THE EFFECTS OF POMIFERIN ON GROWTH RATE AND VASCULARIZATION OF MDA-MB-435 TUMOR XENOGRAFTS IN ATHYMIC NUDE MICE

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ABSTRACT

THE EFFECTS OF POMIFERIN ON GROWTH RATE AND VASCULARIZATION OF MDA-MB-435 TUMOR XENOGRAFTS IN ATHYMIC NUDE MICE

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University of Guelph, 2007

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This thesis is an investigation of the effects of orally-administered pomiferin on tumor growth rate and tumor vascularization, when given to athymic nude mice bearing MDA-MB-435 human breast cancer xenografts. Mice were randomly allocated to either a control, 0.002%, or 0.02% pomiferin (by weight) diet group. The treatment period lasted for three weeks, during which tumor growth rate was monitored. After the treatment period, mice were sacrificed, and tumors removed and subsequently analyzed immuonohistochemically for endothelial cell markers, allowing for the calculation of microvessel density. The data collected showed that there was no significant difference between the control, 0.002%, and 0.02% pomiferin groups in regards to tumor growth rate, and tumor neovascularisation (respective mean MVDs ± standard deviation were 118 ± 34, 111 ± 22 and 93 ± 46). Despite not reaching significance, the data did suggest that increasing amounts of dietary pomiferin could possibly reduce both of these variables.

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1.1. Cancer and Diet

1.1.1. *The Stages of Cancer*

Cancer, now more than ever, captivates the media and public alike. This is likely attributable to the aggressive and incurable nature of some forms of the disease, as well as the many modifiable lifestyle and dietary factors that are suspected to alter individual susceptibility.

On a fundamental level, cancer represents unchecked cellular growth – that is, the escape of cells from the molecular and chemical controls that normally regulate cell division. Mutations of two types of genes typically lead to this state: proto-oncogenes and tumor-suppressor genes. Proto-oncogenes, once mutated, will enhance cellular signaling that favors proliferation; tumor-suppressor genes, once mutated, will remove signals that are normally present to suppress proliferation (1).

To help understand and study its disease process, cancer has been operationally – and experimentally – defined into three stages: initiation, promotion and progression. Initiation involves at least one mutation to a proto-oncogene or tumor-suppressor gene, resulting in signaling changes favorable to proliferation; promotion involves the clonal expansion of this initiated cell, resulting in a benign lesion or focus of cells possessing the same proliferative mutation(s); progression involves the further acquisition of genetic changes that result in the change from a benign to a malignant phenotype (1, 2).

1.1.2. *Breast Cancer*

Breast cancer represents 23% of all female cancers and, as such, is the most prevalent cancer amongst women worldwide with an estimated 4.4 million alive with the

disease (diagnosed during the previous five years) (3). It is the leading cause of cancer mortality amongst women, but ranks only fifth as the cause of death from all cancers, which is the result of breast cancer having a relatively good prognosis compared to some of the other types of cancer (3). More than one-half of breast cancer cases occur in developed countries, which can be partly attributed to better screening in these more affluent areas, and partly attributable to increased exposure to environmental and lifestyle factors that increase risk (3). Much recent interest has come to the role of diet in cancer incidence, as it is a highly modifiable lifestyle factor.

1.1.3. *Diet and Cancer*

The role of diet in the etiology and prevention of cancer has been of great interest to both the general public and the research community. In particular, a great deal of work has been done to investigate the association between fruit and vegetable consumption and cancer incidence. Epidemiological studies have generated mixed results, with some cohort studies showing a decreased incidence of cancer with total vegetable and fruit consumption(4), some showing decreased cancer incidence with fruit consumption only (5), and some showing no association between fruit and vegetable consumption and cancer risk (6). A meta-analysis of the prospective studies showed only a weak, non-significant, association between total vegetable and fruit consumption and cancer incidence (7). However, it has been noted, in a cohort study, that increased variety of fruit and vegetable intake is significantly associated with a decreased risk of total cancers (8), which suggests that this relationship is more complex than simply increasing fruit and vegetable intake to reduce cancer risk. A meta-analysis of case-control studies showed a stronger association between increased fruit and vegetable intake and cancer

incidence, but it has been warned that such an association could be artificially exaggerated due to recall and selection biases in this type of study (7).

When examining breast cancer on its own, epidemiology suggests that produce intake may indeed be related to disease incidence. A meta-analysis of epidemiological studies conducted between 1982 and 1997 showed a strong association between increased vegetable intake and decreased breast cancer incidence (RR = 0.75, 95% CI 0.66-0.85), but the relationship was weaker for fruit consumption and breast cancer incidence (RR = 0.94, 95% CI 0.79-1.11) (9). However, a pooled analysis of prospective cohort studies alone did not show a significant association between fruit and vegetable consumption and breast cancer risk (RR, 0.93; 95% CI, 0.86-1.00) (10), which could, again, be due to the fact that this type of study is less subject to recall and selection bias than case-control studies.

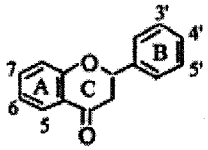
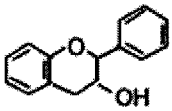
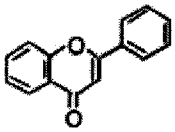
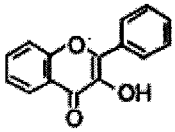
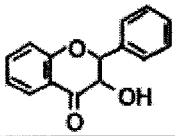
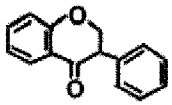
Despite their ability to examine trends in large populations of people, these epidemiological studies lack the ability to look specifically at the plethora of plant constituent chemicals, known collectively as phytochemicals, each of which possessing its own unique chemical properties upon ingestion.

1.1.4. *Flavonoids*

Flavonoids are a class of polyphenolic phytochemicals, constituting more than 4000 compounds, which all share a phenylchromanone structure – that is, fifteen carbon atoms arranged in two benzene rings, attached by a three carbon chain, commonly abbreviated as (C6-C3-C6) (11). In other words, it can be thought of as a three-ring structure, with a chromane ring joining two aromatic (or benzene) rings (see figure 1). The oxidation state of the heterocyclic chromane ring, as well as the position of ring B

(the second aromatic ring), determine which subgroup a particular flavonoid will be part of. Isoflavonoids differ from flavonoids in that ring B is in position 3 on the chromane ring, instead of position 2 (11).

Figure 1. Some of the common plant flavonoids, in their aglycone forms, and corresponding chemical structures (11).

Structural formula	Representative flavonoids	Substitutions					
		5	6	7	3'	4'	5'
Flavanone							
	Eriodictyol	OH	H	OH	OH	OH	H
	Hesperitin	OH	H	OH	OH	OMe	H
	Naringenin	OH	H	OH	H	OH	H
Flavanol							
	Catechin	OH	H	OH	OH	OH	H
	Gallocatechin	OH	H	OH	OH	OH	OH
Flavone							
	Apigenin	OH	H	OH	H	OH	H
	Chrysin	H	H	OH	H	H	H
	Luteolin	OH	H	OH	OH	OH	H
Flavonol							
	Kampherol	OH	H	OH	H	OH	H
	Myricetin	OH	H	OH	OH	OH	OH
	Quercetin	OH	H	OH	OH	OH	H
Flavanonol							
	Taxifolin	OH	H	OH	OH	OH	H
Isoflavone							
	Daidzein	H	H	OH	H	OH	H
	Genistein	OH	H	OH	H	OH	H
	Glycitein	OH	OMe	OH	H	OH	H
	Formononetin	H	H	OH	H	OMe	H

Being present in all terrestrial vascular plants (11), the ubiquitous nature of flavonoids has made estimating their levels in the human diet rather difficult. However, a recent study, which made use of - and expanded - the USDA Flavonoid Database, found that the average daily intake of flavonoids in U.S. adults is 189.7 mg/day, with the greatest mean contributions from tea (157 mg), citrus fruit juices (8mg), wine (4 mg), and citrus fruits (3 mg) (12). The dietary intake of flavonoids does not directly correlate with their levels in the body as flavonoids are typically present as glycosides (attached to sugar moieties) in food form, making absorption predominantly possible after bacterial cleavage – to the aglycone form – in the colon, although some absorption across the small intestine has been proven possible for certain flavonoids (13). Once absorbed, flavonoids can remain in their aglycone form, but more often than not, they are conjugated to glucuronic acid and/or sulfate, altering their biological activity inside the body (14). The matter is further complicated by noting that position, as well as type, of conjugation will determine the ultimate activity of the flavonoid; and, that each flavonoid has a unique conjugation pattern inside the body (14).

Thus, when considering the role of dietary flavonoids in disease incidence and prevention, the differing metabolism of these compounds must be understood, in addition to their respective dietary intakes, in order to get a holistic understanding of their biological activity.

1.1.5. *The Role of Flavonoids in Cancer*

Despite the difficulties inherent in finding a relationship between dietary intake of flavonoids and cancer incidence, there has been – and continues to be – many *in vitro* and

animal model studies looking at the biochemical and physiological roles that these compounds may play in chemoprevention. For instance, a number of flavonoids have been found to modulate cellular signaling pathways – affecting transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), as well as protein kinases such as mitogen activated protein kinases (MAPK) and protein kinase C (PKC) – ultimately resulting in beneficial changes to proliferative gene expression (reviewed in(15)). In addition, some studies show that certain flavonoids may: a) promote cell cycle arrest – via modulation of certain cyclins and cyclin dependant kinases – in certain types of cancer; b) promote apoptosis through modulation of caspase pathways; c) induce phase II enzymes – such as glutathione-S-transferase (GST) – which, in turn, metabolize carcinogenic compounds to readily excretable forms; d) act as potent antioxidants, both by directly quenching reactive oxygen species, as well as by playing an inhibitory role in inflammatory signaling cascades that generate free radicals; and, e) inhibit telomerase, an enzyme implicated in the immortalization of cancer cells (reviewed in (15)). Furthermore, isoflavonoids have a structure similar to estradiol, which allows them to bind to estrogen receptors and exert estrogenic or antiestrogenic effects depending on their specific structures (16). Estrogen metabolism - and its consequent byproducts - is thought to play a contributory role in the etiology of cancers such as breast cancer; therefore, phytoestrogens have been suspected of having a preventive activity against such cancers. Soy is a rich source of two isoflavones, genestein and daidzein, but a direct relationship between increased consumption of soy products and decreased breast cancer risk still remains unclear (16).

These numerous biochemical mechanisms of chemoprevention suggest an important link between flavonoid consumption and cancer risk. One additional mechanism by which flavonoids may act as chemopreventive agents, not mentioned above, is their role as anti-angiogenic agents, a topic that will be covered at great length in forthcoming sections.

1.2. Angiogenesis and Cancer

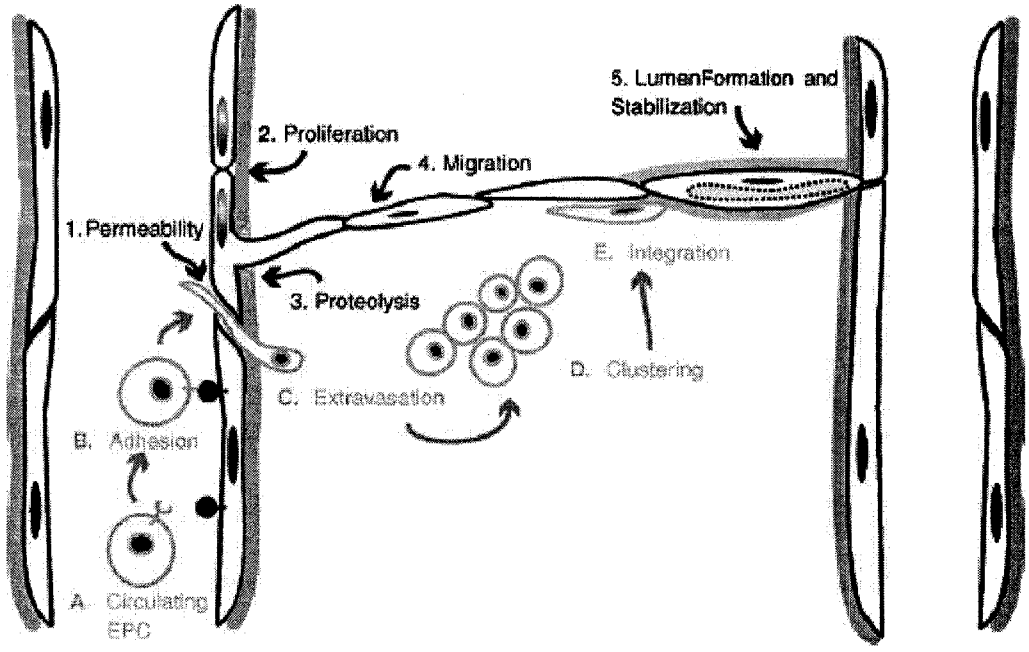
1.2.1. *What is Angiogenesis?*

Angiogenesis is the process by which new capillaries grow from existing blood vessels. Normally, in a healthy individual, it is a process that occurs only during growth and development (as the body grows its complex circulatory system), during wound healing and tissue repair, and as part of the menstrual cycle in women (a necessary part of endometrial remodeling). However, in some diseases, such as cancer and macular degeneration, pathological angiogenesis is required for the disease to run its course.

This process, normally held in a delicate homeostasis, is controlled by a plethora of activator and inhibitor molecules: growth factors and their respective cell-surface receptors, intracellular signaling molecules, and transcription factors (17). However, certain stimuli can trigger angiogenesis to occur – these include hypoxia (such as that found in the core of an unvascularized tumor), inflammation, and mechanical factors (such as those incurred during injury and intense exercise) (17). The "conventional" form of angiogenesis is also known as abluminal sprouting; there are other ways of generating new vessels, but this is the most clearly understood and extensively studied (17).

During the process of abluminal sprouting, the aforementioned stimuli signal the endothelial cells (via autocrine and paracrine production of growth factors) to become activated, to begin proliferating, and to increase permeability by dissolving adherens junctions (17). Then the basement membrane matrix is enzymatically proteolysed, which allows endothelial cells to migrate into the interstitium via formation of a sprouting tip (17). The sprouting tip will form a lumen as it becomes multi-cellular and join, or anastamose, with a pre-existing vessel, connecting the two (17). Once these vessels have joined, the endothelial cells are deactivated, the adherens junctions are reformed, and the basement membrane is reconstructed (17). As well, circulating endothelial progenitor cells can assist in the process, first by adhering to activated endothelial cells, then extravasating into the interstitium where they can either join the new capillary or become perivascular cells (see figure 2) (17).

Figure 2. Depiction of angiogenesis (abluminal sprouting). Autocrine and paracrine growth factors activate endothelial cells, 1) dissolving adherens junctions to increase permeability and 2) initiating proliferation. 3) The basement membrane is enzymatically proteolysed, 4) allowing endothelial cells to migrate into the interstitium in the form of a sprouting tip. 5) As the sprouting tip becomes multicellular, a lumen will form, and eventually, the new vessel will join to a pre-existing vessel, followed by deactivation of endothelial cells, reconstruction of the basement membrane and reformation of the adherens junctions. A) Circulating endothelial cells can assist in the formation of the new vessel by B) adhering to activated endothelial cells, C) extravasating into the interstitium where they will D) cluster, and either E) integrate into the new vessel, or become perivascular cells (17).



1.2.2. *Angiogenesis, Metastasis, and Prognosis in Breast Cancer*

The vascularity of breast tumors – commonly measured as the microvessel density (MVD), which is the number of vessels present per unit area of tumor – has long been scrutinized as a factor in the clinical and pathological course of the disease. Studies with node-negative breast cancer patients and invasive ductal breast carcinomas have shown that MVD significantly correlates with overall survival and disease-free survival (18-21). However, other studies with primary invasive breast cancer tumors were unable to find a positive correlation between MVD and disease-free survival (22-24).

Various reasons have been suggested for this discrepancy, including a high intra-tumoral variation in vascularity (22). As well, it has been observed that MVD is positively correlated with disease-free survival in certain stages (stages I and II) and certain nodal conditions (node negative, or one to three positive nodes in the axilla), but not in other stages (stage IV) or nodal conditions (four or more positive nodes in the axilla) (25). Similarly, another study showed MVD positively correlated with relapse-free survival and overall survival in node-negative patients, but not node-postive patients, and, in addition, with larger T2 and T3 tumors, but not T1 tumors (26). Taken together, it appears that pathologic features of breast cancer tumors might determine the ultimate utility of MVD as a prognositic indicator of disease course.

In addition to pathological features of the tumor, factors related to study design and methodology may also affect these results. For instance, chemotherapeutic drugs, such as tamoxifen, being taken during the course of the study might have affected the outcome (24). As well, different methods of quantifying MVD may affect the ability of angiogenesis to be prognostic. For instance, one study of patients with invasive breast

cancer showed that hot spot counting of microvessels – a method that focuses on areas of highest neovascularization in tumor sections – is a strong prognostic factor in overall survival, whereas global vessel counting – a random sampling of fields throughout the tumor - did not correlate with prognosis (27). Another method, known as the "Chalkley method" of counting, uses a 25-point grid or graticule to quantify area covered by microvessels in a tumor section. This method of calculating vessel density, when examining primary invasive breast tumors, was significantly associated with disease-free, as well as overall survival (28). Furthermore, it has been shown that the Chalkley method has prognostic value when assessing disease-free and overall survival in patients with invasive breast cancer, while traditional methods of counting microvessel density, carried out in the exact same group of subjects, showed no correlation (29). It has been speculated that the strength of the Chalkley method lies in its ability to account for individual vessel size, an aspect overlooked by traditional counting methods, as large vessels could be more important than smaller vessels to disease pathology (29).

The degree of angiogenesis, or MVD, of breast cancer tumors might also predict the likelihood of metastasis in patients with breast cancer. Studies of breast cancer patients with known nodal status have shown that MVD – counted both in a traditional manner and using the Chalkley method - is a strong predictor of metastatic diffusion (28, 30). However, primary tumor MVD could not predict site of metastases, when comparing stage IV breast cancer patients with soft tissue, bone and visceral metastases (31). Also, a high MVD in axillary lymph node metastases was shown to be prognostic of poor disease-free survival and overall survival, even when MVD of the primary tumor failed to be of prognostic value (23).

Overall, it appears that tumor angiogenesis is very important to the pathological course of breast cancer, as well as to the clinical course of patients with the disease. The inconsistency between some of the studies seems to be mostly attributable to methodological differences, which should be further studied to find that with the best prognostic value.

1.2.3. *Antiangiogenic Therapy*

In light of the role angiogenesis may play in breast cancer prognostication and metastasis likelihood, antiangiogenic therapy has become a burgeoning topic of research in breast cancer pharmacotherapy. This interest is heightened by the fact that traditional breast cancer drugs, such as tamoxifen – an antagonist for the estrogen receptor (ER), used in the treatment of ER-positive breast cancer – were shown to have antiangiogenic activity in the chick egg chorioallantoic membrane model (32) as well as in MCF-7 tumor xenografts in nude mice (33).

A novel antigangiogenic approach aims to use a monoclonal antibody, known as Bevacizumab, to bind to VEGF and block its subsequent binding to high-affinity receptors. A phase I/II trial in patients with previously treated metastatic breast cancer showed a modest response to Bevacizumab (9.3% overall response rate, with 16% of subjects having stable disease or an ongoing response at the conclusion of the trial); as well, adverse events observed in this study – namely hypertension – were distinct from those associated with traditional chemotherapies (34). This suggested that Bevacizumab may be particularly beneficial if combined with chemotherapy. A later trial looked at the efficacy of Bevacizumab in combination with the chemotherapy drug, Docetaxel, as a first- or second-line therapy for metastatic breast cancer and found an overall response

rate of 52%, with a median progression-free survival time of 7.5 months (35). Similarly, a phase III trial that examined the efficacy of capecitabine with our without Bevacizumab in patients with previously treated metastatic breast cancer, found that the addition of Bevacizumab increased the response rate (19.8% vs. 9.1%) (36). However, this increased response rate did not translate into a longer progression-free survival, which, the authors speculated, could be because Bevacizumab might work better as a targeted therapy – in individuals with a particular genotype – or, because the drug may be more effective in earlier stages of the disease (36). Bevacizumab, does however show promise as an effective chemotherapeutic agent.

In addition to blocking VEGF from binding to its receptor, it is also possible to inhibit the tyrosine kinase activity of the VEGF receptor (VEGF-R). The novel drug, SU11248, a multitargeted tyrosine kinase inhibitor, when co-administered with docetaxel, has been able to increase the survival-time of mice with breast cancer xenografts, as compared with either drug administered individually (37). As well, ZD6474, an inhibitor of VEGF-R and epidermal growth factor receptor (EGFR), was able to inhibit atypical ductal hyperplasia and mammary tumor formation in mice administered dimethylbenzanthracene (DMBA), a potent carcinogen (38). Thus far, the preclinical evidence for VEGF-R inhibitors appears promising and they may very well have a place in the future clinical pharmacotherapy of cancer.

Another approach to antiangiogenic therapy is to inhibit matrix metalloproteinases (MMPs), enzymes important in the digestion of basement membrane and, hence, the escape of endothelial cells into the interstitium. However, marimastat, a drug designed for this purpose, has shown little potential to prolong progression free-

survival or overall survival in patients with metastatic breast cancer (39). Furthermore, musculoskeletal toxicity – specifically arthralgia and arthritis – is a common side effect to taking marimastat, which precludes the safe clinical use of the drug (39, 40). It has been hypothesized that the inefficacy of marimastat might be due to an upregulation of MMP production or activity, or a loss of MMP-governed regulation of angiostatin and endostatin – both of which are important endogenous inhibitors of angiogenesis (39). Such problems need to be addressed before the drug will show any clinical potential.

1.3. Antiangiogenic Flavonoids

A number of flavonoids have been found to have chemopreventive effects in both *in vitro* and *in vivo* models of cancer. As mentioned previously, these anticancer properties are elicited through a wide variety of mechanisms. This section will focus specifically on the antiangiogenic properties that have been described for various flavonoids.

1.3.1. *Genistein*

Epidemiological studies have shown that high soy and soy-product consumption – as is commonly seen in certain Asian populations – is associated with a decreased risk of some types of cancers (41, 42). This association between soy and cancer is not observed in non-Asian Americans, as soy consumption is extremely low, with an average intake of less than one serving per week in certain areas (43). The anticancer properties of soy are generally attributed to the isoflavonoids, genistein and daidzein, which they contain in abundance. In fact, soy is the primary source of these isoflavonoids in the human diet. Evidence suggests that these isoflavonoids have various anticancer properties, included in which is the strong antiangiogenic nature of genistein.

Studies have consistently shown that genistein can reduce MVD in mouse xenograft models of various forms of cancer, including: bladder (MB49 and 253 J B-V cells, respectively) (44, 45), breast (ER-negative MDA-MB-231, ER-positive MCF-7, and F3II cells) (46, 47), renal (SMKT R-1 cells) (48), oral squamous (HSC-3 cells) (49), and melanoma (B16 cells) (47). In addition, soy phytochemical concentrate (SPC) and soy protein isolate (SPI), both rich in genistein, were shown to decrease MVD in a mouse xenograft model of prostate cancer (LNCaP cells) (50). Taken together, these studies show that genistein has a consistent ability to inhibit angiogenesis, as measured by MVD, across numerous types of cancer. A number of potential molecular mechanisms for this antiangiogenic ability have been observed.

Possible targets for genistein are the MMPs. In both *in vitro* and *in vivo* experiments with MCF-7 and MDA-MB-231 breast cancer cells lines, genistein was able to inhibit the activity of MMP-9 (as measured by zymography) as well as increase the mRNA expression of tissue inhibitors of metalloproteinases (TIMPs), an endogenous family of enzymes that inhibit MMPs (46). Conversely, in another study, genistein did not effect the levels of MMP-2 and MMP-9 secreted by F3II mammary carcinoma cells; it did, however, inhibit urokinase-type plasminogen activator (uPA) secretion – an enzyme which also plays a role in extracellular matrix degradation (47). Genistein was also able to decrease uPA expression in various bladder cancer cells lines (51), and uPA-receptor (uPAR) expression in PC3 prostate cancer cells (52). As well, genistein, when given to mice bearing bladder cancer xenografts (TSGH8301 cells), and to other bladder cancer cells lines *in vitro*, was able to decrease expression and secretion of MMP-2 and MMP-9 (51). In androgen-dependant PC-3 human prostate cancer cells, genistein was

able to decrease mRNA levels of MMP-13 (53) and MMP-9 (52). These studies suggest that down-regulation of the MMP and uPA enzymes may play a role in the anticancer activity of genistein, thus inhibiting digestion of the basement membrane during the angiogenic process.

Another possible target for genistein is the VEGF pro-angiogenic pathway. In mice bearing breast tumor xenografts (both MDA-MB-231 and MCF-7 cells), genistein effectively decreased both serum and tumor levels of VEGF protein (46). Similarly, VEGF protein production was decreased in various pancreatic cancer cell lines (54), as well as in prostate cancer (PC-3) cells (55). Genistein has also been shown to down-regulate *in vitro* VEGF mRNA expression in prostate cancer (PC3) cells (52, 55), renal cancer (SMKT-R-1 and SMKT-R-3) cells (56), oral squamous cancer (HSC-3) cells (49), various pancreatic cancer cell lines (54), various bladder cancer cell lines (51), and HUVECs (55). Reducing the amount of VEGF transcription, and subsequent translation, may very well be an integral part of genistein's antiangiogenic activity.

Genistein's effects on VEGF may be consequent to its regulation of the transcription factor, HIF-1α, which is crucial to the regulation of VEGF expression under hypoxic conditions. Genistein treatment of various pancreatic cancer cell lines led to an impaired HIF-1α activation (54). Similarly, a significant decrease in HIF-1α nuclear accumulation was noted in prostate cancer (PC-3) cells after treatment with genistein (55). In addition to regulating HIF-1α, genistein may also regulate VEGF activity by modulating VEGF-R1 and VEGF-R2. Treatment of HUVECs with genistein decreased the mRNA expression of VEGF-R1 and VEGF-R2, suggesting that this may be another point of regulation by the flavonoid (57).

In addition to the aforementioned effects of genistein on angiogenesis, there are a number of studies, in various types of cancer, showing that it can also modulate various other growth factors related to angiogenesis, including the down-regulation of the pro-angiogenic factors: transforming growth factor beta (TGF-β) (46, 52), basic fibroblast growth factor (bFGF) (56), platelet derived growth factor (PDGF) (51), interleukin-8 (IL-8), and follistatin (53). Furthermore, genistein appears to be able to up-regulate some endogenous inhibitors of angiogenesis in cancer cells: plasminogen activator inhibitor-1, endostatin, angiostatin, and thrombospondin-1 (51). A recent study of HUVECs also showed that genistein can modulate a number of genes related to cell-adhesion, suggesting yet another route via which this isoflavonoid may regulate angiogenesis (58).

Taken together, these studies suggest that there are numerous enzymes, growth factors and transcription factors, all related to angiogenesis, which genistein may be able to regulate in various types of human cancer cells, both *in vitro* and in some *in vivo* models. These interactions may serve as the mechanistic basis that underlies the observed association between high levels of soy consumption and decreased prevalence of cancer, noted in certain Asian populations.

1.3.2. *Green Tea Flavonoids*

Green tea is known to contain a number of flavonoids. Particularly, it is known to be abundant in certain catechins: epicatechin, epicatechin gallate (EG), catechin, and epigallocatechin gallate (EGCG). These catechins are believed to be responsible for the long-purported health benefits of green tea. Included in these health benefits are cancer preventive properties, which are supported by some epidemiology: epidemiological studies suggest a decreased risk of breast and ovarian cancers in relation to green tea

consumption (59-62). However, the epidemiological data is weak for a relationship between green tea consumption and colorectal cancer risk (63, 64). It is worth noting that the protective effect of green tea against breast cancer may be more pronounced in individuals with the low activity catechol-O-methyltransferase (COMT) genotype, an enzyme that rapidly metabolizes green tea catechins for removal (62), and in individuals with the high activity angiotensin converting enzyme genotype (ACE), an enzyme associated with increased risk of breast cancer (65). *In vitro* and *in vivo* studies suggest that an antiangiogenic ability may be amongst green tea's chemopreventive properties.

Studies have shown that green tea, or its constituent catechins, are capable of decreasing MVD in a number of *in vivo* models of cancer. One study showed that green tea, given in the drinking water of A/J female mice, was able to decrease the MVD of NNK-induced lung tumors (66). Similarly, oral administration of green tea to mice bearing ovarian cancer xenografts (HEY cells) resulted in a decrease in tumor MVD (67). When given to C3(1)/SV40 mice (a mouse model of breast cancer that generates spontaneous ductal carcinomas), green tea extract significantly decreased MVD (68). As well, addition of green tea extract to the drinking water of mice bearing breast cancer xenografts (MCF-7 cells) worked synergistically with tamoxifen to decrease MVD (69). These studies suggest a consistent ability of green tea to decrease MVD *in vivo*, and a number of underlying molecular mechanisms for this have been proposed.

Like genistein, certain green tea catechins are suspected to have an inhibitory effect on the MMP proteins. For instance, EGCG elicited a dose-dependant decrease in MMP-2 and MMP-9 activity in human fibrosarcoma (HT1080)-condition-media, analyzed by gelatin zymography (70). Similarly, a mixture of green tea polyphenols

decreased expression of MMP-2 and MMP-9 – and, additionally, increased the expression of TIMP - in female SKH-1 hairless mice that were UVB-irradiated to induce skin cancer (71). Other *in vitro* studies have shown that EGCG can inhibit MMP-2 expression/activity in HUVECs (72), MMP-9 expression/activity in myeloid leukemia cells (HL-60) (73), and both MMP-2 as well as MMP-9 in ovarian carcinoma cells (HEY and OVCA 433 cells) (67). The effect on MMP-2 may be due to the inhibition of membrane-type 1 MMP (MT1-MMP), which is responsible for activation of pro-MMP-2. Using a gelatin zymography assay with human fibrosarcoma (HT1080) cells, EGCG was shown to cause a dose-dependant inhibition of MT1-MMP *in vitro*, and a consequent accumulation of non-activated MMP-2 (74). A similar inhibition of MT1-MMP, and resultant decrease in the active form of MMP-2, was also observed in HUVECs treated with EGCG (75). Furthermore, regulation of the MMPs by EGCG may very well be at the transcriptional level. A model system using rat aortic endothelial cells and HUVECs, grown in a collagen matrix, demonstrated that EGCG can inhibit the levels of certain transcription factors – namely, Ets-1, c-Fos, and c-Jun – which are known to play an important role in the transcription of collagenase/protease genes during angiogenesis (76). As well, EGCG was shown to decrease expression of the mRNA stabilizing factor HuR – a factor important in MMP-9 mRNA expression - in myeloid leukemia cells (HL-60) (73). These numerous observed effects on MMPs, as well as factors that regulate them, likely contribute strongly to the chemopreventive nature of green tea.

Green tea catechins, again like genistein, also appear to target the VEGF pro-angiogenic pathway. EGCG has been shown to decrease *in vitro* VEGF expression in a number of different models, including: swine granulose cells (77), ovarian carcinoma

cells (HEY and OVCA) (67), human cervical cancer cells (HeLa) and human hepatoma cells (HepG2) (78). As well, green tea and green tea polyphenol mixtures have been shown to decrease VEGF expression in lung tumors from female A/J mice exposed to NNK (66), skin cancer from female SKH-1 mice exposed to UVB irradiation (71), ovarian cancer xenografts (67), human cervical cancer cells (HeLa) and human hepatoma cells (HepG2) (78), atherosclerotic plaques obtained from New Zealand white rabbits on hypercholesterolemic diets (79), breast cancer cells (MCF-7 and MDA-MB 231) (68), and in the C3(1)/SV40 mouse model of spontaneous ductal adenocarcinomas (68). As implied by the above observations, modulation of VEGF levels by green tea polyphenols likely plays an important part in its anti-angiogenic properties, as well as the consequent chemopreventive nature of the flavonoids.

There are a number of ways through which green tea flavonoids may modulate VEGF expression, or activity. First of all, there is the modulation of transcription factor HIF-1α. An *in vitro* study using human cervical carcinoma cells (HeLa) and human hepatoma cells (HepG2) showed that green tea and EGCG were able to significantly inhibit hypoxia- and serum- induced accumulation of HIF-1α protein, but not HIF-1α mRNA (78). This inhibitory effect appeared to be consequent to an inhibitory effect of green tea and EGCG on the PI3K/Akt and ERK1/2 regulated kinases, both known to regulate HIF-1α, as well as by increasing the degradation of HIF-1α via the proteasome system (78). However, in contrast to these findings, studies of human prostate cancer cells (PC-3ML) under normoxic conditions showed that EGCG exposure increased HIF-1α-regulated-gene-transcription, as well as HIF-1α protein levels (80). Treatment with ferrous ions – needed for prolyl hydroxylase to hydroxylate HIF-1α, allowing subsequent

interaction with pVHL and consequent ubiquination - eliminated this increase, which implies that EGCG may be a ferrous ion chelator, thus blocking hydroxylation of HIF-1α (80). Based on these two studies, it appears that green tea catechins possess the ability to regulate HIF-1α, but more studies are needed to clarify the exact nature of this interaction.

Another way that green tea catechins may be able to modulate VEGF signaling is by interfering with its receptor, VEGF-R. It has been shown that *in vitro* exposure of HUVECs to green tea extract will decrease the expression of VEGF-R1 and VEGF-R2 (81). As well, EGCG, catechin-3-gallate, and epicatechin-3-gallate were shown to inhibit phosphorylation of the VEGF-R2 receptor in bovine aortic endothelial cells (82). Similarly, treatment of HUVECs with EGCG was shown to prevent the formation of the VEGF-R2 complex - an essential step in transmitting the VEGF signal from receptors to the nucleus – and thus inhibiting the activity of downstream transcription factors such as NF-KB (83). Together, these three studies suggest that green tea catechins, and EGCG in particular, may very well elicit part of their antiangiogenic ability through inhibition of the expression and activity of the VEGF-Rs.

Finally, there is also evidence suggesting that green tea catechins may inhibit the expression of the potent angiogenic factor, IL-8. EGCG was shown to inhibit the *in vitro* expression of IL-8 in human microvascular endothelial cells (84), and in HUVECs (83). More studies are needed to investigate the effects of green tea catechins on IL-8 expression, but it may very well represent a target for angiogenesis regulation.

Overall, there is strong evidence suggesting a role for green tea catechins, most notably EGCG, in the inhibition of angiogenesis. The exact nature, and extent, of the antiangiogenic capacity requires further clarification.

1.3.3. *Quercetin*

Quercetin is a flavonol found in various fruits and vegetables, including, citrus fruits, apples, leafy green vegetables, broccoli, numerous berries, and tea leaves. There is much interest in its health benefits because of its ubiquitous nature in diets high in fruits and vegetables. *In vitro* and *in vivo* studies have shown that quercetin may exert its health benefits via antiangiogenic mechanisms.

A number of studies have shown that quercetin can inhibit vascular tube formation. One such study, using bovine aortic endothelial cells in a three-dimensional culture system, showed that quercetin inhibited vascular tube formation in a dose-dependant manner (85). Similarly, quercetin also inhibited *in vitro* tube formation of human microvascular endothelial cells (86), and in HUVECs (87). These effects on *in vitro* tube formation are paralleled in *in vivo* models. For instance, quercetin was shown to inhibit vascularization in the chicken chorioallantoic membrane assay (86). Also, quercetin decreased MVD in female Balb/c mice bearing EMT6 mammary tumors (88). There are a number of proposed mechanisms by which quercetin may affect these changes in microvessel formation.

One mechanism by which quercetin may inhibit angiogenesis is by interfering with HIF-1α. Using Chinese hamster ovary cells (A4-4), it was shown that quercetin inhibited a luciferase reporter construct under the control of HIF responsive elements (HRE) (89). HREs are the areas located in the promoters to which HIF binds and induces

transcription. Paradoxically, however, quercetin was shown to induce HRE activity in HeLa cells, by stabilizing HIF-1α, allowing for subsequent nuclear localization of the active transcription factor (90). These conflicting observations suggest that additional study of quercetin's influence on HIF-1α regulation is needed.

As well, quercetin has been shown to inhibit MMP-2 activity in a gelatin zymography assay using human microvascular endothelial cells (86). And, it was shown to inhibit endothelial nitric oxide synthase (eNOS) (an enzyme known to be involved in angiogenesis) in bovine aortic endothelial cells (88), and to arrest the same bovine aortic endothelial cells at early M-phase (88). All of these are plausible antiangiogenic mechanisms for quercetin and warrant further investigation.

It is interesting to note that quercetin is extremely water insoluble. However, this problem was solved by encapsulating the flavonoid in polyethylene glycol 4000 liposomes, which significantly improved the solubility of the compound and its intratumoral accumulation in C57BL/6N mice bearing LL/2 lewis lung cancer xenografts and in Balb/c mice bearing CT26 colon cancer cells and H22 hepatoma cells (91). This emphasizes the importance of bioavailability studies when examining the health benefits of flavonoids.

Furthermore, it is of the utmost importance to note that the differential metabolism of quercetin in the body may very well result in divergent effects on angiogenesis. In humans, quercetin has two main conjugates: quercetin-3'-sulphate (Q3'S) and quercetin-3-glucuronide (Q3G). A study using bovine post-capillary coronary venular endothelial cells (CVEC) and porcine aortic endothelial cells (PAEC) found that Q3'S promoted angiogenesis by stimulating the VEGFR-2, resulting in

downstream activation of the PI3K/Akt signaling pathway and NOS pathway, whereas quercetin and Q3G had inhibitory effects on angiogenesis (92). Therefore, the ultimate activity of quercetin in the human body will be determined by its metabolism after absorption. Understanding this, and possibly learning how to modulate its metabolism, will help clarify quercetin's exact role in angiogenesis and disease prevention.

These few studies seem to suggest that quercetin has some antiangiogenic capacity; however, there are a number of factors, such as water solubility and metabolism, which will determine its ultimate fate after consumption. Only after all of these factors are accounted for could quercetin be effectively used as an antiangiogenic agent.

1.3.4. *Apigenin*

Apigenin is a flavonoid found in a wide variety of herbs, fruits and vegetables, including celery, oranges, onions, basil and parsely. It is a member of the flavone subgroup of flavonoids. There is much interest in the health benefits of apigenin, and a number of studies suggest that it has antiangiogenic properties.

Apigenin has been shown to inhibit *in vitro* tube formation by HUVECs (93). Much evidence suggests that this inhibition of angiogenesis may be through an inhibitory effect on VEGF and HIF-1α. Apigenin has been shown to decrease the *in vitro* expression of VEGF in human umbilical artery endothelial cells (94), human ovarian cancer cells (OVCAR-3 and A2780/CP70) (93), lung cancer cells (A549) (95), prostate cancer cells (PC-3, LNCaP and DU145) (96), colon cancer cells (HCT-8) (96), and breast cancer cells (MCF-7) (96). Likewise, apigenin inhibited VEGF expression *in vivo* in male Balb/cA-nu nude mice with lung cancer (A549) xenografts (95), and in the chicken

chorioallantoic membrane assay using prostate cancer (PC-3) and ovarian cancer (OVCAR-3) cells to induce angiogenesis (96). This down-regulation of VEGF appears to be consequent to an inhibition of HIF-1α.

A number of studies – both *in vitro* and *in vivo* – have shown that apigenin can inhibit HIF-1α expression. *In vitro*, apigenin has been observed to inhibit HIF-1α expression in ovarian cancer cells (OVCAR-3 and A2780/CP70) (93), lung cancer cells (A549) (95), prostate cancer cells (PC-3, LNCaP and DU145) (96), colon cancer cells (HCT-8) (96), and breast cancer cells (MCF-7) (96); *in vivo*, apigenin has been observed to inhibit HIF-1α expression in male Balb/cA-nu nude mice with lung cancer (A549) xenografts (95), and in the chicken chorioallantoic membrane assay using prostate cancer (PC-3) and ovarian cancer (OVCAR-3) cells to induce angiogenesis (96). Apigenin may regulate HIF-1α expression in more than one way.

So far, there appear to be two ways in which apigenin is capable of regulating HIF-1α expression. The first is by modulating cellular signaling pathways known to promote oxygen-independent HIF-1α expression. Apigenin has been shown to inhibit HIF-1α by modulation of PI3K/Akt/p70S6K1 signaling – important in the oxygen-independent regulation of HIF-1α - in ovarian cancer cells (OVCAR-3 ad A2780/CP70) (93), in lung cancer cells (A549) (95), and in the chicken chorioallantoic membrane assay using prostate cancer (PC-3) and ovarian cancer (OVCAR-3) cells to induce angiogenesis (96). The second way that apigenin appears to regulate HIF-1α expression is by interfering with heat shock protein 90 (Hsp 90), a protein suspected to be important for HIF-1α stabilization. Apigenin has been shown to interfere with the Hsp 90/ HIF-1α interaction in human umbilical artery endothelial cells (94), and in prostate cancer cells

(PC-3) (96). Therefore, modulation of the oxygen-independent regulation of HIF-1α and the interaction of HIF-1α with Hsp90 appear to be two routes via which apigenin can inhibit HIF-1α function.

Aside from effects on VEGF and HIF-1α, apigenin has been shown to cause endothelial cell cycle arrest. When treated with apigenin *in vitro*, various types of endothelial cells – including human microvascular endothelial cells – were arrested between the G_2 and M phases of the cell cycle (97). As well, apigenin was shown to inhibit MMP-1 and MT1-MMP expression, and pro-MMP-2 activation in HUVECs stimulated with VEGF and bFGF (98). Also in HUVECs, apigenin blocked the expression of 33kDa uPA, and decreased the activity of 55kDa uPA (98). Thus, apigenin appears to have a strong inhibitory effect on enzymes important in the degradation of the basement membrane.

Overall, apigenin appears to be a promising antiangiogenic phytochemical. More research is needed to verify this and to clarify its mechanisms of action, but these few studies give a good indication of the mechanisms by which it may hinder angiogenesis.

2. Study Rationale and Objectives

2.1. Rationale

Treatment of breast cancer typically involves surgery – either removal of the tumor (lumpectomy), a larger area of the breast, or the entire breast (mastectomy) – followed by adjuvant chemotherapy, radiotherapy, or hormonal therapy. In addition to the emotional disquiet caused by the surgery, patients may suffer many additional side effects from adjuvant therapies, such as skin damage from radiation or impairment of the immune system associated with some types of chemotherapy.

As previously mentioned, antiangiogneic pharmacotherapy represents a relatively new area of research in the treatment in breast cancer. In conjunction with the range of pharmaceuticals being developed to target angiogenesis, a number of plant-based compounds have been found to exhibit anti-angiogenic properties. These plant phytochemicals could serve as viable alternative, or adjuvant, treatments for cancer, accompanied by relatively fewer side effects compared to some of the traditional treatment options. Together, these antiangiogenic compounds present themselves as novel tools to be used both in the treatment, and prevention of this devastating disease.

The flavonoids have proven to be a class of plant polyphenols that are abundant in compounds with potential anticancer properties, including the inhibition of angiogenesis. This lab has preliminary *in vitro* evidence suggesting that the novel isoflavonoid, pomiferin – isolated from the osage orange, an inedible fruit from the *maclura pomifera* plant – has anticancer properties against breast cancer cells.

The main purpose of this study was to use a mouse xenograft model to investigate the utility of this novel isoflavonoid to inhibit *in vivo* breast tumor growth, and to investigate its ability to inhibit the neovascularization of these same tumors.

2.2. Specific Objectives

i) To determine the effect of orally administered pomiferin on the growth of MDA-MB-435 (estrogen receptor-negative) breast cancer cell xenografts in female Balb/c, athymic nude mice. Tumor growth will be assessed using calipers and recorded as millimeters of growth per day.

ii) To determine the effect of orally administered pomiferin on the noevasculaization of MDA-MB-435 (estrogen receptor-negative) breast cancer cell xenografts in female Balb/c, athymic nude mice. Neovascularization will be determined using immunohistochemical staining of endothelial markers on tumor sections and the subsequent calculation of MVD.

2.3. Experimental Hypothesis

Pomiferin will decrease the growth rate of MDA-MB-435 tumors xenografts, as well as decrease tumor neovasculatization.

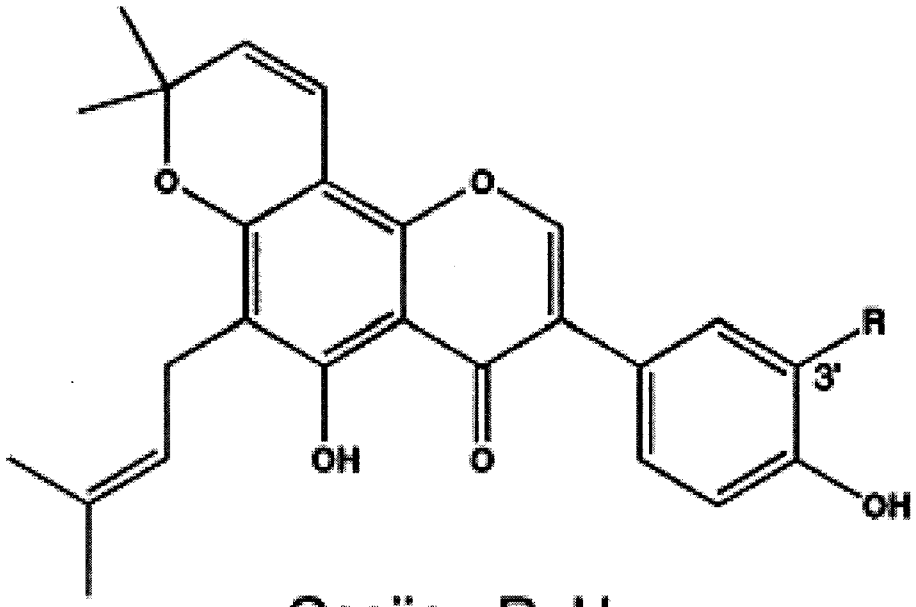
3. The Effects of Pomiferin on MDA-MB-435 Tumor Xenograft Growth Rate and Neovascularization

3.1. Introduction

As discussed extensively above, there is great interest in the use of dietary polyphenolics, specifically flavonoids, to prevent and treat cancer. As such, there is a need to identify and study novel compounds that may be of benefit.

Maclura pomifera is a hardwood tree found in the Midwestern United States and in Ontario, Canada. Its non-edible fruit, known as the osage orange or hedge apple – fittingly named as *Maclura pomifera* is commonly used as a hedge tree – has traditionally been used as an insect repellant and antifungal agent. However, over fifty years ago, the fruit was found to have high levels of the isoflavones, pomiferin and osajin (Figure 3). These compounds evaded investigation for decades until relatively recently, when the health benefits of the soy isoflavones – genistein and daidzein – were being elucidated. The relative rarity of isoflavones in the food supply – as compared to other types of flavonoids – made the osage orange an attractive fruit to study, as the isoflavones it harbors may have uncharacterized health benefits.

Figure 3. Structures of the isoflavones, pomiferin and osajin (100).



Osajin: R=H
Pomiferin: R=OH

A number of studies were conducted to examine the antioxidant potential of these novel isoflavones. It was shown that pomiferin was highly active as an antioxidant, as measured by inhibition of lipid peroxidation and peroxynitrite scavenging ability, whereas osajin showed only a low activity (99). Similarly, pomiferin was shown to be a strong antioxidant using the Ferric Reducing/Antioxidant Power Assay (FRAP) and the beta-carotene-linoleic acid model system (β-CLAMS), whereas osajin, genistein and daidzein showed no antioxidant activity (100). As well, both pomiferin and osajin were able to suppress the oxidative damage caused by ischemia-reperfusion injury in rat hearts, again suggesting antioxidant activity (101). Taken together, these studies strongly suggested that pomiferin acts as an antioxidant, which meant that it may have chemopreventive potential.

Pomiferin has been shown to be effective at inhibiting the *in vitro* growth of some types of cancer cells. It was shown to have cytotoxic effects on human cholangiocarcinoma cells (HuCCA) – a malignant cancer of the bile duct epithelium – by inducing apoptosis (IC_{50} of 0.9μg/mL) (102). As well, an undergraduate student from our lab, Heather Courtney, showed that pomiferin was selectively cytotoxic against MCF-7, human estrogen-receptor positive breast cancer cells (IC_{50} of 5.78 ± 5.16 μM), as compared to MCF-10A breast epithelial cells (IC_{50} of >10μM), wheras osajin did not show this selective cytoxicity (unpublished data). Furthermore, there was no correlation between the antioxidant potential of the isoflavonoids and their antioxidant ability, as measured by the FRAP assay (unpublished data). This selective cytotoxicity against breast cancer cells was a promising discovery and warranted further investigation.

This current study aimed to take the next step by examining the effect of pomiferin on MDA-MB-435 estrogen-receptor negative breast cancer cells *in vivo*, using a mouse xenograft model. In addition to measuring the growth rate of tumors (or rate of cancer cell proliferation), this study also aims to see if pomiferin exerts anti-angiogenic effects on the tumor xenografts. Immunohistochemical staining of tissue sections with an antibody for CD31 platelet/endothelial cell adhesion molecule 1 (PECAM-1 or CD31), and with isolectin GS-IB4 will be done in order to determine MVD - a commonly measured marker of neovascularization in solid tumors. The monoclonal antibody MEC 13.3 (IgG2a) is known to be specific for the murine form of CD31 (or PECAM-1) – found in abundance at the intercellular junctions of endothelial cells- and, hence, efficiently recognizes the endothelial cells of mouse blood vessels (103). Isolectin GS-IB4 is a lectin isolated from the *Griffonia simplicifolia* plant that has been shown to bind alpha-D-galactosyl residues that are predominantly located on mouse blood vessel endothelia (104). Therefore, the co-localization of these two stains should be highly specific for the mouse blood vessels found in the tumors xenografts from this experiment.

3.2. Materials and Methods

This project was a joint effort: PhD student, Raymond Yang, was responsible for materials and methods 3.2.3 and 3.2.4; Raymond Yang and undergraduate student, Lisa Clements, were responsible for materials and methods 3.2.1, 3.2.2, 3.2.5, and 3.2.6; and, MSc student, Matthew Chronowic, was responsible for materials and methods 3.2.7, 3.2.8, 3.2.9, 3.2.10, and 3.2.11.

3.2.1. *Cell Culture*

MDA-MB-435 estrogen receptor-negative breast cancer cells (obtained from Dr. Ken Carroll of the University of Western, Ontario) were cultured in DMEM high glucose medium, which was supplemented with 10% bovine serum and 1% penicillin/streptomyocin. Cell growth – in humidified air at 37°C and 95% air/5% CO_2 – was monitored on a regular basis, passaging cells when appropriate. Counting of cells was done with a haemocytometer prior to xenotransplantation into nude mice. As well, cells were suspended in a 1:1 mixture of media and matrigel (BD Biosciences, Mississauga, ON), and this mixture put onto ice until time of injection. 100µl of this mixture, containing approximately $6.5x10^6$ cells, was injected into the right flank of each mouse.

3.2.2. *Mice*

All mice were female, 5-6 week old, homozygous, athymic nude, Balb/c mice (Simonsen Laboratories, California). After arriving from a pathogen-free isolation facility, they were allowed 8 days to acclimatize, and put into cages in a controlled environment (22°C, 50% humidity, 12 hour light/dark cycle, no more than 6 mice per cage). Ear-hole punching was used to number mice.

3.2.3. *Plant Extracts and Fractionation*

Ethyl acetate (1:4, w/v) extraction of four kilograms of osage orange fruits – picked in late October 2004 – was performed at ambient temperature, followed by passage of the extract through Whatman No. 1 filter paper. After four repetitions of this extraction/filtration process, the filtrates were combined. Approximately 612g of crude

extract was obtained by placing the combined filtrates *in vacuo* at $\leq 40^{\circ}C$, and bringing to dryness.

To purify pomiferin, 50g of crude extract was loaded into a 5x60cm column - column was packed with a slurry of silica gel (70-230 mesh) mixed with solvents (hexane: ethyl acetate, 4:6, v/v) – and eluted with the same hexane and ethyl acetate solvent mixture (hexane: ethyl acetate, 4:6, v/v). After running twelve columns, a total of 5.6g of purified pomiferin was obtained.

3.2.4. *Verification of Pomiferin Purity*

Analysis and quantification of pomiferin was done using an Agilent analytical HPLC serial 1100 system (equipped with a quaternary pump, degasser, thermostatic auto-sampler, and DAD detector). Compounds, at an injection volume of 10µL, were separated using 2% acetic acid in water (solvent A) and acetonitrile (solvent B), passed, at a flow rate of 1 mL/min, through a Phenomenex® 5µ ODS-2 C18 RP (150x4.6mm ID) column - with a C18 guard column (linear gradient elution conditions were: 50% B to 100% B in 15 min, 100% B back to 50% B in 2 min). Pomiferin was identified in two ways: 1) using HPLC equipped with photodiode array detector, and 2) using electrospray ionization-mass spectrometry (ESI-MS), in a negative ion mode. Purity was calculated using the pure standard of pomiferin. Pomiferin prepared in this manner was 88% pure.

3.2.5. *Preparation of Diets*

The control diet was the powdered form of growing rodent diet (AIN-93G) (Research Diets Inc.). Experimental diets (0.02% pomiferin and 0.002% pomiferin) were made by adding purified pomiferin to the control diet in an autoclaved blender; 4.18g and

0.43g of pomiferin were added to 1.83kg and 1.90kg of control diet, respectively. The diets were transferred to separate autoclaved bags and labeled accordingly.

3.2.6. *Tumor Growth-Rate Monitoring*

To ensure that tumor size was not influenced by an inflammatory response associated with injections, tumors were allowed to grow for 12 days. After tumors reached 5.5mm in diameter, mice were randomized into three groups: control diet, 0.002% pomiferin diet, and 0.02% pomiferin diet. Tumor size was measured every two days using calipers for a total of three weeks. The mice received their respective diets *ad libitum* during this 3-week period, and were euthanized after day 21. Whole blood was collected via cardiac puncture, and lung and tumor tissues were harvested. Samples were labeled accordingly and frozen in liquid nitrogen, then stored at -80°C.

3.2.7. *Embedding in OCT*

Tumors were removed from -80°C freezer after eight months and embedded in Shandon Cryomatrix™ (Thermo Scientific) embedding resin. Embedding was accomplished by placing the frozen tumor into a cryotube, covering with Shandon Cryomatrix™, and flash-freezing the capped tube in liquid nitrogen. After flash-freezing, tubes were labeled accordingly and returned to the -80°C freezer.

3.2.8. *Tissue Sectioning*

Individual Shandon Cryomatrix™-embedded tumors were removed from the -80°C freezer and sectioned using a Leica CM3050S cryostat. Sections were cut at 5µm with a carbon steel blade, and mounted on Superfrost Plus® slides (Fisher Scientific). Cryostat object- and chamber- temperatures were both set at -20°C for sectioning. Tumors were initially arbitrarily oriented in the cryostat when sectioning, then removed

from the mount, rotated 180° and sectioned from the opposite side. Therefore, sections were obtained from two distinct sectioning planes per tumor. Slide-mounted sections were then frozen at -80°C.

3.2.9. *Immunohistochemical Staining*

Groups of 10-14 slides were removed from the -80°C freezer and allowed to thaw at ambient temperature for 30 min. A mixture of 50% acetone/ 50% ethanol, cooled for at least 10 min in a -8°C freezer, was used as the fixative. Slides were fixed for 15 min in 50% acetone/ 50% ethanol, in the -8°C freezer. Slides were then washed 3x 3 min in 1x phosphate buffer solution (PBS). Sections were then circled with a Dako pen (Dako) to create a hydrophobic barrier for subsequent incubations. A protein block was done for 35 min using 5% goat serum. Next, sections were incubated with 1:50 CD31 (PECAM-1) rat monoclonal IgG_{2a} (Santa Cruz Biotechnology), raised against mouse endothelioma, for 35 min. Sections were then incubated with 1:200 goat anti-rat IgG-R (conjugated to rhodamine) (Santa Cruz Biotechnology) secondary antibody for 30 min. Slides were then washed 1x 3 min in 1x PBS, and 2x 3 min in 1x PBS with calcium and magnesium. Sections were then incubated with 1:200 isolectin GS-IB4, conjugated to Alexa Fluor 488 (Invitrogen-Molecular Probes) for 60 min. Slides were again washed 3x 3 min in 1x PBS. Sections were then incubated with 1:3600 4',6-diamidino-2-phenylindole (DAPI) dilactate (Invitrogen-Molecular Probes) for 3 min. Finally, slides were washed 3x 3 min with 1x PBS and 1x 3 min with Milli-Q water, and mounted with Dako fluorescent mounting media (Dako).

3.2.10. *Microscopy*

Slides were viewed at 200x magnification using a Leica DMLB microscope, equipped with red, green and blue fluorescent filters. Using a QImaging QICAM Fast1394 camera, twelve images were taken per section: one image of each stain – CD31, isolectin, and DAPI - from four distinct fields. The four fields were chosen randomly from non-necrotic regions of each section. Images were saved using the Q-Capture imaging software. Two sections –from different areas of each tumor - were used, giving a total of eight 200x fields per tumor. Images were saved as TIFF files.

3.2.11. *Calculation of MVD*

Adobe Photoshop was used to analyze, and enhance contrast of images. Corresponding CD31 and isolectin GS-IB4 images were overlaid using Adobe Photoshop to identify co-localization of the stains. The number of distinct, independent microvessels – as identified by co-localization - was counted in each image and recorded in Microsoft Excel. Vessel counts for the eight fields per tumor were totaled and divided by eight to give the average number of vessels per 200x field, for each tumor.

3.2.12. *Statistics*

Tumor growth rate and tumor MVD data are presented as means ± standard deviation. Both tumor growth rate and tumor MVD were analyzed by ANOVA followed by Tukey's Multiple Comparison test for differences between means using GraphPad Prism (version 4.0) for Windows (GraphPad Software Inc., San Diego CA) – this required the assumption that variances in all groups were homogeneous, and that the data from each group was normally distributed. Differences were considered significant if $P < 0.05$.

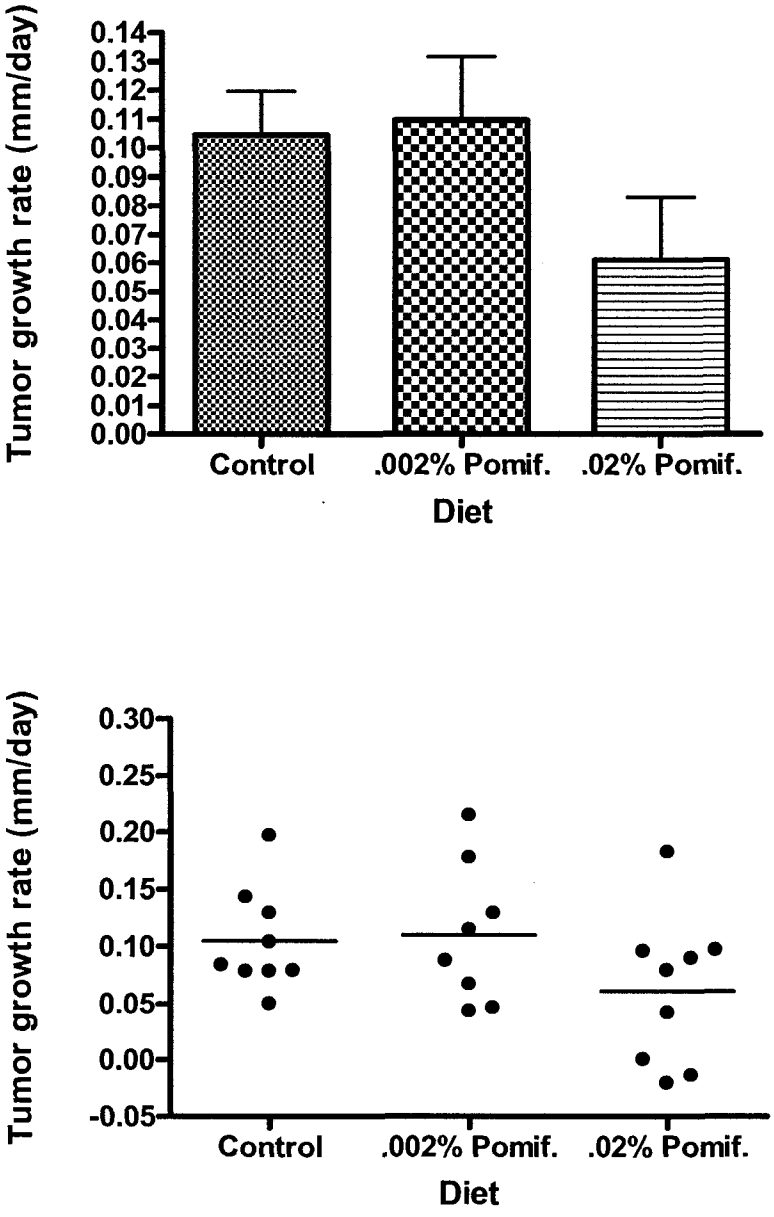
3.3. Results

Oral pomiferin does not significantly decrease MDA-MB-435 tumor xenograft growth rate in athymic nude mice

Approximately 6.5 x 10^6 MDA-MB-435 cells – suspended in a 1:1 mixture of media and matrigel – were injected into the right flank of 5-6 week old, female athymic, nude, Balb/c mice. After tumor xenografts reached 5.5mm in diameter, mice were randomized to one of three groups: control (n = 9), 0.002% pomiferin diet (n = 8), or 0.02% pomiferin diet (n = 9). Respective diets were given *ad libitum* for a three week period, during which tumor diameter was measured every two days using calipers.

This study suggested that there was no significant difference in tumor growth rate between the control mice, the 0.002% pomiferin group and the 0.02% pomiferin group; respective mean growth rates were 0.105 ± 0.045 mm/day, 0.110 ± 0.062 mm/day and 0.061 ± 0.066 mm/day (Figure 4). However, significance was close to being reached between the 0.02% pomiferin and the 0.002% pomiferin groups (p= 0.0981). Furthermore, three mice in the study – all in the 0.02% pomiferin group – actually showed tumor regression during the course of the study.

Figure 4. Tumor growth rate of MDA-MB-435 tumor xenografts in athymic nude mice after 3-week treatment period. Mice bearing MDA-MB-435 tumor xenografts were randomized to a control group (n = 9) or one of two treatment groups – 0.002% pomiferin diet (n = 8) or 0.02% pomiferin diet (n = 9) - for a 3 week period. Tumor diameter was measured using calipers every two days during the treatment period. Data presented as histogram (top) and scatter plot (bottom).



Oral pomiferin does not significantly decrease microvessel density in MDA-MB-435 tumor xenografts in athymic nude mice

Tumors were embedded in Shandon Cryomatrix and frozen at -80°C. 5μm sections were taken from two different regions of each tumor. Sections were stained with an anti-CD31 antibody (raised against mouse endothelioma), isolectin-GSIB4, and with DAPI. Four, random 200x fields of each tumor section were imaged for each of the three stains. Electronic images of CD31 and isolectin-GSIB4 were overlaid to identify co-localization. Vessels, as identified by co-localization, were counted in eight fields from each tumor (four fields from two distinct locations per tumor); these eight counts were subsequently totaled and divided by eight to give the average number of microvessels per 200x field. Figures 5 and 6 are the CD31, isolectin-GSIB4 and overlaid electronic images of a poorly vascularized field; whereas Figures 7 and 8 show the CD31, isolectin-GSIB4 and overlaid electronic images of a well-vascularized field.

Figure 5. Electronic images of a poorly vascularized 200x field from MDA-MB-435 tumor xenograft after 3-week treatment period. The top image shows staining with an anti-CD31 antibody and the bottom with isolectin-GSIB4.

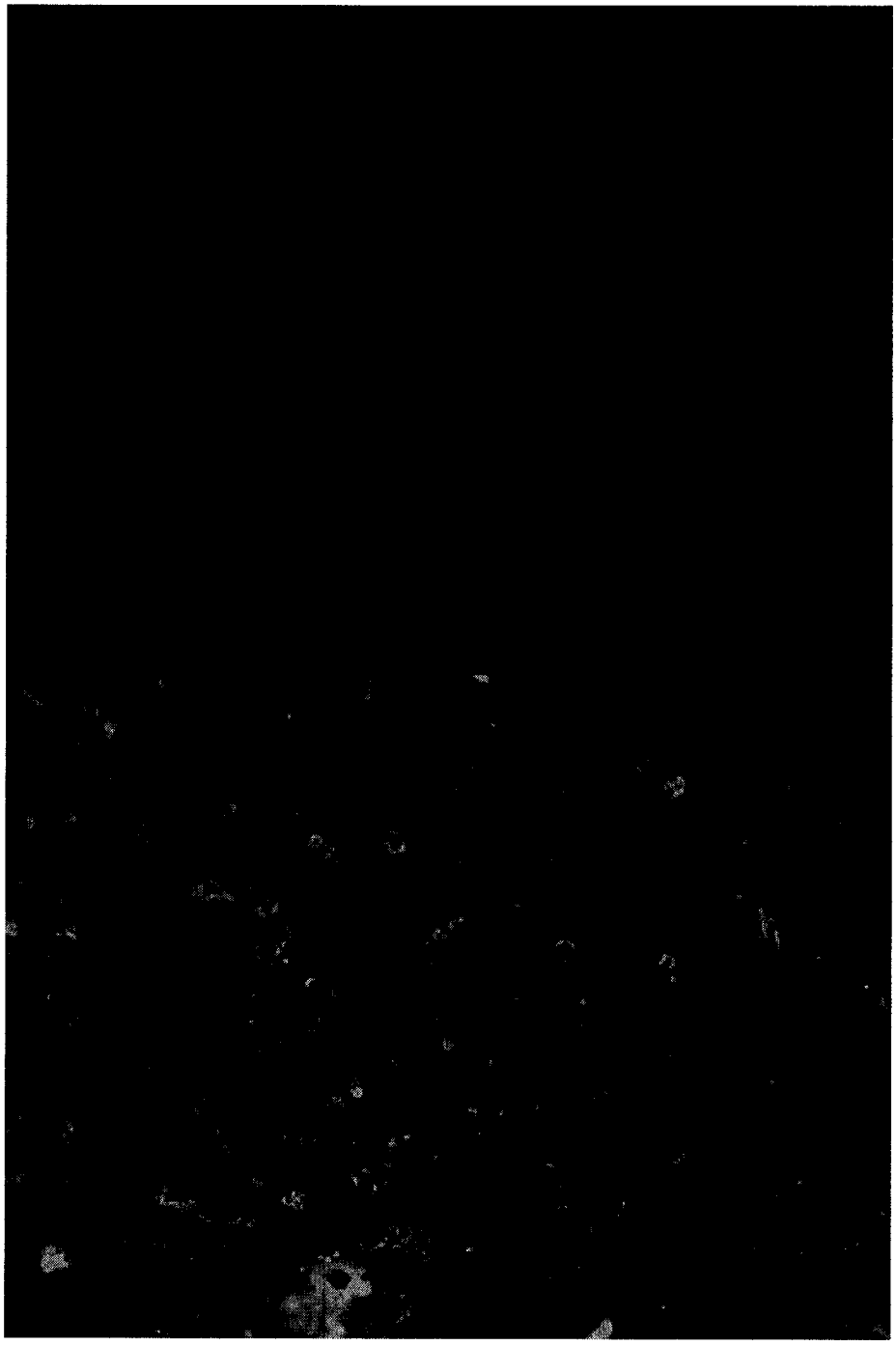


Figure 6. Overlaid electronic images of a poorly vascularized 200x field from MDA-MB-435 tumor xenograft after 3-week treatment period. This image shows the overlaid product of the CD31 and isolectin-GSIB4 images from Figure 5. Co-localization of vessel markers results in a yellow colour.

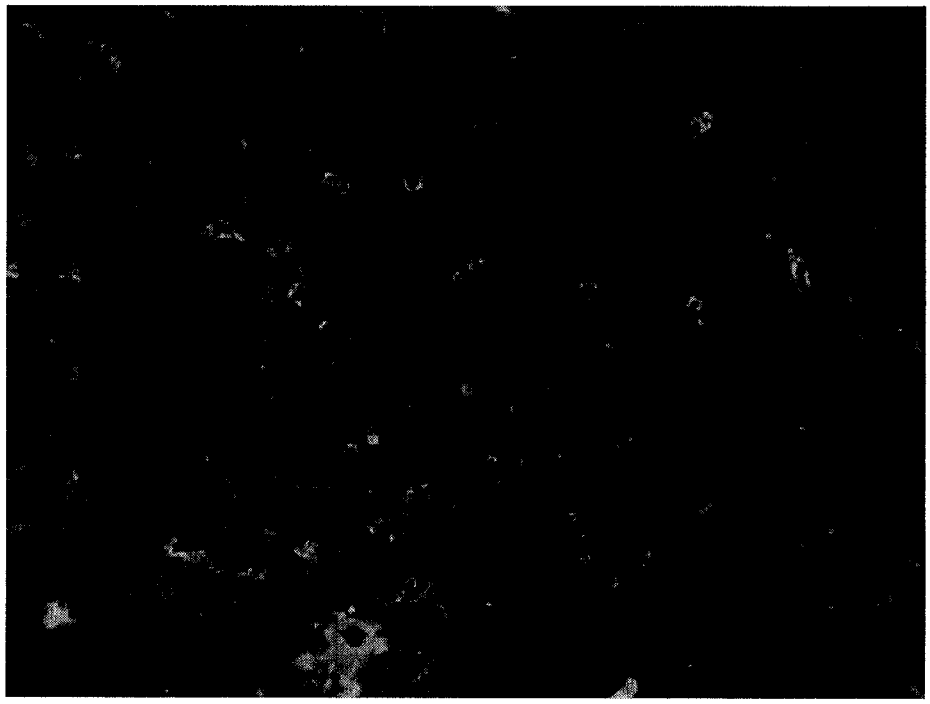


Figure 7. Electronic images of an abundantly vascularized 200x field from MDA-MB-435 tumor xenograft after 3-week treatment period. The top image shows staining with an anti-CD31 antibody and the bottom with isolectin-GSIB4.

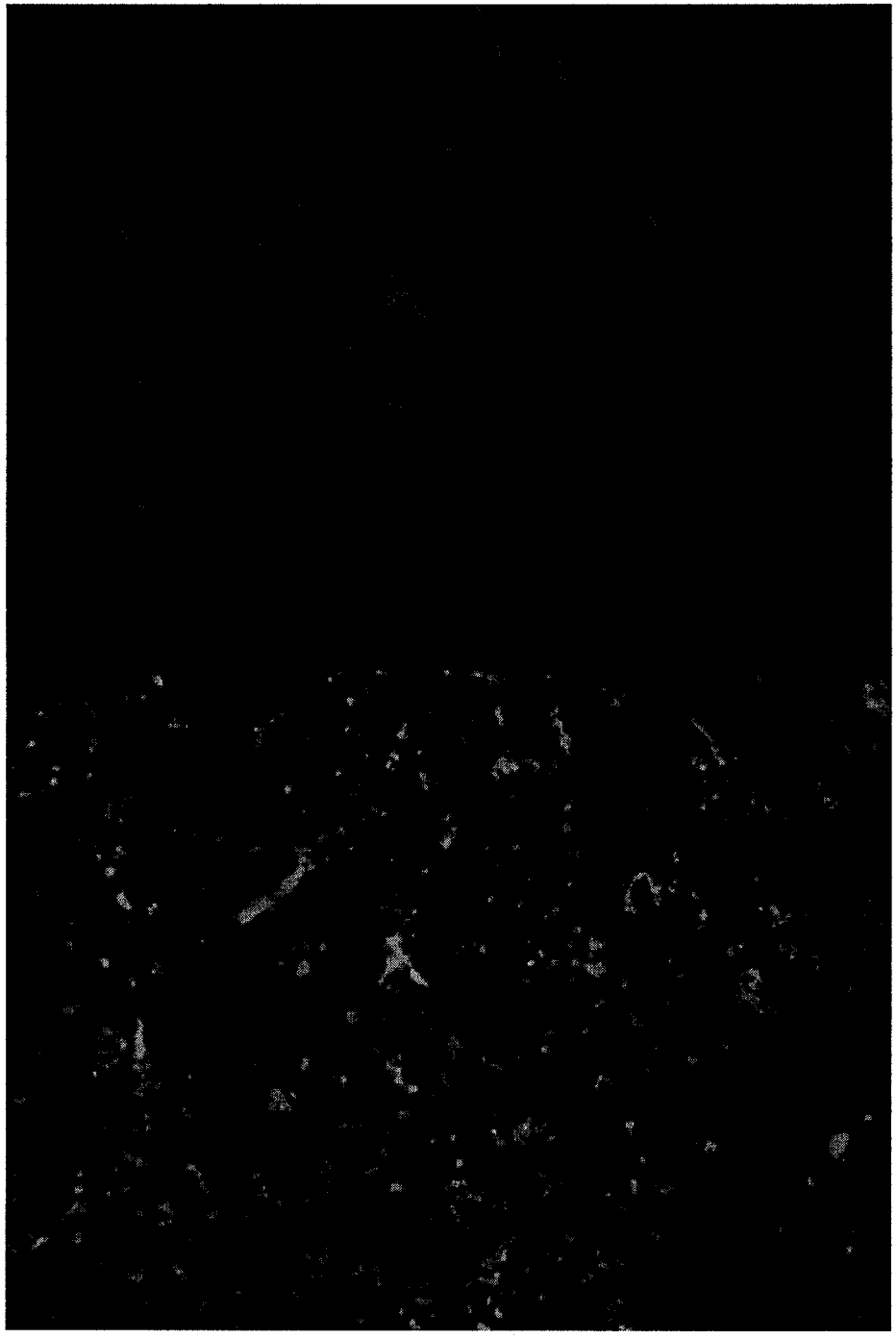
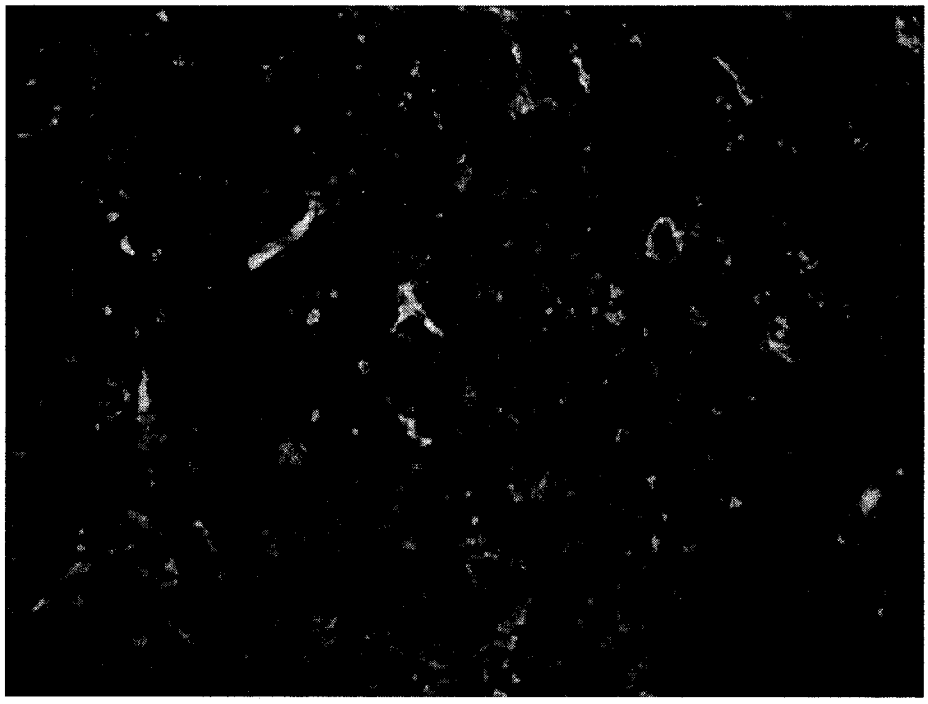


Figure 8. Overlaid electronic images of an abundantly vascularized 200x field from MDA-MB-435 tumor xenograft after 3-week treatment period. This image shows the overlaid product of the CD31 and isolectin-GSIB4 images from Figure 7. Co-localization of vessel markers results in a yellow colour.



Figures 9 and 10 suggest that there was no significant difference in tumor MVD between the control mice, the 0.002% pomiferin group and the 0.02% pomiferin group; respective total MVDs per eight 200x fields were 118 ± 34, 111 ± 22 and 93 ± 46. It should be noted that six of the eight lowest MVD counts (<90 microvessels in eight 200x fields) were in the 0.02% pomiferin group. However, the remaining three tumors in the 0.02% pomiferin group were amongst the seven highest MVD counts (>135 misrovessels in eight 200x fields) in the study, which greatly raised the mean MVD count for the 0.02% pomiferin treatment group.

Figure 9. Microvessel density of MDA-MB-435 tumor xenografts in athymic nude mice after 3-week treatment period. Mice bearing MDA-MB-435 tumor xenografts were randomized to a control group (n = 9) or one of two treatment groups – 0.002% pomiferin diet (n = 8) or 0.02% pomiferin diet (n = 9) - for a 3 week period. Tumors were excised, sectioned at 5μm, and stained with an antibody for CD31, isolectin GS-IB4, and DAPI (all fluorescently labeled). Eight random, 200x fields – four from two different non-necrotic regions per tumor – were imaged using fluorescent microscopy. Microvessles were summed across these eight fields. (Pomif. = pomiferin). See Figure 10 for histogram representation of data.

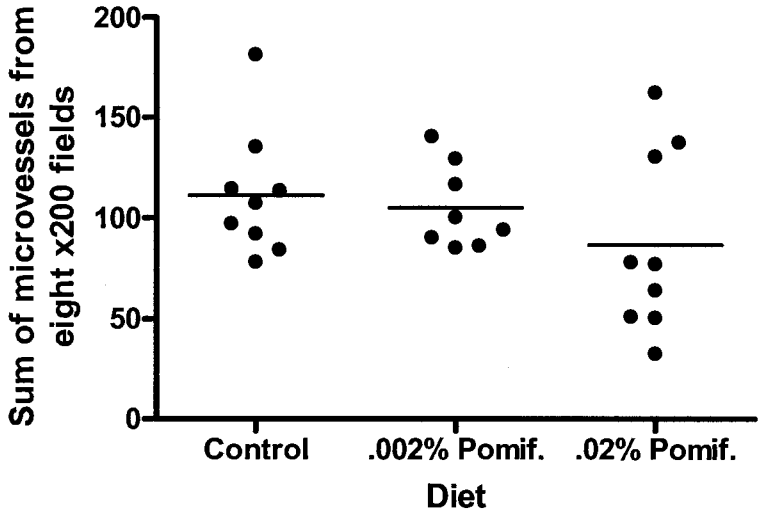
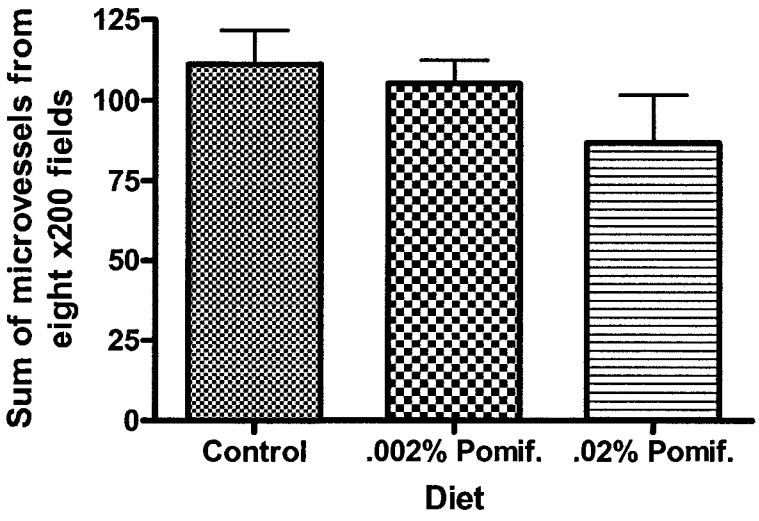


Figure 10. Microvessel density of MDA-MB-435 tumor xenografts in athymic nude mice after 3-week treatment period. Mice bearing MDA-MB-435 tumor xenografts were randomized to a control group (n = 9) or one of two treatment groups – 0.002% pomiferin diet (n = 8) or 0.02% pomiferin diet (n = 9) - for a 3 week period. Tumors were excised, sectioned at 5μm, and stained with an antibody for CD31, isolectin GS-IB4, and DAPI (all fluorescently labeled). Eight random, 200x fields – four from two different non-necrotic regions per tumor – were imaged using fluorescent microscopy. Microvessles were summed across these eight fields. (Pomif. = pomiferin).



3.4. Discussion

In this study, dietary pomiferin had no significant effect on tumor growth rate or MVD in mice bearing MDA-MB-435 tumor xenografts. However, despite not reaching significance, both parameters – tumor growth rate and MVD – did appear to be reduced by dietary pomiferin. There are a number of reasons why significance may not have been reached in this particular study.

A number of studies using breast cancer xenografts in mice have shown that flavonoids can reduce MVD. However, length of treatment, route of flavonoid administration, and breast cancer cell type have varied widely. One study showed that subcutaneous shoulder injections of genistein, given every other day for two weeks, were able to decrease MVD in mice bearing MDA-MB-231 and MCF-7 breast cancer xenografts (46). Another, showed that intra-peritoneal injections of genistein, given daily for five days, were able to decrease MVD in mice bearing F3II and B16FO breast cancer xenografts (47). Yet another showed that intra-peritoneal injections of quercetin, given daily for fourteen days, was able to decrease neovascularization in EMT6 mouse mammary tumor xenografts and in a matrigel plug assay, stimulated by VEGF (88). In addition, green tea extract, given in the drinking water of mice bearing MCF-7 tumor xenografts, only caused a significant decrease in MVD in the green tea plus tamoxifen treatment group, and only after seven weeks of treatment (69). Similarly, green tea extract, given in the drinking water of C3(1)/SV40 transgenic mice, decreased MVD but only after twenty weeks (and not after 8, 12, or 15 weeks) (68). Given that pomiferin treatment in the current study was only three weeks in duration, it is possible that a longer treatment period would further decrease MVD, possibly leading to significant results. As

well, examining the five studies above, only two used an orally administered flavonoid (68, 69), and significant results were only obtained after 7 weeks and 20 weeks in these studies. Other routes of administration – namely intra-peritoneal injection and subcutaneous shoulder injection – elicited a decrease in MVD over a much shorter timeframe: five days (47) or two weeks (46, 88). Differences in metabolism could account for this variation in response time - orally administered flavonoids would be subject to the first-pass effect, where dietary components are passed from the digestive system to the hepatic portal system and to the liver often to be extensively metabolized. Subcutaneous and intra-peritoneal injections, on the other hand, are not subject to the first pass effect, allowing unmetabolized drugs (or flavonoids in this case) to be taken-up by bodily tissues. Given that quercetin and its two different metabolites (quercetin-3'-sulphate Q3'S and quercetin-3-glucuronide Q3G) have differing effects on angiogenesis - with quercetin and Q3G inhibiting the process, and Q3'S promoting the process – it emphasizes the importance of metabolism in the study of flavonoids, as well as their ultimate clinical utility in disease treatment/prevention. It would be of great value to determine how pomiferin is metabolized in the body, and which of its conjugates (or its unmetabolized form) is the most effective at inhibiting cancer and angiogenesis. Furthermore, it is possible that the different breast cancer cell lines (or different models in the case of the C3(1)/SV40 transgenic mice), used in the aforementioned studies, respond differently to antiangiogenic treatment. Therefore, the relatively short 3-week length of the current study, the route of flavonoid delivery (subject to first-pass effect in this instance), and the type of breast cancer cells used in the xenografts may have all contributed to the data being non-significant.

In addition, the small sample sizes of this experiment may have prevented the data from reaching significance. A power calculation suggested that, based on the MVD data collected in this study, treatment groups would have needed to contain 43 mice each to reach significance. Another shortcoming of this study was that tumor sections were only obtained at one time point: the end of the treatment period. This negated the possibility of measuring changes in vessel density that may have occurred over time. Hence, an early decline in MVD followed by subsequent compensation could have been missed.

When tested, antiangiogenic therapies have met a number of physiological hurdles. These shortcomings have been discussed in a recent review article (105). One of the difficulties that antiangiogenic agents face is the heterogeneity observed in tumor blood vessel endothelium (105). For instance, one study showed that approximately 37% of microvascular endothelial cells in B-cell lymphomas carried lymphoma-specific chromosomal translocations (106). This endothelium heterogeneity could, theoretically, result in differing responses to antiangiogenic therapy; in other words, certain endothelial genotypes could be more susceptible to antiangiogenic therapies than others. There is no way to know if the vasculature of the tumors in this current study were homogenous or heterogeneous, but genetic heterogeneity could certainly account inconsistent effects of pomiferin on MVD and tumor growth rate observed in the 0.02% pomiferin treatment group.

Another of the proposed hurdles to antiangiogenic therapy is the compensational response of tumors (105). For instance, the anti-VEGF-R2 monoclonal antibodies, DC101 and RAFL-1, were shown to cause a rapid increase in mouse VEGF, both in non-tumor-bearing-, and tumor-bearing-mice (107). Furthermore, when treated with DC101

(a blocking antibody specific for VEGFR-2), human 253J-BV bladder cancer xeonografts increased the mean vessel size and increased VEGF-R2 levels (associated with HIF-1α expression (108). As well, chronic antiangiogenic treatment of mice bearing Wilms' tumor xenografts resulted in enhanced vascular stability, characterized by significant increases in vessel diameter, vascular cell proliferation, and platelet-derived growth factor-β expression (a factor known to increase integrity of vasculature by recruiting stromal cells) (109). These compensational responses made by tumors may greatly hinder the efficacy of antiangiogenic agents. There is no way of knowing how these factors may have influenced the results in the current study but, the use of a single cell line (MDA-MB-435) to seed xenografts in this experiment should have helped limit the number of differing compensational responses occurring.

Aside from endothelial heterogeneity and compensational responses made by tumors, antiangiogenic therapies are also hindered by the fact that tumors can have angiogenesis-independent methods of acquiring oxygen and nutrients. These methods include vessel cooption (the use of pre-existing blood vessels), intussusception (splitting of an existing blood vessel to create a new one), vasculogenesis (blood vessel formation by *de novo* production of endothelial cells), and vascular mimicry (channels, lined by tumor cells instead of endothelial cells, formed to act as conduits) (105). One study showed that sprouting angiogenesis and intussusception both occur simultaneously in mammary tumors of neuT transgenic mice (110). The importance of these various mechanisms to the multitude of cancers remains to be studied. It is possible, however, that these angiogenesis-independent mechanisms of acquiring nutrients contributed to the inconsistent nature of the data obtained in this particular study.

Measuring the response to antiangiogenic agents is a challenging task. To measure this response directly, the number of new blood vessels per day or the increase in vessel area per day would have to be measured, but this is extremely difficult - if not impossible - to do (111). The most commonly used method to measure this response, and the method used in this study, is to measure the MVD of immunohistochemically-stained tumor sections at one or more time points. However, this method has a number of shortcomings. First, and most importantly of which, is that a decrease in tumor vascularity may be accompanied by a proportional decrease in tumor growth rate, thus giving the appearance of no change in MVD (111). Such an occurrence would greatly reduce the utility of MVD as a biomarker of angiogenesis. Secondly, antiangiogenic agents might remove the less-developed and more inefficient blood vessels, thus creating a more effective vascular network (111). This could be beneficial as a more efficient vascular network may, theoretically, provide other chemotherapeutic agents with easier-access to the center of solid tumors (112). Measuring MVD could not account for the differential elimination of less-efficient blood vessels, as compared to efficient blood vessels. Thirdly, it has been shown that a neutralizing antibody against VEGF (A4.6.1) decreased the vascular permeability in tumor xenografts implanted in mice (113). This decrease in permeability to macromolecules could result in a decrease in interstitial fluid pressure, thus improving the blood flow rate in vessels (111). MVD does not take into account vascular permeability, interstitial fluid pressure, or tumor blood flow rate. All of these could drastically affect tumor perfusion. Finally, MVD is not capable of distinguishing between antiangiogenic effects (preventing the formation of new blood vessels) and antivascular effects (the loss of pre-existing vessels) (111). Both effects

would be beneficial to chemoprevention, but antivascular effects do not necessarily mean that the compound in question has any antiangiogenic ability. While MVD remains the most commonly used method to assess tumor vascularity, it may not accurately represent the physiological effects of the test compound on tumor vasculature. Novel imaging methods incorporating factors such as vascular permeability, tumor interstitial fluid pressure, and tumor blood flow may help clarify the antiangiogenic effects of drugs and phytochemicals.

Dosing is a complicated issue for chemotherapeutic agents, and there is no way to know if the mice in this study received adequate dosing of pomiferin. However, there is much interest in the use of conventional cytotoxic drugs – classically given at or near their maximum tolerated dose (MTD) at infrequent intervals – in metronomic dosing schedules, which provide the drug in doses much lower than the MTD, and much more frequently. Conventional cytotoxic drugs given in these metronomic dosing schedules have been shown to target tumor endothelial cells, thus providing an antiangiogenic effect (107, 114). Since mice in this study were receiving pomiferin in their diet, the dosing schedule would be more representative of a metronomic schedule than a classic MTD schedule. As such, the data collected in this study suggests that pomiferin may be effective in a metronomic dosing schedule, but does not rule out the possibility that it would also be effective, or perhaps more so, if given close to the MTD, which currently remains to be determined.

4. Conclusions and Future Directions

In conclusion, the objective of this study was to determine if pomiferin, administered through the diet, could reduce tumor growth rate and/or tumor MVD in mice bearing MDA-MB-435 breast cancer xenografts. The results of this study suggested that pomiferin could not significantly reduce the tumor growth rate or tumor MVD in this model of breast cancer. Despite not reaching significance, the data did suggest that increasing amounts of dietary pomiferin may reduce both of these variables. A number of study design factors including dose of pomiferin, sample size, treatment duration, route of administration, the number of time-points data were collected, and the limitations of MVD as a biomarker of angiogeneis, could have precluded the data from reaching significance. As well, physiological factors such as endothelial cell heterogeneity, tumor compensation to treatment, and alternative routes of tumor vascularization may, theoretically, have influenced the results.

Future studies should address the shortcomings of this experiment. As well, it is important to determine if pomiferin can modulate the expression of factors related to angiogenesis, including VEGF, HIF-1α, the MMPs (and other proteolytic enzymes), bFGF, and IL-8, in both cancerous cells and endothelial cells. Furthermore, as viable alternatives to MVD arise for the experimental measurement of angiogenesis, the exact role of pomiferin, and other such anti-angiogenic flavonoids, will likely be clarified.

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