MOLECULAR BASIS OF CELL MIGRATION INHIBITION

BY THE DIETARY PHYTOCHEMICAL PIPERINE

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Knowledge is like a baobab tree; one person's arms cannot encompass it.

– Ghanaian proverb

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ABSTRACT

Molecular Basis of Cell Migration Inhibition by the Dietary Phytochemical Piperine

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Metastasis, the migration of cells from a primary tumour site to distant tissues, is associated with the degradation of the epithelial basement membrane and extracellular matrix in connective tissue and often leads to poor prognosis in breast cancer. Matrix metalloproteinases (MMPs) are enzymes that are implicated in the destruction of the extracellular matrix and are overexpressed in breast cancer cells. Naturally occurring plant compounds known as phytochemicals are being investigated as possible anti-cancer agents. Piperine, a major alkaloid component of black pepper, is one such phytochemical that has shown chemotherapeutic effects in early experiments on human and mouse tumour cells. The current study investigates the potential of piperine to prevent cancer metastasis. The overall effect of piperine on MB-MDA-231 breast cancer cell migration was examined using a cell migration (wound-healing) assay. A mechanism of drug action was then explored by determining the effect of piperine on the mRNA transcription, production and activation of the gelatinases, MMP-2 and MMP-9, as well as the stromelysin, MMP-3. The cell migration assay showed that piperine significantly inhibited cancer cell migration at concentrations of 50 μM. RT-qPCR analysis also showed that piperine significantly inhibited MMP-2 and MMP-9 mRNA expression after 48 hours of treatment. Preliminary results from western blot and gelatin zymography analyses indicated that piperine may also inhibit the translation and activity of MMP-2 and MMP-9. Piperine did not appear to affect MMP-3. These results offer further insight into the anti-metastatic action of piperine and suggest a possible novel treatment for aggressive breast cancers.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
ANOVA	Analysis of variance
APS	Ammonium persulfate
β	Beta
BSA	Bovine serum albumin
°C	Degrees celsius
$CaCl_2$	Calcium chloride
cDMEM	Completed Dulbecco's Modified Eagle Medium
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
Ct	Cycle threshold
CXCL12	C-X-C motif chemokine 12
ddH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid

ER	Estrogen receptor
FBS	Fetal bovine serum
h	Hour
HER2	Human epidermal growth factor receptor 2
HRP	Horse-radish peroxidase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
(h)GAPDH	(Human) glyceraldehyde-3-phosphate dehydrogenase
(h)MMP2	(Human) matrix metalloproteinase 2
(h)MMP3	(Human) matrix metalloproteinase 3
(h)MMP9	(Human) matrix metalloproteinase 9
HRP	Horse-radish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
κ	Kappa
kDa	Kilodaltons
ma	Milliamperes
min	Minute
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
NaF	Sodium fluoride
NP-40	Nonyl phenoxypolyethoxylethanol-40
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PMA	Phorbol 12-myristate 13-acetate
PR	Progesterone receptor
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
SYBR®	N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TIMPs	Tissue-inhibitors of matrix metalloproteinases
TNF	Tumour necrosis factor
TPBS	Tween 20 Phosphate-Buffered Saline
TTBS	Tween 20 Tris-Buffered Saline
V	Volts
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Breast cancer continues to be one of the leading causes of cancer-related death in women across the globe, particularly in developed countries including Canada (Jemal, et al. 2011). A 2013 study conducted by the Public Health Agency of Canada, Statistics Canada and the Canadian Cancer Society, estimates that 26.1% of all new cancer cases affecting women will be breast cancer cases (Canadian Cancer Society's Advisory Committee on Cancer Statistics 2013). Meanwhile, by the same study, breast cancer represents only 0.2% of cancer cases in men. The report also stated that in women, 13.9% of those affected with breast cancer succumb to the disease, making it the second leading cause of cancer death among Canadian women. Although treatment options have improved over the past decade, there remain significant limitations when treating more aggressive forms of breast cancer (Al-Hajj, et al. 2003). Moreover, these treatments are often expensive and inaccessible in low-income settings (Bines and Eniu 2008).

Hormonal therapy and chemotherapy are two forms of breast cancer treatment. They comprise of the use of compounds that stop or slow down the progression of cancer. Unfortunately, hormonal therapy is infective in many breast cancer patients (Hayashi and Yamaguchi 2008). Furthermore, patients that undergo chemotherapy often suffer extreme side effects. These include anaemia, changes in appetite, irregular bowel movements, fatigue, nausea and vomiting (Lenz 2012). The surgical excision of a tumour is another option for treating breast cancer. However, studies are showing that this form of treatment may be associated with the potential of an accelerated relapse as a result of the release tumour-promoting proteins during the procedure (Pagani, et al. 2010). Finally, breast

cancer patients may be exposed to high levels of radiation by undergoing radiotherapy. Radiotherapy also has adverse side effects with almost 50% of patients experiencing acute encephalopathy and other nervous system disorders (Soussain, et al. 2009). The development of safe, effective and inexpensive anti-cancer drugs is therefore needed to reduce mortality and increase the quality of life in breast-cancer patients.

1.2 Breast Cancer Metastasis

Perhaps the single most significant contributing factor to poor prognosis in patients is the migration of cells from the primary tumour to a distant tissue (Stasinopoulos, et al. 2010). This process is known as metastasis (Ramaswamy, et al. 2003). According to Nguyen and Massagué (2007), about 90% of deaths from solid tumours are a direct result of metastasis. Tumour cells from different cancers have preferences for metastasis to particular organs (Nicolson 1993). Breast cancer tumours often form secondary sites in osseous, lung and hepatic tissues (Weigelt, Peterse and van't Veer 2005). Preferences for breast cancer cells to these tissues are primarily due to chemokine signalling (Müller, et al. 2001). Although metastasis is a highly complex, multi-event process, many of the key steps involved have been described and are collectively called the metastatic cascade. They include stages of invasion, vascularization and the modification of normal cell adhesion and motility properties (Stasinopoulos, et al. 2010).

At least three of the steps included in the metastatic cascade require some degradation of extracellular matrix (ECM) in order to facilitate cell migration (See Figure 1.1). The ECM is composed of a network of proteins secreted by mesenchymal cells and holds epithelial tissue in place in the organ system (Alberts, et al. 2002). For this reason, the destruction of ECM is particularly relevant in cancers of epithelial origin, termed

carcinomas, of which breast cancer is an example (Kramárová and Stiller 1996). Epithelial cells, by definition, are anchored to a basement membrane, preventing locomotion. They also lack an extensive network of vessels and their main function is to provide protection to the underlying tissues (Alberts, et al. 2002). Depending on the type and tumour location, cancer cell metastasis involves of one or more of several modes. First, cancer cells must detach themselves from the basement membrane in order to become free to move into the circulatory system. This entry of cells into the circulation is known as intravasation. ECM surrounding the epithelium must also be degraded to allow the restricted cells to migrate. Finally, cancer cells leave the circulation to colonize distant tissues by penetrating blood or lymphatic vessels, a process known as extravasation (Deryugina and Quigley 2006). The degradation of ECM to facilitate cancer metastasis is thus an important characteristic of malignancy in carcinomas, including breast cancer.

The development of new blood vessels from established vasculature, known as angiogenesis, is also a significant contributing factor to cancer cell malignancy. In fact, angiogenesis is often described as the rate-limiting step in tumour growth because tumours cannot grow beyond a certain critical size without the necessary supply of oxygen and nutrients by blood vessels (Zetter 1998). While angiogenesis occurs in normal physiological situations such as wound-healing, it is the hypoxic conditions and growth factor milieu in the tumour microenvironment that initiate the transcription of pro-angiogenic factors, including angiopoietin-2 (Ang-2) and fibroblast growth factor-2 (FGF-2) (Carmeliet and Jain, 2000). Vascular endothelial growth factor-A (VEGF-A) however, is the predominant inducer of angiogenesis (Carmeliet and Jain 2011). Research has demonstrated that the interaction between VEGF-A and its receptor tyrosine kinase, VEGF receptor-2 (VEGFR-2,) is primarily responsible for angiogenesis associated with

tumours. VEGFR-2 is one of three related receptor tyrosine kinases, the other two being VEGFR-1 and VEGFR-3 (Clauss 2000). There is growing evidence to suggest that VEGFR-1 exhibits some pro-angiogenic properties in cancer cells by recruiting monocytes and other cells that increase tumour vasculature (Ferrara and Kerbel 2005). In normal cells, signalling to stimulate the formation of blood vessels is highly regulated with the three VEGF receptors being implicated on multiple levels in the pathway (Hanahan and Weinberg 2011). Mechanisms for controlling angiogenesis are largely unregulated in tumour cells and, as a result, tumours are characterized by irregularly shaped blood vessels that are leaky with disrupted blood-flow (Baluk, Hashizume and McDonald 2005). Perhaps not surprisingly, in order for angiogenesis to occur, the ECM must be remodelled to allow for the migration of the endothelial cells that will form the new vasculature (Cheresh and Stupack 2008).

Cancer cells also differ immensely in their adhesion properties in comparison to healthy cells. One characteristic of epithelial cells is that that they possess adhesion molecules that enable them to form adherens junctions with one another and also attach to components of the ECM. Cell-cell adherens junctions such as these restrict the migratory ability of cells and regulate their proliferation (Conacci-Sorrell, Zhurinsky and Ben-Ze'ev 2002). The disruption of normal adhesion in cancer cells is exemplified in the downregulation of E-cadherin in many carcinomas. E-cadherin is a transmembrane glycoprotein that is involved in the formation of adherens junctions between epithelial cells by interacting with catenin and actin components of the cytoskeleton. Decreased expression of E-cadherin is associated with increased invasiveness and metastasis by cancer cells (Hanahan and Weinberg 2011). On the other hand, a related protein, N-cadherin, has been shown to promote invasion and motility in breast cancer cells (Nieman,

et al. 1999). The downregulation of E-cadherin and upregulation of N-cadherin in carcinomas is often termed the cadherin switch and constitutes part of a broader phenomenon known as the epithelial-to-mesenchymal transition, a reference to the transformation of non-motile epithelial cells to cancer cells that possess mesenchymal cell-like motility and properties (Bacac and Stamenkovic 2007). Other cell membrane receptors, known as integrins, primarily associate with glycoproteins in the stromal ECM. Integrins act to regulate the migratory ability of cells by controlling adhesion to the ECM (Hynes 1992). The expression of integrins in cancer cells is complicated and not well understood but does seem to depend on the stage of tumour development (Guo and Giancotti 2004). Nonetheless, integrins and related adhesion molecules play an important role in the regulation of normal physiological processes in the body and their modification is implicated in the development of cancer.

1.3 Matrix Metalloproteinases

Recently, studies investigating cancer metastasis have turned much attention towards a group of peptidases known as matrix metalloproteinases or MMPs (Egeblad and Werb 2002). In humans, MMPs are a group of at least 23 enzymes, characterized by their ability to degrade peptide elements in the ECM (See Table 1.1). They are further classified by their substrates into six different families namely, the collagenases, gelatinases, stromelysins, membrane-type MMPs, matrilysins and other MMPs (Fink and Boratynski 2012). All of these enzymes are similar in that their active sites contain a Zn^{2+} ion, but differ in their location in the cell and substrate specificity. Most MMPs are secreted as zymogens, which are non-functional precursors proteins that are activated extracellularly. MMPs are naturally inhibited by a group of enzymes known as tissue-inhibitors of matrix metalloproteinases (TIMPs) (Bauvois 2012). MMPs are among the

most recently discovered enzymes, with the first MMP being isolated in 1962 (Gross and Lapiere 1962), but are quickly becoming some of the most studied. Despite increased research into their function, MMP mechanism of action and function remains poorly understood and is highly debated. In addition to causing proteolysis of the ECM, which facilitates metastasis and stimulates the release of growth and angiogenic factors, some MMPs can also cleave E-cadherin, thereby modifying cellular adhesion properties and enhancing cell motility (Moss, Jensen-Taubman and Stetler-Stevenson 2012).

The discovery of the link between MMPs and ECM destruction and cancer metastasis led to a strong interest in MMP activity and many researchers worked to develop compounds that could either serve as MMP inhibitors or activators of TIMPs. These compounds tended to act on a broad spectrum and included zinc chelators and MMP antagonists (Noël, et al. 2012). Examples of such drugs include a variety of carboxylic acid, thiole and thioether compounds (Dormán, et al. 2010). Clinical trials for these treatments, however, produced disappointing results with some compounds actually worsening the patient prognosis (Hidalgo and Eckhardt 2001). Moreover, the adverse side effects induced by many of the synthetic MMP inhibitors prevented the administration of adequate doses of drug (Coussens, Fingleton and Matrisian 2002). New research suggests that the "bulldozer" model, which predicts that all MMPs promote cell migration by degrading the surrounding ECM, is flawed. Studies have demonstrated that certain MMPs may in fact have tumour suppressing roles during tumorigenesis. The downregulation of MMP8, for example, is associated with increased risk of tumour development in mice (Balbin, et al. 2003). Likewise, MMP11 inhibits tumour growth while MMP19 reduces angiogenesis in tumour cells (Noël, et al. 2012). Noel et al. (2012) called this paradoxical relationship among MMPs the "Brake and Booster" model in reference to the stimulatory

and inhibitory effects that different MMPs have on cancer metastasis. Although the exact mechanisms involved in MMP activity remain under debate, researchers agree that certain MMPs play a critical role in ECM remodelling and, therefore, cancer metastasis (Balduyck, et al. 2000). These MMPs are overexpressed in cancer cells and compounds that are selective in their inhibition of such MMPs are showing promise as possible treatments for cancer (Syed, et al. 2004).

Included in the list of possible MMP targets are gelatinase A and B, also known as MMP2 and MMP9, respectively, whose increased expression correlates with increased malignancy in breast cancer (Egeblad and Werb 2002). Both MMP2 and MMP9 are characterized by their ability to degrade gelatin (John and Tuszynski 2001), which is denatured collagen, the most abundant protein in the human body (Di Lullo, et al. 2002) and a major polypeptide in the basement membrane of mammary tissue (Monteagudo, et al. 1990). Another enzyme of interest is MMP3, which happens to be one of the least studied MMPs (Klein and Bischoff 2011). MMP3 is believed to degrade a wide range of substrates including fibronectin and laminin (John and Tuszynski 2001). In addition, there is evidence that MMP3 may play a role in activating and deactivating other MMPs including MMP2 (Klein and Bischoff 2011). All three MMPs are implicated in human breast cancer cell angiogenesis and metastasis (Balduyck, et al. 2000; Brasse, et al. 2010; Farina, et al. 2011) and thus merit further study.

1.3.1 Gelatinase A: MMP2

MMP2 is one of only two known gelatinases, the other being MMP9. Liotta et al. (1979) first discovered MMP2. Pro-MMP2 has a cysteine residue coordinated to the Zn^{2+} ion in the active site (Mecham and Parks 1998). Cleavage of this domain leads to activation of MMP2. The activation of pro-MMP2 is of particular interest to researchers

as it differs from that of other MMPs. MMP2 associates with TIMP2 and this interaction is believed to lead to the transformation of the zymogen into a functional protein. In addition to ECM remodelling, MMP2 is believed to be involved in other physiological processes including modifying cell adhesion and mitotic activity (Brooks, et al. 1996). Regulation of MMP2 is largely achieved by affecting the activation of pro-MMP2, specifically by influencing the interaction between MMP2 and TIMP2 (Mecham and Parks 1998). Interestingly, studies have shown that the MMP2-TIMP2 complex is significantly reduced in breast cancers, indicating that the enzyme may be activated by other means in tumour cells (Mecham and Parks 1998).

Substrates for MMP2 are not limited to gelatin alone but also include type V, VII, X collagens and elastin (Mecham and Parks 1998). Gianelli et al. (1997) showed that the degradation of laminin-5 in the basement membrane by MMP2 contributed significantly to the enhanced migration of breast cancer cells. Laminin-5 interacts with epithelial cell integrins, which facilitates cell adhesion. Disruption of this interaction, therefore, reduces cell adhesion and promotes cell motility (Hayashida, et al. 2010). Recent studies also show that MMP2 may play a critical role in the processing of various signalling proteins (Klein and Bischoff 2011). MMP2 does not appear to possess any tumour-suppressing properties unlike some other MMPs. Rather, a high correlation of MMP2 overexpression and cancer metastatic potential suggests that MMP2 promotes cancer metastasis (Qin, et al. 2008). Many studies have shown that increased MMP2 expression in cells leads to increased tumour invasiveness and angiogenic activity (Egeblad and Werb 2002).

1.3.2 Gelatinase B: MMP9

At 92kDa, pro-MMP9 is the largest of all known MMPs and also one of the most structurally complex. MMP9 is activated by the proteolysis of a prodomain, in which the

Zn^{2+} active site is bound to a cysteine residue (Van den Steen, Dubois, et al. 2002). This is known as the cysteine-switch mechanism, as described by van Wart and Birkedal-Hansen (1990). The hemopexin-domain in MMP9 is important for regulation by TIMPs (Mecham and Parks 1998). MMP9 is most effective at degrading gelatin, although MMP9 can also cleave collagen type II and collagen type V with some efficiency (Van den Steen, Proost, et al. 2002). Interestingly, MMP9 has been shown to affect other proteins not found in the ECM, as it can cleave transforming growth factor – β (Yu and Stamenkovic, 2000) and interleukin (IL)-2 receptor-α (Sheu, et al. 2001) and may also affect the activation of MMP3 (Olson, et al. 2000).

MMP9 is regulated by changes to gene expression as well as protein secretion, activation, glycosylation and the presence of inhibitor proteins (Van den Steen, Dubois, et al. 2002). In epithelial cells, transcription of MMP9 is stimulated by several molecules including phorbol 12-myristate 13-acetate (PMA) in conjunction with IL-1-α and tumour necrosis factor (TNF)-α (Van den Steen, Dubois, et al. 2002). Like most MMPs, MMP9 is released as a zymogen and activated extracellularly by TIMP1 and proteinases, like trypsin, and even other MMPs including MMP3 (Van den Steen, Dubois, et al. 2002). MMP9 is also involved in angiogenesis and can induce the release of VEGF-A, making it one of the most researched MMPs by oncologists (Bergers and Benjamin 2002).

1.3.3 Stromelysin-1: MMP3

In the literature, MMP3 appears to be one of the least studied of all MMPs. Active MMP3's molecular weight is 54kDa and it was first discovered in 1974 (Sapolsky, Howell and Woessner Jr. 1974). MMP3 is transcribed in the cell as a pre-proenzyme, which is then secreted into the extracellular environment as a proenzyme, where it is subsequently activated. Like the gelatinases, MMP3 is activated by the cysteine switch

mechanism. MMP3 seems to have a broad spectrum of substrates, including different types of collagen, fibronectin, laminin, and gelatin (Klein and Bischoff 2011). MMP3 has been shown to activate other MMPs such as MMP1, MMP8, MMP9 and MMP13 (Mecham and Parks 1998). Regulation of MMP3 is generally achieved by influencing transcription and translation rather than activation (Mecham and Parks 1998).

The role of MMP3 in cancer metastasis has not been clearly established. MMP3 is overexpressed in certain cancers such oesophageal carcinomas but is absent in colon and gastric carcinomas (Mecham and Parks 1998). MMP3 is upregulated in mammary tissue after the lactation period and may induce apoptosis in certain cells (Witty, Wright and Matrisian 1995). Some studies also show that MMP3 may influence cell adhesion properties by inducing the release of adhesion molecules such as E-cadherin (Noë, et al. 2001).

1.4 Piperine: A Dietary Phytochemical

Phytochemicals are defined as the bioactive non-nutrient compounds that are found naturally in foods from plants (Liu 2003). They include carotenoids, alkaloids, phenolics, nitrogen-containing compounds and organosulfur compounds (Liu 2004). The clinical benefits of a diet rich in phytochemicals is well documented. Antioxidants in apples reduce the incidence of cardiovascular disease, cholesterol levels and lipid oxidation (Boyer and Liu 2004). Recently, there has been increased research into phytochemicals as possible agents in cancer therapy. Curcumin, found in tumeric, possesses chemopreventive properties including cytotoxicity in tumour cells (Teiten, et al. 2010). Thymoquinone from black caraway seeds reduce breast cancer cell viability (Sutton, Doucette and Hoskin 2012). [8]-Gingerol and [6]-gingerol from ginger have inhibit murine melanoma tumour progression (Huang, et al. 2013). Phytochemicals are

particularly attractive to scientists and clinicians as medications because they tend to be well tolerated, have minimal side effects and can be produced at a lower cost when compared with other pharmaceuticals (Kadam, et al. 2012).

One of the more promising phytochemical drugs is piperine, the active ingredient in black pepper (*Piper nigrum*) and long pepper (*Piper longum*) (See Figure 1.2). Piperine is responsible for the pungency associated with black pepper, as it constitutes 5-7% of the total mass (Graham 1965). It is classified as an alkaloid and is derived from the amino-acid, lysine (Koleva, et al. 2012). The range of physiological benefits of piperine is broad. Piperine possesses antioxidant effects, stimulatory effects on pancreatic digestive enzymes and the ability to increase digestive capacity (Srinivasan 2007). In addition, piperine increases the bioavailabilty of drugs and nutrients in the body (Jin and Han 2010) and is effective as an anti-inflammatory drug by inhibiting cyclooxygenase-2 expression in murine macrophages (Kim, et al. 2012).

Piperine potentially possesses some chemotherapeutic properties in different cancers. One study demonstrated that piperine caused apoptosis in rectal cancer cells by inducing reactive oxygen species (Yaffe, et al. 2013). Piperine has also been reported to suppress the development of lung (Selvendiran, Banu and Sakthisekaran 2004) and breast cancers (Kakarala, et al. 2010). Piperine inhibits angiogenic activity in human umbilical vein endothelial cells (Doucette 2012). Piperine may have potential as a chemotherapeutic agent with both anti-angiogenic (Doucette, Hilchie, et al. 2013) and pro-apoptotic activity (Lin, et al. 2014). The effects of piperine on the human body do not seem to be detrimental to human health (Srinivasan 2007). For the reason that cancer metastasis is indicative of more severe disease and poor clinical prognosis, it is important to examine

the anti-metastatic properties of piperine in human breast cancer cells and investigate a molecular basis of this action.

1.5. Studying Cancer Metastasis *In Vitro*

While *in vivo* studies are usually best for establishing concrete conclusions about the behaviour of cancer cells in a more complex system, they are often costly and time-consuming (Welch 1997). *In vitro* studies are therefore a good first-step in studying the effect of a drug on cancer cells as they are not only cost-effective, but also allow for the elimination of confounding variables. Many molecular and non-molecular techniques for studying cancer cells *in vitro* have been reported in the literature. A few are described here.

1.5.1 Cell Migration Assays

A common method for investigating the metastatic potential of carcinoma cells is by observing their migration relative to normal cells (Valster, et al. 2005; Doucette, et al. 2013). The technique that is often used is a wound-healing assay, whereby a monolayer of epithelium is "wounded" by scratching with a pipette tip thus creating an area devoid of cells (Peng, et al. 2012; Doucette, et al. 2013). The wound is then observed overtime and the rate at which migrating cells fill the void is calculated. By comparing the rates among cells under different conditions, one can determine how much more migratory one set of cells is as compared to another (Rodriguez, Wu and Guan 2005). The wound-healing assay is appealing to scientists because it is simple and inexpensive (Liang, Park and Guan 2007). However, because the size of the void created by the pipette tip is dependent on the pressure applied, which tends to vary, wound-healing assays tend to yield results that are sometimes not reproducible. Furthermore, an added complication arises when the void takes longer than 24 h to be filled by migrating cells as the migratory ability of cells

becomes confounded with cell proliferation. This problem can be at least partially resolved by treating cells with compounds such as mitomycin C that inhibit mitosis (Tomasz 1995).

1.5.2 RT-qPCR

Changes in gene expression are particularly important in understanding the molecular basis of cancer progression. Many cancer-causing agents act by controlling transcription in cells. Drugs that inhibit, stop or reverse these effects may therefore hold promise as anti-cancer agents. Real time – quantitative polymerase chain reaction (RT-qPCR) is a method used to study changes in cellular transcription (Bustin 2000). The technique measures the amount mRNA by first isolating mRNA transcripts and then using the enzyme, reverse transcriptase, to synthesize complementary strands. The new, double-stranded molecules are essentially DNA molecules that mimic the DNA molecule from which the mRNA was originally derived. DNA polymerase can then amplify the newly synthesized cDNA. In RT-qPCR this amplification is monitored over time. The number of cycles taken for a transcript of interest to reach a threshold number is noted. A higher number of cycles required is associated with fewer mRNA transcripts present.

1.5.3 Western Blot

Western blotting was first described by Towbin et al. (1979) and is still commonly used by scientists to monitor changes in the expression of protein in cells. Various proteins, including MMPs, are implicated in several pathways that are associated with the development of cancer. The upregulation or downregulation of specific proteins can yield valuable information concerning the pathology of certain cancers. Western blots are developed by performing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on proteins isolated from cells. This technique separates proteins based on

their molecular weight. The separated proteins are then transferred to a nitrocellulose membrane and probed with antibodies that are specific to the protein of interest. Recently, chemiluminiscent reagents have been developed to help quantify isolated proteins (Kurien and Scofield 2006). Blots are washed in these reagents and exposed to X-ray film in order to visualize protein bands. The intensity of each band corresponds to the quantity of protein in that sample.

1.5.4 Gelatin Zymography

When considering the role that the gelatinases play in cancer metastasis, gelatin zymography is a powerful technique that can be used to determine gelatinase activity in cells (Woo, et al. 2005). In this assay, proteins in tissue culture supernatants are collected and separated using SDS-PAGE in a polyacrylamide gel enriched with gelatin. The gel is then stained and destained until clear bands where the gelatin has been degraded appear against a darkened background. These bands represent gelatinolytic activity and their intensity corresponds to the level of activity. Gelatin zymography has been shown to be sensitive to not just MMP2 and MMP9, but other MMPs, such as MMP3, which are able to degrade gelatin albeit at a lower efficiency (Troth and Fridman 2001).

1.6 Summary

Considering the prevalence and high mortality rates associated with breast cancer, there is an urgent need to develop novel, more effective treatments for the disease. Perhaps more importantly, these treatments should be cost-effective and well tolerated in order to ensure that they are accessible to patients regardless of their socioeconomic status. The dietary phytochemical piperine has potential as an anti-cancer treatment. The effects of piperine on components breast cancer metastasis therefore merit further investigation.

1.7 Objectives

This study will determine the effect of piperine on cancer cell migration by investigating MMP mRNA transcription, MMP protein expression and MMP activation in MDA-MB-231 breast cancer cells. This research is also aimed at understanding the mechanism of how piperine effects breast cancer cell migration. Piperine has been reported to exhibit cytotoxic effects on cancer cells at concentrations of 250 μM and higher (Sulina and Kuttan 2004). Therefore, the current study will focus on cell migration using piperine at the non-cytotoxic concentrations of 25 μM and 50 μM. The effect of piperine at these concentrations will be tested on three different MMPs, namely MMP2, MMP9 and MMP3.

This research will determine the effect of piperine on the following physiological processes.

1. Cell migration
2. MMP mRNA expression
3. MMP protein expression
4. MMP activation

It is expected that piperine will inhibit breast cancer migration by inhibiting MMP mRNA expression and therefore protein expression and activation. Results from this study will provide further support in the development of piperine as a treatment for breast cancer.

Figure 1.1. The Metastatic Cascade. Figure shows some of the key steps involved in breast cancer metastasis that require ECM degradation. Figure modified from Onkal and Djamgoz (2009).

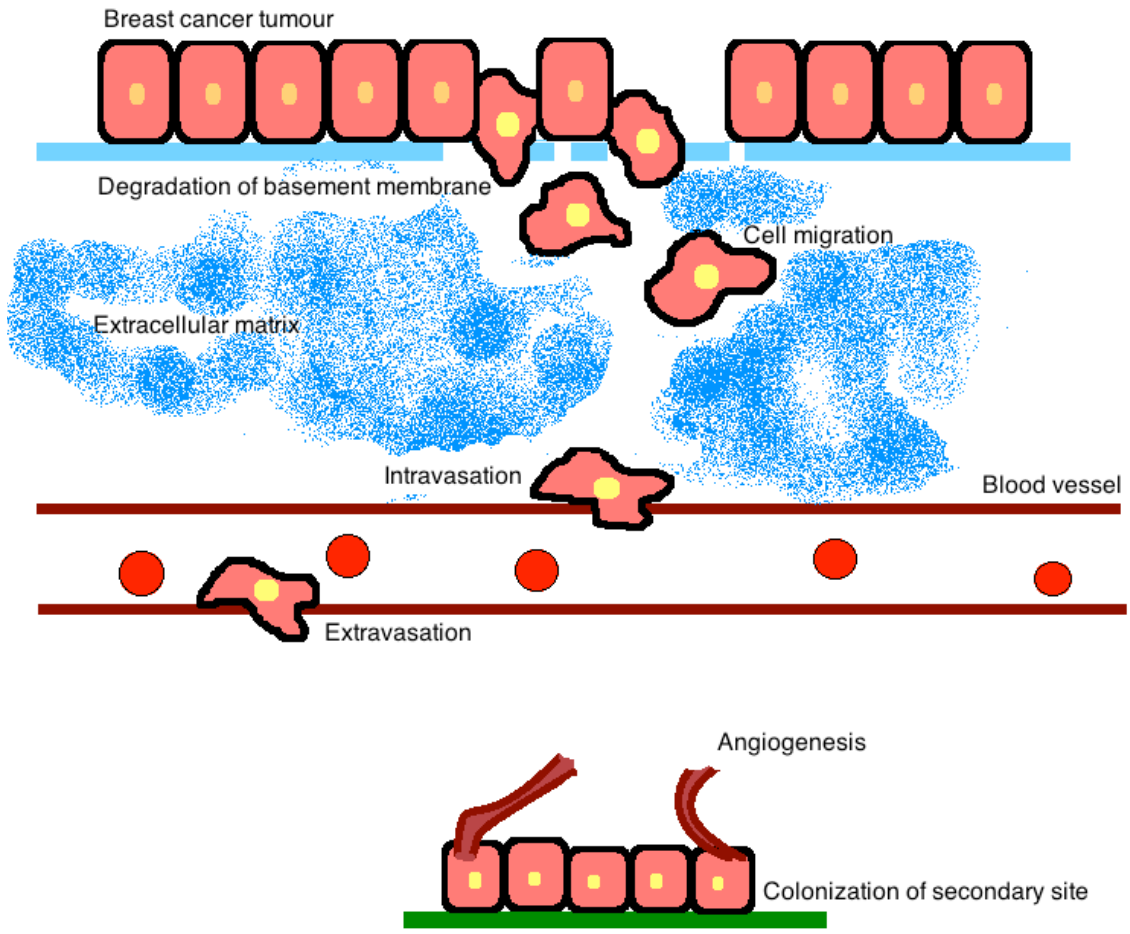
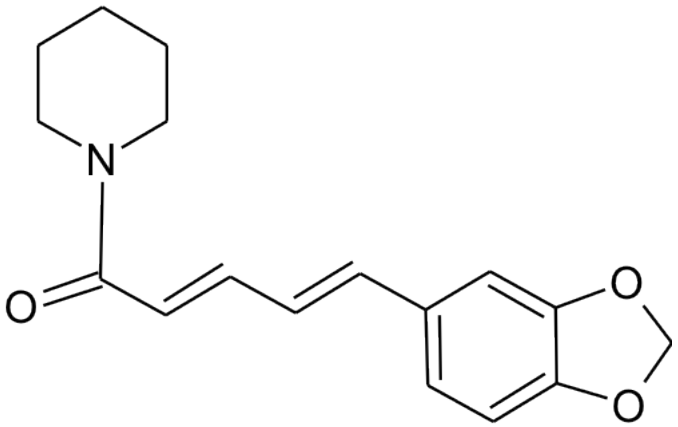

Breast cancer tumour
Degradation of basement membrane
Cell migration
Extracellular matrix
Intravasation
Blood vessel
Extravasation
Angiogenesis
Colonization of secondary site

Table 1.1 MMP families and their substrates. This table lists some MMPs and some of their substrates. List was adapted from John and Tuszynski (2001) and Verma and Hansch (2007).

FAMILY	MMP	SUBSTRATE
Collagenases	MMP1, MMP8, MMP13, MMP18	Fibrillar Collagens
Stromelysins	MMP3, MMP10, MMP11, MMP27	Laminin, fibronectin, non-fibrillar collagens
Matrilysins	MMP7, MMP26	Laminin, fibronectin, non-fibrillar collagens
Membrane-Type MMPS	MMP14, MMP15, MMP16, MMP17, MMP24, MMP25	Gelatinase A
Gelatinases	MMP2, MMP9	Type IV, V and fibrillar collagens, gelatin
Other MMPS	MMP12, MMP19, MMP20, MMP21, MMP22, MMP23, MMP28, MMP29	Various

Figure 1.2. Piperine is the major alkaloid in black pepper and long pepper. Figure A shows the molecular structure of piperine. Figure B shows an image of long pepper, while Figure C shows an image of the black pepper plant. Figures B and C were taken from the online database of free stock photos, Foter.com.

A



B

C

CHAPTER 2

MATERIALS & METHODS

2.1 Materials

MDA-MB-231 cancer cells were generously provided by Dr. S. Drover of Memorial University of Newfoundland. Bovine serum albumin (BSA), bromophenol blue solution, Coomassie Brilliant Blue Dye, Dulbecco's Modified Eagle's Medium (DMEM), dimethyl sulfoxide (DMSO), β-mercaptoethanol, mitomycin C, piperine, phenylmethylsulfonyl fluoride (PMSF), phosphate buffered saline (PBS), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, and Triton X-100 were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Dithiothreiotol (DTT), deoxyribonucleotide triphosphates (dNTP), fetal bovine serum (FBS), L-glutamine, penicillin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, oligo(dT), streptomycin, SuperScript® reverse-transcriptase, trypan blue dye solution, TRYPLE™ dissociation reagent, trypsin-EDTA and 5x First-Strand buffer were obtained from Invitrogen Canada (Oakville, ON, Canada). Acrylamide/bis-acrylamide, ammonium persulfate (APS), sodium dodecyl sulphate (SDS), tetramethylethylenediamine (TEMED), tris base and Tween-20 were obtained from Bio-Shop Canada Inc. (Burlington, ON, Canada). Anhydrous ethanol was obtained from Commercial Alcohols (Brampton, ON, Canada). Ecolite™ scintillation fluid and tritiated thymidine and were purchased from MP Biomedicals (Santa Ana, CA, USA). Bio-Rad protein assay dye reagent and gelatin powder were obtained from Bio-Rad Laboratories Inc. (Mississauga, ON, Canada). Acetic acid and methanol were purchased from Fisher Scientific (Ottawa, ON, Canada). Amersham™ ECL™ chemiluminescent reagent was obtained from GE Healthcare Life

Sciences (Uppsala, Sweden). Powdered Carnation® skimmed milk was obtained from the local grocery store. All RNA primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Rabbit anti-human MMP9 polyclonal and mouse anti-MMP3 monoclonal primary antibodies were obtained from EMD Millipore (Temecula, CA, USA). Rabbit anti-human MMP2 polyclonal primary antibody as well as goat anti-mouse IgG- horse-radish peroxidase (HRP), donkey anti-rabbit IgG-HRP and anti-actin (I-9) goat polyclonal secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Quantifast SYBR® Green was obtained from Qiagen (Toronto, ON, Canada).

2.2 Maintenance of MDA-MB-231 Cell Line

MDA-MB-231 cells were cultured in complete Dulbecco's Modified Eagle Medium (cDMEM) made up of 100 U/mL penicillin, 100 μg/mL streptomycin, 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 5 mM HEPES in DMEM at 37°C under 10% humidified CO_2. The following procedure was performed for cell subculturing. Cells cultured in Corning® (Corning Incorporated Life Sciences, Tewksbury, CA, USA) 75 cm^2 vented flasks were viewed under a Unico® compound light microscope (Unico, Dayton, NJ, USA) to confirm adhesion to the bottom of the flask's inner surface. The cDMEM was then carefully pipetted out of the flask and discarded. Trypsin (3 mL) was added to the adherent cells and left to stand for approximately 2 min. The sides of the flask were tapped to release the adherent cells and complete detachment of the cells confirmed by observation under compound light microscope. A volume of 7 mL of cDMEM was then added to the detached cells and 2 mL of the cell suspension pipetted into 8 mL of cDMEM in a new vented-flask to create a new culture. Cultures were incubated at 37°C under 10% humidified CO_2. The subculturing procedure was repeated each time the cells

reached approximately 90% confluence as determined by inspection under the microscope. As a result, cells were split approximately every 3 days.

2.3 Cell Migration Assay

Four sterile, adhesive, Ibidi® two-well culture-inserts (Ibidi, Ingersoll, ON, Canada) were placed in Thermo Scientific® 6-well plates at one insert per well. MDA-MB-231 cancer cells were seeded in each culture-insert well at a concentration of 10,000 cells per 100 µL cDMEM. The cells were incubated overnight in order to ensure sufficient cell adhesion to the plate surface. After the incubation period, cDMEM in each culture-insert well was replaced with 100 µL of 10 µg/mL mitomycin C in cDMEM and cells were cultured for 2 h. Treatment of cells with mitomycin C was used to inhibit proliferation of the cancer cells. Following mitomycin C treatment, cells were allowed to recuperate for 12 h. Piperine solutions of 25 µM and 50 µM were prepared in cDMEM from a 150 mM stock solution of piperine in DMSO. The concentration of DMSO in each treatment solution was kept constant, including the vehicle control. Culture-inserts were lifted from plates using sterile forceps and a clear void was observed to be separating the two cell populations. Cells were washed with 1 mL of cDMEM and treated with 2 mL of piperine or its vehicle control. A well with 2 mL cDMEM served as the medium control. Images of the void were captured using a Nikon® Digital Sight camera (Nikon Canada Inc, Mississisauga, ON, Canada) head connected to a Nikon® Eclipse t5100 microscope immediately after treatment, 9 h after treatment and then every 2 h until the void in the medium control was observed to have fully closed (33 h). The raw values of the area covered by the void at 33 h as determined by Image J software were subtracted from the values at 0 h for each treatment. The differences for the vehicle control as well as the

piperine treated cells were then divided by the difference for the medium control to obtain a value for relative inhibition.

2.4 Tritiated Thymidine Incorporation Assay

A tritiated incorporation thymidine assay was performed to confirm the inhibitory effect of mitomycin C on cell proliferation. MDA-MB-231 breast cancer cells were seeded into Thermo Scientific® 96-well plates (Fisher Scientific®, Ottawa, ON, Canada) at concentrations of 5,000 cells per well in 200 µL cDMEM then incubated overnight. After incubation, supernatants were removed and wells were treated with 10 µg/mL mitomycin C or its cDMEM control for 2 h after which the mitomycin C solution was replaced with 200 µL cDMEM. Twenty millilitres of a 1:1,000 dilution of tritiated thymidine in cDMEM was added to each well and then cells were cultured for 6 h. Plates were then incubated overnight at -20°C. The following day, the plate was thawed for 20 min at 37°C and the cells harvested onto filter mats using a Titerek® Cell Harvester (Skatron Instruments, Sterling, VA, USA). The mats were placed in scintillation vials and 1 mL scintillation fluid was added per vial. A Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc., Mississauga, ON, Canada) was used to measure the amount of tritiated thymidine incorporated into cellular DNA. The difference in tritiated thymidine incorporation into DNA between samples treated with or without mitomycin C was measured 8, 24, 48 and 72 h after initial treatment. The samples were treated with tritiated thymidine 6 h before the completion of their respective time-points. All scintillation counts were normalized to the 6 h treatment, as this was the reference, and each time-point was analyzed for statistical significance using Student's t-test analysis.

2.5 Western Blot Analysis

2.5.1 Protein Isolation

MDA-MB-231 cells were seeded into Corning® 75cm^2 canted-neck vented culture flasks at 700,000 cells per flask then incubated overnight. Cells were then treated with piperine (25 or 50 μM), medium, or vehicle control and cultured for 24 or 48 h. Following culture, the media from each flask was pipetted into separate 15 mL centrifuge tubes. Adherent cells were harvested using TrypLE, added to its respective media tube and centrifuged at 500 x g at 4°C for 5 min. The supernatants were discarded and pellets were resuspended in 1 mL PBS. The cell suspensions were then transferred into micro-centrifuge tubes and centrifuged at 500 x g for 5 min at 4°C. The supernatants were discarded, the remaining pellets were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing 1% Nonyl phenoxypolyethoxylethanol-40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 20 mM Tris, 150 mM sodium chloride (NaCl), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA) at pH 7.5 with 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10 μg/mL apotinin, 100 μM sodium orthovanadate, 1 mM DTT, 10 mM NaF, 10 μM phenylaesine oxide and 1 mM PMSF. Cells were lysed on ice for 15 min and then centrifuged at 14,000 x g and 4°C for 10 minutes. Supernatants were then harvested and stored at -80 °C. Protein concentration was determined via a Bradford assay.

2.5.2 Protein Quantification (Bradford Method) & Denaturation

One mL diluted (1:4 v/v in ddH_2O) Bio-Rad® protein assay dye solution was added to 5 μL of each protein supernatant sample. A set of protein standards was prepared by mixing increasing volumes of BSA (0, 2.5, 5, 10, 20, 40, 80 μg/mL) with 1 mL of the dye. Standards and samples were loaded in quadruplicate into Thermo

Scientific® 96-well plates at 150 µL per well and analyzed spectrophotmetrically using an ASYS Expert 96 microplate reader (Montreal Biotech, Dorval, QC) and DigiRead software at a wavelength of 570 nm. The software program, SoftMax® Pro 4.3 LS (Molecular Devices, Sunnyvale, CA, USA), was used to calculate sample concentrations using a 4-parameter regression on the standard curve. All samples were diluted with the appropriate volume of inhibitor cocktail solution to achieve an equal protein concentration in each sample for a final volume of 25 µL. In order to denature the protein samples, 12.5 µL 3x SDS-sample buffer containing 15% β-mercaptoethanol (v/v), 0.01% bromophenol blue (w/v), 30% glyverol (v/v), 6% SDS and 200mM Tris HCl (pH 6.8), was added to each sample, mixed and heated at 95°C for 5 min.

2.5.3 SDS-PAGE Gel Electrophoresis

Gel electrophoresis was run on a 12 % acrylamide separating gel containing 0.15% TEMED (v/v), 0.1 % SDS (w/v), 0.1 % APS (w/v) and 375 mM Tris-HCl (pH 8.8). A 4% acrylamide stacking gel was prepared containing 0.3% TEMED (v/v), 0.1% SDS (w/v), 0.1% APS (w/v) and 125 mM Tris-HCl (pH 6.8). Protein samples dissolved in SDS sample buffer solution were loaded into the wells of the stacking gel at a load rate of 10 µg of protein per well. A volume of 5 µL of Bio-Rad® protein ladder was loaded into one well to serve as a MW reference. Electrophoresis was performed at a voltage of 200 V and 120 mA for 60 min in SDS-PAGE running buffer.

2.5.4 Gel Transfer and Development of Blots

Proteins from SDS-PAGE gels were transferred onto a nitrocellulose membrane using an iBlot® Gel Transfer Device (Life Technologies™, Burlington, ON, Canada). A single gel was placed between an anode and a sheet of filter paper wetted with deionized water and a cathode was placed on top of the paper. The sandwiched gel was then placed

in the transfer device with a foam sponge inside its lid. The device was run for 7 min. The nitrocellulose membrane was trimmed and blocked with 5% (w/v) skim milk in Tween-20 Tris Buffered Saline (T-TBS) for 60 min with gentle rocking. The skim milk solution was then discarded and the membrane was washed 6 times over a 30-min period in T-TBS. The blot was then probed with primary antibody overnight at 4°C with gentle shaking. The next day, primary antibody was removed and the blot was washed as described above. After washing, the blot was placed in secondary antibody for 60 min with gentle rocking at room temperature. Secondary antibody was then removed and the blot was washed as described above. Primary antibodies against MMP2, actin as well as all secondary antibodies were prepared as 1:10000 dilutions in 5% skimmed milk and 1x T-TBS. Primary antibodies against MMP3 and MMP9 were prepared as 1:1000 dilutions in 3% skimmed milk and T-PBS. Blots were soaked in 2 mL of chemiluminescent reagent and developed on Fujifilm® medical x-ray film (Fujifilm, Mississauga, ON, Canada) using a Kodak X-OMAT 1000A X-ray developer (Eastman Kodak Company, Rochester, NY, USA). Densitometry analysis of the resulting bands was performed using the Image J 1.47 (National Institutes of Health, Bethesda, MD, USA) software program for Mac OSX.

2.6 Analysis of mRNA Expression

2.6.1 RNA Extraction

MDA-MB-231 cells were seeded into Corning® 75cm^2 canted-neck vented culture flasks at 700,000 cells per flask then cultured overnight. The following day, media was replaced with medium control, vehicle control, 25 μM and 50 μM piperine treatments and cultured for 24 or 48 h. Following culture, RNA was extracted from cells using a Qiagen RNeasy® minikit (Qiagen, Toronto, ON, Canada). Centrifugation was performed at 8,000 x g and 4°C for 15 s. Cells were lifted from culture flasks by replacing their treatment

solutions with 3 mL TrypLE™ and then incubating them at 37°C. The solution containing lifted cells was centrifuged at 500 x g for 5 min and the supernatant was discarded. Buffer RLT (350 µL) and 70% ethanol (350 µL) were added to the cell pellet and mixed thoroughly to lyse the cells after which 700 µL was transferred into a 2 mL collection tube. The solution was centrifuged in an RNeasy® Mini spin column and the flow-through was discarded. Buffer RW1 (350µL) was added to the column and centrifuged and the flow-through was discarded. An incubation mix of 50 µL DNase and 350 µL Buffer RDD was prepared and centrifuged using a minifuge for 5 s. The incubation mix (80 µL) was added to the column membrane and incubated at room temperature for 15 minutes. Buffer RW1 (350 µL) was then added to the spin column, centrifuged, and the flow-through was discarded. Buffer RPE (500 mL) was added to spin column and centrifuged and flow-through was discarded. This step was repeated after which the spin column was placed in a new 2 mL collection tube and centrifuged for 1 min. The spin column was placed in a 1.5 mL collection tube and 40 µL of RNase-free water was directly added to the column membrane and centrifuged for 1 min. The resulting filtrate, which contained RNA was stored at -80°C. Extracted RNA purity was analyzed by loading 1.5 µL onto a Thermo Scientific® Nanodrop 2000 spectrophotometer.

2.6.2 First-Strand cDNA Synthesis

RNA (667 ng) from each treatment group was added to RNase-free water in microcentrifuge tubes and diluted to a final volume of 10 µL. A volume of 1 µL of 50 µM oligo(dT) and 1 µL of 100 mM dNTP was then added to each tube. The mixtures were heated to 65°C in a Bio-Rad® T100™ thermal cycler and cooled immediately on ice for 5-10 min. 4 mL of 5X First-Strand Buffer and 2 µL of 0.1 M dithiothreiotol (DTT) were added to each sample, then samples were incubated for 2 min at 42°C. SuperScript®

reverse-transcriptase (1 μL) was then added and the reaction mixtures were incubated at 42°C for 50 min followed by another incubation at 70°C for 15 min. The reaction products were stored at -20°C.

2.6.3 RT-qPCR

PCR reaction mixtures contained 5 μL of Qiagen Quantifast SYBR® Green, 1 μL of 1 μM sample cDNA, 2 μL RNase-free water and 1 μL each of 1 μM forward and reverse primers. The primer sequences are given below.

- hMMP2 – Forward: 5'-TGG CAA GTA CGG CTT CTG TC-3'; Reverse: 5'-TTC TTG TCG CGG TCG TAG TC-3'
- hMMP3 – Forward: 5'-GGA GGT TCG TGA AGG-3'; Reverse: 5'-TCC TGG CAG AAA TAG GCT TTC-3'
- hMMP9 – Forward: 5'-GAA GAG AAA TTC CAT GGA GCC AGG-3'; Reverse: 5'-AGA AAT AAA AGA ACC CAA ATT CTT CAA AAA CA-3'
- hGAPDH - Forward: 5'-GAG TCA ACG GAT TTG GTC GT-3'; Reverse: 5'-TTG ATT TTG GAG GGA TCT CG-3'

Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) served as the loading control. The PCR reactions were performed in triplicate using a Corbett Research Rotor Gene 6000 series analyzer (Corbett Life Science, Concorde, NSW, Australia). The cycling conditions were as follows: 5 min initial heat activation at 95°C, 10 s denaturation at 95°C and 30 s extension at 60°C. Forty cycles were used and a melt curve analysis was performed. Data was analyzed using the Rotor-Gene 6000 series software program.

2.7 Gelatin Zymography Analysis

2.7.1 Sample Preparation

The protocol for the gelatin zymography assay was modified from Toth et al. (2012). MDA-MB-231 cells in cDMEM were seeded in Corning® 75cm^2 canted-neck vented culture flasks at 1,000,000 cells per flask and incubated overnight. Cells were then washed with PBS then treated with medium, vehicle control, or piperine (25 or 50 μM) for 24 or 48 h in serum-free cDMEM. After the appropriate incubation period, the medium from each flask was harvested into separate 15 mL centrifuge tubes and centrifuged at 400 x g for 5 min at 4°C. The supernatant was transferred to a 15 mL Amicon® centrifugal unit (Billerica, MA, USA) and centrifuged at 14,000 x g for 30 minutes in order to concentrate the protein. The supernatant (75 μL) was mixed with 25 μL of 4x sample buffer containing 0.25M Tris/ HCl (pH 6.8), 40% glycerol (v/v), 8% SDS (w/v) and 0.01% bromophenol blue (w/v). The mixture was allowed to sit at room temperature for 15 minutes. A volume of 75 μL of regular cDMEM (i.e. cDMEM with FBS) were also mixed with 25 μL of 4x sample buffer to serve as a positive control.

2.7.2 Zymography Gel Electrophoresis

Protein samples were run by electrophoresis on an 8% acrylamide separating gel containing 0.1% gelatin (w/v), 0.18% TEMED (v/v), 0.1% SDS (w/v), 0.06% APS (w/v) and 300 mM Tris-HCl (pH 8.8). A 4% acrylamide stacking gel was used, containing 0.2% TEMED (v/v), 0.1% SDS (w/v), 0.6% APS (w/v) and 65mM Tris-HCl (pH 6.8). Samples dissolved in sample buffer were pipetted into gel wells at a load rate of 30 μL protein sample per well. Bio-Rad® protein ladder (5 μL) was loaded into one well to use as MW reference. Electrophoresis was performed at a voltage of 125 V and 40 mA for 120 min in

SDS-PAGE running buffer containing 200 mM glycine, 0.1% SDS (v/v) and 20mM Tris-HCl (pH 8.3).

2.7.3. Development of Gel

Gels were washed for 30 min in 100 mL renaturing solution containing 25% Triton X-100 (v/v) in deionized water, with gentle rocking. The renaturing solution was then decanted and the gel was rinsed with 300 mL of deionized water. After rinsing, the gel was washed with 100 mL 1x developing buffer containing 0.05 M Tris-HCl (pH 7.8), 0.2 M NaCl, 0.005 M $CaCl_2$ and 0.1% Triton X-100 in deionized water, for 30 min with gentle rocking. This buffer solution was then replaced with 100 mL of fresh 1x developing buffer solution and incubated at 37°C for 16 h. After incubation, the gel was removed from the developing buffer and allowed to sit in staining solution containing 0.5% Coomassie Blue (w/v), 5% methanol (v/v) and 10% acetic acid (v/v) in deionized water, for 60 min until a uniform blue background developed on the gel. Staining solution was then decanted and replaced with destaining solution containing 5% methanol (v/v) and 10% acetic acid (v/v) in deionized water. The gel was destained until white bands appeared, indicating gelatinolytic activity.

2.8 Statistical Analysis

All statistical analyses were performed using the GraphPad Prism® 5 software program (GraphPad Software, La Jolla, CA, USA) for Mac OSX. The difference in proliferation between mitomycin C treated and untreated cells was determined using a paired t-test with α=0.05. T-tests were also used to analyze the difference in void widths for the cell migration assays. All other statistical analyses were carried out using one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test. Differences were said to be significant when the p-value was less than 0.05.

CHAPTER 3

RESULTS

3.1 Mitomycin C Inhibits MDA-MB-231 Breast Cancer Cell Proliferation

A tritiated thymidine assay was used to determine whether mitomycin C at 10 ng/mL inhibited MDA-MB-231 breast cancer cell proliferation. It was necessary to perform the assay in order to establish that the filling of the void as observed in the cell migration assay was due to cell migration alone and not cell proliferation. The tritiated thymidine assay showed that cells treated with mitomycin C demonstrated less tritiated thymidine incorporation into DNA as compared with mitomycin C untreated cells indicating that cell proliferation was indeed inhibited by mitomycin C. It is important to note that scintillation counts in the 10 μg/mL mitomycin C treated cells remained fairly constant over time while counts in untreated cells increased. This demonstrated that tritiated thymidine incorporation remained the same in mitomycin C treated cells. Significant differences in tritiated thymidine incorporation were observed for 6, 24, 48 and 72 h time-points. Graphical representation of these results is given in Figure 3.1.

3.2 Piperine Inhibits MDA-MB-231 Breast Cancer Cell Migration

A cell-migration assay was used to determine the effect of non-cytotoxic concentrations of piperine on MDA-MB-231 breast cancer cell migration. Treatment of the breast cancer cells with 10 μg/mL mitomycin C ensured that cell migration calculations were not confounded with cell proliferation. Piperine at a dosage of 50 μM significantly reduced MDA-MB-231 breast cancer cell migration into the void created by the culture-insert (Figure 3.2). Cells with medium and/or vehicle consistently migrated to fill the void created by the culture-insert after 33 h.

It was also noted that piperine seemed to change the overall morphology of the cells. Piperine-treated cells tended to appear smaller than the untreated cells. The exact cause of this change was unknown, however it was not believed to have been a result of cell death. Floating cells as a result of a loss in adhesion properties is typically indicative of cell death in tissue culture (personal observation). There was no significant number of floating cells observed in this experiment. Additionally, patchiness in the cell monolayer as a result of detachment was not observed in this experiment, giving further evidence of continued viability in the piperine-treated cells.

3.3 Piperine Inhibits the mRNA Expression of MMP2 and MMP9 but Not MMP3 in MDA-MB-231 Breast Cancer Cells

For the reason that MMPs play a significant role in cell migration, the possible effects of piperine on MMP expression were tested. RT-qPCR showed lower expression of MMP2 and MMP9 mRNA in piperine-treated MDA-MB-231 breast cancer cells when compared with the vehicle and medium controls. Piperine at 50 μM concentrations significantly inhibited MMP2 (Figure 3.3) and MMP9 (Figure 3.5) mRNA expression after 48 h of treatment. Significance was determined by one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. Piperine treatments after 24 h showed slight dose-dependent decreases in MMP2 and MMP9 mRNA expression. Conversely, piperine concentrations of 25 and 50 μM at 24 and 48 h time-points had no effect on MMP3 mRNA expression (Figure 3.4) as determined by one-way ANOVA with Bonferroni post-hoc test.

3.4 Piperine Does Not Decrease MMP2, MMP3 or MMP9 Protein Levels in MDA-MB-231 Breast Cancer Cells.

Analysis by western blot showed no significant inhibition of MMP2, MMP3 or MMP9 protein expression by piperine in MDA-MB-231 breast cancer cells. Distinct bands for MMP2 and MMP3 at sizes of 72 kDa and 59 kDa, respectively, were observed on the western blots. Blots for MMP9 showed no bands that corresponded to the protein's expected molecular weight (data not shown). Densitometry analysis showed a slight dose-dependent decrease in MMP2 expression 24 h after treatment with piperine but not after 48 h (Figure 3.6). As with the RT-qPCR analysis, piperine did not affect MMP3 protein expression (Figure 3.7).

3.5 Analysis of the Effect of Piperine on Gelatinase Activity in MDA-MB-231 Breast Cancer Cells Yielded Inconclusive Results.

Gelatin zymography was used to evaluate the effect of piperine on the activity of MMP2 and MMP9 produced by breast cancer cells. Active MMPs break down gelatin in the Coomassie blue-stained gel leading to a clear, unstained band. Unfortunately, zymograms were inconclusive in measuring gelatinolytic activity, as they did not produce bands that could be measured for the lanes containing sample supernatants. However, faint bands corresponding to MMP2 could be seen with the naked eye. These bands seemed to show decreased clearance of dye for piperine treated cells compared to vehicle-treated supernatants, suggesting that piperine could potentially inhibit the conversion of pro-MMP2 to active MMP2. No bands were observed that corresponded to the MW of MMP9. It is of note that the supernatant from control cultures showed gelatinolytic bands corresponding to MMP2 and MMP9. Gelatinases are present in the serum contained in cDMEM, thus the gelatinolytic activity by these samples in the zymograms was expected.

A representative image of the resulting zymogram is given in Figure 3.8. Better methods of concentrating supernatant gelatinases are needed in future experiments in order to obtain conclusive results from the gelatin zymography assay.

Figure 3.1. Mitomycin C inhibits the proliferation of MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells treated with 10 μg/mL mitomycin C were tested for thymidine incorporation and compared to untreated cells. Scintillation counts were normalized to the 6 h time-point. Results are shown for 6, 24, 48 and 72 h after mitomycin C treatment in Graphs A, B, C and D respectively. Column graphs show mean counts with error bars representing the standard error of the means. A Student's t-test showed significant differences between the mitomycin C treated and untreated cells for each time-point ($p<0.05$, $n=3$). Significance is shown by an (*).

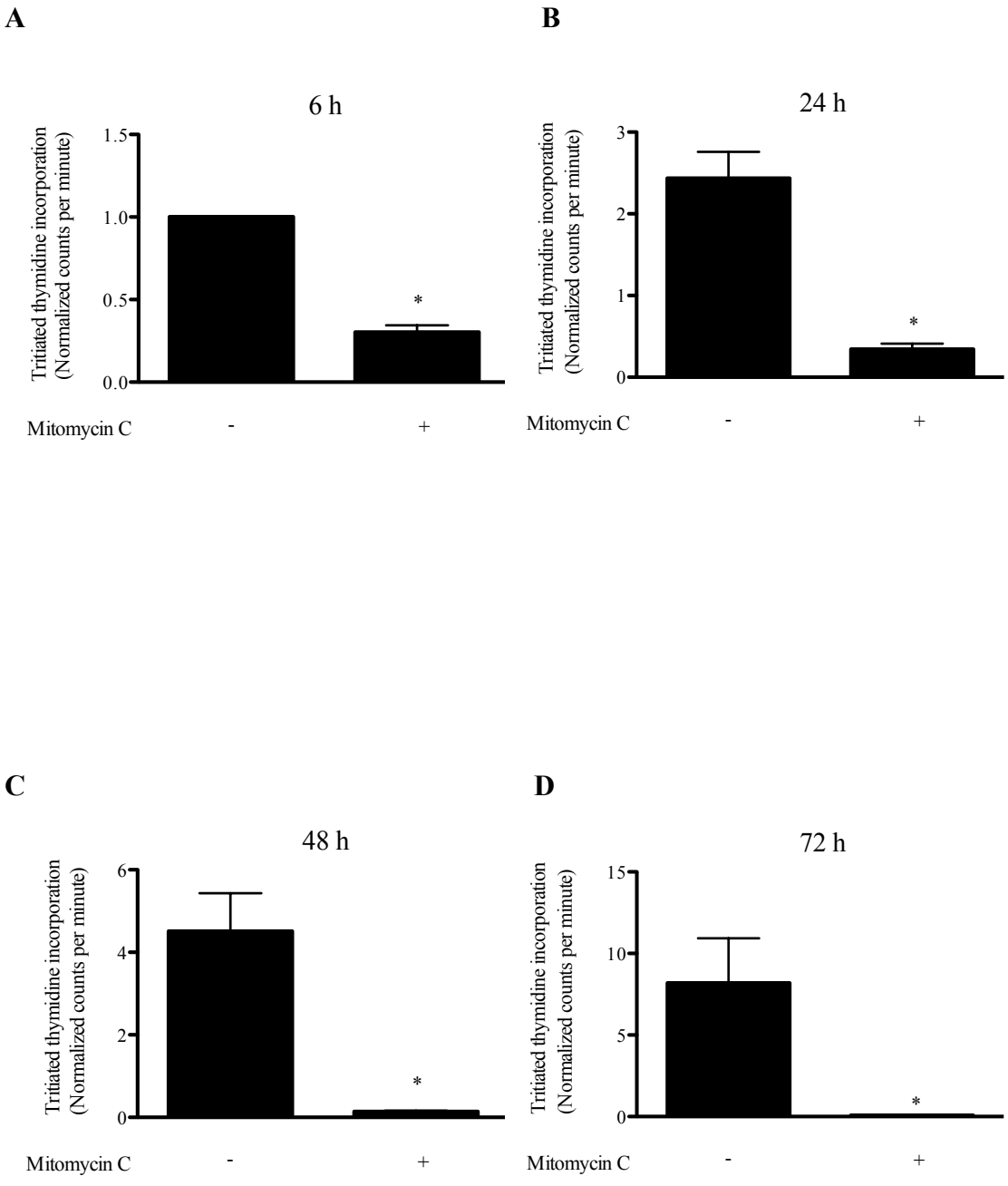
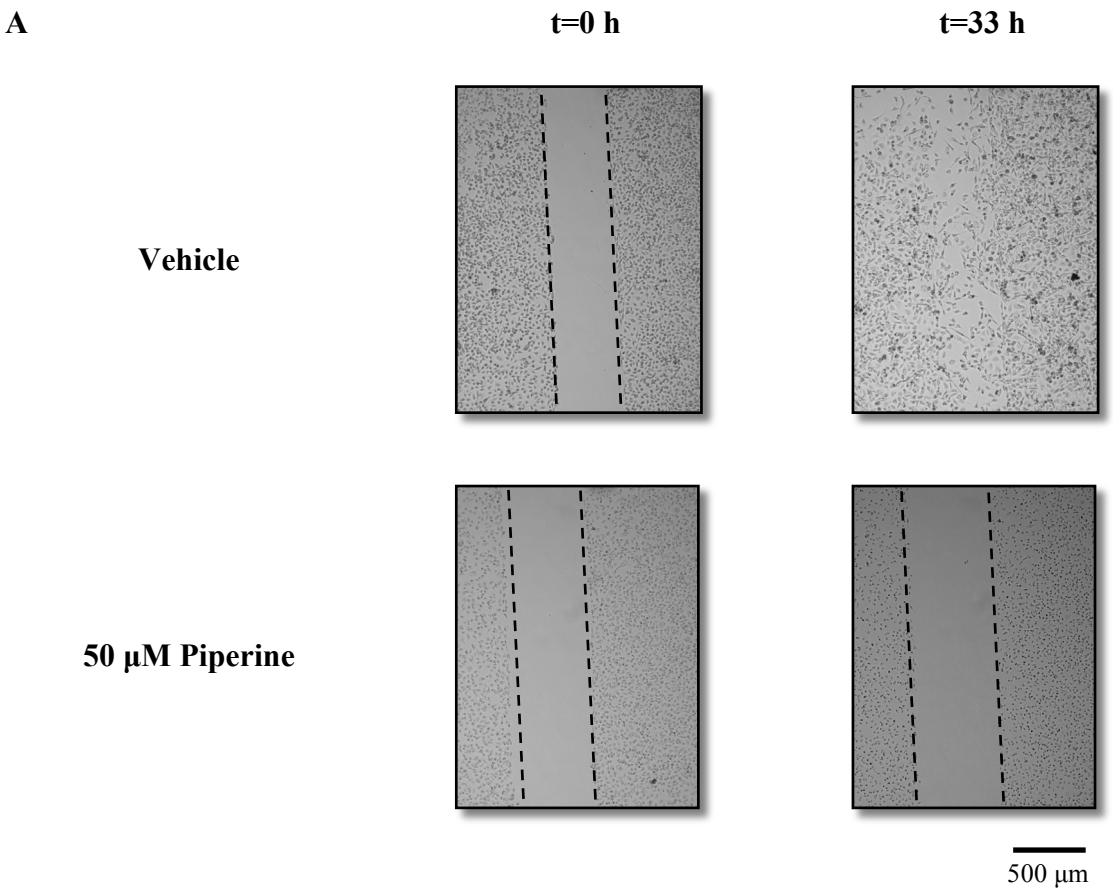

A
6 h
Tritiated thymidine incorporation (Normalized counts per minute)
1.5
1.0
0.5
0.0
*
Mitomycin C
-
+
B
24 h
Tritiated thymidine incorporation (Normalized counts per minute)
3
2
1
0
*
Mitomycin C
-
+
C
48 h
Tritiated thymidine incorporation (Normalized counts per minute)
6
4
2
0
*
Mitomycin C
-
+
D
72 h
Tritiated thymidine incorporation (Normalized counts per minute)
15
10
5
0
*
Mitomycin C
-
+

Figure 3.2. Piperine inhibits cell migration of MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells were treated with piperine or vehicle and observed for 33 h. Representative images for vehicle and 50 μM piperine treatments are shown at 0 and 33 h after treatment (A). The void in control cells was consistently observed to be filled by migrating cells 33 h after treatment. Graph shows mean width values normalized to medium control with error bars representing the standard deviation (B). A Student's t-test showed a significant difference between the vehicle control and 50 μM piperine ($p<0.05$, $n=3$). Significance is shown by an (*).


A
t=0 h
t=33 h
Vehicle
50 µM Piperine
500 µm

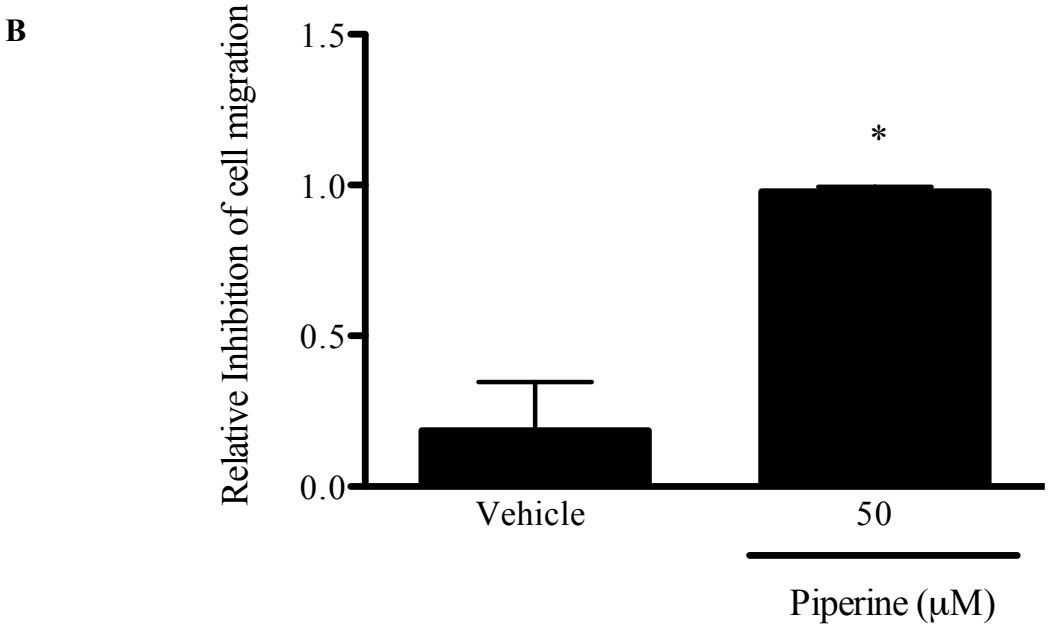
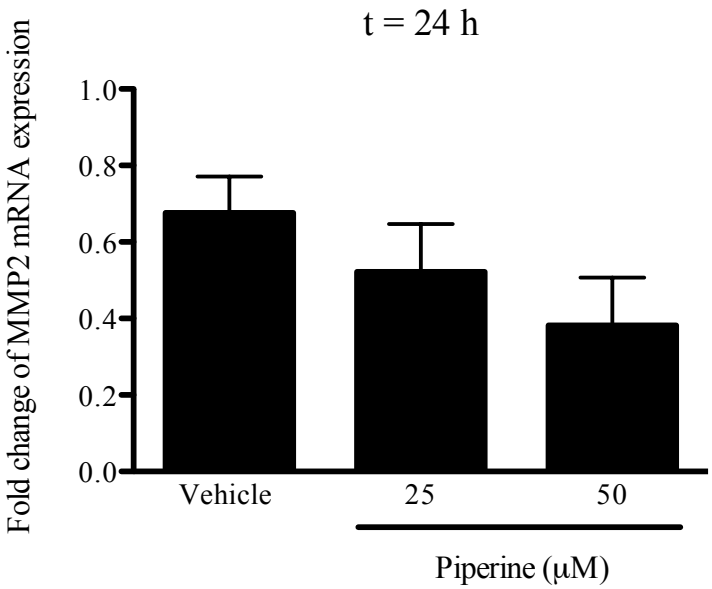

B
Relative Inhibition of cell migration
1.5
1.0
0.5
0.0
*
Vehicle
50
Piperine (µM)

Figure 3.3. Piperine reduces MMP2 mRNA expression in MDA-MB-231 breast cancer cells 48 h after treatment. MDA-MB-231 breast cancer cells were treated with vehicle or piperine at doses of 25 μM or 50 μM. The mRNA expression for MMP2 was determined by RT-qPCR. Cycle threshold (Ct) values were subtracted from GAPDH values to obtain a value called the dCt. Each difference was then subtracted from the value for the medium control to obtain a ddCt. Fold changes were calculated using the formula $y=2^{-x}$, where y is the fold change value and x is the ddCt. Column graph shows mean fold change in MMP2 mRNA expression with error bars representing the standard error of the mean. Graph A shows the results for the 24 h time-point while Graph B shows the results for the 48 h time-point. A one-way ANOVA with Bonferroni post-hoc test showed significant inhibition with 50 μM piperine for the 48 h time-point ($p<0.05$, $n=3$). Each experiment was performed in triplicate and repeated three times. Significance is shown by (*).

A


t = 24 h
Fold change of MMP2 mRNA expression
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
25
50
Piperine (μM)

B

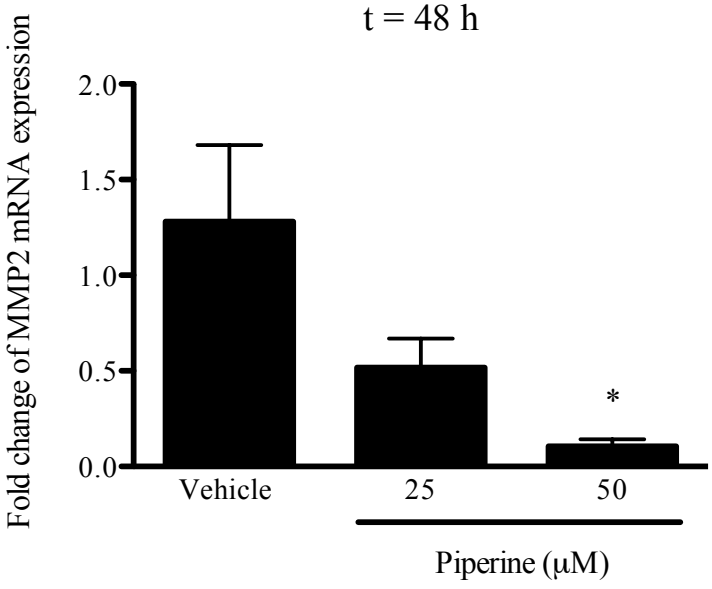
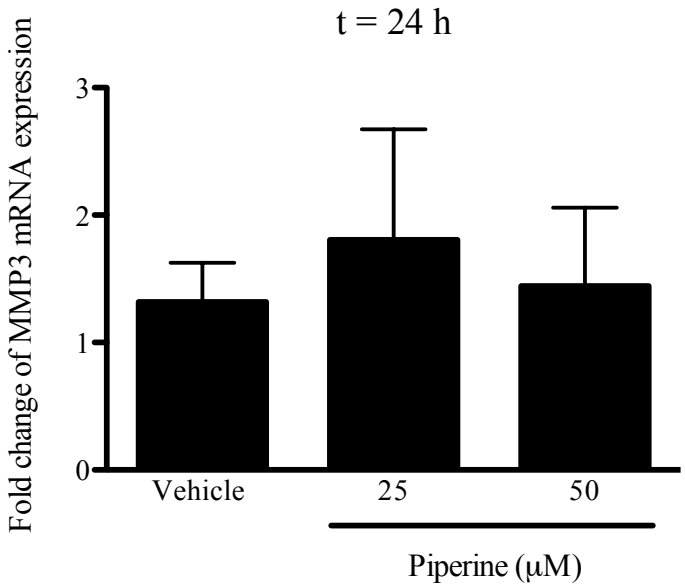

t = 48 h
Fold change of MMP2 mRNA expression
2.0
1.5
1.0
0.5
0.0
*
Vehicle
25
50
Piperine (μM)

Figure 3.4. Piperine does not affect MMP3 mRNA expression in MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells were treated with vehicle or piperine at doses of 25 μM or 50 μM. The mRNA expression for MMP3 was determined by RT-qPCR. Cycle threshold (Ct) values were subtracted from GAPDH values to obtain a value called the dCt. Each difference was then subtracted from the value for the medium control to obtain a ddCt. Fold changes were calculated using the formula $y=2^{-x}$, where y is the fold change value and x is the ddCt. Column graph shows mean fold change in MMP3 mRNA expression with error bars representing the standard error of the mean. Graph A shows the results for the 24 h time-point while Graph B shows the results for the 48 h time-point. A one-way ANOVA with Bonferroni post-hoc test showed no significant differences in treatment conditions ($p<0.05$, $n=3$). Each experiment was performed in triplicate and repeated three times.

A


t = 24 h
Fold change of MMP3 mRNA expression
3
2
1
0
Vehicle
25
50
Piperine (μM)

B

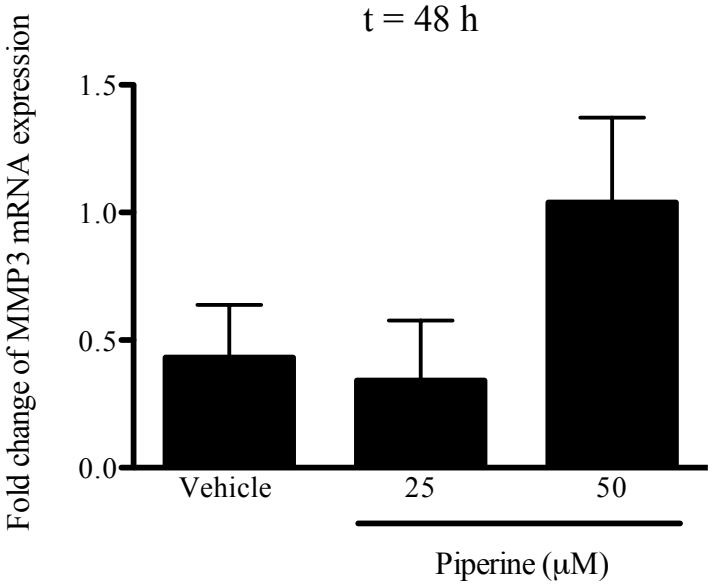
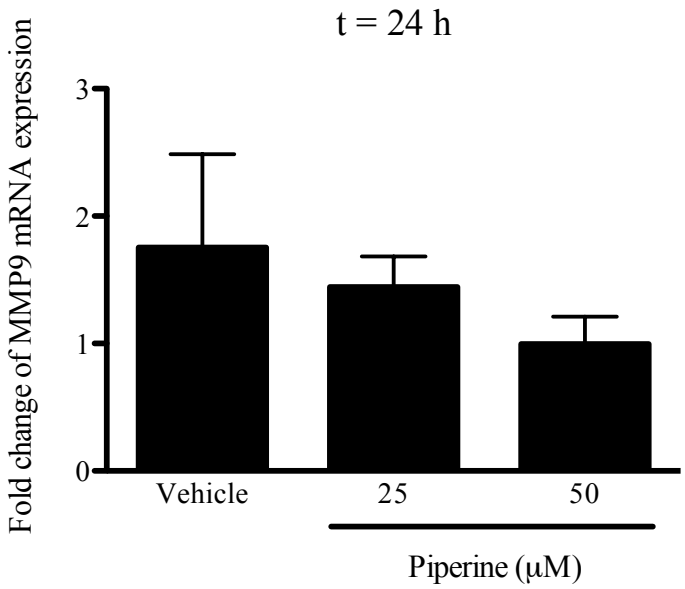

t = 48 h
Fold change of MMP3 mRNA expression
1.5
1.0
0.5
0.0
Vehicle
25
50
Piperine (μM)

Figure 3.5. Piperine reduces MMP9 mRNA expression in MDA-MB-231 breast cancer cells after 48 h. MDA-MB-231 breast cancer cells were treated with vehicle or piperine at doses of 25 μM or 50 μM. The mRNA expression for MMP3 was determined by RT-qPCR. Cycle threshold (Ct) values were subtracted from GAPDH values to obtain a value called the dCt. Each difference was then subtracted from the value for the medium control to obtain a ddCt. Fold changes were calculated using the formula $y=2^{-x}$, where y is the fold change value and x is the ddCt. Column graph shows mean fold change in MMP9 mRNA expression with error bars representing the standard error of the mean. Graph A shows the results for the 24 h time-point while Graph B shows the results for the 48 h time-point. A one-way ANOVA with Bonferroni post-hoc test showed significant inhibition at 50 μM for the 48 h time-point ($p<0.05$, $n=3$). Each experiment was performed in triplicate and repeated three times. Significance is shown by (*).

A


t = 24 h
Fold change of MMP9 mRNA expression
3
2
1
0
Vehicle
25
50
Piperine (μM)

B

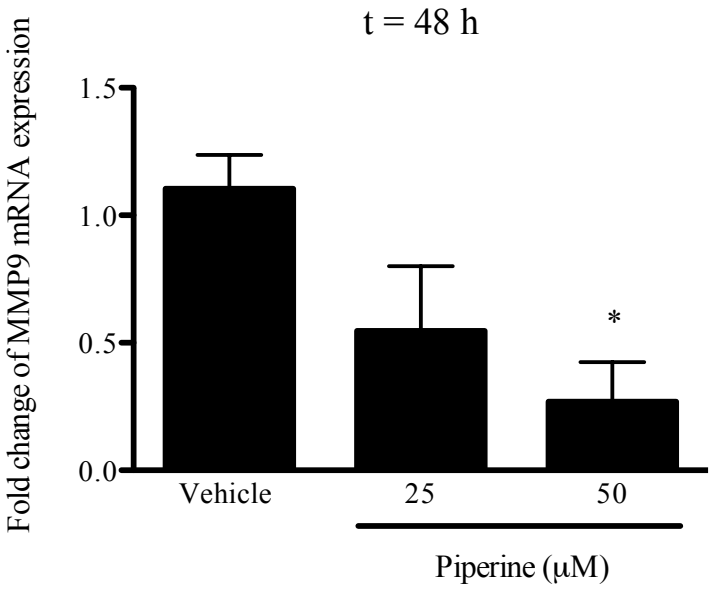
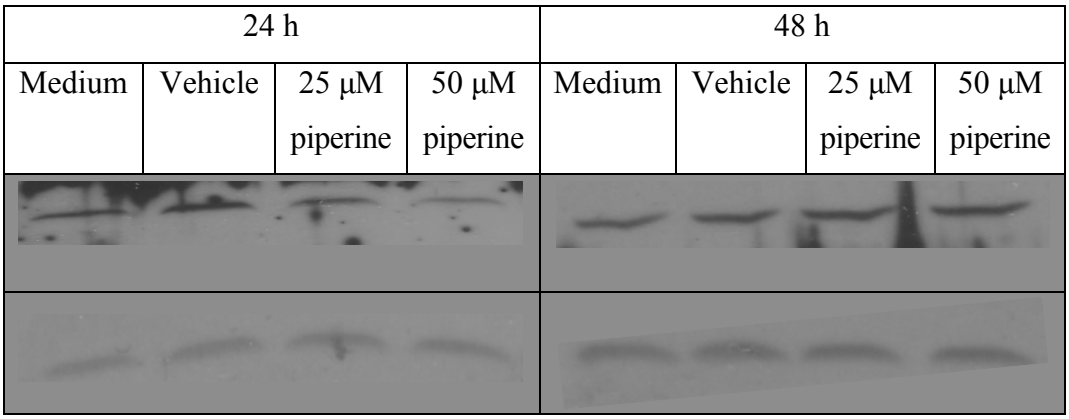
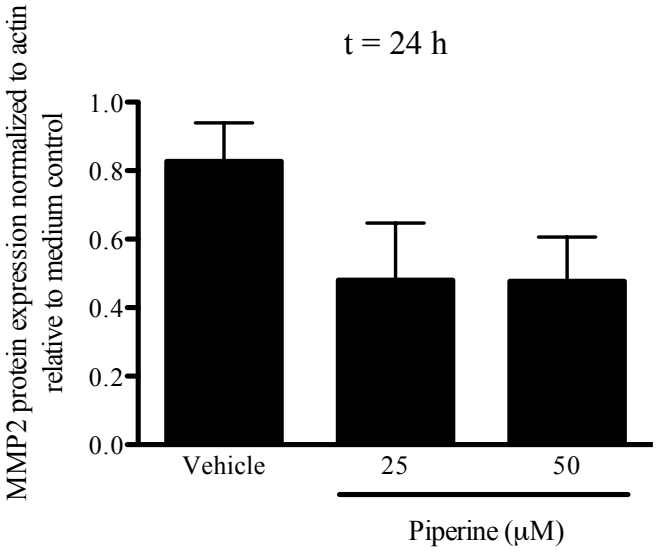

t = 48 h
Fold change of MMP9 mRNA expression
1.5
1.0
0.5
0.0
*
Vehicle
25
50
Piperine (μM)

Figure 3.6. Piperine does not significantly inhibit MMP2 protein expression in MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells were treated with vehicle or piperine at 25 μM or 50 μM. MMP2 protein expression was determined by western blot analysis. Figure A shows representative blots for MMP2 with β-actin control bands are shown for 24 and 48 h time-points. Western blot protein band densities were normalized to β-actin and then compared to the medium control. Column graph shows mean values with error bars representing the standard error of the mean. Graph B shows the results for the 24 h time-point while Graph C shows the results for the 48 h time-point. A one-way ANOVA with Bonferroni post-hoc test showed no significant differences in treatment conditions ($p<0.05$, $n=3$).

A


24 h
48 h
Medium
Vehicle
25 μM piperine
50 μM piperine
Medium
Vehicle
25 μM piperine
50 μM piperine
MMP2
Actin

B


t = 24 h
MMP2 protein expression normalized to actin relative to medium control
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
25
50
Piperine (μM)

C

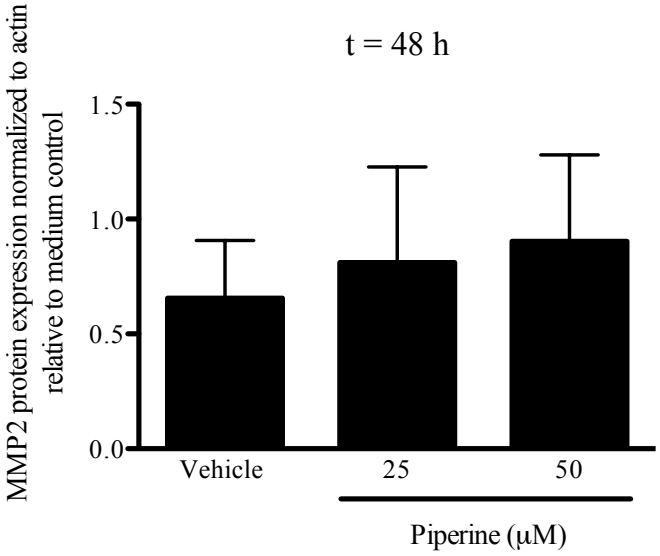
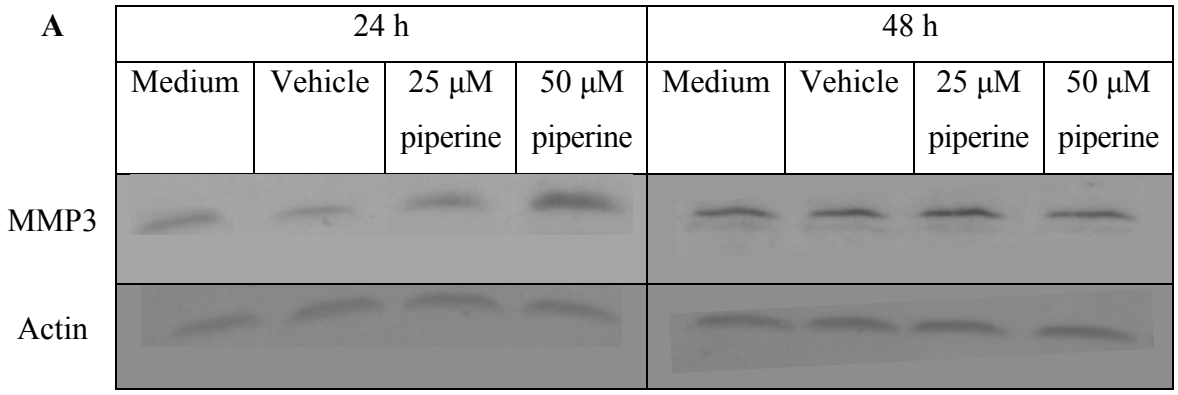
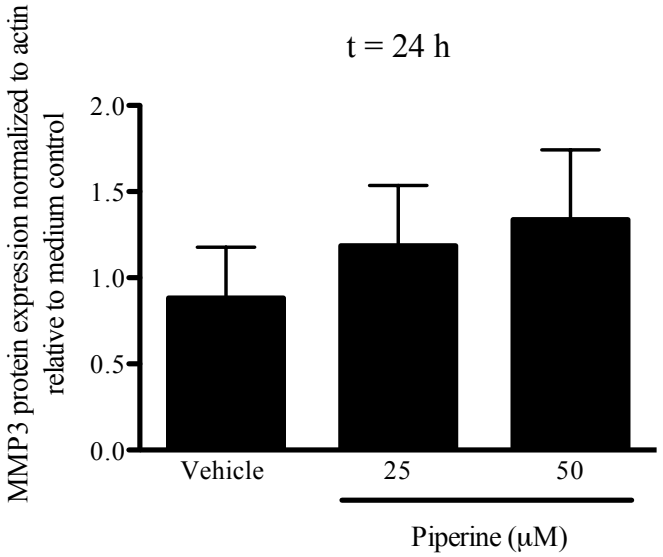

t = 48 h
MMP2 protein expression normalized to actin relative to medium control
1.5
1.0
0.5
0.0
Vehicle
25
50
Piperine (μM)

Figure 3.7. Piperine does not decrease MMP3 expression in MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells were treated with vehicle or piperine at 25 μM or 50 μM. MMP3 protein expression was determined by western blot analysis. Figure A shows representative blots for MMP3 with β-actin control bands are shown for 24 and 48 h time-points. Western blot protein band densities were normalized to β-actin and then compared to the medium control. Column graph shows mean values with error bars representing the standard error of the mean. Graph B shows the results for the 24 h time-point while Graph C shows the results for the 48 h time-point. A one-way ANOVA with Bonferroni post-hoc test showed no significant differences among treatment conditions ($p<0.05$, $n=3$).

A


24 h
48 h
Medium
Vehicle
25 μM piperine
50 μM piperine
Medium
Vehicle
25 μM piperine
50 μM piperine
MMP3
Actin

B


t = 24 h
MMP3 protein expression normalized to actin relative to medium control
2.0
1.5
1.0
0.5
0.0
Vehicle
25
50
Piperine (μM)

C

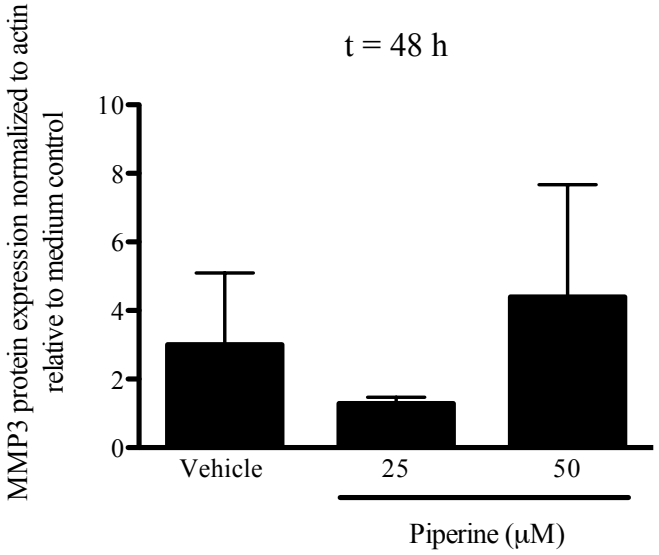
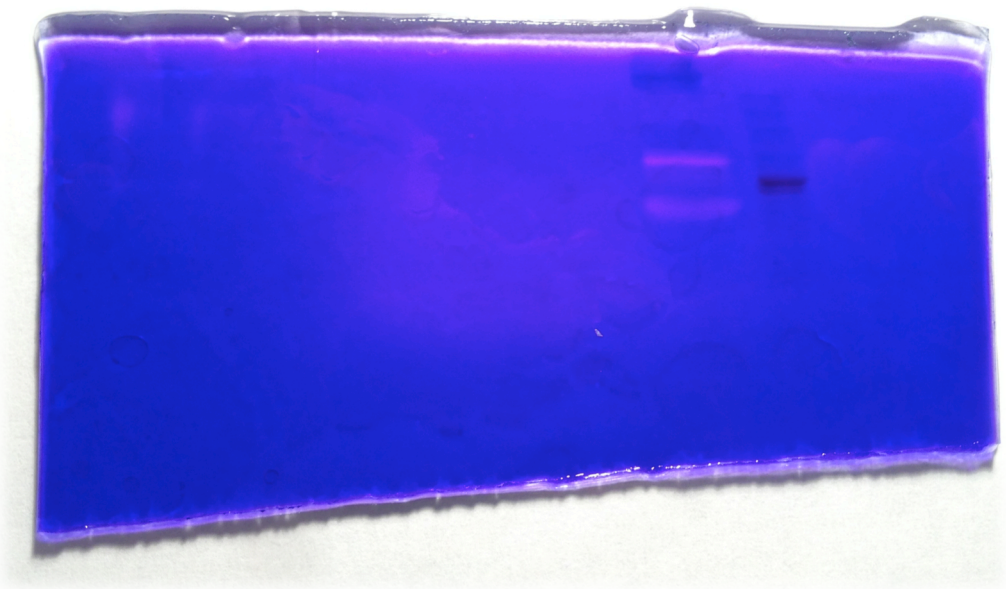

t = 48 h
MMP3 protein expression normalized to actin relative to medium control
10
8
6
4
2
0
Vehicle
25
50
Piperine (μM)

Figure 3.8. Analysis of the effect of piperine on gelatinase activity in MDA-MB-231 breast cancer cells yielded inconclusive results. Gelatin zymography was used to determine the activity of the gelatinases, MMP2 and MMP9, in MDA-MB-231 breast cancer cells. Gels were stained with Coomassie-Blue dye and destained with an acetic acid/methanol solution. Gelatinase-containing serum medium was used as a positive control. The resulting zymogram showed clear bands for the positive control and faint bands for the samples corresponding to MMP2. Although piperine treated samples showed reduced clearance, these bands were very faint and were not detectable on the photograph. The figure shows a representative photograph of a resulting zymogram.

Medium	Vehicle	25 µM piperine	50 µM piperine	Positive control

MMP9

MMP2



CHAPTER 4

DISCUSSION

4.1 Effect of Mitomycin C on MDA-MB-231 Breast Cancer Cell Proliferation

In this study, it was important to ensure that the proliferation of MDA-MB-231 breast cancer cells was being inhibited so that the filling of the void by cells in the migration assay was due to cell migration alone. The results of the tritiated thymidine incorporation assay, which measures the amount of thymidine incorporated into cellular DNA, indicated that 10 μg/mL mitomycin C was sufficient to significantly inhibit mitotic activity in MDA-MB-231 breast cancer cells (Figure 3.1). Mitomycin C is an antibiotic that was first isolated from the bacteria *Streptomyces caespitosus* (Szybalski and Iyer 1964). It inhibits mitotic activity in mammalian cells by crosslinking complementary strands of DNA (Schwartz, Sternberg and Philips 1963). Specifically, this action of mitomycin C prevents cells from proceeding past the S-phase of the cell cycle (Tomasz 1995). These properties of mitomycin C prompted investigations into its use as a possible anti-tumour drug (Mao, Varoglu and Sherman 1999).

Mitomycin C is commonly used to treat cells in migration assays. Fronza et al (2009) treated 3T3 fibroblasts cells with the 5 μg/mL mitomycin C prior to observing their migration in a wound-healing assay. Similarly, Basu et al (2001) used 10 μg/mL mitomycin C to inhibit proliferation of dermal fibroblasts. To observe migration of keratinocytes, Stojadinovic et al (2005) treated cells with 8 μg/mL mitomycin C. Differences in mitomycin C concentrations used to prevent proliferation are likely due to different responses to the antibiotic by different cell types.

4.2 Effect of Piperine on MDA-MB-231 Breast Cancer Cell Migration

Piperine at a concentration of 50 μM significantly inhibited the migration of MDA-MB-231 breast cancer cells as demonstrated by the results of the cell migration assay (Figure 3.2). In a recent article, 25 and 50 μM piperine were found to inhibit migration of SKBR3 breast cancer cells, a cell-line that is characterized by the overexpression of HER2 (Do, et al. 2013). The current study, however, investigated the effects of 25 and 50 μM piperine on a triple-negative human breast cancer cell-line, which lacks the human epidermal growth receptor-2 (HER2), as well as progesterone (PR) and estrogen receptors (ER) (Foulkes, Smith and Reis-Filho 2010). Triple-negative breast cancers are difficult to treat because they often do not respond to chemotherapy and hormone therapy, two of the most common forms of breast cancer treatment (Kawashima, et al. 2011). The results of the cell migration assay therefore, not only support the findings of Do et al (2013), but also further suggest that piperine may be able to treat metastasis in forms of breast cancers that are unresponsive to conventional therapeutic methods.

While the degradation of ECM is involved in the migration of cells, it is important to note that the modification of chemokine signalling and adhesion properties of cells also play a role in breast cancer cell migration. Determining the exact physiological process that is affected by piperine is beyond the scope of this study but other research indicates that piperine may have affected each of these processes to some degree. Kumar, Singhal et al. (2007) reported that piperine reduces the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, thereby inhibiting the migratory ability of neutrophils. The same effect was observed in human umbilical vein endothelial cells (Kumar, Arya, et al. 2005). Additionally, piperine

was shown to modulate cytokine signalling in C57BL/6 murine tumour cells (Sulina and Kuttan 2006). The modification of cell adhesion properties as well as chemical communication modes may have thus increased the migratory ability of cells in this study.

In addition to the degradation of ECM, MMPs are implicated in chemokine signalling and the regulation of adhesion molecules in cancer cells. MMP9 stimulates cell migration by inducing epidermal growth factor (EGF) in epithelial cells (McCawley, O'Brien and Hudson 1998) while MMP2 cleaves C-X-C motif chemokine 12 (CXCL12) (Zhang, et al. 2003). CXCL12 is partially responsible for preferential breast cancer metastatsis to certain tissues in the body (Müller, et al. 2001). MMP2 also cleaves P-cadherin, resulting in an increase in the invasive character of cancer cells (Ribeiro, et al. 2010). MMP2 also modulates cell adhesion by interacting with integrins (Ray and Stetler-Stevenson 1995). On the other hand, MMP3 is associated with the cleavage of E-cadherin, resulting in enhanced cell motility (Lochter, et al. 1997). Piperine may also inhibit cell migration by directly influencing processes that affect cell motility such as cytoskeleton remodelling (Khajuria, Thusu and Zutshi 2002). The modification of cytoskeleton components contributes to breast cancer metastasis (Barkan, et al. 2008).

In the current study, it was noted in the cell migration assay that the matrix that allowed cells to adhere to the plate surface may not have contained significant amounts of collagen and gelatin as would be observed *in vivo*. This is because fibroblasts and other mesenchymal cells are responsible for secreting the collagens and other proteins that comprise much of the ECM (Kalluri and Zeisberg 2006). FBS in cDMEM contains nutrients and growth factors such as platelet-derived growth factor (PDGF), which is implicated in blood clotting (Andrae, Gallini and Betsholtz 2008). Fibroblasts were absent in the cell migration assay used in the current study and so did not contribute to

matrix formation. Studies by Smith et al. (1979) showed that human epithelial cells in culture are capable of producing fibronectin to form an extracellular matrix on which they can adhere. Epithelial cells of rat intestines have also showed an ability to synthesize fibronectin in culture (Quaroni, Isselbacher and Ruoslahti 1978). Fibronectin may also be contained in FBS in appreciable amounts and may be used by cultured cells to form a matrix (Hayman and Ruoslahti 1979). These studies suggest then that the migration of MDA-MB-231 breast cancer cells in the current study may have occurred via the degradation of fibronectin. Fibronectin is a substrate of MMP3, but not MMP2 or MMP9. Therefore, while MMP2 and MMP9 may have played significant roles in cell migration by regulating cell adhesion and signalling properties, MMP3 and other stromelysins may have been the primary source of ECM degradation. Immunocytochemical analysis with fluorescent antibodies that are specific to fibronectin would confirm this hypothesis. It may be necessary for future studies to monitor cell migration in pre-formed ECM containing diverse proteins such as collagens and gelatin. It is also important to note that the cell-migration assay observed cell migration in two-dimensions. Future experiments may use other analytical methods such as transwell migration assays to determine the effect of piperine on cell migration in three-dimensions.

4.3 Effect of Piperine on MMP2 Expression in MDA-MB-231 Breast Cancer Cells

Piperine at concentrations of 50 μM significantly inhibited MMP2 mRNA expression in MDA-MB-231 breast cancer cells after 48 h of treatment, as determined by RT-qPCR analysis (Figure 3.3). However, this inhibitory effect was lost at the point of protein expression as western blot analysis showed no significant inhibition of MMP2 by piperine. Although western blots showed a slight dose-dependent decrease in MMP2 protein expression after 24 h of piperine treatment, protein levels recovered by 48 h. This

was in contrast to RT-qPCR data, which showed that MMP2 mRNA inhibition by piperine increased at the 48 h time-point as compared with the 24 h time-point. One possible reason for this result is that pro-MMP2 is stored in cells in very minimal amounts, with a majority of the protein being secreted upon translation. In this scenario, the western blots performed in this study would not have been representative of the actual protein expression. Significant differences would thus be expected in the amount of secreted MMP2. Sternlicht and Werb (2001) reported that while most MMPs are secreted upon translation, some MMPs such as MMP8 and MMP9 are stored in certain immune cells. The authors, however, did not comment on MMP2 storage in the cell and it is unclear whether the storage of MMPs within the cell is characteristic of immune cells only. In future experiments, determining the changes in secreted MMP2 levels may be a more accurate method of measuring the effect of piperine on MMP2 protein expression.

MMP2 mRNA may also experience post-transcriptional modifications that regulate the amount of genetic material that is translated into protein. Post-transcriptional modifications are common in cancer cells and often involve the silencing of mRNA transcripts (Thomson, et al. 2006). MMP13 mRNA, for example, undergoes significant post-transcriptional modifications in tumour cells as compared with normal cells (Yu, et al. 2003). A similar mechanism may be involved in MMP2 post-transcriptional regulation and may be linked to the threshold quantity of intracellular pro-MMP2 that a cell may allow. These modifications may therefore have been reflected in the loss of significant MMP2 inhibition as determined by western blot analysis in the current study.

Nonetheless, the fact that piperine significantly inhibited MMP2 mRNA levels indicated that piperine does influence the regulation of MMP2. This effect of piperine may therefore contribute to the inhibition of cell migration as observed in the cell

migration assay. Investigations into the effect of piperine on the amount of secreted MMP2 and after longer hours of treatment may further clarify the results obtained in this study.

4.4 Effect of Piperine on MMP9 Expression in MDA-MB-231 Breast Cancer Cells

Similar to the results for MMP2 mRNA expression, RT-qPCR analysis also showed that 50 μM piperine significantly inhibited MMP9 mRNA expression after 48 h of treatment. It was to be expected that MMP9 would show a similar response to piperine as MMP2, given that both MMPs belong to the same MMP family and there is considerable overlap in their substrates (Klein and Bischoff 2011). This result also corresponds with a study showing that piperine inhibits mRNA expression of MMP9 in 4T1 murine breast cancer cells (Lai, et al. 2012). Western blot analysis, however, failed to show any bands that corresponded to the MMP9 protein. A likely cause for this result was that the primary MMP9 antibody used in the western blot analysis was not sufficiently specific or sensitive to detect MMP9. This experiment should be performed again using a different antibody to verify this hypothesis.

Another possible reason for the discrepancy between MMP9 mRNA and protein expression levels is that the MMP9 protein is not expressed in high quantities in cancer cells. In similar studies, Lee et al (2007) noted that the basal levels of MMP9 in MCF-7 human breast cancer cells were negligible and required phorbol 12-myristate 13-acetate (PMA) to induce MMP9 expression. PMA was also used to induce MMP9 expression in renal carcinoma cells (Hong, et al. 2005). It may therefore be necessary to induce MMP9 protein expression in future experiments so that the protein can be detected by western blot analysis.

As was suggested in the case of MMP2 protein expression, pro-MMP9 may also be secreted rapidly after translation. In this case, the results from this study would suggest that MMP9 is secreted at a significantly faster rate than MMP2. Jackson and Nguyen (1997) reported faster secretion rates of MMP9 relative to MMP2 in human umbilical vein endothelial cells. Other investigations suggest that unlike most MMPs, intracellular MMP9 may be activated under certain conditions to facilitate quick expulsion from the cell (Nguyen, Arkell and Jackson 1998). Activated intracellular MMP9 may not have been observed in the current study because the western blot method used was designed to detect pro-MMP9 alone. There may be merit, therefore, in testing the effects of piperine on secreted MMP9 protein. Using an antibody that can detect both latent and active forms of MMP9 may also help to determine if intracellular activation of MMP9 does occur in MDA-MB-231 breast cancer cells.

Piperine-mediated inhibition of MMP9 mRNA expression may contribute to cell migration inhibition observed in 50 μM piperine treated MDA-MB-231 breast cancer cells. The effect of piperine on secreted MMP9 should be investigated in order to correlate the results between RT-qPCR and western blot analyses. Dose-dependent differences in secreted MMP9 protein levels in such an experiment would indicate that the inhibitory effect of piperine as observed in mRNA expression also translated to protein expression.

4.5 Effect of Piperine on MMP3 Expression in MDA-MB-231 Breast Cancer Cells

The results from the current study showed that piperine affected neither the mRNA expression nor the protein expression of MMP3 in MDA-MB-231 breast cancer cells (Figures 3.4 and 3.7). Both the data from RT-qPCR and western blot analyses showed high variability in mRNA and protein expression values, respectively, as

expressed in the large error bars on graphs. Statistical analysis showed that piperine did not significantly inhibit MMP3 mRNA expression or protein expression.

Selective inhibition of MMPs is preferred in anti-metastatic drugs because non-specific inhibition of MMPs has yielded poor results in the past (Hidalgo and Eckhardt 2001). The inability of piperine to significantly inhibit MMP3 expression, despite inhibiting the expression of MMP2 and MMP9, therefore demonstrates selectivity in drug action. Furthermore, inhibition of MMP3 may be a disadvantage when treating breast cancer metastasis because although MMP3 is not very well studied in the literature, at least one study shows that MMP3 may induce apoptosis in cells (Si-Tayeb, et al. 2006). Another study suggested that MMP3 may inhibit MDA-MB-231 breast cancer invasiveness (Farina, et al. 2002). The anti-metastatic role of MMP3 may therefore be exploited in the future by drugs that increase intracellular levels of MMP3.

MMP3 is also involved in activating and deactivating other MMPs. Ramos-DeSimone et al (1999) showed that in MDA-MB-231 breast cancer cells, MMP3 activates and deactivates MMP9, depending on the amount of TIMP1 present. When MMP9 is present in lower quantities than TIMP1, MMP3 deactivates MMP9. When TIMP1 is present in lower quantities than MMP9, activation of MMP9 occurs. This finding offers another reason why the lack of MMP3 inhibition by piperine may be beneficial. In the current study, RT-qPCR showed that piperine significantly inhibits the mRNA expression of MMP9, which may also result in reduced levels of secreted MMP9. Therefore, by keeping MMP9 levels low and leaving MMP3 levels unchanged, piperine may be exploiting this convergent pathway of MMP9 activation in inhibiting MDA-MB-231 breast cancer cell migration. Future studies into the effect of piperine on TIMP1 levels may determine if piperine decreases MMP9/TIMP1 ratios in breast cancer cells.

Therefore, non-inhibition of MMP3 expression may be an advantage in breast cancer treatments because of the pro-apoptotic and anti-migratory roles MMP3 may play in a cell. Piperine does not inhibit MMP3 expression in MDA-MB-231 breast cancer cells and may thus contribute to cell migration inhibition. Further studies are required to verify these results.

4.6 Effect of Piperine on MMP Activity

Gelatin zymography failed to produce any bands corresponding to gelatinase activity by MMPs in samples from breast cancer cells (Figure 3.8). Serum-supplemented medium, which contains gelatinases, did produce bands of gelatin clearance, indicating that the samples did not contain enough active gelatinases to be detected by this method of analysis. Faint bands corresponding to MMP2 could be seen on the zymogram with the naked eye, however, which confirmed the presence of some active gelatinase in culture medium. These bands were too faint to be quantified and thus the results of the gelatin zymography were deemed inconclusive. There is some evidence in the literature to suggest that piperine inhibits MMP activity in some cancers. For example, Pradeeep and Kuttan (2004) reported inhibition of MMP2 and MMP9 activity by piperine in B16F-10 melanoma cells, as detected by gelatin zymography.

One possible reason for the absence of clear bands in gelatin zymography analysis was that the concentration of MMP protein in the culture supernatant may have been too low to be detected. Even though efforts were made in this study to concentrate supernatant proteins, higher concentration of protein may still be needed in order to increase the visibility of gelatinase bands. In order to achieve this, cells may be seeded at higher concentrations in culture flasks so that more secreted protein will be present in the medium. However, if the concentration of seeded cells varied too significantly from the

concentration used for the other experiments in this study, i.e. western blot and RT-qPCR, the results may not have correlated with the observations made in these other parts of the study. More effective spin filters may also be required to further concentrate secreted MMPs to make their action more visible on the gelatin zymogram. Lyophilization of the culture medium may also help to concentrate the MMPs.

Another possible reason for the absence of observable gelatinolytic activity is the lack of gelatin and collagen in the matrix of cultured cells. The presence of these proteins in the extracellular matrix may be necessary to induce gelatinase activation. Zhuge and Xu (2001) have suggested that the presence of type I collagen is implicated in the activation of MMP2. Similarly, Bannikov et al (2002) showed that MMP9 binding to gelatin or type IV collagen may be necessary to induce enzyme activation. Since the quantity of gelatin and collagens in the culture matrix was likely negligible in the current study, the gelatinases may not have been activated in appreciable amounts. Future studies may therefore require that cells to be sampled for gelatinolytic activity be cultured on a coating mixture of gelatin and collagens.

4.7 Future Directions

Given the evidence that MMPs are secreted quickly after translation, it is important for future studies to look at the effect of piperine on secreted MMPs. Further experiments to investigate the effect of piperine on other MMPs should also be conducted. Overexpression of the collagenase MMP13, for example, is known to be associated with aggressive forms of breast cancer (Zhang, et al. 2008). It would also be interesting to study the effects of piperine on other members of the stromelysin family, such as MMP10, to see how they compare with the effects of piperine on MMP3, as determined in current study. Other phytochemicals, such as piperlongumine, may also have anti-metastatic

properties. Determining if such phytochemicals inhibit cell migration and elucidating a mechanism for this action may help scientists better understand the processes involved in breast cancer metastasis as it relates to MMP expression. Finally, *in vivo* studies will help to further confirm the results of this study.

4.8 Conclusions

Piperine was found to be a potent inhibitor of MDA-MB-231 human breast cancer cell migration. Piperine also inhibited the mRNA expression of the gelatinases, MMP2 and MMP9. However, piperine did not inhibit the mRNA expression of the stromelysin, MMP3. Piperine also did not inhibit the protein expression of MMP2, MMP3 or MMP9. It was concluded that analysis of secreted MMPs may offer more accurate information about the effect of piperine on MMP protein expression. The effect of piperine on MMP activation also did not yield conclusive results. The overall findings in this study, however, indicate that piperine inhibits migration of a triple negative breast cancer cell line and could potentially be used as an effective drug to inhibit metastasis in breast cancer cells.

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