plates, with ampicillin, and incubated at 37°C for 12-14 hours. Blue/white screening was used to select transformed colonies. Two colonies were selected (from the 1:100 dilution plate) from each shRNA plasmid sequence, and placed in LB miller broth (with 50 μg/ml ampicillin); tubes were incubated with shaking 4 hours at 37°C. 2ml of transformed E. coli suspension was added to 200 ml LB miller broth (with 50 μg/ml ampicillin) in 500 ml flasks and incubated overnight at 37°C with shaking (150 rpm).

Plasmid Purification. Plasmids were purified using the EndoFree Plasmid Maxi Kit (Qiagen) by collecting two, 45 ml aliquots from each flask of shRNA in 50 ml conical centrifuged tubes. Tubes were centrifuged for 20 minutes at 4500 rpm at 4°C. Supernatant was discarded and the pellet re-suspended in 5 ml of buffer 1, tubes were condensed from 2 to 1, resulting in one tube per clone. P2 Buffer (10 ml) was added to each tube, mixed by inversion and incubated on ice for 10 minutes. Chilled P3 buffer was added to each tube, mixed and immediately added to the QIAfilter cartridge and incubated undisturbed for 10 minutes at room temperature. A plunger was used to force the solution through the filter and lysate was collected. Buffer ER (2.5 ml) was added to lysate and incubated on ice for 1.5 hours. Qiagen tips were equilibrated with Buffer QBT, filtered lysate was poured into the Qiagen tips and flow through was discarded. Columns were washed twice with 30 ml Buffer QC (wash was discarded). DNA was

diluted with 15 ml of Buffer QN and collected in 50 ml conical centrifuge tube. DNA was precipitated out by adding 70% isopropanol to the mix which was then centrifuged for 60 minutes at 5000g at 4°C. Supernatants were decanted and the pellet washed with 5ml endotoxin-free 70% ethanol. Tubes were centrifuged for 60 minutes at 5000g at 4°C. Supernatant was decanted and pellet dried for 10 minutes. Pellets were dissolved in 600 µl TE buffer and stored at -20°C until transfection.

Enzyme Digestion (Pst1). DNA plasmids were thawed on ice. In a PCR reaction tube, 1 µg of DNA, 16.3 µl ddH_2O, 2 µl RE 10X buffer, 2 µl acetylated BSA, and 0.5 µl Pst1 restriction enzyme were added. A second set of tubes was prepared without Pst1 restriction enzyme. Samples were incubated at 37°C for 1.5 hours. Loading dye (4 ml of 6X dye) was added to each tube and samples were separated on a 1% agarose gel.

Lipofectamine Transfection of T47D cells with shRNA plasmid DNA. Plasmid DNA (48 µg) was diluted in 3ml of serum-free DMEM/F-12 media (one for each clone). Lipofectamine (120 µl) was diluted into 3ml of serum-free DMEM/F-12 media (one for each clone). The two preparations were combined, and 3 ml of lipofectamine mixture was added to one plate (performed in duplicate), T47D cells were 60-70% confluence in 100 mm cell culture plates. Plates were incubated for 5-6 hours at 37°C, after which 3 ml of 20% FBS DMEM/F-12 was added to each plate and incubation proceeded for a further 48 hours at 37°C. Cells were grown in 10% FBS DMEM/F-12 with 600 µg/ml G418 for 2 weeks. Three colonies from each plate (6 colonies per clone), were selected and grown in a 6-well plate with 10% FBS DMEM/F-12 with 200µg/ml G418. As each well reached confluence, cells were harvested and grown in 100mm cell culture plates with 10% FBS DMEM/F-12 media with 200 µg/ml G418. As plates reached confluence,

clones were saved and kept in liquid nitrogen for later screening for HIF-1α protein expression.

VEGF ELISA. After treatment of cells (16-18 hours), 1 ml of cell culture medium was collected from plates for assay using a Quantikine human VEGF enzyme-linked immunosorbent assay kit (ELISA), obtained from R&D systems, Inc. (Minneapolis, MN). Experiments were performed in triplicate, and analyzed according to the manufacturer's protocol. According to the manufacturer, the minimum detectable concentration of VEGF is less than 5 pg/ml, and the intra-assay precision has a coefficient variance (CV%) between 3.5-6.5%, while the CV% of the inter-assay ranges from 5.0-8.5%.

Bicinchoninic Acid Protein Assay. Cells were harvested and cell pellets were resuspended in 300 μl of lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, and 1% NP-40) and incubated on ice for 30 minutes. Samples were centrifuged for 20 minutes at 12,700 rpm at 4°C. Collected supernatant was used for protein determination by bicinchoninic acid (BCA) assay using bovine serum albumin as a standard. Absorbance was measured at 562 nm using a SpecTRA MAX 190 microplate reader (Sunnyvale, CA). All samples were analyzed in duplicate.

Preparation of Nuclear Extract. Nuclear extract was collected from treated cells using the ActiveMotif Nuclear Extract Kit (Carlsbad, CA). Following treatment, cells were washed with PBS/Phosphatase inhibitors, scraped from cell culture dishes, collected in a conical centrifuge tubes and centrifuged for 6 minutes at 1000 rpm. Supernatants were removed, pellets were re-suspended in 500 μl hypotonic buffer and incubated for 15 minutes on ice. Twenty-five μl of detergent was added to samples which were vortexed

for 10 seconds and centrifuged for 1 minute at 13,000 rpm. Supernatants were removed and nuclear pellets were re-suspended in complete lysis buffer, vortexed for 10 seconds and incubated on ice for 30 minutes. Samples were vortexed for 30 seconds and centrifuged for 12 minutes at 13,000 rpm. Supernatant (nuclear fraction) was collected. Supernatant fractions are 80% pure according to the manufacturer's specifications.

Western Blotting. Proteins, 20 μg per lane, were separated on a 7.5% Tris-HCl Criteron pre-cast gel from Bio-Rad Laboratories (Hercules, CA). Separated proteins were transferred to a PVDF membrane from Bio-Rad Laboratories (Hercules, CA). The blots were blocked for 1 hour at room temperature (or overnight at 4°C) in 5% non-fat dry milk in TBS containing 1% Tween-20 (TBS-T). Blots were incubated with primary anti-body at the specified dilution for 2 hours at room temperature (or overnight at 4°C): anti-PR-AB (52) (1:200 dilution), anti-HIF-1α (28b) (1:100). Blots were washed 5 times for 5 minutes each in TBS-T, and then incubated with secondary anti-body for 1 hour at room temperature. Blots were washed again, 5 times for 5 minutes each in TBS-T, and immuno-reactive bands were visualized following 5-minute incubation with the ECL plus detection kit from Amersham Pharmacia Biotech (Arlington Heights, IL). Membranes were stripped and re-blotted for β-actin (Sigma Aldrich, St. Louis, MO). Bands were normalized to the β-actin signal in the same sample.

Chromatin Immunoprecipitation Assay. A ChIP Assay kit was purchased from Millipore (Billerica, MA) and reagents were used to perform the ChIP Assay. Cells were treated for 30 minutes; media was removed and replaced with 1% formaldehyde for cross-linking, and incubated for 10 minutes at 37°C. Formaldehyde was removed and cells were washed with ice-cold PBS, containing phosphatase inhibitors. 3ml of ice-cold

PBS containing phosphatase inhibitors was added to plates and cells were gently scraped. Cells were centrifuged for 6 minutes at 2000 rpm at 4°C, pellets re-suspended in 600 µl of SDS lysis buffer and incubated on ice for 10 minutes. Cell lysates were sonicated 15 times, for 10 continuous seconds each. Sonicated samples were centrifuged for 10 minutes at 13,000 rpm at 4°C. 20 µl of sonicated material was kept at -20°C for concentrated input, and remaining samples were diluted 10-fold. 20 µl of diluted sample was removed and kept at -20°C for diluted input. Remaining diluted sample was pre-cleared with Protein A Agarose/Salmon Sperm DNA for 1 hour at 4°C with rotation. Samples were centrifuged 1 minute at 1000rpm; supernatant was collected and incubated with 2 µg of HIF-1α or IgG antibody overnight at 4°C with rotation. Protein A Agarose/Salmon Sperm DNA was added to each sample and incubated for 2 hours at 4°C with rotation. Samples were centrifuged for 1 minute at 1000rpm at 4°C. Supernatant was discarded, and pellets were washed for 5 minutes each at 4°C in low salt, high salt, LiCl salt and two times in TE buffer. After each wash, samples were centrifuged 1 minute at 1000 rmp at 4°C and supernatant was discarded. 500 µl of freshly prepared elution buffer (84 mg NaHCO3, 500 µl 20% SDS, 9.5 ml nuclease-free H2O) was added to each sample which were incubated at room temperature for 15 minutes with rotation. Samples were centrifuged for 1 minute at 1000 rpm. Supernatant was collected and 20 µl of 5M NaCl was added to each sample. 1 µl 5M NaCl was added to input material and all samples were incubated overnight at 65°C. 15µg proteinase K was added to all samples (including input), 20 ml Tris-HCl +10 µl EDTA was added to each sample, while 1 µl Tris-HCL and 1 µl EDTA was added to input material. All samples were incubated at 45°C for 2 hours. Samples and inputs were purified for PCR analysis using Qiagen

Immunoprecipitation Assay. After at least 24 hours in 5% DCC DMEM F-12 media, T47D cells were pretreated with 1 μM RU-486 or 100 μM YC-1 for 30 minutes then treated with 10 nM MPA the specified time. Cells were harvested and lysed for 30 minutes on ice in 1X RIPA Buffer (Santa Cruz Biotechnologies, Inc, Santa Cruz, CA). Lysate was centrifuged and supernatant collected for BCA protein analysis to determine protein concentration. Five hundred (500) μg of protein was incubated with 2 μg of antibody (HIF-1α or IgG) overnight at 4°C. Forty (40) μl of Protein G Agrarose beads (Santa Cruz Biotechnologies, Inc, Santa Cruz, CA) was added to samples which were incubated overnight at 4°C. Samples were centrifuged for 30 seconds at 10,000 rmp and beads were washed three times with cold PBS. Forty μl of sample buffer (with β-mercaptoethanol) was added to beads and boiled for 10 minutes. Samples were centrifuged for 10 minutes at 12,000 rpm and proteins in the supernatant separated on a 7.5% SDS-PAGE gel. Immunoblotting was carried out with the PR-AB and HIF-1α antibody.

RNA Extraction. Cells were co-treated with progestin (10 nM) and 100 μM YC-1 for 6 hours at 37°C. One (1) ml of Ultraspec RNA reagent (Biotecx Products, Oxon, UK) was added to plates and cells were transferred to a 2 ml microcentrifuge tube and incubated for 5 minutes at 4°C. Cholorform (300 μL; Acros Organics, New Jersey, USA) was added to the cells which were vortexted for 15 seconds and incubated on ice for 5 minutes. Samples were centrifuged at 12,000g for 15 minutes at 4ºC. The aqueous phase was carefully transferred to a fresh tube without disturbing the interphase, an equal volume of isopropanol (Sigma Aldrich, St. Louis, MO) added and the solution incubated for 10 minutes at 4ºC. RNA precipitate was formed as a pellet at the bottom of the tube

after centrifugation at 12,000g for 10 minutes. Pellets were washed with 75% ethanol by vortexing and subsequent centrifugation. Ethanol was decanted and pellets dried before being re-suspended in DEPC treated water.

RT-PCR. RNA was DNase treated before RT-PCR, SuperScript III one-step RT-PCR kit was used with 1 µg of RNA and 1 µl of 10 µM forward and reverse primers. VEGF and β-actin primer pairs were purchased from R&D Systems, Inc (Minneapolis, MN). Forward 5'-3': ATGAGAAGTATGACAACAGCC, and reverse 5'-3': TGAGTCCTTCCACGATACC were used for GAPDH. The cycle used was 60°C for 30 minutes for cDNA synthesis, 94°C for 2 minutes for denaturing, and 40 cycles of 94°C for 15 seconds, 50-55°C for 30 seconds, and 68°C for 1 minute, and final extension was 7 minutes at 68°C.

Statistical Analysis. The mean average value for VEGF levels (pg/mg) in the untreated (control) samples was calculated from the number of determinants in that group and used to calculate VEGF levels in untreated and treated groups as a percentage value. Within each group, results were plotted as bar graph representing mean $\pm$ SEM. Data were analyzed by one-way ANOVA and all groups compared with controls. Data sets with inhibitors were compared with VEGF values in progestin treated samples and differences considered significant when p values were <0.05. SRB cell viability assay was performed in multiples of six. All data sets were compared to controls for significant difference following treatment. All data was checked for normality and homogeneity of variance. Since a number of analyses did not satisfy these assumptions, data was ranked and ANOVA performed as outlined by Conover and Iman [109]. Fisher's protected least significant difference (LSD) was performed to determine treatment differences, as

suggested by Chew [110]. All significance was based on the ranked transformations; however treatment values were presented as actual values. For DMBA studies, a difference in the growth curve of tumor size was compared via t-test. Curves were considered significantly different when p values <0.05. For nude mice studies, points along the tumor volume curve were compared via One-Way ANOVA, where differences were considered significantly different when p values <0.05. FoveaoPro 3.0 ® software analysis was used to determine positive staining by area in immunohistochemical studies. One-Way ANOVA was used to statistically compare VEGF, CD34, and PR staining differences among experimental groups. For all statistical comparisons, $p<0.05$ was regarded as statistically significant.

4.3 Results

2ME2 and YC-1 inhibit progestin-induced VEGF secretion from T47-D cells. To determine if 2ME2 was able to inhibit progestin-dependent VEGF secretion from T47-D human breast cancer cells, cells were pre-treated for 30 minutes with 1, 10, or 100 μM 2ME2, or 1 μM RU-486 prior to the addition of 10 nM MPA, as described in the methods section. It was previously established via SRB cell proliferation assay that 100 μM 2ME2 was not cytotoxic to T47D cells. As expected we observed a 4-fold increase in VEGF secreted from T47-D cells with 10 nM MPA treatment and this effect was blocked by pre-treatment with RU-486. We also observed that 2ME2 inhibits MPA- and progestin-induced VEGF secretion from T47-D breast cancer cells.

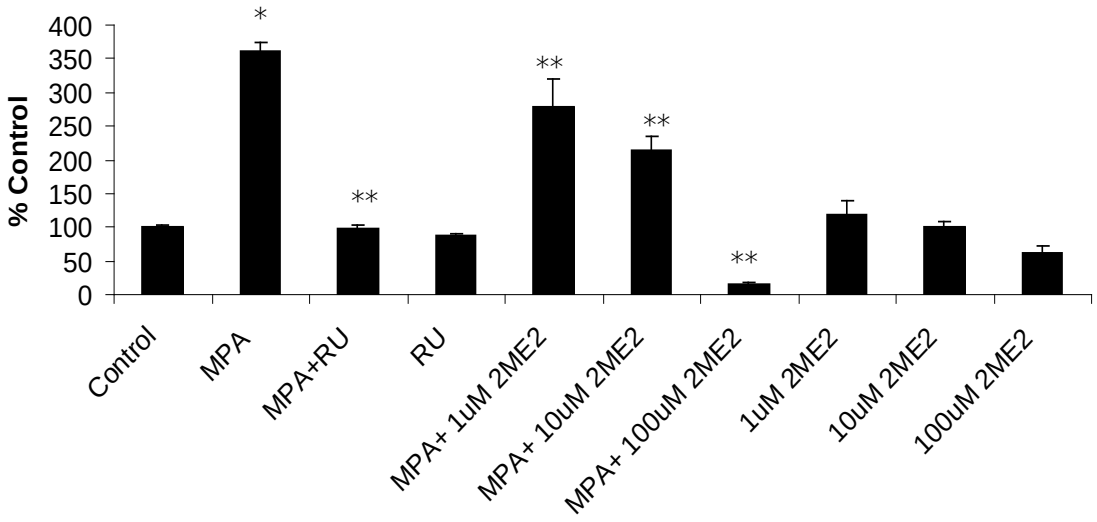

Figure 4.1: VEGF ELISA. (T47D): 2ME2 inhibits MPA-induced VEGF secretion. As described in Methods and Materials, cells were pre-treated for 30 minutes with 1 μM RU-486 or 1, 10, or 100 μM 2ME2. Cells were then treated with 10 nM MPA for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expressed as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyze data, Control = 271 ± 35, n=3-9. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$.

To determine if 2ME2 was able to block the effects of the natural hormone, progesterone, we pre-treated T47-D cells with 1 μM RU-486 or 100 μM 2ME2, as previously described, before treatment with10 nM progesterone.

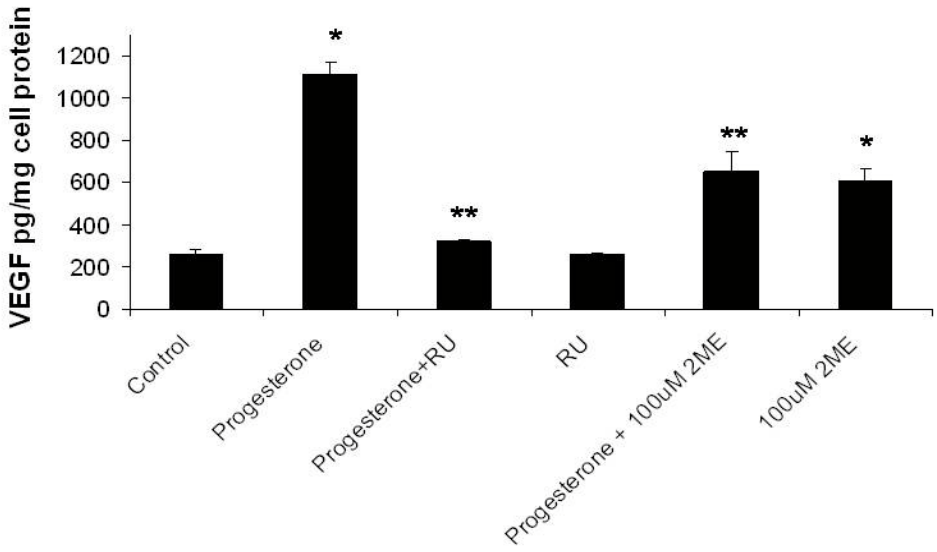

Figure 4.2: VEGF ELISA (T47D): 2ME2 inhibits progesterone-induced VEGF secretion. As described in Methods and Materials, cells were pre-treated for 30 minutes with 1 μM RU-486, or 100 μM 2ME2. Cells were then treated with 10 nM progesterone for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expresses as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyze data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$.

We then wanted to examine if the inhibitory effects of 2ME were due to HIF-1α inhibition. Because 2ME2 has targets other than HIF-1α, YC-1 was used to confirm the involvement of HIF-1α in this progestin-dependent pathway. Like 2ME2, it was previously established via SRB cell proliferation assay that 100 μM YC-1 was not cytotoxic to T47D cells. Results from VEGF ELISAs show that YC-1 inhibited MPA- and progesterone-induced VEGF secretion from T47-D cells (Figure 4.3). YC-1 also inhibited induction from T47D cells as a result of treatment with other progestins including norgestrel and norethindrone (Figure 4.4). Because YC-1 confirmed HIF-1α involvement in the inhibition of progestin-dependent VEGF induction, this agent was used in subsequent experiments to determine the role of HIF-1α in progestin-dependent VEGF induction.

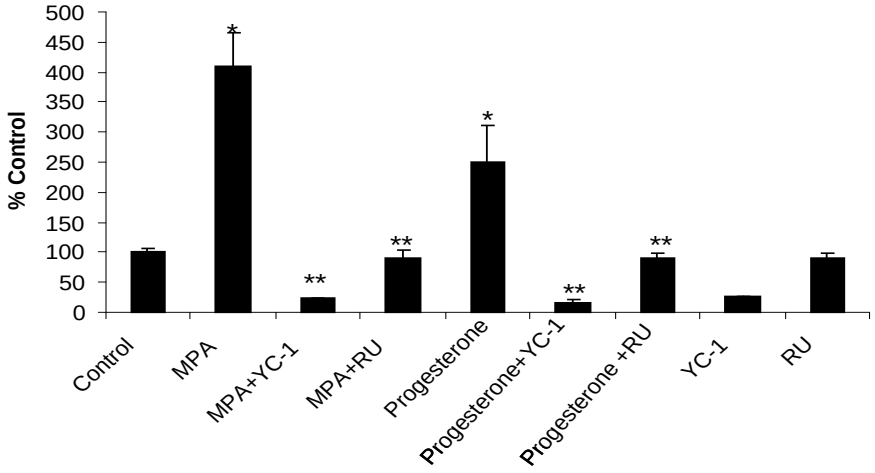

Figure 4.3: VEGF ELISA (T47D): YC-1 inhibits MPA-induced VEGF secretion. As described in Methods and Materials, cells were pre-treated for 30 minutes with 1 μM RU-486, or 100 μM YC-1. Cells were then treated with 10 nM MPA or progesterone for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expressed as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyze data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$.

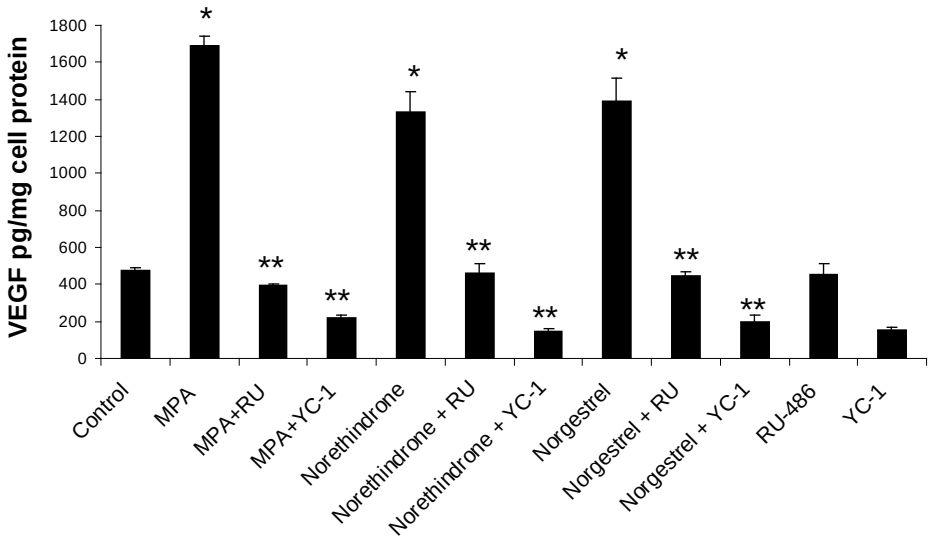

Figure 4.4: VEGF ELISA (T47D). YC-1 inhibits progestin-induced VEGF secretion. As described in Methods and Materials, cells were pre-treated for 30 minutes with 1 μM RU-486, or 100 μM YC-1. Cells were then treated with 10 nM MPA, norethindrone, or norgestrel for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expresses as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyze data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$.

YC-1 did not inhibit progestin-induced VEGF secretion from BT474 cells. Based on SRB cell proliferation assay, showing cytotoxic effects of YC-1 in BT474 at 100 μM (Figure 4.5), BT474 cells were treated with 10 nM MPA and/or 50 μM YC-1 for VEGF secretion analysis via ELISA, as opposed to the 100 μM YC-1 used with T47D cells. This was to help determine if the involvement of HIF-1α in progestin-dependent VEGF induction is cell type-specific. 50 μM of YC-1 was unable to block MPA-induced VEGF secretion from BT474 cells. This suggests that there may be a threshold concentration that is required to significantly inhibit HIF-1α activity.

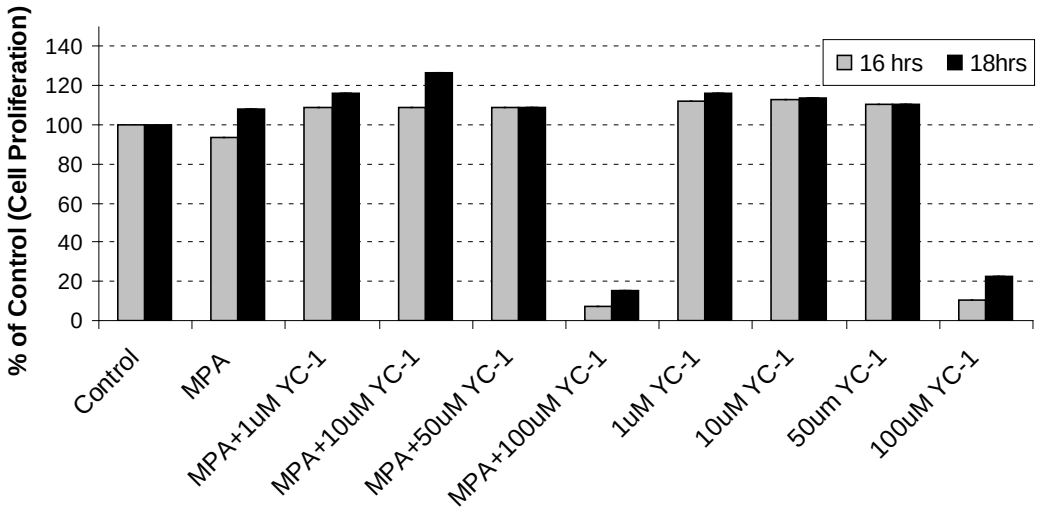

Figure 4.5: SRB Assay (BT474): Cells were treated with vehicle or with 10 nM MPA with and without 1-100 μM YC-1 for 18 and 16 hours at 37°C. Cell viability was measured using SRB Assay as described in the Materials and Methods.

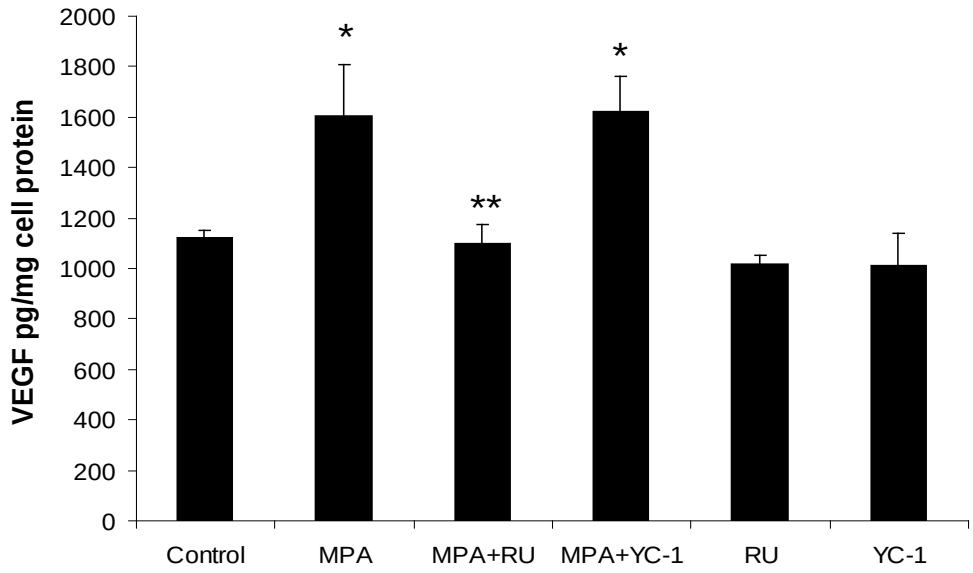

Figure 4.6: VEGF ELISA (BT474). YC-1 failed to inhibit MPA-induced VEGF secretion in BT474 cells. As described in Methods and Materials, cells were pre-treated for 30 minutes with 1 μM RU-486, or 50 μM YC-1. Cells were then treated with 10 nM MPA or progesterone for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expresses as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyze data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$.

2ME2 and YC-1 block progestin/hypoxia-mediated super induction of VEGF secretion from T47D cells. Because growing tumors have a hypoxic microenvironment, we treated T47D cells with both MPA (10 nM) and $CoCl_2$ (1 mM), which mimics hypoxia by inhibiting the proteosomal degradation of HIF-1α. This combination treatment resulted in a 'super' induction of VEGF section from T47D cells (Figure 4.7). This super induction was partially blocked by 1 μM RU-486, suggesting that factors other than PR are required for this induction.

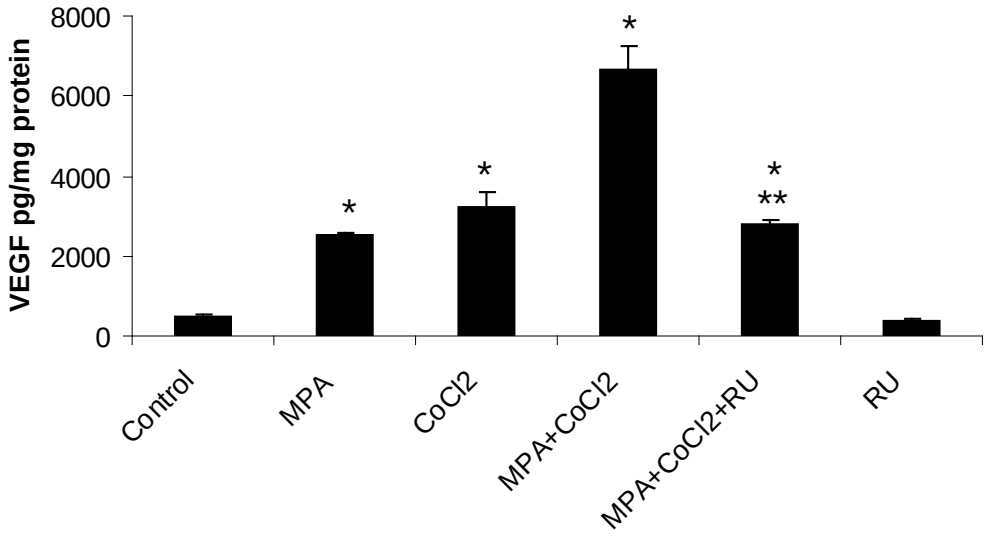

Figure 4.7: VEGF ELISA (T47D). RU-486 (1 μM) partially inhibits MPA + $CoCl_2$-induced VEGF secretion. As described in Methods and Materials, cells were pre-treated for 30 minutes with 1 μM RU-486. Cells were then treated with 10 nM MPA and/or 1 mM $CoCl_2$ for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expresses as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyze data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$. RU=RU-486

Importantly, the super induction was completely abolished by 2ME2 and YC-1 treatment (Figures 4.8 and 4.9). This provides strong evidence that HIF-1α is required for progestin-induced VEGF secretion from human breast cancer cells.

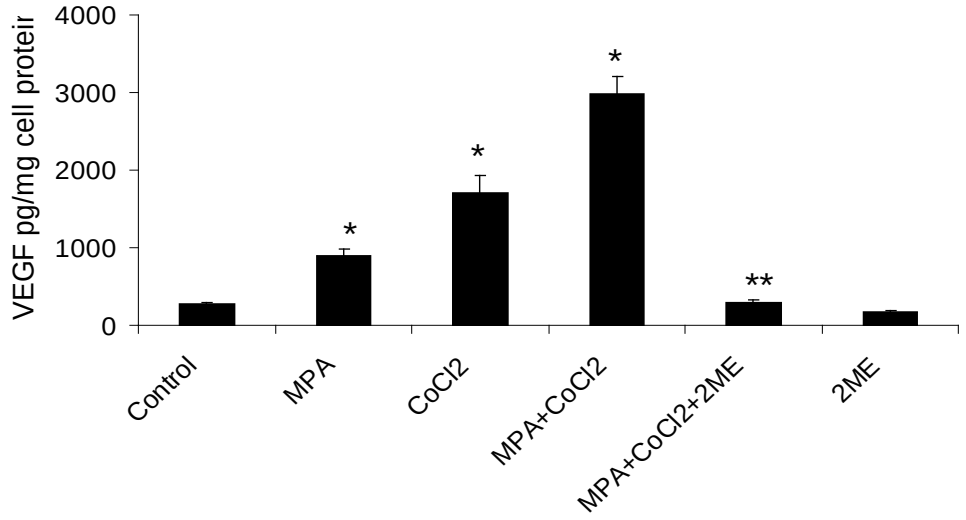

Figure 4.8: VEGF ELISA (T47D). 2ME2 inhibits MPA + $CoCl_2$-induced VEGF secretion. As described in Methods and Materials, cells were pre-treated for 30 minutes with 100 μM 2ME2. Cells were then treated with 10 nM MPA and/or 1 mM $CoCl_2$ for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expresses as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyze data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$.

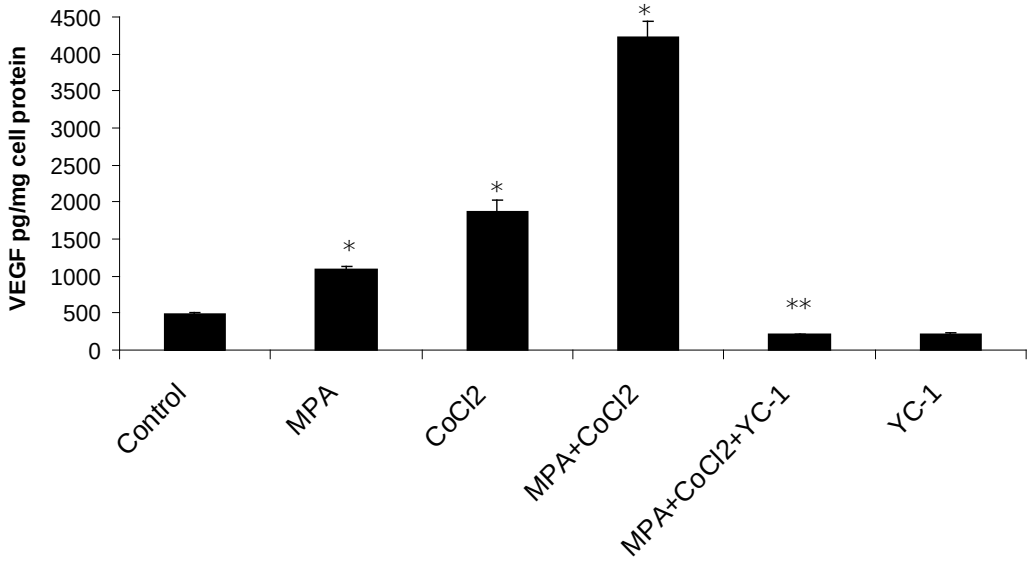

Figure 4.9: VEGF ELISA. YC-1 inhibits MPA + $CoCl_2$-induced VEGF secretion. As described in Methods and Materials, cells were pre-treated for 30 minutes with 100μM YC-1. Cells were then treated with 10 nM MPA and/or 1 mM $CoCl_2$ for 16-18 hours. Cell culture supernatant was collected and analyzed for VEGF presence as described in Methods and Materials. Secreted VEGF levels are expresses as pg/mg cell protein based on BCA Protein Assay results. One-way ANOVA was used to statistically analyzed data. * = Significant difference from control, ** = significant difference from appropriate progestin control, $p \leq 0.05$

T47D cells may require HIF-1α protein for Cell Survival. To confirm that HIF-1α is required for progestin-induced VEGF secretion from human breast cancer cells, we used shRNA in T47-D cells to stably knock down gene expression of HIF-1α. Interestingly, screened clones showed no significant inhibition of HIF-1α. It is however, noteworthy that there were clones that did not adhere to the cell culture plate, thus not surviving. These clones, if they did adhere, grew slowly, and had a high rate of cell death, suggesting that HIF-1α may be vital to cell survival. It was later discovered the cell culture plates used during this time were defective resulting in low adherence of cells to plates. As a result, the shRNA experiments were not carried out to completion.

Progesterone receptor and HIF-1α protein expression are not affected by 2ME2 and YC-1 treatment. To assure that the loss of progestin-dependent affects were not mediated by a loss of PR and/or HIF-1α expression, nuclear extracts were collected from treated cells. Immunoblotting was conducted to determine protein levels. Neither PR nor HIF-1α protein levels were significantly decreased by 2ME2 at 16-18 hours (Figure 4.10) or 2ME2 or YC-1 treatment after 6 hours (Figure 4.11). The presence of PR suggests that 2ME2 and YC-1 were not inhibiting progestin activity by down-regulating PR. Interestingly, because HIF-1α levels were not significantly affected, as shown in Figures 4.10 and 4.11, we suggest that the inhibition of VEGF in response to progestin treatment, may take place at the transcriptional level by inhibiting HIF-1α from binding to the VEGF promoter.

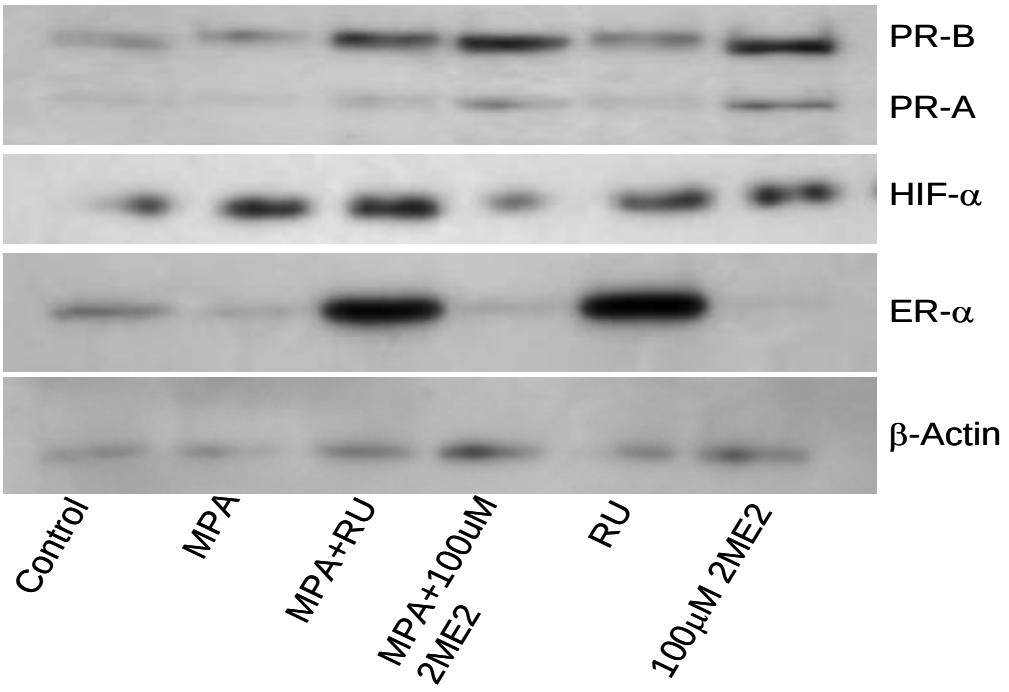

Figure 4.10: Western Blot of T47D Cell Nuclear Extract. T47D cells were treated with 10 nM MPA, 1 μM RU-486 or 100 μM 2ME2 for 16-18hrs. Nuclear extracts were collected as described in Materials and Methods and 10-15μg of protein was loaded and probed for HIF-1α, PR-AB, ER-α, and β-actin. RU=486.

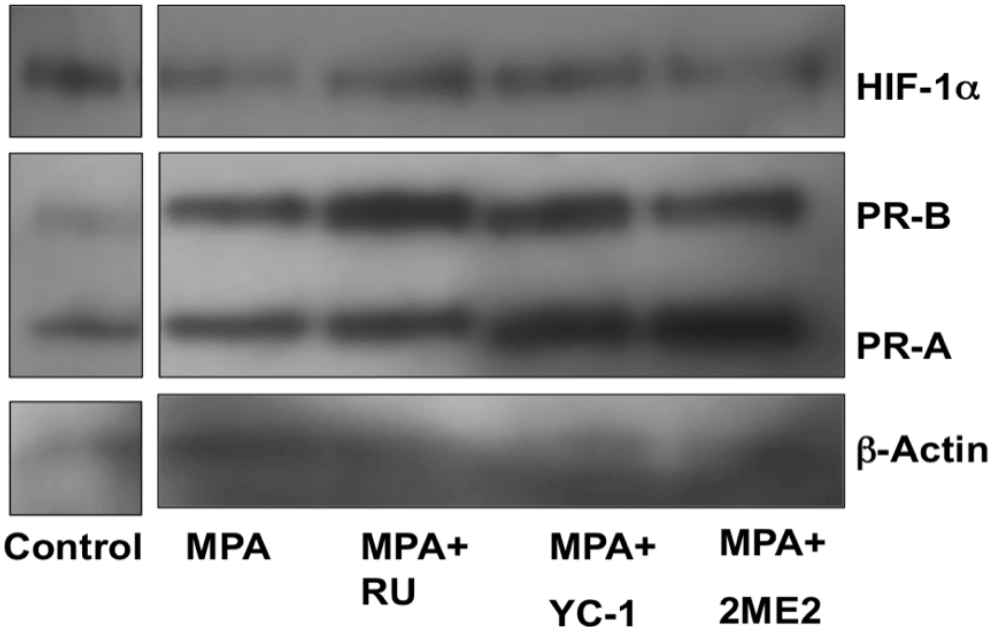

Figure 4.11: Western Blot of T47D Cell Nuclear Extract. T47D cells were treated with 10 nM MPA, 1 μM RU-486, 100 μM YC-1 or 100 μM 2ME2 for 6hrs. Nuclear extracts were collected as described in Materials and Methods and 20 μg of protein was loaded and probed for HIF-1α, PR-AB, and β-actin. RU=RU-486.

VEGF transcript levels are decreased with YC-1 treatment. YC-1 has been reported to inhibit HIF-1α from binding to the DNA of its target genes. We wanted to determine if YC-1 affected VEGF mRNA transcript levels. This would allow us to determine if the inhibitory effects of YC-1 was transcriptional or translational. After a 30 minute pre-treatment with 1 μM RU-486 or 100 μM YC-1, cells were treated for 6 hours with 10 nM progestin. Then RNA was extracted RT-PCR was performed using VEGF primers. The results show that VEGF transcript levels were increased with MPA treatment, while this induction was blocked with YC-1 treatment (Figure 4.12). This suggests that YC-1 may prevent HIF-1α from binding to VEGF DNA.

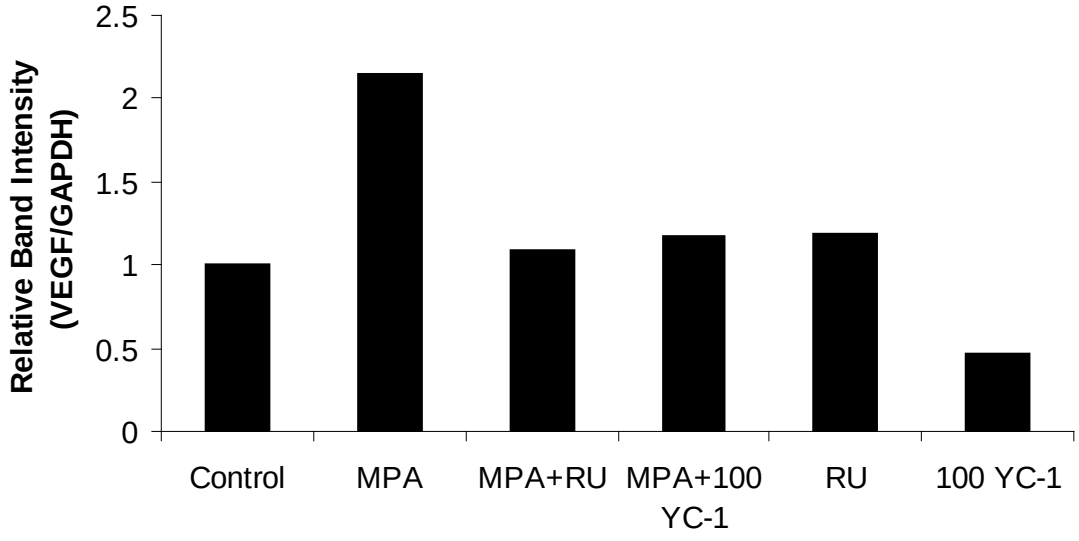

Figure 4.12: T47D cells were treated for 6 hours and RT-PCR for VEGF mRNA was performed using 1 μg of RNA from treated cells, as described in Materials and methods. RU=RU-486

YC-1 and 2ME2 inhibits HIF-1α DNA Binding to VEGF Promoter. The mRNA transcript levels of VEGF were reduced in cells treated with YC-1 and 2ME2, suggesting that the inhibition of VEGF secretion occurs before transcription. As a result, a ChIP assay was performed to study the role of HIF-1α on the VEGF promoter in the presence of MPA with and without treatment of YC-1 or 2ME2. Interestingly, we observed increased HIF-1α binding to the VEGF promoter in response to MPA treatment (-1149 to -794) in T47D cells, compared to control (Figure 4.13). This increased binding, however, was significantly blocked with YC-1 treatment and partially blocked by 2ME2 treatment (Figure 4.13).

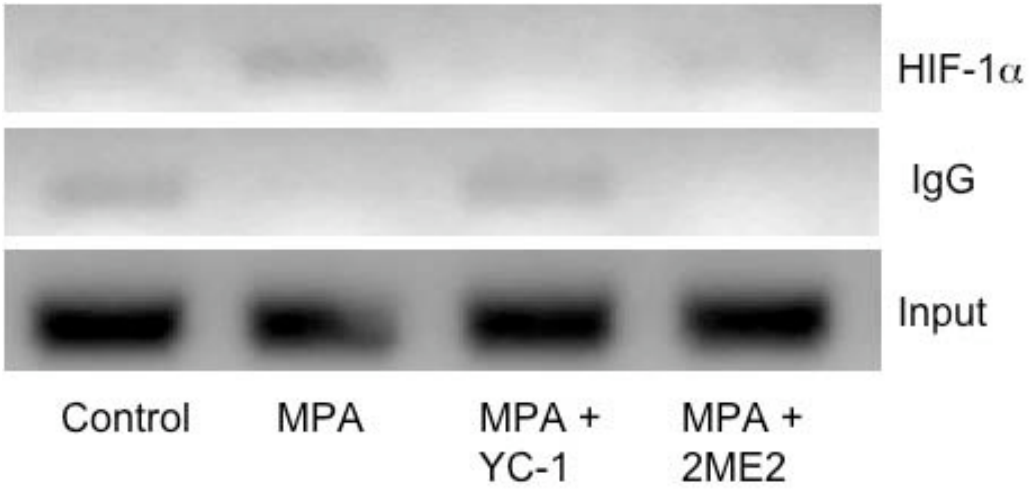

Figure 4.13: T47D cells were treated for 30 minutes, as described in Materials and Methods. ChIP assay was performed and PCR using *VEGF* promoter primers (-1149 to -794) was conducted.

Progestin-mediated VEGF induction may not mediated by direct binding of PR to the VEGF promoter. It has been hypothesized that PR directly binds to the VEGF promoter [7, 32, 35, 36], thus directly regulating progestin-mediated VEGF induction. However, no consensus PRE has been discovered on the VEGF promoter. In an effort to locate the region on the VEGF promoter to which PR binds, a ChIP assay was performed

on 4 regions of the VEGF promoter, scanning regions from -2263 to +50, in relation to the transcription start site. Interestingly, after 30 minutes of MPA treatment (lane 2, Figure 4.15), no binding of PR was observed to the VEGF promoter, whereas binding of HIF-1α was observed with only 30 minutes of treatment (Figure 4.13). This suggests that HIF-1α may be a critical transcription factor for progestin-mediated VEGF induction, in that HIF-1α is required for PR to interact, indirectly, to the VEGF promoter.

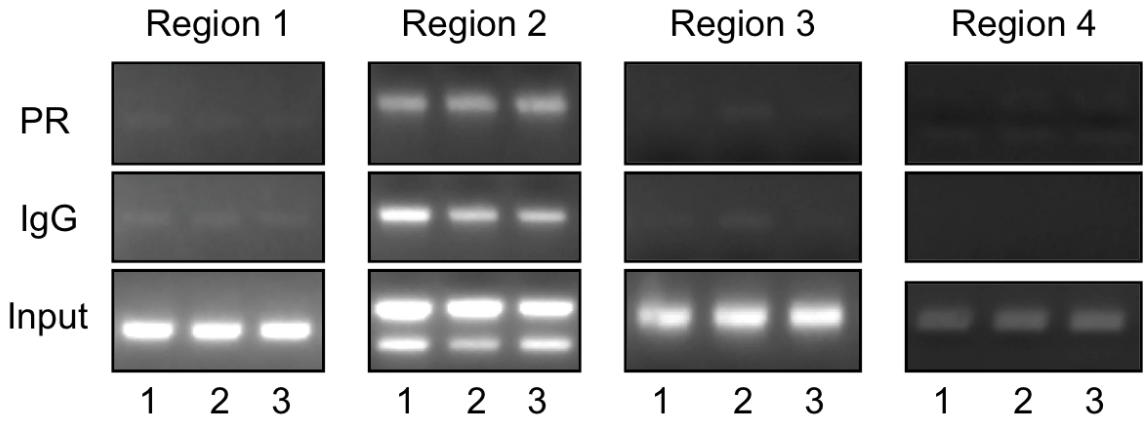

Figure 4.14: ChIP Assay in T47D cells. Cells were treated for 30mins with vehicle (lane 1), 10 nM MPA (lane 2), or 10 nM MPA + 1 μM RU-486 (lane 3). Anti-PR was used to immunoprecipitate DNA/protein complex from the cell lysate, as described in Materials and Methods. PCR was conducted to scan the VEGF promoter to visualize PR binding to the VEGF promoter via PCR. Region 1 (-2263 to -1810); Region 2 (-1810 to -794); Region 3 (-794 to -415); Region (-415 to +50)

HIF-1α and PR directly interact in T47D cells. An immunoprecipitation assay was conducted in which anti-HIF-1α precipitates the HIF-1α protein from the cell lysate of T47D cells after the designated treatment. PR protein levels were determined via immunoblotting. As shown in Figure 4.15, after 5 minutes or 30 minutes of treatment, PR was precipitated with HIF-1α, with no changes in response to treatment. Though the current treatment does not influence the interaction under the present conditions, this is

the first report to our knowledge that shows HIF-1α and PR have a direct protein-protein interaction.

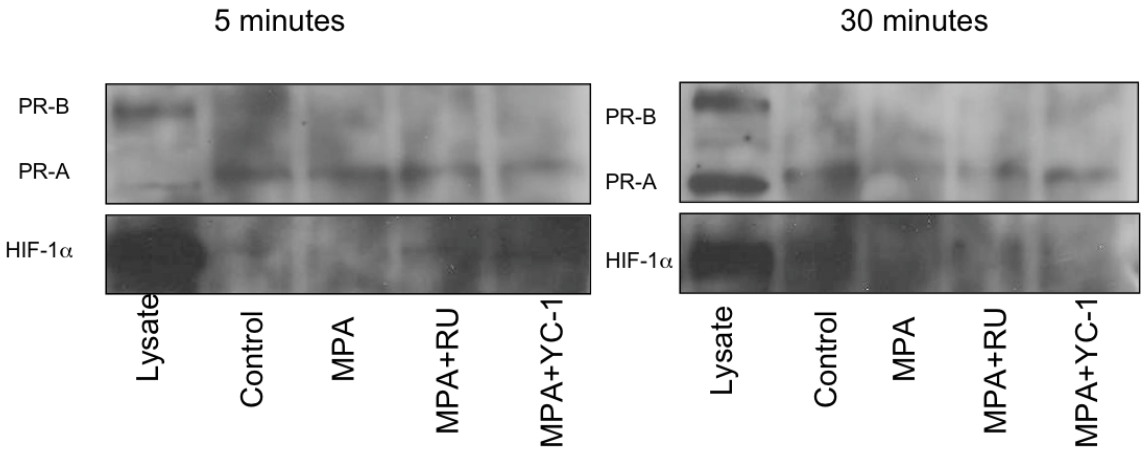

Figure 4. 15: Co-immunoprecipitation assay shows direct interaction between HIF-1α and PR. After the indicated treatment, T47D lysate was incubated with 2 mg HIF-1α antibody overnight followed by overnight incubation with Protein G Agarose beads. Eluate was collected and run on an SDS-PAGE gel, and immunoblotted for the presence of both PR-AB and HIF-1α. RU=RU-486

YC-1 inhibits progression of DMBA-induced, MPA-driven mammary tumors. We have previously shown that MPA accelerates tumor development and progression of DMBA-induced mammary tumors in female Sprague Dawley rats [61]. DMBA was administered to female, Sprague Dawley rats (45-50 days old) on day 0. MPA pellets (25 mg, 60-day timed release) were placed subcutaneously on day 30. On day 68, tumor-bearing animals were treated with YC-1 (3.75 mg/rat) via tail vain injection for 5 consecutive days, control animals were treated with vehicle. Tumors ranged in size from 2 mm^2 to 100 mm^2. Animals were weighed to monitor toxicity and tumors measured and palpated daily during injections, and 2-3 times per week after daily administration of YC-1. As hypothesized, we observed a continuous increase in tumor size with vehicle-treated animals, while YC-1 inhibited further progression of tumors with an original size less

than 100 mm^2 (Figure 4.16). Based on animal weights YC-1 seemed to be well tolerated at this dose.

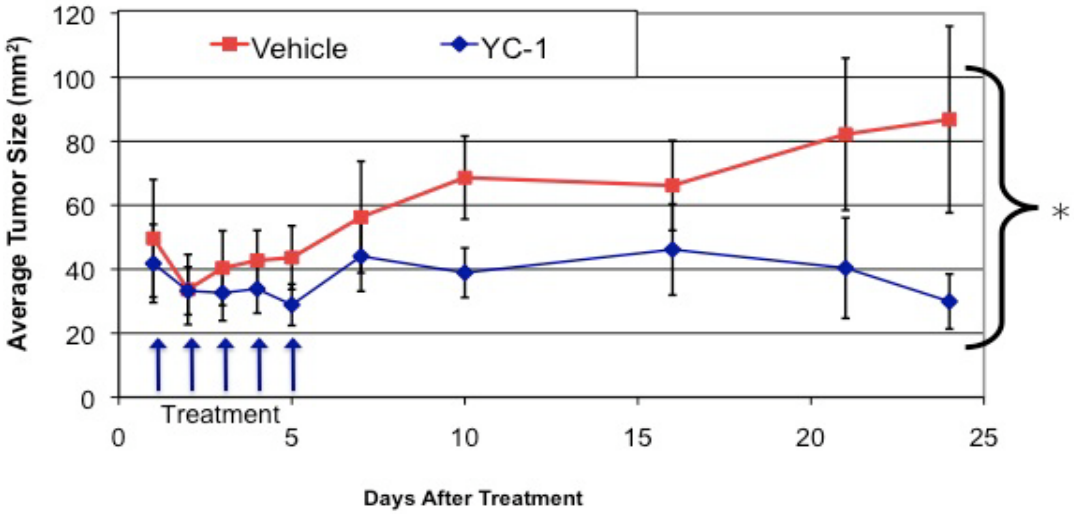

Figure 4.16: Rats were given 20 mg DMBA via gavage. After 4 weeks, 25 mg/60-day timed release pellets of MPA were implanted. 3.75 mg YC-1, or vehicle, was administered to animals for 5 days via tail vein injection. Error bars represent SEM, n=3-4 animals; 7-9 total tumors/group treated with a tumor size less than 100mm^2. * Represents significance as analyzed by t-test, $p<0.05$.

Though there was no distinct difference in histology between the tumors from treated and untreated animals (Figure 4.17), immunohistochemical analysis showed a significant inhibition of VEGF expression in the mammary tumors in YC-1-treated animals, especially in the stroma (Figure 4.18).

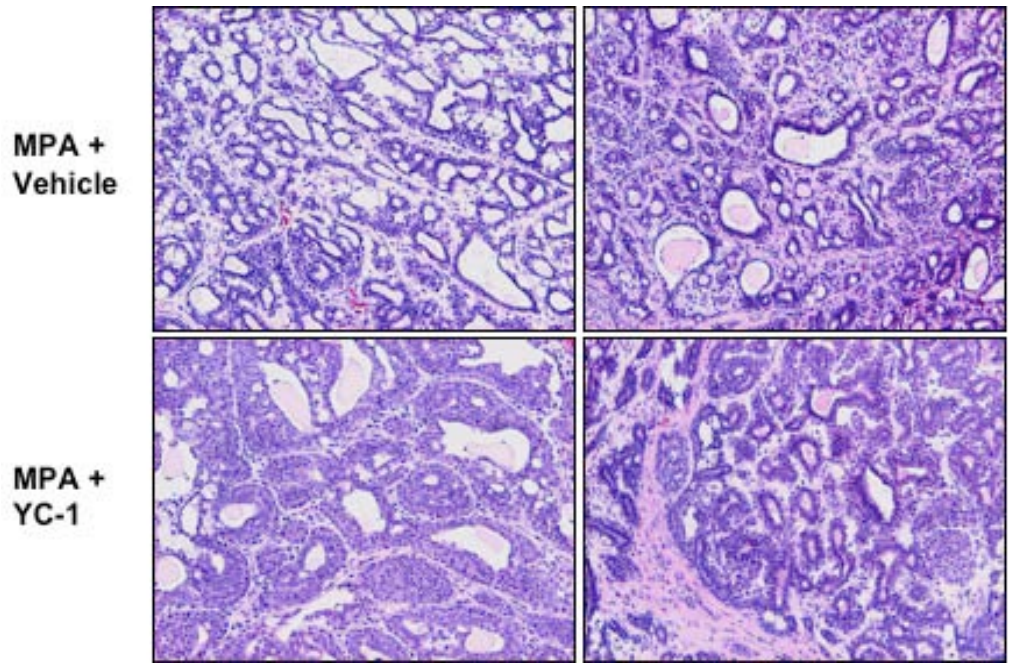

Figure 4.17: H&E staining of tumor tissue. Original magnification was 20X

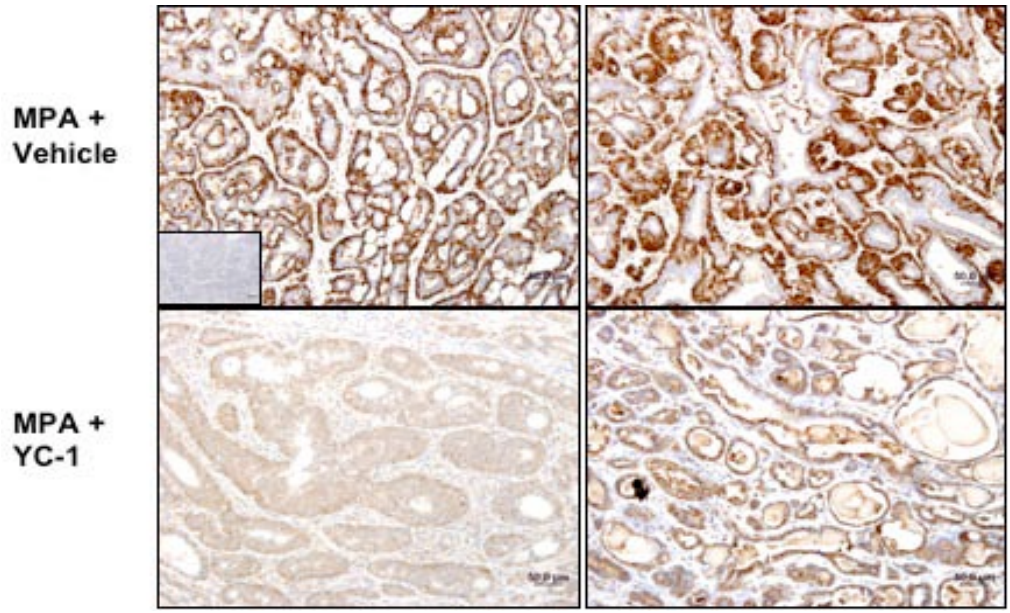

Figure 4.18: VEGF staining of tumor tissue shows a marked reduction in VEGF levels in animals treated with YC-1. Insert represents no antibody control. Original magnification was 20X.

The decreased VEGF expression coincided with a decrease in the number of blood vessels, as stained with the antibody CD34 (Figure 4.19). There was 38.7%

reduction in the number of positively stained blood vessels in the YC-1 treated tumors, compared to the vehicle-treated tumors (Figure 4.19). Though this decrease was not statistically significant analyzed via t-test (Figure 4.20), the vessels appeared to be smaller in diameter suggesting a decrease in blood flow to the tumors, as depicted in Figure 4.21.

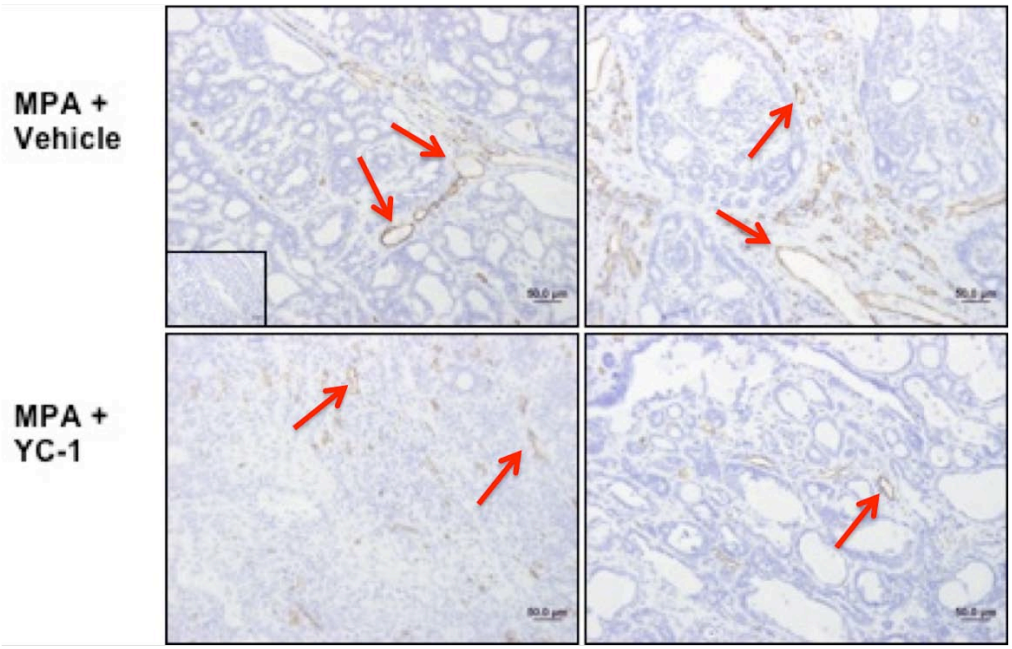

Figure 4.19: CD34 staining shows a slight reduction in vascular density. Arrows show positively stained blood vessels. Insert represents no antibody control. Original magnification was 20X.

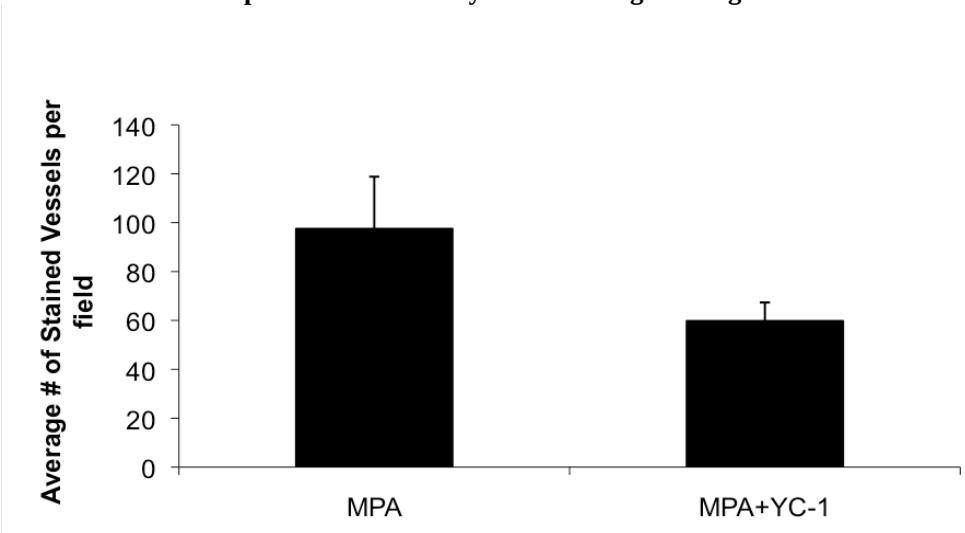

Figure 4.20: Vascular Density based on 3 fields per section. Reduction was not statistically significant as analyzed via t-test.

To determine blood perfusion to the tumors, animals were administered Texas red-conjugated tomato lectin (0.5 mg) vial tail vein injection 10 minutes prior to sacrificing. Sections show a significant decrease in blood perfusion in tumors from animals treated with YC-1 compared to animals that only received vehicle (Figure 4.21).

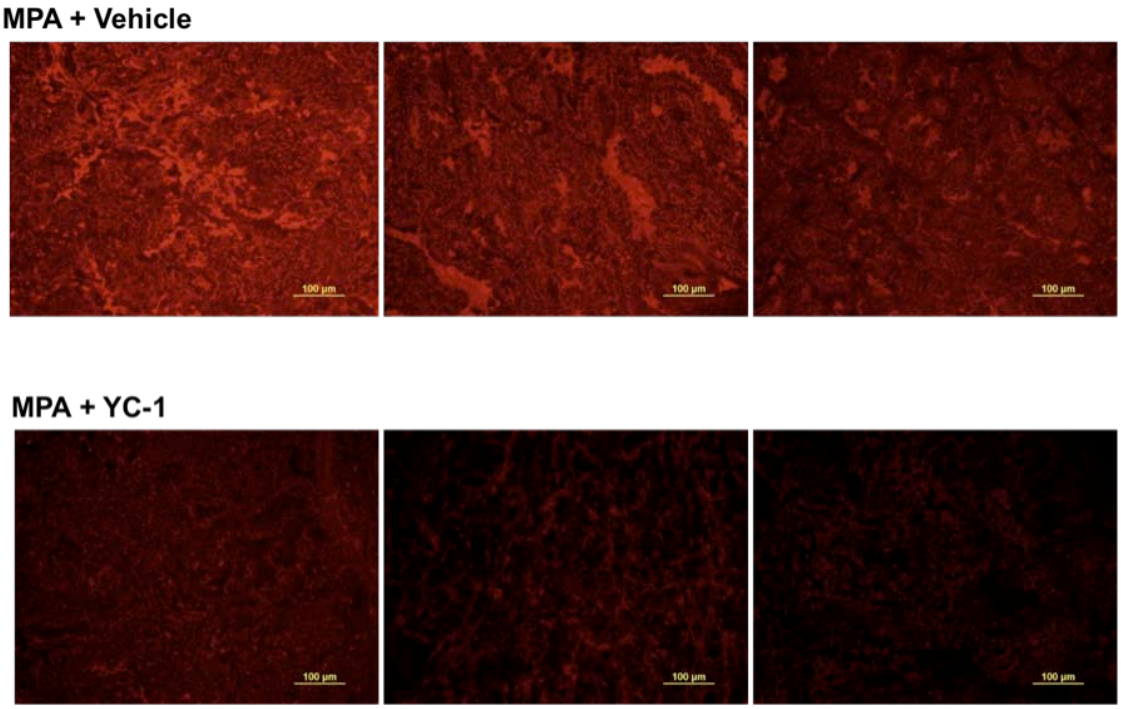

Figure 4.21: Texas red tomato-conjugated letcin staining in tumor tissue. Top panel shows tumors from animals treated with MPA and vehicle, bottom panel shows tumors from animals treated with MPA + YC-1 (3.75 mg).

IHC was also conducted to determine progesterone and estrogen receptor α and β levels in tumor tissue (Figures 4.22, 4.24, and 4.25). There were no significant changes or decreases in the expression of PR in the YC-1 treated tumors, compared to that of the vehicle-treated controls (Figures 4.22 and 4.23). This supports the *in vitro* data shown via western blot, suggesting that YC-1 is not inhibiting the PR protein expression.

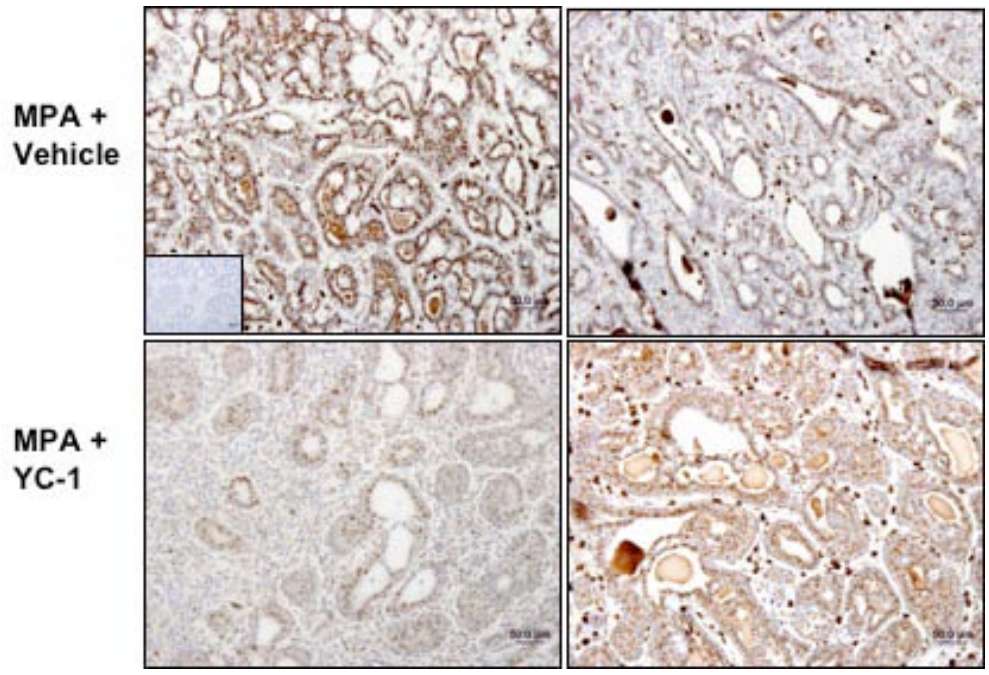

Figure 4.22: PR staining in tumor tissue. Insert represents no antibody control. Original magnification was 20X.

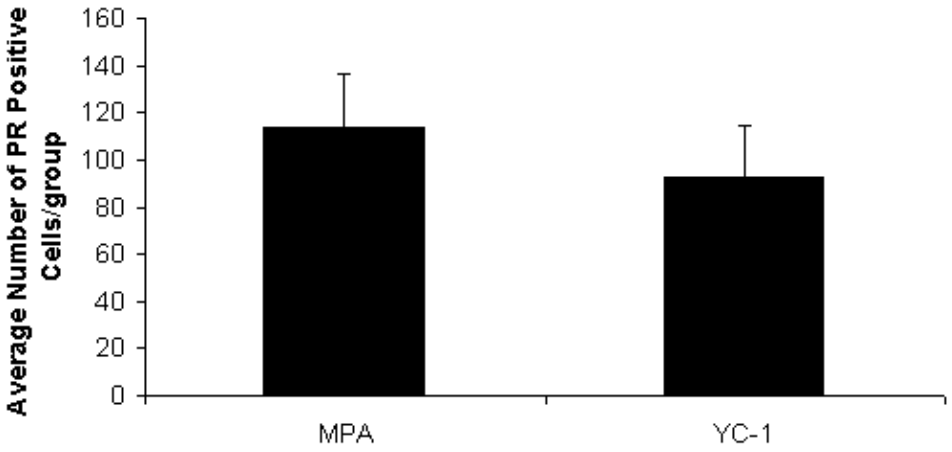

Figure 4. 23: Average number of PR-positively stained cells from 6 fields. MPA averaged a total 114 ±22 positively stained cells. MPA+YC-1 averaged a total of 92 ±24 positively stained cells. No statistical significance was found when analyzed via t-test.

ER expression and signaling is vital to the expression and function of the PR [148, 149], which is critical for P-dependent VEGF induction. As a result, we sought to determine the expression levels of ERα and ERβ after YC-1 treatment. As shown in

Figure 4.24, ERα levels were unchanged after YC-1 treatment. Nonetheless, there appeared to be a change in the location of ERα expression in the mammary tumors with YC-1 treatment. In the vehicle treated mammary tumors, the ERα-positive cells were generally closer to the stromal component of the mammary tumor, as compared to the YC-1 treated tumors; in which the ER-positive cells generally did not have contact with the stromal component of the mammary tumor. Though this event was not seen in all sections, the proximity of ERα to the stroma, may play a role in the progression of P-dependent mammary tumors, since ER is critical to PR function [150]. YC-1 may be inhibiting this stroma-epithelial interaction.

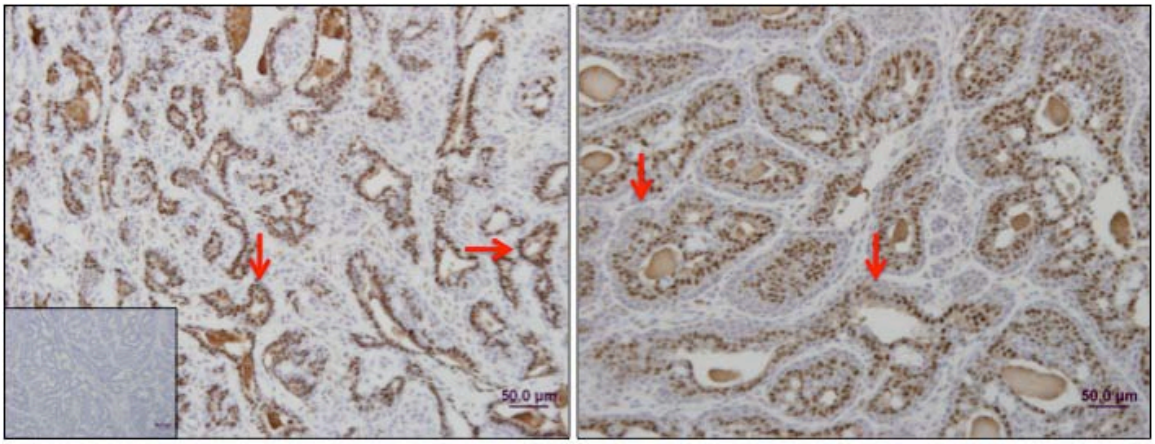

Figure 4.24: ERα staining in tumor tissue. Arrows show cells in contact with stroma. Insert represents no antibody control. Original magnification was 20X.

As seen with ERα, ERβ showed no significant changes in expression in YC-1 treated mammary tumors, compared to those of the vehicle-treated control (Figure 4.25). However, unlike ERα, ERβ had a more evenly distributed expression, suggesting that YC-1 has little, or no effect on ERβ expression levels in the mammary tumor.

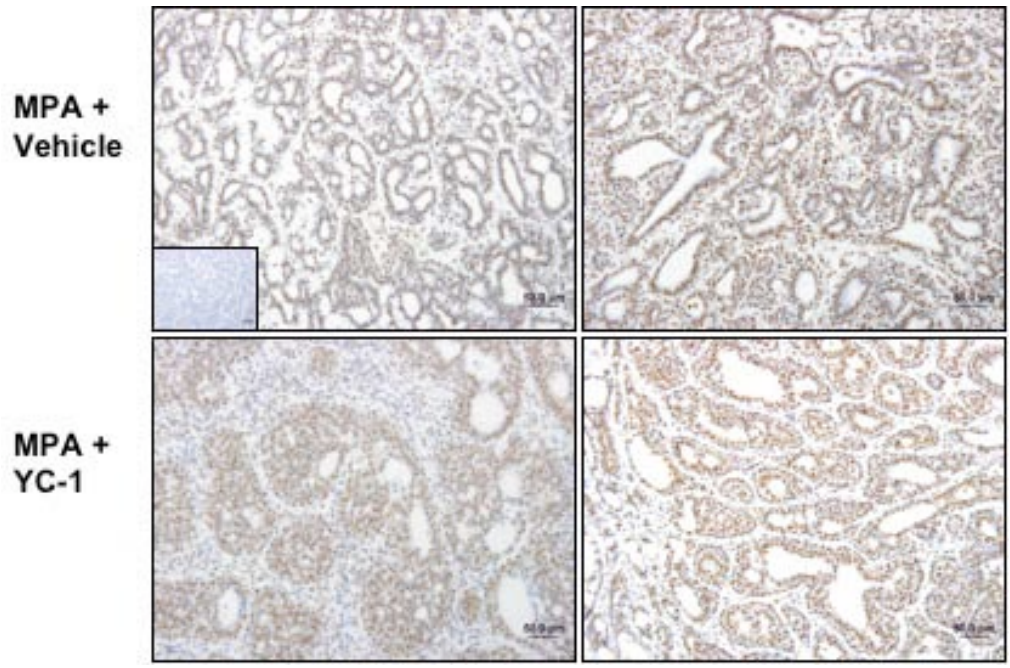

Figure 4.25: ERβ staining in tumor tissue. Insert represents no antibody control. Original magnification was 20X.

YC-1 inhibits progression and induced regression of MPA-driven, T47D- and BT-474- initiated tumors in Nude Mice. Nude mice with established MPA-driven tumors derived from T47D and BT-474 breast cancer cells were treated with YC-1 (600 μg/mouse/day) for 7-10 days, as described in Methods and Materials. Tumor volume was measured throughout the study, as well as animal weight to monitor possible toxicity.

T47D derived tumors showed a significant decrease in tumor volume after 7 treatments of YC-1 (600 μg/mouse/day). Based on animal weight, there was no sign of toxicity after 7 days of treatment. As a result, we continued treatment for a total of 10 days to investigate the possibility of complete tumor regression. As shown in Figure 4.26, complete tumor regression was not observed within the given time, however, significant tumor regression did occur in response to YC-1 treatment.

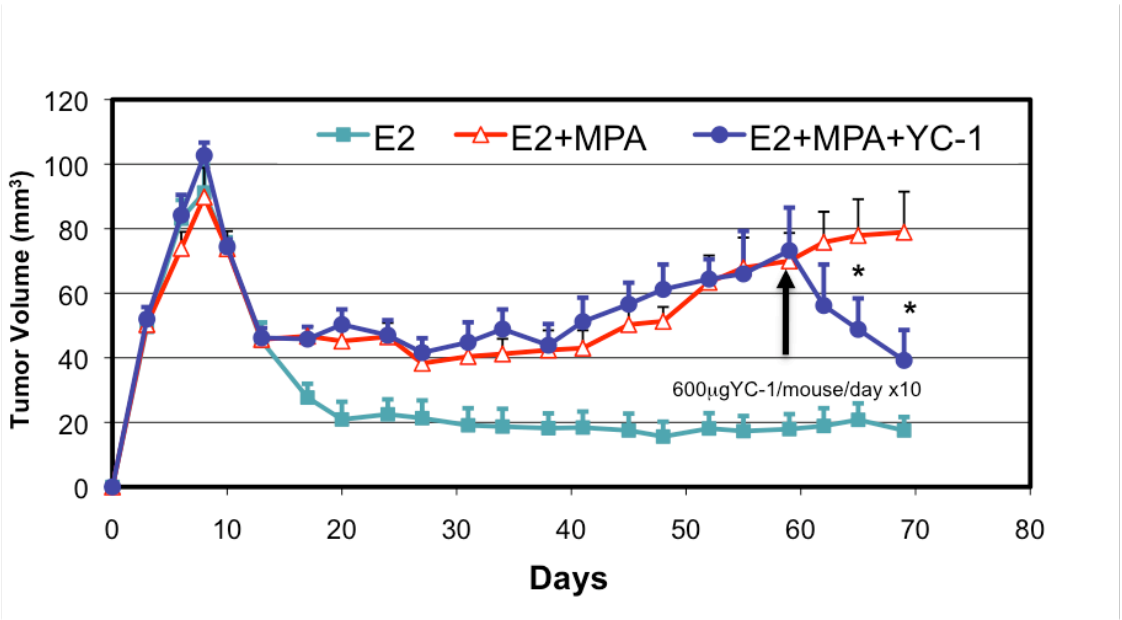

Figure 4.26: T47D-derived, MPA-driven tumors in intact nu/nu mice. 600 μg YC-1/mouse/day was administered via i.p. injection from day 61 to 72 and day 82 to 91. Averaging the tumor volumes from day 82-91 with tumor volumes from day 61-72, based on treatment day, combined tumor volumes from both treatment sets. * Represents significance as analyzed by One-Way ANOVA, $p<0.05$, n=4-5

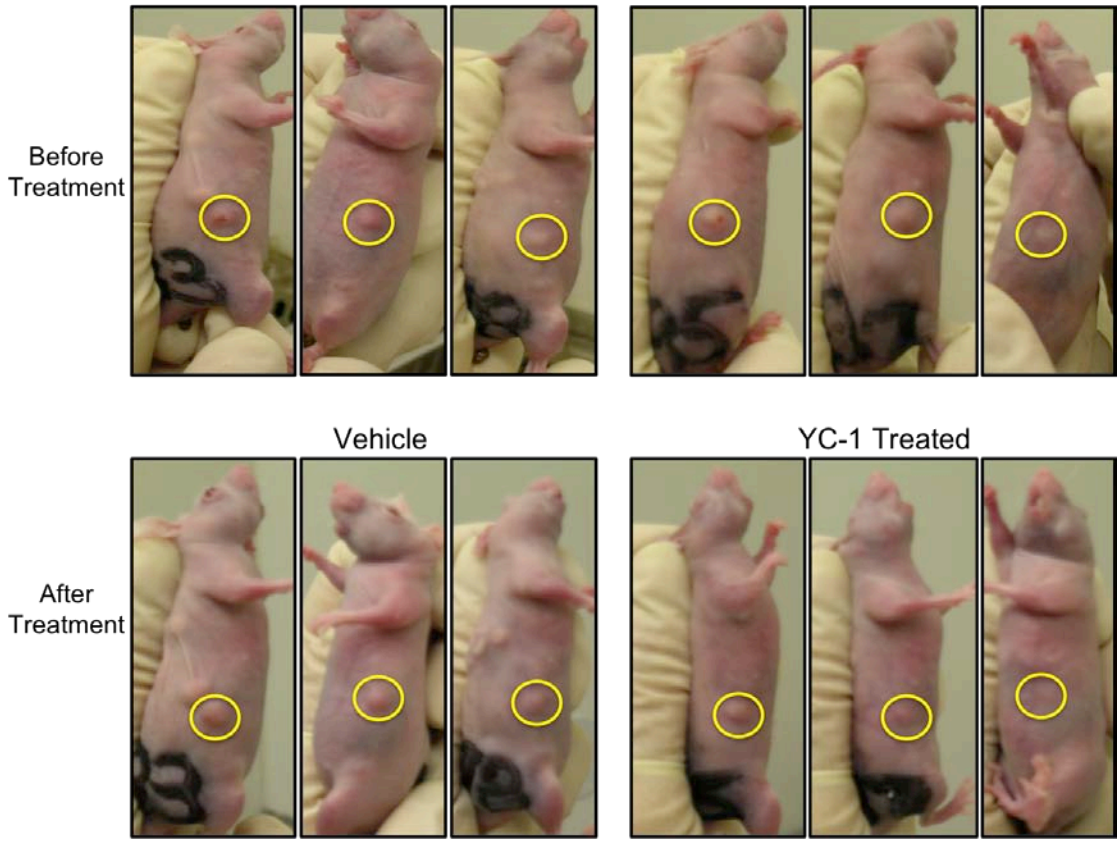

Figure 4.27: Tumor sizes before (upper panel) and after (lower panel) YC-1 or vehicle treatment of individual mice shown.

Two hours after the final (tenth) treatment of T47D-derived tumors, tumor tissues were collected from animals at the time of sacrifice for immunohistochemical analysis. At time of tissue collection, we observed a marked reduction in blood vessel number and size surrounding the tumors in the MPA + YC-1 treated animals, compared to that of those animals who only received MPA (Figure 4.28).

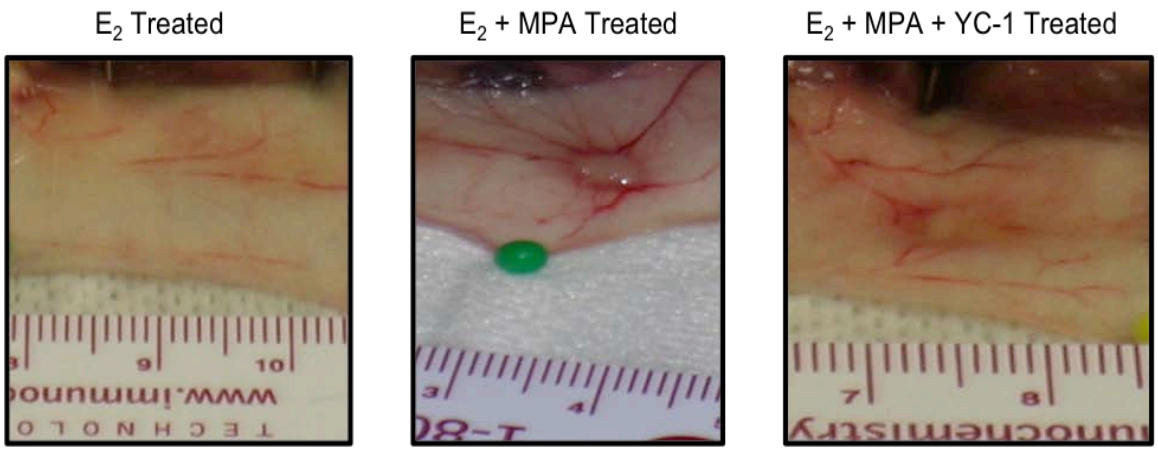

Figure 4.28: Comparison of vascularization of E2, E2+MPA+Vehicle, and E2+MPA+YC-1 treatment.

Immunohistochemistry from T47D-derived tumors showed a similar trend compared to the DMBA studies. Although not significantly different, CD34 staining revealed a decrease in blood vessels in the MPA + YC-1-treated animals (16 $\pm$ 2.3) n=3, compared to MPA + vehicle-treated animals (20 $\pm$ 5.5) n=5 (Figure 4.29).

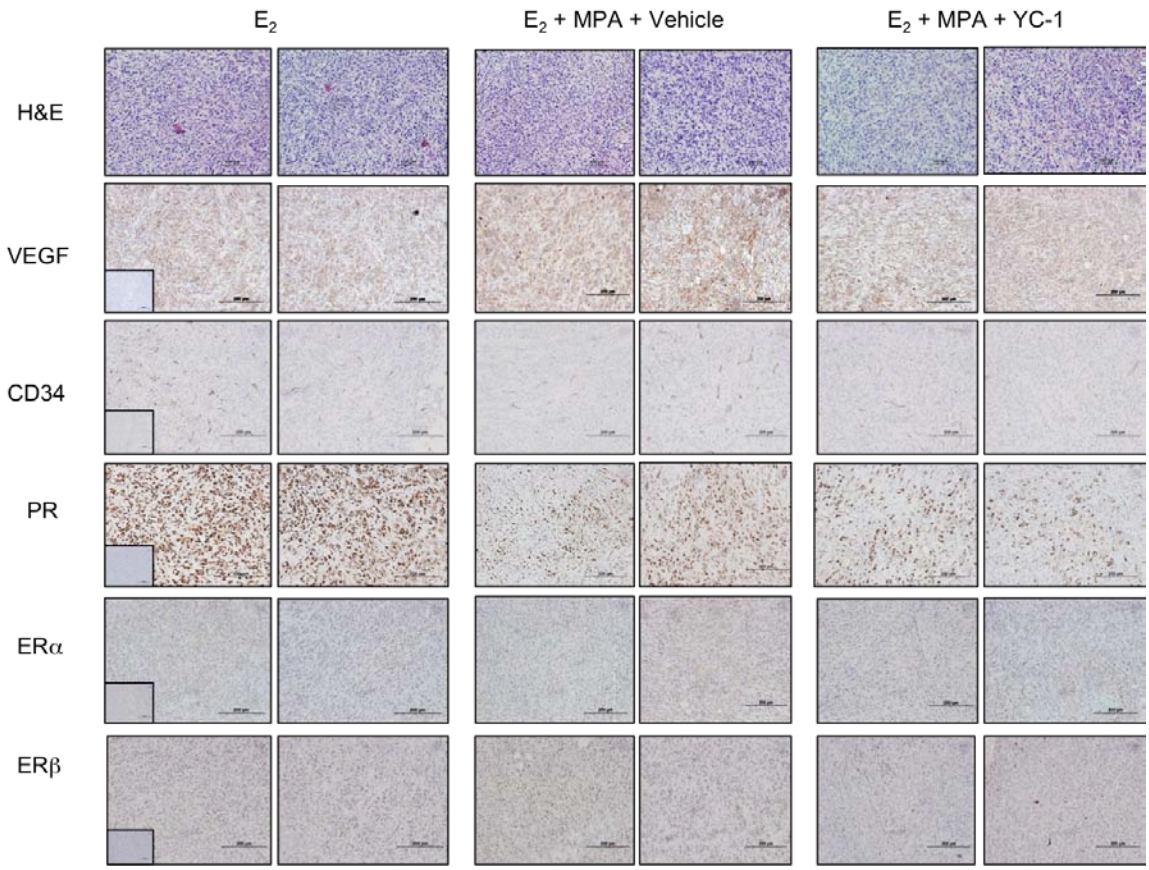

Figure 4.29: Immunohistochemical analysis of MPA-dependent, T47D-derived tumors. H&E, VEGF, CD34 PR and ER expression were analyzed in control (E_2), untreated (E_2 + MPA + Vehicle) and treated (E_2 + MPA + YC-1) tumors. Collected tumor tissues were sectioned and immunostained for the designated protein as described in the Methods section. Insets represent negative controls with no primary antibody staining for each antibody. Original Magnification is 20X.

As expected, VEGF staining showed a statistically significant decrease in staining among the three treatment groups (Figure 2.29). VEGF staining in the MPA + Vehicle group was significantly increased compared to VEGF staining in the E_2 group. Tumors collected from MPA + YC-1 treated animals showed a significant decrease in VEGF staining compared to MPA + Vehicle-treated animals. (Figure 4.30).

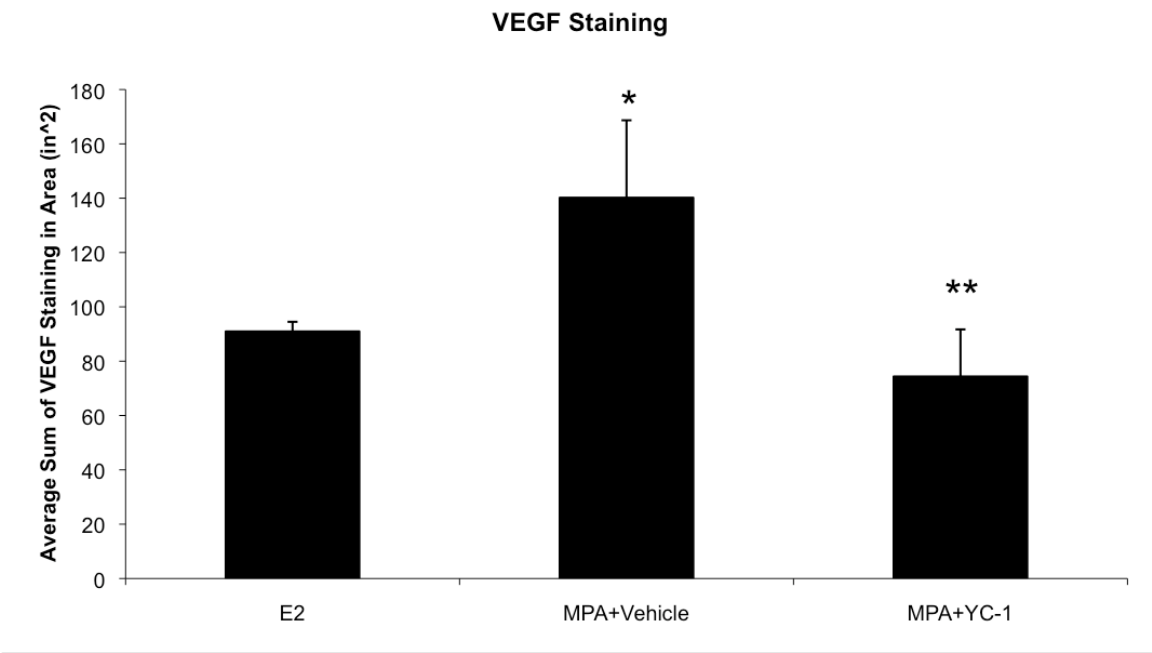

Figure 4.30: VEGF Staining of T47D-derived tumors. Six areas from each slides were analyzed, the area of positively stained pixels is summed from each slide and the graph depicts the average of the sum. Photographs of slides were analyzed using FoveaoPro 3.0 analysis software. Positive VEGF staining was quantified as the number of VEGF-positive pixels in six different fields. Error bars represent SEM, and * represents statistical significance compared to E2, as determined by One-Way ANOVA, Fishers LSD; while ** represents statistical significance compared to MPA + Vehicle, as determined by One-Way ANOVA, Fishers LSD.

While ERα and ERβ levels remained unchanged, Figure 4.29 shows that PR levels were significantly decreased in the MPA + Vehicle-treated animals compared to E_2 treated animals, as well as MPA + YC-1-treated animals compared to both E_2 and MPA + Vehicle-treated animals, as shown in Figure 4.31. This decrease in PR levels was not observed in DMBA studies, this may be attributed to the time elapsed between the last YC-1 treatment and tumor collection in DMBA studies, allowing time for PR levels to be recovered.

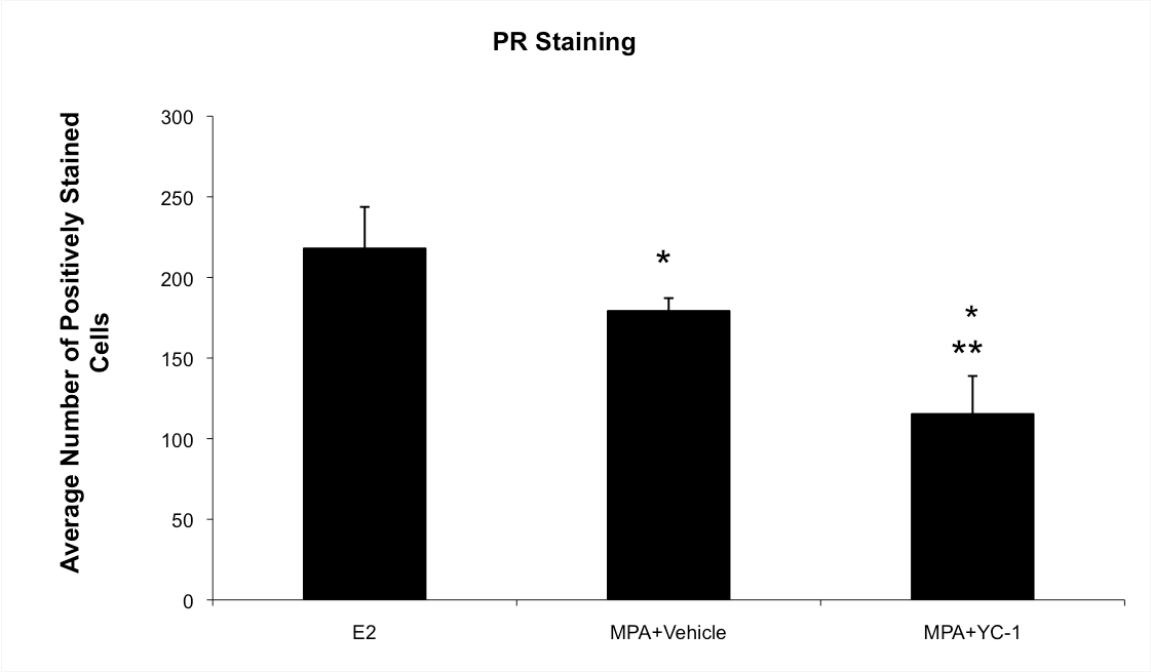

Figure 4. 31: Average number of positively stained cells from T47D Nude Mouse Tumors. Average of 6 fields per slide, error Bars represent SEM. E2, n=3 tumors; MPA + Vehicle, n=2 tumors; MPA + YC-1, n=5 tumors. One-Way ANOVA, Fisher's (LSD) was conducted, showing statistical significance at p=<0.001).

To determine the effectiveness of YC-1 on a second progestin-responsive cell line, we established tumors derived from BT-474 breast cancer cells in nude mice. After only 7 days of YC-1 treatment (600 μg/mouse/day) tumor volume decreased after one dose of YC-1, with statistical significance after the second dose (Figure 4.32). At day 54, animals were re-inoculated with new MPA pellets which resulted in a slight increase in tumor size in the treatment group, however the regression continued after this data point, while untreated animals experienced continued tumor growth (Figure 4.32).

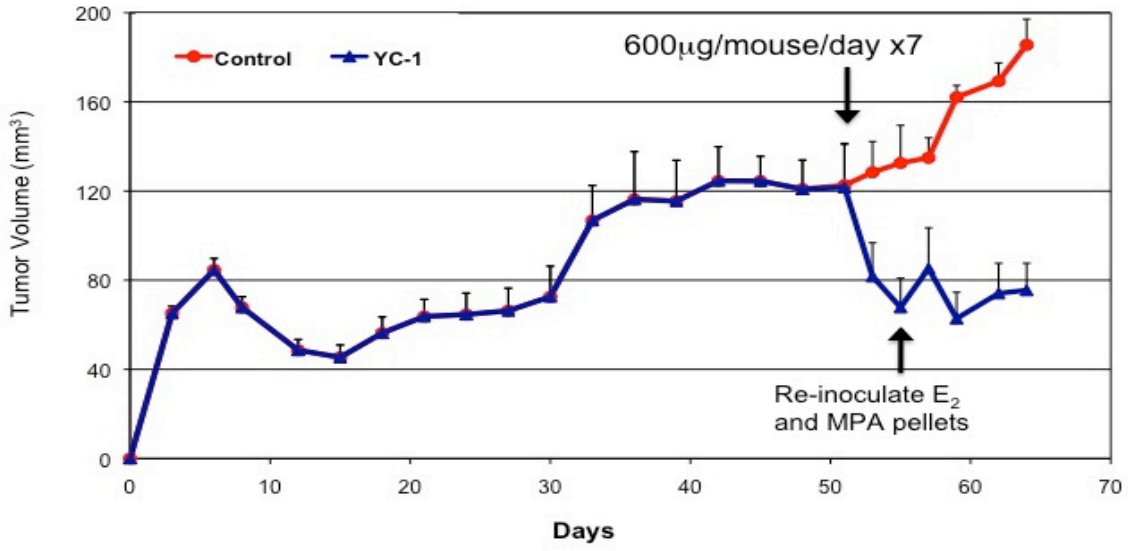

Figure 4.32: BT474 derived, MPA-driven tumors in ovariectimized nu/nu mice. 600μg YC-1/mouse/day was administered via i.p. injection from day 50 to day 57. New MPA pellets (10 mg/60-day timed release) were placed on day 54. * Represents significance as analyzed by One-Way ANOVA, $p<0.05$, n=3-4.

At the conclusion of the study, animals were sacrificed and tumors photographed and collected for IHC analysis. Though a significant regression in tumor size was observed in the BT474-derived tumors, there was no observed inhibition in VEGF levels in the analyzed tumors that were collected on day 71 of the study (data not shown). The delay between the time of the last YC-1 treatment and the collection of tumors may have allowed the recovery of VEGF expression within the tumor, which would explain why we observed no change in VEGF levels in the YC-1-treated, BT474-derived tumors.

4.4 Discussion

Previous reports provide strong evidence that progestins used in HRT increase the risk of breast cancer in postmenopausal women by increasing the production of VEGF, thus driving tumorigenesis [2, 18]. Since this discovery, compounds, specifically anti-hormones, have been tested in an effort to combat the negative effects of progestins in

HRT as it relates to breast cancer [60]. Unfortunately, investigators encounter numerous problems that prevent the success of these compounds, including low/no bioavailability [145] and lack of tissue or receptor specificity [60], which leads to the interference of other body systems. HIF-1α has been termed a potential target for multiple cancer types [58, 151, 152], however the role of HIF-1α in hormone-dependent cancers has not been well studied. The current study investigates the role of HIF-1α in progestin-dependent VEGF induction, a novel hypothesis that revealed a significant and mandatory role for HIF-1α in the present model. It was previously reported that the human VEGF promoter contains at least two hypoxia response elements (HyRE) [153], one of which has been shown to be essential to hypoxia-induced VEGF transcription. The current study shows that HyRE is essential for progestin-induced VEGF transcription. This is the first study to show that progestins increase HIF-1α binding to the VEGF promoter, and that both YC-1 and 2ME2 prevent such binding. This is also the first report to show that HIF-1α and PR have a direct protein-protein interaction. Under the current conditions of IP, the HIF-1α/PR complex is not influenced by YC-1 treatment, which may suggest the involvement of other transcription factors in HIF-dependent VEGF transcription. A recent study reports that YC-1 inactivates HIF-1 by stimulating Factor Inhibiting HIF (FIH) as well as inhibiting the recruitment of p300 to HIF-1 in Hep3B cells [154]. This may also occur in the present model.

The present study also shows that co-treatment of T47D cells with $CoCl_2$ and MPA results in a super-induction of VEGF with higher levels secreted from cells. This induction was partially blocked with anti-progestin, RU-486, but completely abolished when HIF-1α activity was blocked with YC-1 or 2ME2. Though this study shows that

HIF-1α is required for progestin-induced VEGF induction, the results also suggest that HIF and PR interact with one another. The current data suggests the formation of a HIF-1α/PR complex and binding to HyRE on the VEGF promoter. Though PRE sequences are thought to be present on the VEGF promoter [35], the current study shows that direct binding of PR to the VEGF promoter may not occur. Because YC-1 is able to inhibit MPA-induced VEGF mRNA levels, it is possible that PR and HIF form a complex and induce VEGF transcription activity by binding to the HyRE on the VEGF promoter.

Previous studies have provided strong evidence that both YC-1 and 2ME2 have strong potential to target HIF-1α in an effort to inhibit angiogenesis [151, 152, 155]. The present study, however, suggests that HIF-1α can be targeted by YC-1 and 2ME2 to inhibit hormone-driven angiogenesis as well. This is the first study to demonstrate inhibition of progestin-dependent angiogenesis of the mammary gland by targeting HIF-1α with YC-1. Animal studies conducted in this study provide strong evidence that HIF-1α is a worthwhile molecular target to inhibit progestin-driven angiogenesis and tumor progression of the mammary gland. This study suggests that HIF-1α may also be used as a molecular target to combat progestin-dependent breast cancer, and potentially other hormone-dependent cancer types.

The present study also investigated the effect of YC-1 on PR expression, *in vitro* and PR and ER *in vivo*. There was no significant change in the expression of ovarian hormone steroid receptors *in vitro* or *in vivo* in our DMBA studies. Although a significant decrease in PR levels was observed in the T47D-derived xenograft model, based on PR levels in DMBA studies, PR levels may be recovered after YC-1 treatment has ceased. Due to the inability of YC-1 to maintain altered receptor expression, it may prove

beneficial to explore combination treatment with YC-1 and an anti-hormone, such as RU-486 or tamoxifen. Having multiple drug targets may prove more to be a more effective treatment for receptor positive tumors, thus providing a more promising outcome for cancer patients.

In conclusion, progestins cause an increase in HIF-1α binding to the VEGF promoter, and blocking the DNA binding results in decreased VEGF mRNA levels and protein secretion. Thus, HIF-1α is required for progestin-induced VEGF induction in breast cancer cells. Also, YC-1 blocks progestin-driven angiogenesis and tumor progression in DMBA-induced tumors in Sprague-Dawley rats and progestin-driven T47D- and BT474-derived human xenograft mammary tumors in nude mice. This study provides strong evidence that HIF-1α may be a useful molecular target through which to treat progestin-dependent mammary tumors.

CHAPTER 5

CONCLUSIONS AND DISCUSSION

As previously discussed, progestin-dependent mammary cancer is prevalent among post-menopausal women who receive, or have received, combined HRT [2]. Studies have revealed that the progestin component of this treatment is a major factor in the increased risk of breast cancer among these women [2, 18]. The findings in these reports have led to many studies aiming to elucidate the mechanism through which progestins accelerate mammary cancers [7, 61, 117]. These studies have linked progestins to the production of VEGF in breast cancer cells [28, 35, 156], providing a target for inhibiting progestin-dependent breast cancer. Previous studies and findings in this field have led to the work described in the aforementioned chapters. The initial studies, described in chapter 2, included a screening of natural compounds that had the potential to inhibit progestin-induced VEGF secretion from human breast cancer cells, in an effort to block progestin-dependent angiogenesis of the breast. Based on the results from initial studies, both curcumin (chapter 3) and 2ME2 (chapter 4) showed promise as chemotherapeutic agents and were investigated in detail utilizing *in vitro* and *in vivo* models. As a result, this following discussion is focused primarily on results accumulated from curcumin and 2ME2/HIF-1α studies. Also discussed, are the potential future studies that may be conducted for further investigation of curcumin and 2ME2, as it relates to YC-1 and HIF-1α.

5.1 Curcumin

Previous studies show that progestins stimulate secretion of VEGF from human breast cancer cells expressing PR and mutant p53 protein [28]. VEGF in turn stimulates proliferation of PR-positive and PR-negative breast cancer cells that express receptors for VEGF [28]. VEGF also suppresses apoptosis and stimulates survival of endothelial and breast tumor cells [29]. In DMBA-treated rats, progestins accelerate PR- and VEGF-dependent development of mammary gland tumors [61]. Thus, inhibition of progestin-dependent secretion of VEGF may be an effective strategy by which to control the growth and spread of progestin-dependent breast cancer.

The studies described in chapter 3 show that curcumin specifically reduces MPA-induced secretion of VEGF from T47-D cells, but has no inhibitory effect with other progestins. This suggests that PR is not directly targeted by curcumin, which is consistent with earlier observations suggesting that progesterone and MPA induce secretion of VEGF by distinct pathways [93]. Chapter 3 also investigates the androgen receptor and the transcription factor NF-κB as possible targets of curcumin in this system. It was concluded that the androgen receptor is not activated by MPA, either *in vitro* or *in vivo*, and DNA binding of NF-κB was not altered in the presence of curcumin. It may be speculated that curcumin interacts with or interferes with extra-nuclear pathways that are specific to MPA and not to progesterone [128]. It is also possible that curcumin activates an inhibitory pathway that is selective in its suppression of MPA-induced VEGF but does not interfere with VEGF induction by other progestins. Because curcumin failed to inhibit MPA-induced VEGF secretion in BT474 cells, it is possible that the MPA-specific pathway may also be cell-type specific and that the inhibitory pathway activated by curcumin may not be present or critical in other cell types. In this

context it is interesting to note that recent studies identify membrane progesterone receptors [129, 130], which may also be responsible for some of the differential curcumin effects observed in the present *in vitro* studies. Nevertheless, studies discussed in chapter 3 imply that curcumin may be useful as a preventive agent to minimize the cancer risk associated with MPA-containing HRT. Curcumin is under investigation as an anti-tumor agent in a Phase II clinical trial [131] and appears to be well-tolerated and lacks dose-limiting effects. Such studies should encourage future clinical trials designed to evaluate the efficacy of curcumin in lowering breast cancer risk in women exposed to MPA in the context of HRT.

Chapter 3 shows that curcumin delays MPA-accelerated, DMBA-induced tumor formation by approximately one week, and markedly inhibits morphological changes in MPA-exposed mammary glands, suggesting that curcumin may block MPA effects in the process of tumorigenesis, which could prove beneficial to women receiving combined HRT. Because no change in ER or PR levels was observed, it may be concluded that curcumin may exert anti-cancer properties on the mammary gland with little to no effect on ovarian hormone receptor expression. Although no significant changes were observed in ovarian hormone receptor levels, VEGF staining revealed reduced VEGF expression in the ductal epithelial cells of the mammary gland from animals treated with curcumin, which may help elucidate the inhibitory mechanism of curcumin on DMBA-induced, MPA-accelerated tumor formation in the rat. This is the first study, to our knowledge, that utilizes an animal model to provide evidence that curcumin has the ability to exert chemopreventive properties on progestin-dependent breast cancer. Based on a brief pilot study—it was concluded that curcumin may not show as much promise as a therapeutic

agent as it does as a chemopreventive agent. Curcumin appears to be safe with no toxicity at high doses. A phase I clinical trial with curcumin reports that no toxicity was observed when patients were given up to 8000mg/day [136]. The dose administered in the current study elicited little to no toxic effects on the animals, based on animal weight.

The specific mechanism by which curcumin inhibits angiogenesis is unknown; however, based on the results described in chapter 3, the inhibition of VEGF may play a critical role. Nonetheless, there are studies that suggest cyclin D [111] and p21 [137] are involved in the anti-cancer effects of curcumin, however, no studies provide information on how curcumin interacts with progestin-dependent mammary cancers. Some studies that led to the investigation described in chapter 3 include a report in which NF-κB is a target for curcumin [125, 138, 139]. Curcumin was reported to inhibit NF-κB activation, thus decreasing VEGF mRNA expression in breast cancer cells and ovarian cancer cells, and to reduce the expression in matrix metalloproteinases (MMPs) as a result of decreased NF-κB activity and transcriptional down-regulation of AP-1 [140]. Although it is possible that curcumin inhibits NF-κB activity in the DMBA study previously described, it was also reported that curcumin specifically induces apoptosis in cancer cells and causes cell cycle arrest in G2 phase [111]—which may also be a possible mechanism for curcumin inhibition. Nonetheless, literature reports a vast number of molecular targets affected by curcumin that may result in suppression of tumor growth or progression [141], as depicted in Figure 3.17. Due to the numerous possible targets of curcumin, the inhibitory mechanism in this model remains unknown.

5.1.1 Potential Future Studies with Curcumin

Curcumin studies conducted herein did not result in the elucidation its mechanism of inhibition. However, multiple mechanisms were explored and results suggest that the proposed transcription factors do not play a role in this system. To further prove that HIF-1α, NF-κB, and AR are not involved in the inhibitory mechanism of curcumin, a ChIP assay should be conducted to show that treating breast cancer cells with curcumin does not inhibit binding of these ligands to the VEGF promoter. Positive results from this study would confirm that these transcription factors do not play a role in the inhibitory mechanism of curcumin in the present system.

To investigate the molecular differences in MPA-accelerated, DMBA-induced mammary tumors and non-tumor mammary tissue in animals treated with and without curcumin, a micro array with tissue samples from the animals should be performed. Results from this experiment would determine which genes are up/down regulated. These genes could play a major role in determining the mechanism by which curcumin decreases VEGF and delays DMBA-induce/MPA-driven mammary tumorigenesis in the rat and prevent MPA-driven morphological changes of the mammary gland.

Though the pilot study with curcumin as a treatment did not provide clear or conclusive results, a full study may be worthwhile to determine a better, more concise conclusion as to whether curcumin would be a beneficial treatment for progestin-dependent breast cancer. By administering curcumin to an appropriate number of animals with established MPA-accelerated, DMBA-induced mammary tumors we may be able to better conclude whether this turmeric root derivative has potential as a therapeutic agent for progestin-dependent mammary tumors.

Studies may be conducted to determine if curcumin is beneficial in treating or preventing of progestin-driven angiogenesis utilizing the nude mouse model, previously established in our lab. Results from this study will provide data about the potential to treat established tumors or prevent the establishment of tumors derived from human breast cancer cells. Previously proposed studies, utilizing the rat model, are useful in determining how heterogeneous tumors respond to treatments, while the currently proposed study investigates how homogenous tumors, derived from human breast cancer cells respond to the treatment. Positive results from this study would provide strong evidence that curcumin has the potential to treat and/or prevent progestin-dependent human breast cancer by inhibiting angiogenesis of the breast, and thus may prove beneficial to ingest alongside HRT.

In conclusion, in this pre-clinical study we report that curcumin has inhibitory properties on MPA-driven mammary tumorigenesis. We were unable to elucidate the specific mechanism by which curcumin elicits these inhibitory properties; however, we show that AR, PR, and ER are not involved. Lastly, we show that curcumin blocks the morphological changes in the mammary gland caused by exposure to MPA in the process of tumorigenesis and inhibits MPA-induced VEGF expression in the mammary gland exposed to DMBA, suggesting that curcumin may decrease the risk of breast cancer associated with HRT. This study provides strong evidence that curcumin may be an excellent candidate as a chemopreventive agent in clinical trials for postmenopausal women who are prescribed combined hormone replacement therapy that includes both estrogens and progestins.

5.2 2ME2/YC-1

Previous reports provide strong evidence that progestins used in HRT increase the risk of breast cancer in postmenopausal women by increasing the production of VEGF, thus driving tumorigenesis [2, 18]. Since this discovery, compounds, specifically anti-hormones, have been tested in an effort to combat the negative effects of progestins in HRT as it relates to breast cancer [60]. Unfortunately, investigators encounter numerous problems that prevent the success of these compounds, including low/no bioavailability [145] and lack of tissue or receptor specificity [60], which leads to the interference of other body systems. HIF-1α has been termed a potential target for multiple cancer types [58, 151, 152], however the role of HIF-1α in hormone-dependent cancers has not been well studied. The current study investigates the role of HIF-1α in progestin-dependent VEGF induction, a novel hypothesis that revealed a significant and mandatory role for HIF-1α in the present model. It was previously reported that the human VEGF promoter contains at least two hypoxia response elements (HyRE) [153], one of which has been shown to be essential to hypoxia-induced VEGF transcription. The study described in chapter 4 depicts the HyRE as an essential factor to progestin-induced VEGF transcription. This is the first study to show that progestins increase HIF-1α binding to the VEGF promoter, and both YC-1 and 2ME2 reduced the binding of HIF-1α to the VEGF promoter. This finding may be beneficial in the discovery of a more efficient drug for progestin-dependent breast cancer therapies in that HIF-1α may be a new, more effective target for therapy for this disease.

It was also reported in chapter 4 that co-treatment of T47D cells with $CoCl_2$ and MPA, results in a super-induction of VEGF being secreted from the cells. This induction

was partially blocked with anti-progestin, RU-486, but completely abolished when HIF-1α activity was blocked with YC-1 or 2ME2. Though this study shows that HIF-1α is required for progestin-induced VEGF induction, the results also suggest that HIF and PR interact with one another. The current data suggests the formation of a HIF-1α/PR complex and binding to HyRE on the VEGF promoter. Though PRE sequences are thought to be present on the VEGF promoter, the current study shows that direct binding of PR to the VEGF promoter may not occur. Because YC-1 is able to inhibit MPA-induced VEGF mRNA levels, it is possible that PR and HIF form a complex and induce VEGF transcription activity by binding to the HyRE on the VEGF promoter. This hypothesis was also tested in chapter 4, as this is the first report to show that HIF-1α and PR have a direct protein-protein interaction. Though not influenced by the treatment of YC-1 under the described IP conditions, the HIF-1α/PR complex may include other transcription factors that are influenced by YC-1 treatment. It has been reported that YC-1 inactivates HIF-1α by stimulating Factor Inhibiting HIF (FIH) as well as inhibiting the recruitment of p300 to HIF-1 in Hep3B cells [154]. This may also occur in the model utilized in chapter 4.

Previous studies have provided strong evidence that both YC-1 and 2ME2 have strong potential to target HIF-1α in an effort to inhibit angiogenesis [151, 152, 155]. Results described in chapter 4 however, suggest that HIF-1α can also be targeted by YC-1 and 2ME2 to target hormone-driven angiogenesis. This is the first demonstration showing inhibition of progestin-dependent angiogenesis of the mammary gland by targeting HIF-1α with YC-1. Animal studies described in chapter 4 provide strong evidence that HIF-1α is a worthwhile molecular target through which to inhibit

progestin-driven angiogenesis and tumor progression of the mammary gland. This study suggests that HIF-1α may also be used as a molecular target to combat progestin-dependent breast cancer, and potentially other hormone-dependent cancer types. Interestingly, there was no significant change in the expression of ERα *in vitro* or *in vivo*. Although PR levels were decreased in T47D-derived tumors, this decrease was not observed in DMBA studies, suggesting that PR levels may recover after YC-1 treatment has ceased. Because of this, it may prove beneficial to explore combination treatment with YC-1 and an anti-hormone, such as RU-486 or tamoxifen. Having multiple drug targets may prove to be a more effective treatment for receptor positive tumors, thus providing a more promising outcome for cancer patients.

In conclusion, in chapter 4 we report that MPA causes an increase in HIF-1α binding to the VEGF promoter, and blocks the DNA binding with YC-1, resulting in decreased VEGF mRNA levels and protein secretion. Thus, HIF-1α is required for progestin-induced VEGF induction in breast cancer cells. Also, YC-1 blocks progestin-driven angiogenesis and tumor progression in DMBA-induced tumors in Sprague-Dawley rats and progestin-driven T47D- and BT474-derived human xenograft mammary tumors in nude mice. In chapter 5 we provide strong evidence that HIF-1α may be a useful molecular target for progestin-dependent mammary tumors.

5.2.1 Potential Future Studies with 2ME2/YC-1

The mechanistic studies conducted herein show that YC-1 and 2ME2 inhibit/reduce HIF-1α from binding to the VEGF promoter. However, the method by which YC-1 and 2ME2 may differ should be investigated. This study could improve how

drugs are developed and would assist in creating the most effective method of inhibiting HIF-1 activation. A recent study reports that YC-1 inactivates HIF-1 by stimulating FIH as well as inhibiting the recruitment of p300 to HIF-1 in Hep3B cells [154], a scenario that may also occur in the present model. By decreasing the protein expression of Factor Inhibiting HIF (FIH) with siRNA, we will be able to determine if FIH is required for YC-1- and/or 2ME2-dependent inhibition. Also, performing an IP assay, in which p300 is precipitated from the lysate of treated cells, and probing for the presence of HIF-1α protein would prove vital in determining if there is a reduction in complex formation due to treatment.

Combination therapies of cancers are more and more common as more research develops that elucidates the involvement of different cellular mechanisms in different cancer types. Because YC-1 did not down regulate ER and PR expression, combination treatments with YC-1 may prove beneficial. Targeting HIF-1α with a selective estrogen receptor modulator (SERM) or selective progesterone receptor modulator (SPRM) may prove extremely effective in the inhibition of progestin-induced VEGF secretion, thus reducing angiogenesis of the mammary gland. By pairing YC-1 or 2ME2 with tamoxifen the activation of both HIF-1α and estrogen receptors in the breast, but not the uterus, would be inhibited. This may prove beneficial in the case that the cells become tamoxifen resistant, HIF-1α is still being targeted and thus tumor regression would still occur. In theory, this deactivation would block angiogenesis by inhibiting two required components in the pathway of angiogenesis, thus bringing about more effective tumor regression. Another potential study would be targeting HIF-1α with YC-1 paired with a second molecular target, such as mt-p53 with PRIMA-1, which may increase the efficacy

of YC-1 and PRIMA-1 to decrease progestin-driven angiogenesis of the mammary gland. Our lab has shown in unpublished data that PRIMA-1 decreases HIF-1α protein expression, as well as allowing mtp53 to function as wt-p53. In theory, such combination therapy would eradicate the progestin response and target HIF-1α to decrease VEGF levels and reduce angiogenesis and tumor formation/progression.

Due to the effectiveness of YC-1 in the inhibition of MPA-dependent angiogenesis in DMBA-induced tumors and a human breast cancer xenograft model, YC-1 treatment with other progestins should be investigated. Our lab has demonstrated that all progestins do not elicit the same biological responses, in regards to tumorigenesis and angiogenesis. As a result, the effectiveness of the ability of YC-1 to inhibit angiogenesis induced by other progestins should be determined. A positive result from this study would prove that HIF-1α is a common required component of progestin-dependent angiogenesis and progestin-driven tumor formation. A negative result from this study would suggest that different progestins elicit different pathways that ultimately lead to tumor formation and/or progression. Though unlikely based on *in vitro* data, a negative result would also suggest that HIF-1α is not a common mechanism among the investigated progestins and a treatment targeting HIF-1α could only be used for the progestins that utilize a HIF-1α pathway.

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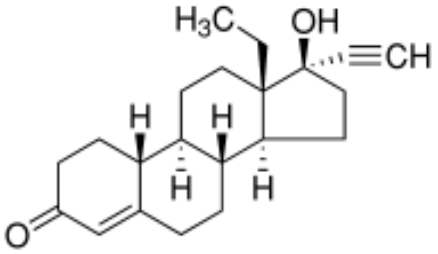
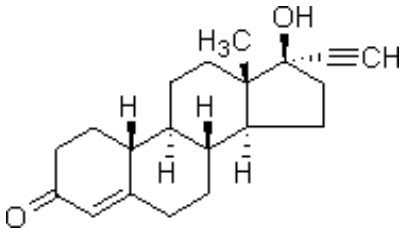
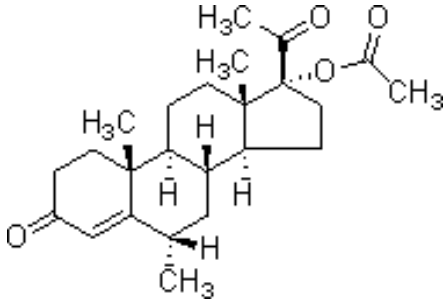
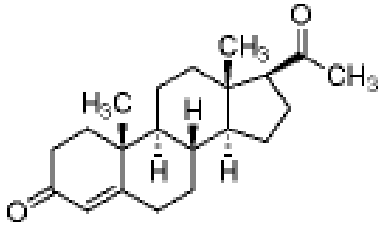
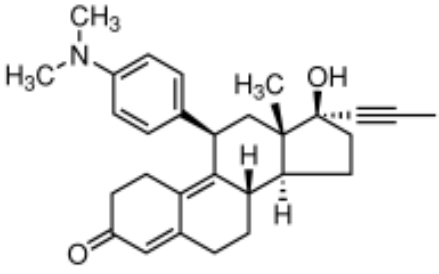
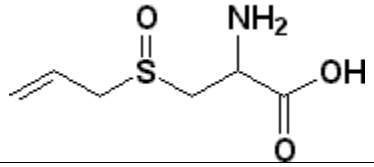
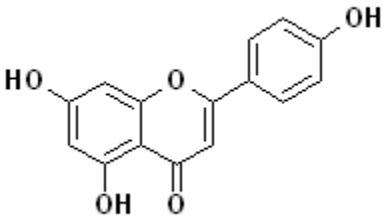
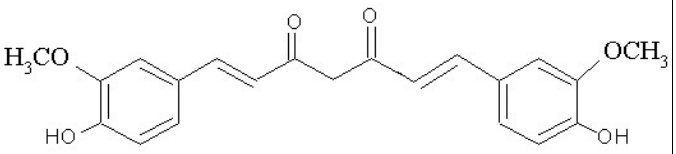
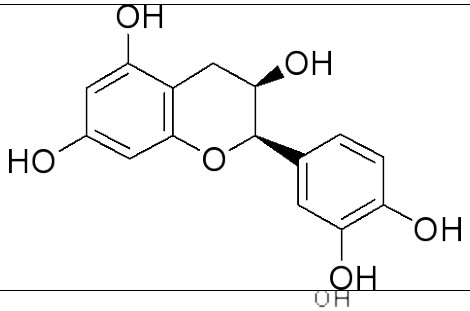
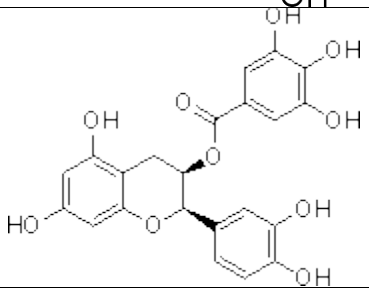
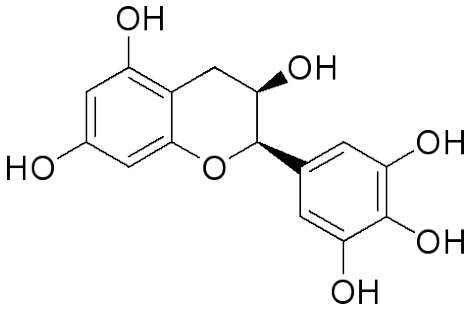
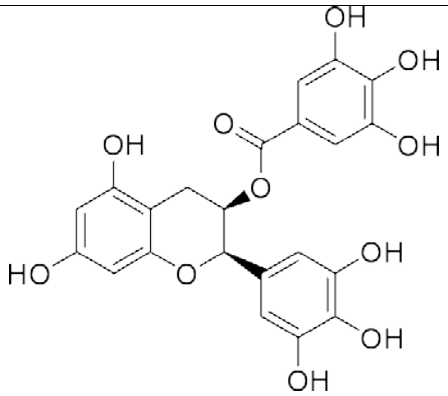
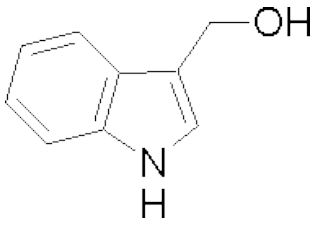
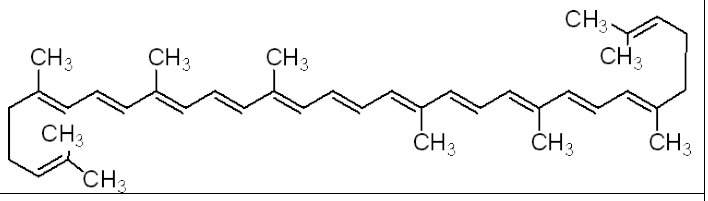
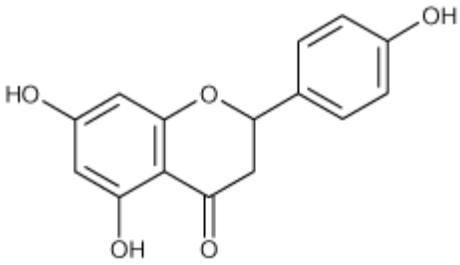
APPENDIX

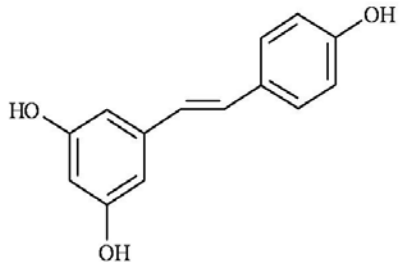
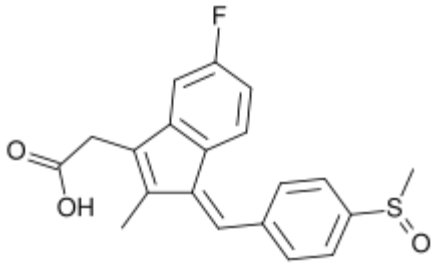
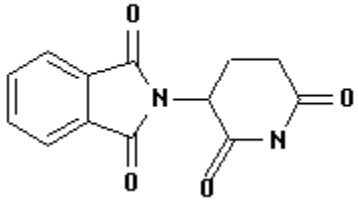
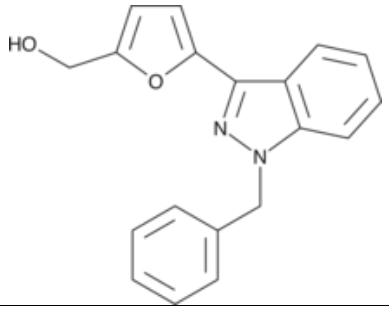
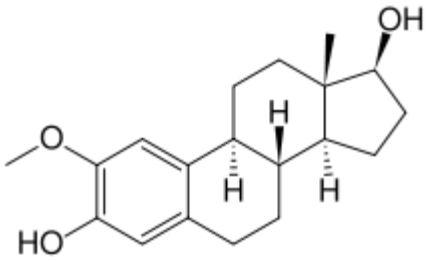
I. CHEMICAL STRUCTURES OF PROGESTINS AND ANTI-PROGESTINS

COMPOUND	TYPE	STRUCTURE
Norgestrel	Synthetic progestin	
Norethindrone	Synthetic progestin	
Medroxyprogesterone Acetate (MPA)	Synthetic progestin	
Progesterone	Endogenous progestin	
RU-486 (mifepristone)	Synthetic anti-progestin	

II. CHEMICAL STRUCTURES OF SCREENED COMPOUNDS

Compound	Source	Structure
Alliin	Garlic	
Apigenin	Leafy plants/veggies	
Curcumin	Turmeric Root (phytochemical)	
Epicatechin Ec	Phytochemical (Green Tea)	
Epicatechin gallate EcG	Phytochemical (Green Tea)	

Epigallocatechin Egc	Phytochemical (Green Tea)	
Epigallocatechin Gallage EGCG	Phytochemical (Green Tea)	
Indole-3-carbinole	Cruciferous Vegetables	
Lycopene	Tomato	
Naringenin	Grapefruit, other citrus fruits	

Resveratrol	Phytochemical (grapes, wine, etc)	
Sulindac Sulfide	Synthetic, NSAID	
Thalidomide	Synthetic, anti-nausea medication and teratogen	
YC-1	Synthetic, soluble guanylate cyclase activator, specific HIF-1α inhibitor	
2-Methoxyetradiol	Natural, Endogenous estrogen metabolite	

III. LAB COLLABORATIVE PROJECTS

Role of synthetic progestins in progression of DMBA-induced mammary tumors in Sprague-Dawley rats

Indira Benakanakere[1], Candace E. Carroll[1], Cynthia Besch-Williford[2], and Salman M. Hyder[1]

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[2]*Department of Pathology, University of Missouri, Columbia, MO, 65211*

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Abstract: Progestins have been under observation for their effects on breast cancer incidence in postmenopausal women undergoing Hormone replacement therapy. However, the efficacy, as well as the different mechanisms of action on prevention and proliferation of breast cancer remains largely unknown. This study seeks to address and examine the cancer prevention or proliferation effects of medroxyprogesterone acetate (MPA), norgestrel, norethindrone, and Megestrol acetate (MGA) in DMBA induced Sprague Dawley rats. We found that while MPA accelerated the incidence (60%) and latency period (35 days after DMBA)of tumors compared to controls(33% incidence and latency period 45 days after DMBA), norgestrel and norethindrone were protective, norgestrel animals were 100% tumor free and Norethindrone animals were 90% tumor free, and MGA was similar to placebo animals. Further norgestrel was protective at 10 and 25 mg doses. Histological data shows that norgestrel treated animals have preneoplasia, suggesting that norgestrel could block the DMBA tumors at preneoplastic stages. Immunohistochemical data suggests that all animals exposed to progestins expressed VEGF in their preneoplastic mammary gland. As norgestrel exposure blocked tumors we stained for proteins like FLK, FLT, ER and PR to see if they were affected. Norgestrel and norithindrone treatments that blocked the DMBA tumors had down-regulated FLT, less than 3% of cells expressed ERalpha, PR was also down-regulated in these tumors. Serum hormone levels indicated that in animals, MPA exposure down-regulated progesterone (0.92±0.51 compared to controls 22.50±11.87) and prolactin (6.0±2.09 compared to controls 31.2±6.22). Norgestrel exposure upregulated prolactin to150.7± 32.32 compared to 31.2±6.22 in controls. Norgestrel introduced at late time points of 90 days after DMBA once tumors were present, failed to offer protection to the

progressing tumors. Norgestrel could accelerate these tumors from size 29.83±13.6 to 78.8±14.5 compared to control sizes from 28.4±3.7 to 7.1±2.0. In summary, different progestins have different effects in the breast. Norgestrel being safest of the lot, when exposed at earlier time points and MPA the most dangerous in this model of DMBA induced breast cancer in rats.

IV. VITA

Curriculum Vitae of:

Candace E. Carroll

Education:

2005-present	Ph.D. Candidate, Biomedical Sciences, College of Veterinary Medicine University of Missouri, Columbia, MO 65211, USA
2001-2005	B.S. Biology, Tougaloo College, Tougaloo, MS 39174, USA
1997-2001	High School Diploma, Callaway High School, Jackson, MS 39206

Research Experience:

2006-present	University of Missouri-Columbia, Graduate Dissertation Topic: *The inhibition of progestin-dependent angiogenesis in breast cancer in postmenopausal women by inhibiting progestin-induced VEGF induction*. Supervised by Dr. Salman M. Hyder
2004-2005	Tougaloo College/University of Mississippi Medical Center, Project: *Effect of Obesity on HIF-1 α Levels in the Kidney*. Supervised by Dr. Jeff Henegar, Assistant Professor of Pathology
Summer 2004	University of Georgia-Athens, (Summer Undergraduate Research Program), Project: *Effect of hydration during long duration exercise on cardiovascular drift and maximal oxygen.* Supervised by Kirk Cureton, PhD, Professor and Head of Exercise Science Department
Summer 2003	Cornell University (The Leadership Alliance), Project: *Correlation of Airway Impedance and Upper Airway Noise in Horses with Laryngeal Hemiplegia*—the research is ongoing. Supervised by Norm Ducharme, DVM, MSc, Professor of Large Animal Surgery
Summer 2002	University of Missouri-Columbia, (Gateways to Veterinary Medicine), Project: *The Effects of Urine pH on Veterinary Bladder Tumor Antigen Test*--the research is ongoing. Supervised by Carolyn Henry, D.V.M., M.S., Assistant Professor of Oncology

Teaching Experience & Training:

Spring 2009 (present)	Adjunct Faculty Instructor for Conservation Biology for non-biology majors (Bio105), Moberly Area Community College
Spring 2008	Teaching Assistant for Laboratory Investigations for Elements of Physiology (MMP 3202), University of Missouri
Summer 2005	Summer Science Program/MedCorp Biology Tutor, Tougaloo College
Fall 2002	Teaching Assistant/Tutor for Discovering Biology (Bio110), Tougaloo College

National Organization Memberships:

Since 2006	American Association for Cancer Research *AACR-Women in Cancer Research; AACR-Minorities in Cancer Research*
Since 2003	The Leadership Alliance
Since 2003	Zeta Phi Beta Sorority, Incorporated

University of Missouri Organization Memberships and Local Affiliations:

April 2009	8th Annual National Pan-Hellenic Council MO-Money Step Show Judge
March 2009	CCAA (Cooper County Activities Association) Conference Science Fair Judge
2008-present	NGA (Nexus Graduate Association), Founding Member
2007-2008	GSA (Graduate Student Association) Departmental Representative
2007-2008	NEXUS: Minority Science Network; Planning Committee (Fall 2007)
2006-2008	Department Co-Chair of Student Communications and Social Activities
Fall 2006	A Way with Words & Numbers Tutoring Program (Volunteer)

Honors, Awards, and Fellowships:

2009	AACR Minority Scholar in Cancer Research Award, NCI Funding to attend the 100th Annual AACR national meeting in 2009, Denver, CO.
2009	Invited participant at the 2009 St Jude National Graduate Student Symposium, *42 selected from 1100 applicants* (March 31-April 4, 2009)
2008-2010	NIH/NCI, Ruth L Kirschstein National Research Service Award, Individual Fellowship
2007	GPC (Graduate Professional Council) Travel Award, $400
2007	Biomedical Sciences Departmental Travel Award, $900
2007	1st Place Oral Presentation Award (2nd & 3rd Year Residents and Graduate Students) 30th Annual Phi Zeta Research Day, Columbia, MO
2005-2007	MBRTI (Minority Biomedical Research Training Initiative) Fellowship (Graduate)
2003-2005	MARC (Minority Access to Research Careers) Scholar (Undergraduate)
2002-2003	CBS (Central Broadcasting Systems) Scholar HBCU-UP (Historically Black College/University-Undergraduate Program) Scholar
2001-2005	Who's Who Among American Colleges
2001	Valedictorian of Callaway High School c/o2001

Grant Support:

4/2008-5/2010	**NIH, Ruth L. Kirschstein National Research Service Award Individual Fellowship**: To Promote Diversity in Health Related Research, 1F31CA130167 PI: Candace E. Carroll Title: Inhibition of Progestin-Dependent Angiogenesis in Breast Cancer Funding: Includes salary, tuition, insurance, travel, research costs for 25 months
01/2007-12/2007	**COR Grant** Co-PI: Dr. Salman M. Hyder and Candace E. Carroll Title: Therapeutic Potential of 2-Methoxyestradiol and Curcumin for the Prevention and Treatment of Progestin-Accelerated Breast Cancer Funding: $18,000 for 12 months
10/2006-9/2007	**Phi Zeta Research Grant** PI: Candace E. Carroll Title: The Inhibition of Progestin-Induced Vascular Endothelial Growth Factor Secretion from Human Breast Cancer Cells by Curcumin Funding: $750.00 for 12 months

Publications:

Carroll CE, Benakanakere I, Besch-Williford C, Liang Y, Hyder SM, HIF-1α is required for progestin-dependent VEGF induction in breast cancer cells and progestin-dependent tumor progression. *(In preparation)*

Benakanakere I, **Carroll CE**, Hyder SM, Effect of natural and synthetic progestins on DMBA-Induced Mammary Tumors in Rats. *(In preparation)*

Carroll CE, Benakanakere I, Besch-Williford C, Ellersieck MR, Hyder SM, Curcumin Delays MPA-Accelerated DMBA-Induced Mammary Tumors. Menopause. 2010 Jan/Feb. *Accepted*

Carroll CE, Hyder SM, Curcumin Inhibits MPA-Induced Secretion of VEGF from T47-D Human Breast Cancer Cells, Response to Letter, Menopause. 2008 13(6):1195

Carroll CE, Ellersieck MR, Hyder SM, Curcumin Inhibits MPA-Induced Secretion of VEGF from T47-D Human Breast Cancer Cells. Menopause. 2008 May/June; 15(3):570-574

Ganio, M.S., Wingo, J.E., **Carroll, C.E.**, Thomas, M.K., Cureton, K.J. Fluid Ingestion Attenuates the Decline in VO_{2max} Associated with Cardiovascular Drift. Medicine and Science in Sports & Exercise. 2006 May; 38(5):901-909.

Accepted Abstract:

Indira Benakanakere, **Candace E Carroll**, Cynthia Besch-Williford, Rachel L Ruhlen, Salman M Hyder. University of Missouri, Columbia, MO. **The synthetic progestin norgestrel prevents the progression of pre-neoplastic lesions in a rat model of DMBA-induced breast cancer.**

- *ENDO 2009: The Endocrine Society's 91st Annual Meeting, June 10-13, 2009*

Candace E Carroll, Indira Benakanakere, Cynthia Besch-Williford, Yayun Liang, Salman M Hyder. University of Missouri, Columbia, MO. **HIF-1{alpha} is required for both progestin-induced VEGF secretion by breast cancer cells and in vivo progression of progestin-dependent mammary tumors.**

- *2009 St. Jude National Graduate Student Symposium, March 31-April 4, 2009*
- *100th Annual Meeting of American Association for Cancer Research, April 18-22, 2009*

Candace E. Carroll, Indira Benakanakere, Salman M. Hyder. University of Missouri, Columbia, MO. **Curcumin: A Potential Chemopreventive Agent for Progestin-dependent Breast Tumors.**

- *ENDO 2007, The Endocrine Society's 89th Annual Meeting, June 2-5, 2007.*
- *Life Science Week 2008, University of Missouri-Columbia, April 2008*
- *Annual NOBCChE Conference held at Eli Lilly, October 24-25, 2008.*

Candace E. Carroll, Indira Benakanakere, Mark Ellerseick, Salman M. Hyder. University of Missouri, Columbia, MO. **Curcumin inhibits progestin-induced vascular endothelial growth factor secretion from human breast cancer cells.**

- *30th Annual Phi Zeta Research Day hosted by the Pi Chapter, March 23, 2007.*
- *100th Annual Meeting of American Association for Cancer Research, April 14-18, 2007.*
- *Bi -Annual Midwest Regional Molecular Endocrinology Conference, May 17-18, 2007.*
- *Annual Cardiovascular Research Day, March 17, 2008*

Jeffrey J Whyte, Angela M Davis, Kristie M. Grimm, Cory M Weimer, **Candace E**

Carroll, R Michael Roberts and Cheryl S Rosenfeld. University of Missouri-Columbia, Columbia, MO. **Differences in Serum Estradiol, Testosterone, Progesterone and Free Fatty Acids at Conception in NIH Swiss Mice Relative to Fat and Carbohydrate Content of Maternal Diet.**

- *39th Annual Meeting of the Society for the Study of Reproduction, July 29-August 1, 2006.*

Presentations and Conferences Attended:

April 2009	**Annual American Associate for Cancer Research** (AACR) held in Denver, CO. Poster Presentation: *HIF-1{alpha} is required for both progestin-induced VEGF secretion by breast cancer cells and in vivo progression of progestin-dependent mammary tumors.*
March/April 2009	**National Graduate Student Symposium at St Jude Children's Research Hospital** held in Memphis, TN. Poster and Oral Presentations: *HIF-1{alpha} is required for both progestin-induced VEGF secretion by breast cancer cells and in vivo progression of progestin-dependent mammary tumors.*
November 2008	**Annual Biomedical Research Conference for Minority Students** (ABRCMS) held in Orlando, FL. I assisted in recruitment for the University of Missouri as well as attended career development seminars, networking luncheons, and participated as a panelist discussing the preparation for graduate school and graduate school applications.
October 2008	**National Organization for the professional advancement Black Chemists and Chemical Engineers (NOBCChE) Conference** held at Eli Lilly in Indianapolis, IN. Poster Presentation: *Curcumin: A potential chemopreventive agent for progestin-dependent breast tumors*
April 2008	**Life Sciences Week** held at University of Missouri-Columbia, Life Sciences Center. Poster Presentation: *Curcumin: A potential chemopreventive agent for progestin-dependent breast tumors*
July 2008	**Leadership Alliance National Symposium *"The Promise of Leadership: Celebrating the First 100 PhDs"*** held in Hartford, CT. As a LA graduate student, I attended career development seminars and moderated undergraduate oral presentation sessions.
May 2007	**Bi-Annual Midwest Regional Molecular Endocrinology Conference** held at the Indiana University, Bloomington, Indiana. Oral Presentation: *Curcumin Inhibits Progestin-Induced Vascular Endothelial Growth Factor Secretion from Human Breast Cancer Cells*
April 2007	**100th Annual American Association for Cancer Research** held at Los Angles, CA. Poster Presentation: *Curcumin Inhibits Secretion of Progestin-Induced Vascular Endothelial Growth Factor in Human Breast Cancer Cells*
March 2007	**30th Annual Phi Zeta Research Day, Pi Chapter** held at the University of Missouri-Columbia, College of Veterinary Medicine, in Columbia, MO. Oral Presentation: *Inhibition of MPA-Dependent VEGF Secretion from Human Breast Cancer Cells*
April 2005	**Annual Mississippi College/Tougaloo College Research Symposium** held at Mississippi College in Clinton, MS. Poster Presentation: *Effect of hydration during long duration exercise on cardiovascular drift and maximal oxygen uptake*

April 2004	**First Annual Mississippi College/ Tougaloo College Research Symposium** held at Tougaloo College in Tougaloo, MS. Poster Presentation: *Correlation of Airway Impedance and Upper Airway Noise in Horses with Laryngeal Hemiplegia*
August 2004	**Annual Summer Undergraduate Research Symposium** held in Athens, GA. Poster Presentation: *Effect of hydration during long duration exercise on cardiovascular drift and maximal oxygen uptake*
Fall 2004	**Annual Student Research Symposium at Tougaloo College** in Tougaloo, MS. Oral Presentation: *Effect of Hydration during long duration exercise on cardiovascular drift and maximal oxygen uptake.*
November 2004	**Annual Biomedical Research Conference for Minority Students** (ABRCMS) held in Dallas, TX. Poster presentation: *Effect of hydration during long duration exercise on cardiovascular drift and maximal oxygen uptake*
October 2003	**Annual Biomedical Research Conference of Minority Students** (ABRCMS) held in San Diego, CA. Poster Presentation: *Correlation of Airway Impedance and Upper Airway Noise in Horses with Laryngeal Hemiplegia*
July 2003	**Annual Leadership Alliance Symposium** held in Chantilly, VA. Poster Presentation: *Correlation of Airway Impedance and Upper Airway Noise in Horses with Laryngeal Hemiplegia*
Fall 2002	**Annual Student Research Symposium at Tougaloo College** in Tougaloo, MS. Oral Presentation: *The Effects of Urine pH on Veterinary Bladder Tumor Antigen Test*. (Awarded Best Biological Presentation)
Fall 2003	**Annual Student Research Symposium at Tougaloo College** in Tougaloo, MS. Oral Presentation: *Correlation of Airway Impedance and Upper Airway Noise in Horses with Laryngeal Hemiplegia*. (Awarded Best Biological Presentation)
August 2002	**Annual Gateways to Veterinary Medicine Research Symposium** held in Columbia, MO. Oral Presentation: *The Effects of Urine pH on Veterinary Bladder Tumor Antigen Test*.