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Inhibition of Hormone Dependent and Independent Breast Cancer Cells by Oyster(Crassostrea virginica) Ceramide

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INHIBITION OF HORMONE DEPENDENT AND INDEPENDENT BREAST CANCER CELLS BY OYSTER (*CRASSOSTREA VIRGINICA*) CERAMIDE

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By
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Dedicated to my beloved father

Mr. Rachakonda Ganapathi Rao

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ABSTRACT

The purpose of this research was to investigate the *in vitro* and *in vivo* anti-angiogenic properties of ceramide isolated from oyster (*Crassostrea virginica*). Ceramide was isolated from oyster by silicic acid chromatography, purified by alkaline hydrolysis, and analyzed by electrospray ionization mass spectrometry (ESI-MS). *In vitro*, the activity of ceramide on human hormone-dependent (MCF-7) and hormone-independent (MDA-MB-435s) breast cancer cells was evaluated for cell viability and proliferation, VEGF-induced cell migration and invasion, apoptosis, and autophagy. The activity of ceramide was also evaluated for VEGF-induced human umbilical vein endothelial cells (HUVEC) tube formation. The protein levels of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) in MCF-7 and MDA-MB435s breast cancer cells treated with ceramide were examined by ELISA. Autophagy was observed by the presence of autophagic vacuoles, and monodansylcadaverine was used as a marker for the detection of autolysosomes. The levels of the transcription factor NF-kB and the activity of phosphatidylinositol kinase were determined by ELISA and Western blot respectively from the cellular extracts of control and ceramide treated breast cancer cells. The activity of ceramide, *in vivo*, was evaluated for inhibition of bFGF-induced angiogenesis with a matrigel plug assay.

The yield of ceramide methyl aminoethylphosphonate from deshelled oysters was 0.16% on a dry weight basis. The molecular size of isolated ceramide was 413 daltons. The viability of MCF-7 and MDA-MB-435s cells exposed to 125μM of ceramide for 48 h was reduced to 76% and 85%, respectively. The viability of MCF-7 and MDA-MB-435s cells exposed to 250 μM of ceramide for 48 h was reduced to and 38% and 45%, respectively. Ceramide at 50 μM inhibited tube formation by HUVEC in the presence of VEGF at 10ng/ml. Ceramide at 125 μM inhibited VEGF-induced MDA-MB-435s cell migration and invasion and also decreased VEGF, EGF

levels, and NF-kB activity in the conditioned media. Ceramide from oyster mediated MCF-7 and MDA-MB-435s breast cancer cell death by autophagy, respectively. *In vivo*, ceramide at 30mg/kg body weight caused a 57% reduction in hemoglobin levels in the matrigel plug assay within seven days. Ceramide from oyster inhibited angiogenesis *in vitro* and *in vivo*.

Key words: ceramide, angiogenesis, breast cancer cells, matrigel plug assay, autophagy, VEGF

CHAPTER: 1
INTRODUCTION

Sporadic breast cancer accounts for more than 95% of all breast cancer cases regardless of ethnic groups suggesting that primary prevention should be the ultimate goal for breast cancer control. Women who are carriers of BRCA1 or BRCA2 gene mutations have about 90% lifetime risk of developing breast cancer and represent 5% of all cases of breast cancer. The inability to predict the majority of breast cancer cases suggests that the prevention would be imporant as part of a broad program to reduce the incidence of breast cancer. It is thought lifelong dietary habits have a large influence on cancer development thus dietary changes may prevent breast cancer development (Hanf and Gonder, 2005; Tsubara et al., 2005; Kotsopoulos and Narod, 2005).

Resistance to chemotherapy is often the result of defects in the apoptotic cell death cascade and a major reason for failure of cancer treatment. Bioactive compounds that can prevent the progression of breast cancer or overcome resistance while inducing breast cancer cell death would improve patient outcomes and prolong patient survival.

Ceramides are sphingolipid-derived bioactive second messengers. These bioactive compounds are being actively studied as potential chemopreventive or chemotherapeutics because they are intimately involved in the regulation of cancer cell growth, differentiation, senescence, and cell death (Ogretmen and Hannun, 2004). Evidence shows that primary and metastatic cancer cells contain less endogenous ceramides compared to normal mucosa from the same patient indicating that ceramide biosynthesis process may be disrupted in cancer cells (Struckhoff et al., 2004). It has also been shown that radiation-resistant and multidrug resistant tumor cells do not generate or accumulate ceramides suggesting an alteration in sphingolipid pathway (Cai et al., 1997). As a proof of concept, treating cancer cells *in vitro* with exogenous ceramides almost always produce cell cycle arrest, senescence, differentiation, apoptosis or

autophagy (Radin, 2003; Scarlatti et al., 2004; Ogretmen and Hannun, 2004). Systemic delivery of exogenous C_6-ceramide to syngeneic Balb/c mice models of breast adenocarcinoma significantly limited tumor growth (Stover et al., 2005). Putative mechanisms responsible for ceramide-induced apoptosis of mammary cancer cells include the accumulation of ceramides within the mitochondria leading to mitochondrial dysfunction, cytochrome *c* release and caspase activation, dephosphorylation of ceramide-activated protein phosphatase, control of calcium levels, and activation of cathepsin D and protein kinase C (Struckhoff et al., 2004; Bourbon et al., 2002; Radin, 2003; Chalfant et al., 2004; Ogretmen and Hannun, 2004; Stover et al., 2005). These data suggest that addition of exogenous ceramides to tumor cells represents an important mechanism for decreasing both tumor cell survival and makes ceramide an attractive bioactive compound for cancer prevention and/or treatment.

While search for naturally occurring bioactive ceramides that may inhibit the progression of breast cancer, we have identified ceramide methyl aminoethylphosphonate in oyster (Matsubara et al, 1975) as an inhibitor of breast cancer cell growth and proliferation. The objectives of this study were to:

1) Isolate and purify ceramide from oysters.
2) Determine the anti angiogenic activity of ceramide *in vitro* using human umbilical vein endothelial cells and hormone-dependent and independent breast cancer cells, and
3) Determine the anti angiogenic activity of ceramide *in vivo* using the Matrigel plug assay.

CHAPTER: 2
LITERATURE REVIEW

2.1. Sphingolipids

2.1.1. Introduction

Sphingolipids belong to the family of membrane lipids that have important structural roles in controlling the fluidity and subdomain structure of the lipid bilayers (Futerman and Hannun, 2004). Sphingolipids represent the most chemically diverse class of biologically active lipids with a wide variety of biological activities. Eukaryotes and prokaryotes contain sphingolipids that are characterized by a common backbone sphingosine. The major sphingolipids in mammalian tissues include ceramides, sphingomyelins, cerebrosides, and gangliosides (Vesper et al., 1999). Sphingolipids are located in cellular membranes, lipoproteins and other lipid rich structures. They are very important for the maintenance of membrane structure and modulate the behavior of growth factor receptors and extracellular proteins. Sphingolipids act as bioeffector molecules, which are very important in various aspects of cancer biology such as cell growth, migration, survival, differentiation, senescence and apoptosis. As all these processes are very important in cancer development and progression, which ceramides having a great impact on anti-cancer therapy (Ogretmen and Hannun, 2004; Reynolds et al., 2004; Modrak et al., 2006). The amino group of sphingosine base is substituted with a long chain fatty acid to produce ceramide, a major molecule in sphingolipid metabolism. Ceramide is the central molecule of the sphingolipid metabolism and is very important for cell regulation activities (Vesper et al., 1999; Ogretmen and Hannun, 2004).

2.1.2. Importance of Sphingolipids to Nutrition

Sphingolipids are constituents of most foods, but in very small amounts (Vesper et al., 1999). There is no evidence for the importance of sphingolipids for growth. But sphingolipids

and their digestion products like ceramide and sphingosine are very important bioactive compounds that are important in cell regulation (Hakamori, 1991). Ceramides are taken up by the intestinal cells and degrades into sphingosine and fatty acids. Dietary sphingolipids do no contribute much to the calories of the body but they are essential nutrients against cancer and some other diseases. Due to their potent biological activities and occurrence in food, sphingolipids can be designated as functional components of food (Hannun and Obeid, 1995; Jaydev et al., 1995; Sweeney et al., 1998; Vesper et al., 1999).

2.2. Ceramides

2.2.1. Definition and Sources

Ceramides belong to the family of sphingolipids composed of N-acylated sphingosine and a fatty acid. Ceramides are associated with wide variety of biological responses to extracellular stimuli, including proliferation, differentiation, growth arrest, cell cycle arrest, immune responses, senescence, and apoptosis (Hannun and Obeid, 2002; Kolesnick, 2002; Stover et al., 2003).

In sphingolipid metabolism, the major modification takes place at the C_1 hydroxyl group of ceramide (Fig 2.1). This hydroxyl group serves as acceptor for glucose and phosphorylcholine to form glycosphingolipid and sphingomyelin, respectively (Perry and Hannun, 1998).

Ceramides are present in trace amounts in dairy products, vegetables, and marine animals. The amount of ceramide present varies from source to source and is generally less than 0.1% (Ziesel et al., 1994; Whitaker, 1996; Vesper et al., 1999). The occurrence of ceramide aminoethylphosphonate and methylaminoethylphosphate in marine animals has been reported (Matsubara, 1975; Matsubara et al., 1990). Ceramide aminoethylphosphonate is a type of phosphonolipid that contains aminoethylphosphonic residue, with a phosphorus carbon bond

attached to lipid backbone sphingosine. Phosphonolipids belong to the class of sphingolipids and the first phosphonolipid identified was ceramide aminoethylphosphonate, which was found in sea anemone (Moschidis, 1985; Matsubara, 1990; Mukhamedova and Glushenkova, 2000). The structures of ceramide aminoethylphosphonate and ceramide methylaminophosphonate are given in fig 2.1a and 2.1b.

2.2.2. Structure and Biochemical Properties

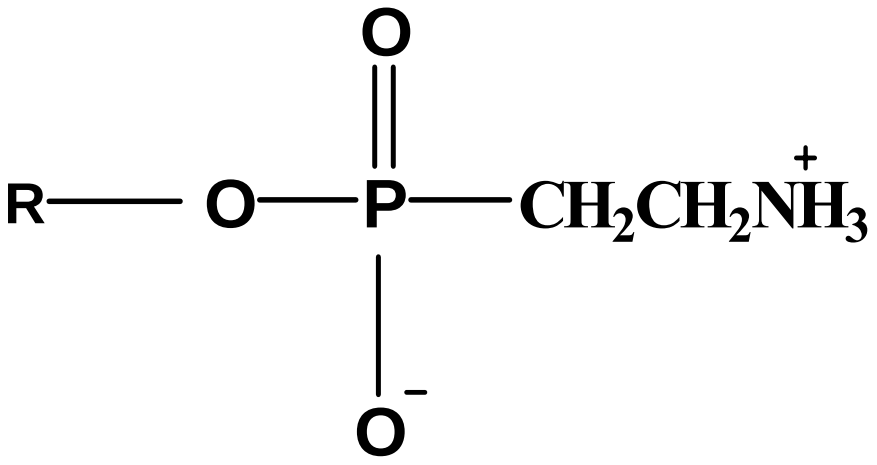

Figure 2.1a: Structure of Ceramide 2-aminoethylphosphonate

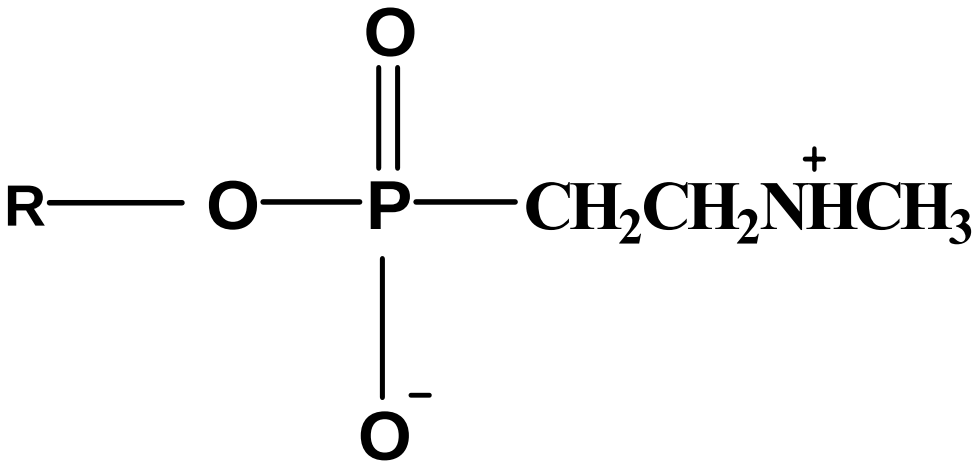

Figure 2.1b: Structure of Ceramide 2-methylaminoethylphosphonate
R- Basic structure of ceramide (Fig 2.1c)

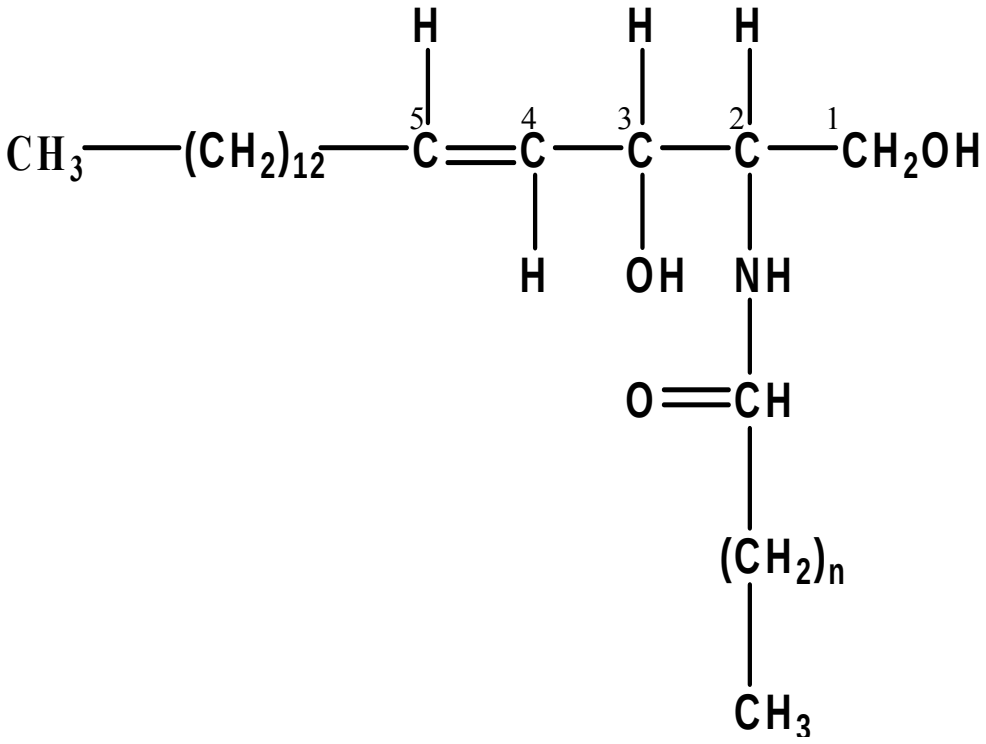

Figure 2.1c: Basic Structure of Ceramide
n=number of carbon atoms

The carbon atoms from 1-5 on sphingosine backbone are biologically important and consist of a primary and secondary hydroxyl group at C_1 and C_3. The amino group at C_2 helps in the formation of amide bond to the fatty acid. The fatty acid portion is highly saturated in ceramide. There is a double bond located between C_4 and C_5, which is responsible for the induction of apoptosis (Perry and Hannun, 1998). And also the replacement of carbonyl group of the amide bond with a methylene group makes the molecule a potent inducer of apoptosis (Perry and Hannun, 1998; Pettus et al., 2002).

2.2.3. Synthesis and Metabolism

Ceramides, the basic structure of sphingolipids can be formed either by *de novo* synthesis or by the hydrolysis of sphingomyelin (Senchenkov et al., 2001; Radin, 2003).The *de novo*

synthesis (Fig 2.3) starts on the surface of endoplasmic reticulum by condensation reaction between serine and palmitoyl Coenzyme A. The condensation reaction is the rate-limiting step, which is followed by reduction, acylation and desaturation. Condensation between serine and palmitoyl Coenzyme A forms ketosphinganine, catalyzed by the enzyme serine palmitoyl transferase. Ketosphinganine reduces the ketone group in presence of ketosphinganine reductase and forms sphinganine (Perry and Hannun, 1998).

Sphinganine is acylated to dihydroceramide. This reaction is followed by the addition of double bond and converts acylated sphinganine to ceramide in presence of dihydroceramide saturase. Drugs such as vincristine, etoposide and paclitaxel and some ionizing agents generally stimulate the *de novo* synthesis of ceramide (Perry and Hannun, 1998; Pettus et al., 2002; Reynolds et al., 2004).

Sphingomyelinases are very important enzymes in sphingolipid metabolism. These enzymes are activated by a wide variety of stress stimuli, resulting in increased ceramide levels. Sphingomyelinase translocates to outer cell membrane after activation by several surface receptors and hydrolyzes sphingomyelin to form ceramide (Segui et al., 2000; Kirschnek et al., 2000; Grassme et al., 2002; Reynolds et al., 2004).

Ceramides can accumulate in the cell or may be converted into a variety of metabolites. The anabolic and catabolic enzymes of ceramide can control ceramide metabolism (Fig 2.3)**.** Phosphorylation of ceramide forms ceramide-1 phosphate and deacylation by ceramidase generates the sphingosine. Sphingosine can be further phosphorylated to sphingosine phosphate by sphingosine kinase. It can also be converted into sphingomyelin by sphingomyelin synthase by transferring the phosphoryl choline from phosphatidyl choline. Glycosylation of ceramide forms glucosylceramide (Mathias et al., 1998; Hannun et al., 2001; Reynolds et al., 2004).

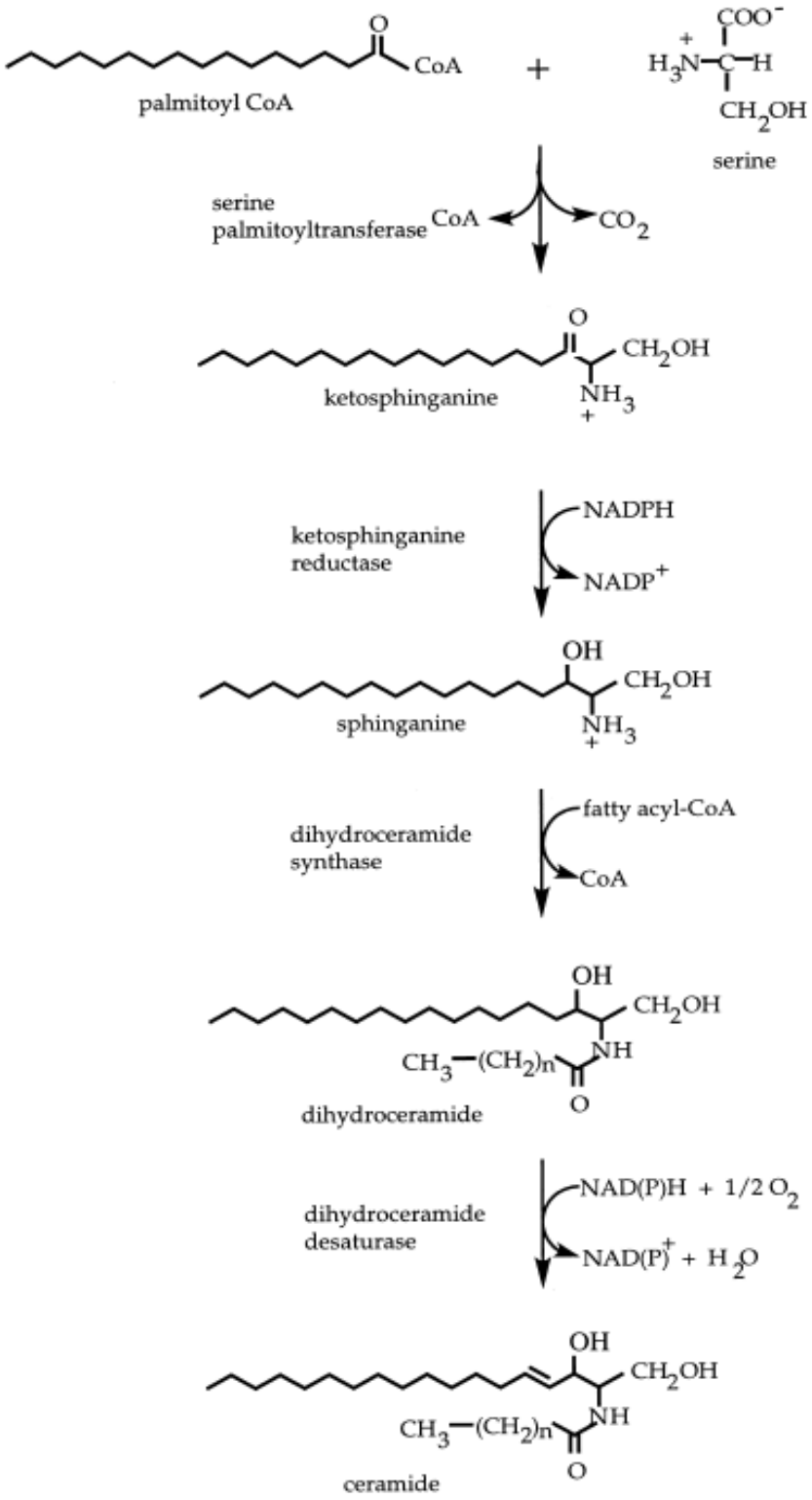

Figure 2.2: *De novo* Synthesis of Ceramides (Perry and Hannun, 1998).

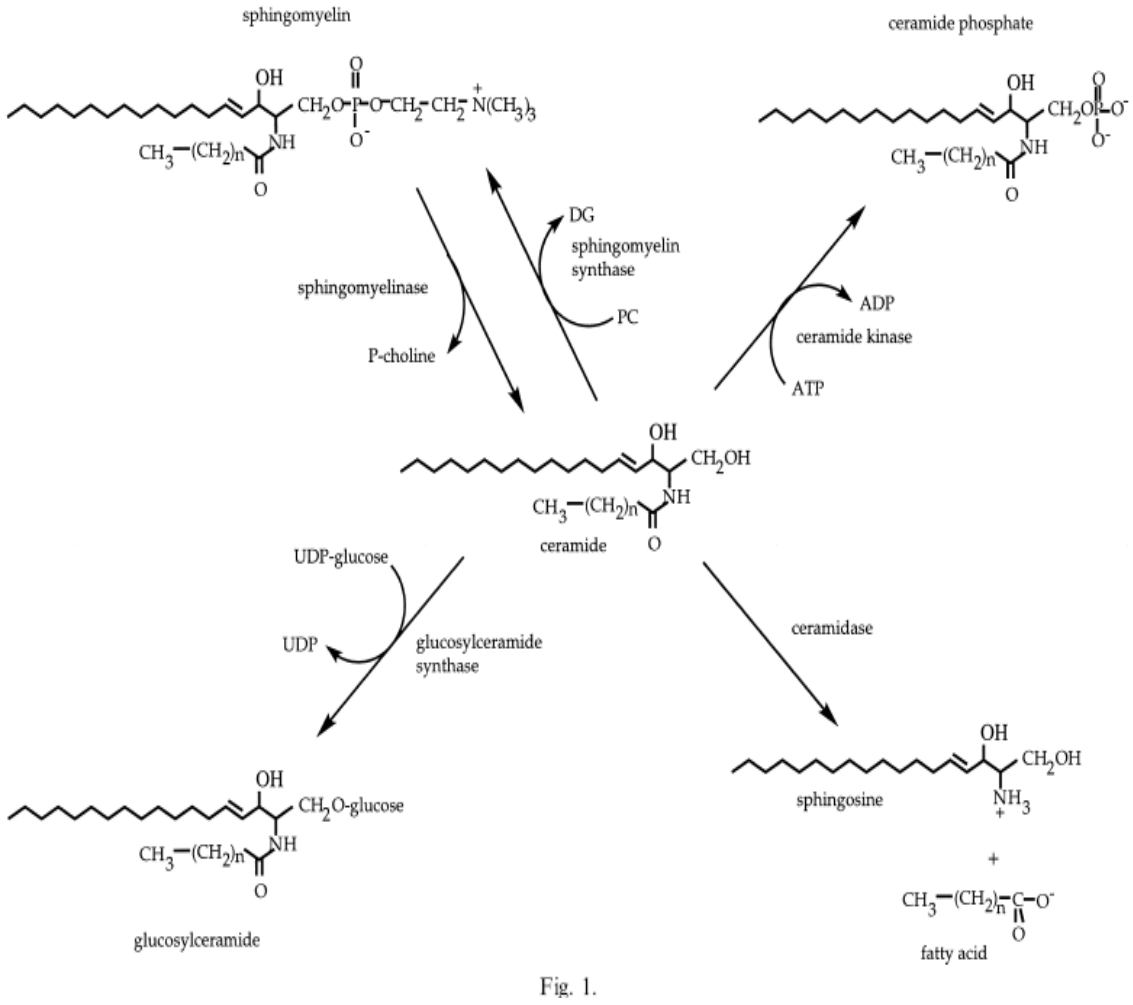

Fig. 1.

Figure 2.3: Ceramides Metabolism (Perry and Hannun, 1998).

2.3. Ceramides in Health and Disease

2.3.1. Introduction

Ceramide is central to sphingolipid metabolism and confers biological functions like anti proliferation, inhibition of migration, growth arrest, and cell death in cancer cells. Due to ceramides cell regulating effects on cancer cells, it has been called tumor suppressor lipid (Futerman and Hannun, 2004; Ogretman and Hannun, 2004; Ogretman, 2006). Ceramides play an important role in preventing diseases like cancer, diabetes and some neurodegenerative diseases. Ceramides have been shown to have wide range of biological effects ranging from cell

proliferation to cell death (Crawford et al., 2003). Endothelial cell proliferation and migration, inflammation, angiogenesis, metastasis are the hallmarks of degenerative disease such as diabetes, heart disease, age related macular degeneration, and cancer (Carameliet and Jain, 2000).

2.3.2. Ceramides under Physiological and Pathological Conditions

Ceramides participate in cell regulating properties like cell death, differentiation, and cell cycle arrest (Hannun and Obeid, 2002; Kolesnick, 2002). Due to these properties ceramides have been extensively studied in the treatment of diseases like cancer, diabetes, heart disease, and other neurodegenerative diseases (Stover et al., 2005; Struckhoff et al., 2004). Ceramide metabolism is altered in tumor cells, and the enzymes targeting ceramide metabolism are expressed differently between cancer cells and normal cells (Crawford et al., 2003).

Under normal physiological conditions angiogenesis is tightly regulated. Angiogenesis is altered in diseases like cancer and can lead to excessive cell proliferation and migration or insufficient blood vessel formation. In diseased conditions like cancer and diabetes, and some other diseases, angiogenesis is imbalanced and leads to production of new blood vessels. In others like infertility and diabetic wounds, angiogenesis is characterized by insufficient blood vessel formation (Carameliet and Jain, 2000). Ceramides mediate anti proliferative pathways, inhibits cell survival mechanisms by regulating signaling cascades controlled by protein phosphatases and also induces cell death by autophagy or apoptosis. Increasing ceramide production in cancer cells leads to increased cytotoxicity in cancer cells (Reynolds et al., 2004; Ogretmen and Hannun, 2004; Ogretmen, 2006).

2.3.3. Ceramides and Cancer

Cancer cells are structurally and functionally abnormal and have highly disorganized

vasculature with excessive branching. Ceramides plays an important role in cell death signaling by cytokines, chemotherapeutic agents, and radiation (Radin, 2001). Exposure of cancer cells to radiation therapy leads to apoptosis and a decrease in Bcl-2 proteins. Ceramides inhibit the antiproliferative pathways by regulating multiple signaling cascades controlled by phosphatases, kinases or cathepsin (Pettus et al., 2002; Simstein et al., 2003; Mochizuki et al., 2004; Scaralatti et al., 2004 ;Takai et al., 2005).

Ceramides are generated in response to chemotherapeutics and radiation by *de novo* synthesis or by hydrolysis of sphingomyelin (Struckhoff et al., 2004; Modrak et al., 2006). Recent investigations have shown that the metabolism of ceramides has influence on the effectiveness of various cancer therapeutics (Senchenkov et al., 2001). Novel ceramide analogs have been used as potential chemotherapeutic agents in breast cancer cells (Stover and Kester, 2003). Chemotherapeutic drugs such as vincristine, etoposide, paclitaxel, and doxorubicin are used to increase intracellular ceramide concentration (Struckhoff et al., 2004). Agents that affect the production or accumulation of ceramides can change the response to chemotherapy as ceramides mediate diverse biological process (Olshefski and Ladisch, 2001; Litvat et al., 2003; Struckhoff et al., 2004). An increase in the levels of ceramides in tumor cells represents an important mechanism for decreasing tumor survival (Struckhoff et al., 2004).

2.3.3.1 Breast Cancer

Breast cancer is the most common cancer of women. It is the second leading cause of death from cancer after lung cancer in women and occurs with 100-fold greater incidence in females than males (Chu et al., 1996; Tsubara et al., 2005; Williams et al., 2006). Breast cancer is a multistep disease characterized by the up regulation of multiple signal pathways that promote growth, proliferation, inhibit apoptosis, and evoke the formation of new blood vessels. It is a

heterogeneous disease that differs from woman to woman. Breast cancer cells escape normal growth control by molecular alterations involving tumor suppressor gene and oncogene products (Coradini and Daidone and 2004). Mutations in BRCA1 and BRCA2 disrupt the breast cell homeostasis and leads to cancer. Women who are carriers of BRCA1 and BRCA2 gene mutations have about 90% lifetime risk of developing breast cancer and represent 5% of all cases of breast cancer (Adem et al., 2004). There are several factors associated with breast cancer such as age, family history, late menopause, obesity, and alcohol and tobacco consumption. Breast cancer can be divided into two types: hormone dependent and hormone independent. Hormone dependent breast cancer cells express receptors to estrogen and progesterone hormones (Williams et al., 2006).

Chemotherapy is the main approach to treat breast cancer cells. Chemotherapeutic compounds produce their cytotoxic effects by interfering with the function of DNA or RNA, or increase the concentration of some bioavailable compounds to act against cancer cells (Kitano, 2004). Chemotherapeutic drugs cause damage to the adjacent cells. Chemoresistance is also accompanied by the progression of breast cancers from hormone dependent, non metastatic, anti estrogen-sensitive phenotype to a hormone independent, invasive, metastatic, antiestrogen-resistant phenotype (Campbell et al., 2001; Simstein et al., 2003). Cancer cells often generate their own growth signals leading to metastasis, angiogenesis, and cellular immortality. (Kitano, 2004). Tumors are highly self-sufficient in generating their own proliferative growth signals, invasion and metastasis, evasion of apoptosis, and leading to neo- angiogenesis (Hanahan and Weinberg, 2000). Tumor cells respond well to the drugs initially, but may become resistant over time. Addition of exogenous ceramide to tumor cells represents an important mechanism for cell death and limited cell growth and makes ceramide an attractive bioactive compound for cancer

treatment (Vesper et al., 1999; Samsel et al., 2004; Ogretmen and Hannun, 2004; Stover et al., 2005; Struckhoff et al., 2005).

2.3.4. Mode of Action of Ceramides on Breast Cancer Cells

2.3.4.1. Inhibition of Angiogenesis

2.3.4.1.1. Physiological and Pathological Angiogenesis

Angiogenesis is the formation of new blood vessels from preexisting vessels. The formation of blood vessels is essential for organ growth and repair and important in both physiological and pathological conditions (Papetti and Herman, 2002).

Vascular endothelial growth factor (VEGF) is the major stimulator in angiogenesis. VEGF stimulates the formation of blood vessels and their permeability. Some other factors thought to promote angiogenesis are fibroblast growth factor (FGF), angiopoietin (Ang-2), platelet-derived growth factor (PDGF), and tumor necrosis factor alpha (TNF-α) and hormones like estrogen, progesterone, and androgens. Metalloproteinase and membrane bound proteins also stimulate angiogenesis. Table 2.1 lists some of the stimulators and inhibitors of angiogenesis known to date.

Table 2.1: List of Angiogenesis Stimulators and Inhibitors (Losso and Bansode, 2004)

Stimulators of Angiogenesis	**Inhibitors of Angiogenesis**
Vascular endothelial growth factor (VEGF)	Angiostatin
Fibroblast growth factors: acidic (aFGF) and basic (bFGF)	Interferon alpha/beta/gamma
Platelet-derived endothelial cell growth factor (PD-ECGF)	Transforming growth factor-beta
Platelet-derived growth factor-BB (PDGF-BB)	Human chrionic gonadotropin
Tumor necrosis factor-alpha (TNF-alpha	Heparinases
Interleukin-8 (IL-8)	Endostatin
Angiogenin	Metalloproteinase inhibitors
Angiopoietin-1	Platelet factor

There are two types of angiogenesis: physiological and pathological angiogenesis. Physiological angiogenesis plays an important role in tissue development, reproduction, wound healing, oxygen delivery to muscles, and tissue repair. In physiological conditions there is a balance between stimulators and inhibitors of angiogenesis. Physiological angiogenesis is important for reproduction, for connective tissue formation, development of child in a mother's womb, maintenance of vascular integrity during wound healing, repair of damaged tissue, oxygen delivery to working muscles, and bone growth and repair (Papetti and Herman, 2002). The mechanism of physiological angiogenesis includes the following steps:
1) pericyte removal from endothelium and angiopoietin-2 (Ang-2)-catalyze transformation of endothelial cells from a stable to a proliferative phenotype; 2) growth factors, VEGF and VE cadherin catalyze vessel hyper-permeability and matrix remodeling by the action of serine and matrix metalloproteinase; 3) growth factor (VEGF, FGF, and EGF) catalyze endothelial cell proliferation; 4) integrin ($\alpha v\beta 3$) and growth factors, VEGF, and FGF; catalyze endothelial cell migration 5) Vecadherin (Vecad) and ephrin B2/ephrin B4 catalyze cell-cell contact; 6) FGF, PDGF, TNF-α, and Eph-2A catalyze tube formation as blood conduits; 7) PDGF- and Ang1/Tie2-catalyze proliferation and migration of mesenchymal cells along the new vessels and TGF-β catalyze pericyte differentiation into mature pericytes; and finally 8) Ang1/Tie2, PDGF, VE cadherins and TGF-β catalyzed vessel stabilization (Papetti and Herman and 2002; Losso and Bansode, 2004).

Pathological angiogenesis occurs when there is imbalance between angiogenesis inhibitors and stimulators. There are two types of pathological angiogenesis: excessive and insufficient angiogenesis. Excessive angiogenesis occurs when angiogenic stimulators out balance angiogenic inhibitors. Insufficient angiogenesis occurs when there is a deficiency of

stimulators. The mechanism of excessive angiogenesis involves: 1) retraction of pericytes from the abluminal surface of the capillary, 2) release of proteases from the activated endothelial cells, 3) protease-catalyzed dissolution of the basement membrane surrounding the pre-existing vessels, 4) endothelial cell migration toward an angiogenic stimulus and their proliferation, 5) formation of tube-like structures, 6) fusion of the formed vessels, and 7) initiation of blood flow(Carameliet and Jain, 2000; Papetti and Herman, 2002).

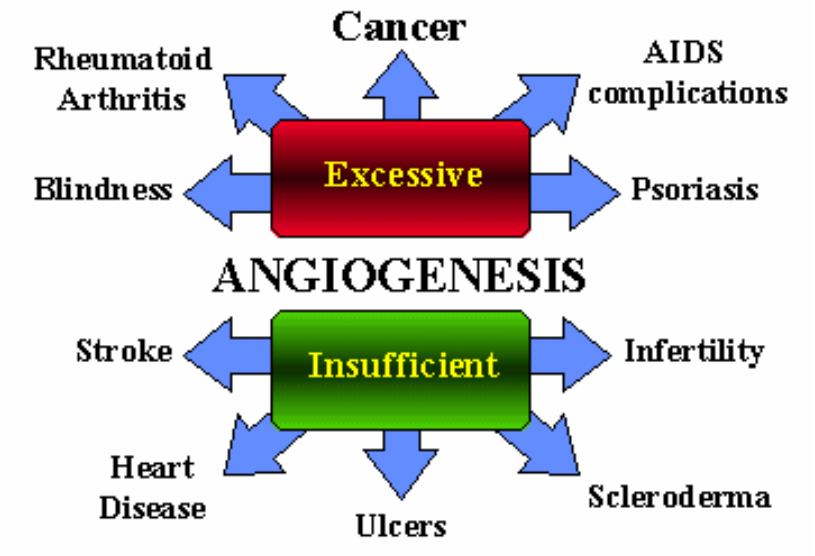

Figure 2.4: Degenerative Diseases Associated with Pathological Angiogenesis (Losso, 2002)

Insufficient angiogenesis is characterized by 1) insufficient vascularization, 2) delayed formation of granulation tissue, 3) decreased collagen content, 4) low breaking strength compared to normal tissue, loss of vascular tone, 5) higher content of oxidized lipids, 6) absence of microtubular structures, 7) impaired collateral vessel formation, and 8) impaired blood vessels (Papetti and Herman, 2002).

Understanding angiogenesis and its characteristics in tumor growth has provided ways to block the process. Antiangiogenic compounds have been used in the treatment of cancer (Ferrara

and Kerbel, 2005). Angiogenesis requires tumor cells to release stimulators and proteolytic enzymes to degrade extracellular matrix for migration and proliferation. Depending on how they function, angiogenesis inhibitors can be divided into the following categories: 1) agents that inhibit the vascular endothelial cell activation; 2) agents that inhibit the vascular endothelial cell migration, proliferation, and survival; 3) compounds that inhibit the extracellular matrix degradation; and 4) compounds that inhibit integrin activation (Kerbel et al., 2001; Viloria-Petit et al., 2001). Depending on the target, angiogenesis inhibitors can be categorized into two types: direct and indirect inhibitors. Direct angiogenesis inhibitors prevent vascular endothelial cell proliferation and migration. Direct inhibitors either block or decrease growth factors, which are necessary for migration, proliferation, and cell death. Indirect angiogenesis inhibitors block the expression activity of growth factors or its receptor that activate angiogenesis in tumor. As these growth factors are the product of genes, indirect inhibitors not only control proliferation and migration but also disrupt the production of angiogenic factors (Albo et al., 2004, Kerbel and Ferrara, 2005).

It has been hypothesized that antiangiogenesis would be an effective strategy to treat human cancer (Folkman, 1971; Folkman, 1995; Carameliet and Jain, 2000; Ferrara and Kerbel, 2005). Ceramides are associated with several biological processes like cell proliferation, differentiation, growth arrest (Hannun and Obeid, 2002; Kolesnick, 2002; Struckhoff et al., 2004). Ceramides such as C6 and C8 ceramides inhibited angiogenesis in breast cancer cells (Crawford et al., 2003; Struckhoff et al., 2004; Takai et al., 2005).

2.3.4.1.2 Angiogenesis and Breast cancer

Pathological angiogenesis a hallmark of cancer and various other diseases. Angiogenesis is essential for growth and progression of breast cancer cells (Toi et al., 2002). VEGF is the key

molecule in breast cancer cells which stimulates endothelial cell proliferation and migration within the tumor (Toi et al., 1995; Toi et al., 2002).

Biomarkers associated with the highest impact on breast cancer development and progression include *c-myc*, HER-1/EGFR, HER-2/neu, estrogen receptor-ß, cyclin A, B, D, and E, p53, bcl-2, telomerase activity, hTERT/hTR, CD31/PECAM-1, VEGF, HIF-1α(Toi et al., 2002). However, other biomarkers of breast cancer associated with the aggressiveness of the disease include matrix metalloproteinase -2 and -9, cathepsin D, NFk-B, Hsp90, COX-2, PI3K, AKT, MAPK, P-glycoproteins, Ki67, Wnt-1, and AP-1 (Coradini and Daidone, 2004). There are other markers, considered as indirect biomarkers such as heparanase and MMP-1 for which the correlation with breast cancer has not been clearly established. However, most cancer biomarkers have been identified in the invasive and proliferative stages of the disease are related to cell proliferation, invasion, migration, hormone-dependence, apoptosis, and metastasis (Jong et al., 2002).

2.3.4.1.3. Ceramides and Breast Cancer

Ceramide analogs have been extensively used in treating breast cancer cells (Struckhoff et al., 2004). Treating breast cancer cells with chemotherapeutic agents such as vincristine, daunorubicin, and gemcitabine increases the intracellular concentration of ceramides by inducing the *de novo* pathway (Struckhoff et al., 2004). Exogenous ceramides can also be utilized to treat breast cancer cells. Ceramides can be delivered to the cancer cells by using liposomal vehicles to target the breast cancer cells. It has been reported that liposomal delivery of ceramide analogs caused significantly greater accumulation of ceramide in breast cancer cells (Radin, 2001; Stover and Kester, 2003). The bioactive ceramides can be incorporated into conventional liposomal vehicles for improved drug delivery and release. Exogenous delivery of ceramides in liposomes

is more effective and has shown inhibitory effects on breast cancer cells (Stover and Kester, 2003). Delivery of ceramides to breast cancer cells caused *in vitro* cell death and *in vivo* delivery of ceramide slowed tumor growth in breast cancer cells (Stover et al., 2003; Futerman and Hannun, 2004; Ogretman, 2006). Novel ceramide analogs showed cytotoxic effects in drug resistant breast cancer cells (Crawford et al., 2003). Naturally occurring ceramide found in oysters may have similar biological activity against cancer cells but this hypothesis has never been investigated.

2.3.4.2. Cell Death Mechanisms

Cell death in cancer cells is caused by the activation of various enzymes that are stimulated by some bioactive compounds. Ceramide is a bioactive compound that plays an important role in cell death in cancer cells by autophagy and or apoptosis (Hannun and Obeid, 1995; Mochizuki et al., 2002). In general there are three different types of cellular death that occurs in cells to maintain cellular homeostasis: autophagy, apoptosis, and necrosis. Ceramides cause cell death in cancer cells by autophagy or apoptosis.

2.3.4.2.1. Autophagy

Autophagy is a degradative pathway, which involves the removal of cytoplasmic portions and intracellular organelles in a vacuole called autophagosome. It is also called as programmed cell death II (PCD II). These vacuoles fuse with lysosome and organelles are degraded and resulting macromolecules recycled. It can be initiated by conditions like starvation, stress, and hormone treatment (Van Sluijters et al., 2000). There are three types of autophagy: macroautophagy, microautophagy, and chaperone mediated autophagy.

Macroautophagy also called autophagy plays an important role in diseases like cancer, muscular disorders, and neurodegenerative diseases (Shintani and Kilonsky, 2004). Autophagy is

a complex process that occurs in several steps. This PCD II is initiated by the formation of an autophagosome. Cytoplasm and various cell organelles such as endoplasmic reticulum, mitochondria and peroxisomes are sequestered by a membrane to form an autophagosome. A smooth membrane from a ribosome free region of the endoplasmic reticulum covers the autophagosome. Thereafter the autophagosome fuses with the lysosome and finally degrades the cell (Munafo and Colombo, 2001). Autophagy protects against cancer cells by removing the damaged cells, increasing protein degradation, and promoting autophagic cell death (Levine and Yuan, 2005). The main characteristic feature of autophagy is its dependence on tumor suppressor genes acting in the formation of the autophagosome and in signaling pathways regulating autophagy. Beclin-I is one of the tumor suppressor genes that plays an important role with class III phosphatidylinositol-3-kinase complex in the autophagic process. Beclin-I inhibits cellular proliferation in the autophagic process. Up regulation of Beclin-I and inhibition of phosphatidylinositol-3-kinase are very important in autophagic cell death in cancer cells (Lavieu et al., 2006). Ceramides have been used to inhibit the phosphotidylinositol-3-kinase and stimulate the beclin-I against breast cancer cells (Scarlatti et al., 2004; Lavieu et al., 2006). Beclin-I expression is reduced in invasive breast cancers. Many drugs have been reported to trigger cell death by autophagy in cancer cells by increasing levels of ceramide (Scaralatti et al., 2004).

2.3.4.2.2. Apoptosis

Apoptosis is one of the main types of cellular death and also called programmed cell death I (PCD I). Apoptosis is an active process that depends on the network of a sequence of signaling events that lead to cell death. Apoptosis is a discrete sequence of morphological changes causing cell death with DNA cleavage by chromatin segregation with nuclear

membrane. It maintains cellular homeostasis in organisms, which is very important in the aspect of disease development in humans (Simstein et al., 2003; Hetz et al., 2005).

Programmed cell death I is characterized by the following steps: a) cells become circular due to the action of caspases that have been activated inside the cell. b) chromatin degrades and condenses into small compact patches c) the nuclear envelope becomes discontinuous and the DNA inside it is fragmented and the cell breaks into apoptotic bodies, which are then phagocytosed. During apoptosis, membranes are well maintained and do not affect the behavior of other cells and the resulting fragments (apoptotic bodies) are phagocytosed by surrounding cells (Simstein et al., 2003).

The key molecules that control PCD are members of the death associated protein kinase (DAPK) family. Death associated protein kinase –I (DAPk) mainly activates apoptosis through a caspase dependent pathway (Shintani and Kilonsky, 2004). The molecular events of apoptosis can be divided into three steps: 1) apoptosis initiation by inducing agent, 2) caspase activation by a signal transduction cascade, and 3) cellular degradation by proteolytic cleavage. There are several genes, which are controlled by extracellular factors that are involved in apoptosis. Caspases are a group of proteases, the initiators in apoptotic pathway, which contain a cysteine active site with aspartate substrate specificity. Activation of caspases involves both extrinsic and intrinsic pathways. Intrinsic pathway involves Bcl-2 proteins and the release of cytochrome *C* from mitochondria. Cytochrome *C* activates the adaptor protein, followed by the activation of procaspase-9, which causes the activation of effector caspases. Extrinsic pathway is induced by death receptor protein complexes that cleave procaspase-8 (Simstein et al., 2003; Reynolds et al., 2004). Caspase-8 cleavage and activation initiates the caspase cascade. After the induction of caspase 8, the extrinsic and intrinsic pathways unite at the level of effector caspases. These

effector caspases cleave cellular signaling molecules; cleave DNA repair enzymes, cellular, and nuclear proteins (Simstein et al., 2003). Additional markers of apoptosis include DNA fragmentation, phosphatidylserine exposure at the cells surface, and the characterization of key molecules, such as cysteine proteases of the caspase family and members of Bcl-2 family of proteins (Danial and Korsmeyer, 2004; Hetz et al., 2005).

It has been reported ceramides mediate cell proliferation, differentiation, cell cycle arrest, and apoptosis (Reynolds et al., 2004). Ceramides are secondary messenger of apoptosis manifested by significant increase in ceramide levels before the onset of apoptosis and activation of caspases. Ceramides play an important role in the execution of apoptosis (Pettus et al., 2002; Simstein et al., 2003; Reynolds et al., 2004; Modrak et al., 2006). Ceramides activate the key molecules like protein kinase, and mitochondria for the induction of apoptosis. Chemotherapeutic drugs have been used to induce the synthesis of ceramide, cause the activation of protein kinase and down regulate the antiapoptotic signals leading to apoptosis (Stover and Kester, 2003).

2.3.4.2.3. Necrosis

Necrosis also called ‘accidental cell death’ is characterized by rapid swelling of the dying cell, rupture of the plasma membrane, and release of cytoplasmic content into the cell environment. Although necrotic cell death has great impact in pathological conditions such as heart diseases and several neurodegenerative diseases, the molecular mechanism of necrotic cell death is poorly understood. Necrosis occurs under conditions of cellular injury and is related to the loss of homeostasis and drastic decreases in ATP levels (Hetz et al., 2005). In recent years it has been reported that necrotic cell death occurs under normal physiological conditions. Additional evidence shows that traditional apoptotic pathways lead to necrotic cell death under certain conditions (Hetz et al., 2002, Leyton and Quest, 2004). Nonapoptotic cell death plays an important

role in the control of cell viability under normal physiological conditions, when injury or other disease triggers cell death. The understandings of molecular approaches to cell death are important as these different forms of cell death share some common features at the morphological level. Understanding of the cell death mechanisms will help in the development of more efficient therapies for the treatment of human diseases like cancer, autoimmune, and neurological diseases (Hetz et al., 2005).

CHAPTER: 3
MATERIALS AND METHODS

3.1. Isolation and Purification of Ceramide from Oyster

3.1.1. Introduction

Ceramide aminoethylphosphonate (CAEPn) and ceramide methylaminophosphonate (CMAEPn) have been isolated from the abductor, gills, and viscera of the oysters (Matsubara, 1975). Studies have shown that CAEPn and CMAEPn are widely distributed in a variety of fresh-water and marine bivalves, and edible mollusks (Kariotoglou and Mastronicolis,2003), snails (Hori et al, 1996; 1967), gastropods (Komai et al, 1973) sea anemones (Karlsson and Samuelsson, 1974; Adosraku et al., 1996), and sea stars (Mukhamedova and Glushenkova, 2000).

3.1.2. Materials

Oyster samples were purchased from P & J New Orleans, LA. Silica gel (32-63 mesh size) was purchased from Bodman Industries, Aston, PA. The Column for chromatography was purchased from Bio Rad Laboratories, Hercules, CA. Organic solvents used were of analytical grade.

3.1.3. Isolation and Purification of CMAEPn

Ceramide methylaminoethylphosphonate was extracted using a modification of the method of Matsubara et al. (1975). Oysters were blended and lyophilized. The lyophilized samples were extracted with acetone (1:1 w/v) with shaking for 1 h at 4°C and the mixture was left at 4°C for two days. Acetone extract removed neutral lipids and water from the oyster samples. The residue was extracted five times with acetone. The remaining residue, which was free of neutral lipids, was extracted with chloroform: methanol (2:1 v/v) twice. Extracts were dried and applied to a silicic acid column. Crude ceramide was eluted with chloroform: methanol

(9:1 v/v) from the silicic acid column. Crude ceramide was purified further by alkaline hydrolysis to remove glycerophospholipids and again passed through silicic acid column. The column was eluted with a series of chloroform: methanol (98:2 v/v) to remove fatty acid methyl esters and then acetone: methanol (9:1 v/v) to obtain ceramide methylaminoethylphosphonate.

3.1.4. Identification of Ceramide by Electrospray Ionization (ESI)-Mass spectrometry (MS)

The isolated ceramide methylaminoethylphosphonate was analyzed by ESI-MS. The samples were dissolved in methanol: water with 1% acetic acid. ESI-MS of ceramide methylaminoethylphosphonate was recorded in the positive-ion mode using a PE Sciex Qstar (Quadrupole-TOF hybrid, Applied Biosystems, Foster City, CA). The spectrum was recorded at an accelerating voltage of 4500 V.

3.2. *In Vitro* Anti- angiogenic Activity of Ceramide

3.2.1. Introduction

Angiogenesis is the formation of new blood vessels from the existing vasculature. The principal cells involved are endothelial cells. First, endothelial cells escape from their location by breaking through the basement membrane to form new blood vessels. Then endothelial cells migrate toward a stimulus that might be released by tumor cells or wound associated macrophages. The migrated endothelial cells then proliferate and organize into three-dimensional tubular structures ultimately forming new blood vessels. Each of these steps, basement membrane disruption, cell migration, cell proliferation, and tube formation can be tested *in vitro* (Auerbach et al., 2003; Staton et al., 2004).In vitro assays of angiogenesis are tested predominantly on migration, proliferation, and tube formation in response to exogenous inhibitory compounds. Cell proliferation assays are easy to perform and reproducible, imparting themselves to accurate quantification (Auerbach et al., 2000; Auerbach et al., 2003). Generally

there are two classes of proliferation assays: some of which determine cell number and the other measure cell cycle kinetics. Cell numbers can be counted using a haemocytometer or by electronic counter. Another method to measure the cell proliferation is by using tetrazolium salt, which is cleaved by active mitochondria to form a dark blue product. The product is measured colorimetrically as a measure of proliferation as living cells produce the formazan blue product (Denizot and Lang, 1986; Wemme et al., 1992; Staton et al., 2004).Cell migration and invasion are also very important for embryonic development and functioning of adult organisms (Valster et al., 2005). There are several tests that can be used to determine the migration response of endothelial cells to angiogenesis stimulating or inhibiting factors. Endothelial cells move by a process called chemotaxis, along a gradient of angiogenesis-stimulating factors such as VEGF. A modified Boyden chamber assay has been used to assess the migration of endothelial cells. In this assay, endothelial cells are placed on top of a filter and migrate across the filter in response to an angiogenic factor placed in the lower chamber. The major advantage of this assay is the high sensitivity to small differences in concentration (Auerbach et al., 2003; Staton et al., 2004). A tube formation assay is the most specific test for angiogenesis. The tube formation assay measures the ability of endothelial cells to form three-dimensional structures. Endothelial cells form tubules spontaneously with the appropriate extracellular matrix components. The formation of tubes can be observed under a microscope. Tube formation can be improved by using collagen or fibrin clots on the culture discs (Auerbach et al., 2003).

3.2.2. Materials

Ceramide was dissolved in DMSO to obtain 20 mM stock, stored at -20^0C in aliquots, and diluted according to the required concentrations. CellTiter 96 AQ_{ueous} One solution was obtained from Promega (Madison, WI). Chemicon in vitro Angiogenesis kit ECM 625 was used

for tube formation assay. Cell migration and cell invasion kits were purchased from Cell Biolabs (San Diego, CA). TransAM Nuclear Extract and TransAM-NFκB kits were purchased from Active Motif (Carlsbad, CA). BCA Protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). Caspase–Glo 9 Assay was purchased from Promega (Madison, WI.). Human VEGF and EGF ELISA kits were purchased from Peprotech, Rock Hill, NJ.

3.2.3. Cell Cultures and Harvesting

Estrogen dependent MCF-7 and estrogen independent MDA-MB 435s cell lines were used. MCF-7 cell line is a BRCA1-competent hormone-sensitive and non-invasive luminal breast cancer cell line that expresses ER+ and p53. The MDA-MB 435 s breast cancer cells are spindle shaped and established from pleural effusion. MDA-MB 435 s cell line is estrogen independent and BRCA1 incompetent. MCF-7 and MDA-MB 435s breast cancer cell lines were obtained from American Type Culture Collection (Rockville, MD) and harvested according to ATCC procedure. The cells were grown as a monolayer and attained 100% confluence.

3.2.4. Cell Proliferation and Viability Assay

Confluent cultures of MCF-7 and MDA-MB435s breast cancer cells were seeded on 96-well plates at a density of approximately 2x 10^4cells/well in a total volume of 100 µl. The cells were grown for 24 h prior to treatment. MCF-7 cells were treated by addition of 10 ng/ml of 17β-estradiol. Ceramide was dissolved in dimethylsulfoxide to achieve final concentrations of 50-500 µM while making sure that DMSO concentration did not exceed 1%. Untreated control cancer cells received less than 1% of dimethylsulfoxide. All experiments were carried out in triplicates. Replicates of culture plates were prepared and incubated for 24, 48, or 72 h in a humidified incubator containing 5% CO_2 at 37 ºC. At the end of the incubation period, the CellTiter 96 AQ_{ueous} One solution (Promega, Madison, WI) was used as follows. Twenty micro liters of the

tetrazolium solution was added to each well and the plate was incubated in a humidified 5% CO_2 incubator at 37^0C for one hour. The plate was read at 490 nm using a *Spectra Max Plus* ELISA plate reader (Molecular Devices, Sunnyvale, CA). Each concentration of the inhibitor was done in triplicates. Cell viability and proliferation were normalized to the levels in untreated control cells to determine the percentage of viable cells.

3.2.5. Cell Migration Assay

The effect of ceramides on MDA-MB435s cell motility, an important pre-requisite for angiogenesis, was investigated by performing endothelial cell migration assay. The assay was carried in a 96-well disposable chamber (Cell Biolabs Inc, San Diego, CA) following the manufacturer's instructions. MDA-MB 435s (1.0 x 10^6 cells) in 100 µl of media was added to the membrane. The wells of the feeder tray were loaded with 150 µl of media containing 10 ng/ml VEGF. The plate was incubated in a cell culture incubator at 5% CO_2, 37^0C for 4 h. MDA-MB-435s cells were treated with concentrations of 50-125 µM ceramide or DMSO (vehicle). At the end of incubation period, the cells were dislodged from the underside of the membrane, using cell detachment buffer and lysed with lysis buffer along with the dye and 150 µl of the mixture was transferred to a 96-well plate suitable for fluorescence measurement. The plate was read at 480nm/520 nm using Perkin Elmer LS 50B spectro fluorimeter.

3.2.6. Cell Invasion Assay

Endothelial cell invasiveness was assayed using the fluorometric format CytoSelect™ 96-well Cell Invasion Assay (Cell Biolabs Inc, San Diego, CA) following the manufacturer's instructions. MDA-MB 435s (2.0 x 10^6 cells) in 100 µl was added to the membrane. The wells of the feeder tray were loaded with 150 µl of media containing 10 ng/ml of VEGF. The plate was incubated in a cell culture incubator at 5% CO_2, 37°C for 4 h. Cells were treated with ceramide at

concentrations of 50-125 μM and controls were treated with 0.1% DMSO (vehicle). At the end of incubation period, the cells were dislodged from the underside of the membrane, using cell detachment buffer and lysed with lysis buffer along with the dye and 150 μl of the mixture was transferred to a 96-well plate suitable for fluorescence measurement. The plate was read at 480nm/520 nm using Perkin Elmer LS 50B fluorometer.

3.2.7. Tube Formation Assay

For the tube formation assay human umbilical vein endothelial cells (HUVEC) were used. After preparing the gel matrix, as per the manufacturer's instructions, and allowing it to solidification. HUVEC cells (1.0×10^4 cells in 100 μl) were seeded onto the surface of the polymerized ECmatrix (Chemicon, Temecula, CA), alone or mixed with 50 μM of ceramide dissolved in DMSO in the presence of 10 ng/ml of VEGF. The plate was incubated at 37°C for 6 h. Tube formation was inspected and photographed using a Leitz phase contrast inverted microscope

3.2.8. Quantification of Apoptosis

Detection of apoptosis was performed using the Caspase Assay (Promega, Madison, WI). Briefly, MCF-7 cells and MDA-MB-435s cells were treated with125 μM 250 μM of ceramide, respectively and incubated for 48 h. At the end of the incubation period, the cells were treated with 100 μl of Caspase- Glo 9 reagent incubated for 1 h and the luminescence of the solution was measured using a Perkin Elmer LS 50B luminometer.

3.2.9. Quantification and Visualization of Autophagic Vacuoles

MCF-7 cells were treated with 125 μM of ceramide and MDA-MB 435s cells were treated with 250 μM of ceramide for 48 h. For visualization of autophagic vacuoles cells were grown on cover slips for 48 h and labeled with 0.05 mM monodansylcadaverine (MDC) in PBS

at 37°C for 10 minutes. After incubation cells were washed four times with PBS and analyzed by fluorescence microscopy. For quantification of autophagic vacuoles cells, were plated in 96-well plates and treated with ceramide followed by incubation with 0.05 mM MDC in PBS at 37°C for 10 minutes. After incubation, cells were washed with PBS four times and collected in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100. Intracellular MDC was measured at 380/520 nm using Perkin Elmer LS 50B spectro fluorometer. DNA fluorescence was measured by adding ethidium bromide to a final concentration of 0.2 μM and measured at 530/590 nm using Perkin Elmer LS 50B spectro fluorometer (Munafo and Colombo, 2004).

3.2.10. Protein Extraction, Immuno Precipitation, and Western Blotting

MCF-7 and MDA-MB 435s cells were plated on 6-well dishes and treated with the 125 μM and 250 μM of ceramide, respectively for 48 h. After the treatment, supernatant was collected for the quantification of growth factors VEGF and EGF. The cells were washed with ice cold PBS. Cytoplasmic and nuclear fractions were obtained using Nuclear Extraction kit (Active Motif, Carlsbad, CA). The protein concentrations for supernatant, cytoplasm, and nuclear fraction were obtained using BCA protein assay kit (Pierce, Rockford, IL).

Cytoplasmic proteins were immunoprecipitated using immunoprecipitation/western blot protocol (Santa Cruz Biotechnology, CA). For immunoprecipitation, 300 μg of cell extract was incubated with anti-PI3K at 4°C on an end-to end rotator. The supernatant was centrifuged at 10,000x *g* for 10 min, and protein A/G beads were added. The mixture was rotated for 3 h at 4°C and the immunocomplexes were washed with lysis buffer three times. SDS sample buffer was added to the beads, and the beads were incubated at 95°C for 5 min and the supernatant subjected to western blot analysis. Electrophoretic separation was performed using 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane. Membranes were

blocked with 5% nonfat dry milk in PBS/Tween 20 (0.05%), followed by incubation with an anti PI3K antibody (1:1000 in 10% milk/PBS-T). Visualization of the bound primary antibody was done by probing with horseradish peroxidase-conjugated secondary antibodies (1:1000), and exposure to chromogenic detection reagent.

3.2.11. ELISA

3.2.11.1. Quantification of VEGF

VEGF levels in the conditioned medium were determined by ELISA using the VEGF ELISA protocol from Peprotech (Rock Hill, NJ) following the manufacturer's instructions. Samples of control MCF-7 and MDA-MB435s breast cancer cells and ceramide treated samples were analyzed. The amount of VEGF was detected using the VEGF standard curve, established with purified VEGF in the range of 0-4000 pg/ml.

3.2.11.2. Quantification of EGF

EGF in the conditioned media was quantified by using Human EGF ELISA kit (Peprotech, Rocky Hill, NJ). In this assay purified rabbit anti human EGF antibodies were used to determine the levels of EGF in ceramide treated cells and control. The amount of EGF was calculated using a standard curve established with purified EGF in the range of 0-4000pg/ml.

3.2.11.3. Quantification of Nuclear Factor-kappa B (NF-κB)

NF-κB levels in the nuclear extract of controls and ceramide treated breast cancer cells were determined using the NF- κB ELISA kit (Active Motif Carlsbad, CA).

3.3. *In Vivo* Anti-angiogenic Activity of Ceramide

3.3.1. Introduction

Angiogenesis, the formation of new blood vessels is very important for tumor growth. Hence the inhibition of tumor angiogenesis has become an important approach in cancer research

(Folkman, 1995; Carmeliet and Jain, 2000; Kragh et al., 2004). One of the important challenges in angiogenesis studies is the selection of the appropriate assay. It has been reported that a compound affecting cell proliferation and migration may not necessarily regulates endothelial cell activity in *vivo* (Staton et al., 2004; Liekens et al., 2001). So the evaluation of *in vivo* angiogenesis is very important to assess anti-angiogenic activity as a prerequisite for the discovery and characterization of anti angiogenic compounds (Staton et al., 2004; Kragh et al., 2003; Ribatti and Vacca, 1999; Jain et al., 1997).

There are many assays developed for *in vivo* angiogenesis. Examples include the matrigel plug assay, chick chorioallantoic membrane (CAM) assay, the window models, rabbit corneal assay, and the disc angiogenesis system (Auerbach et al., 2003; Kragh et al., 2003 ;Kragh et al., 2004). The optimal *in vivo* angiogenesis assay should be able to promote the induction of significant and reproducible amount of new blood vessels, the induced vessels should easily be quantified by more than one method. The matrigel plug assay originally utilized for rabbits. However, due to costs it has been shifted to small animals such as rodents like rat and mouse (Kragh et al., 2003; Kragh et al., 2004). In this assay, angiogenesis inducing compounds such as basic fibroblast growth factor (bFGF) are introduced into the matrigel and injected subcutaneously into the animals. The matrigel is liquid at 0° C and solidifies at room temperature and above. After injection, the matrigel solidifies and allows penetration by host cells that causes vascularization. After 7 days angiogenesis is assessed by determining the hemoglobin concentration in the matrigel or by histological examination of newly formed blood vessels (Kragh et al., 2003; Akhtar et al., 2002).

Matrigel plug assay was used to assess the antiangiogenic activity of ceramide *in vivo.* Rats were used in the assay.

3.3.2. Materials and Methods

3.3.2.1. Animals

Female six-week old Sprague-Dawley rats were purchased from an in-house breeding colony and housed in the Vivarium of the School of Veterinary Medicine at Louisiana State University. The facility is operated by the Division of Laboratory Animal Medicine and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. The Louisiana State University Institutional Animal Care and Use Committee approved the study.

Animals were serologically determined to be free of the following organisms: Sendai virus, sialodacryoadenitis virus, Kilham's rat virus, H-1 virus, rat parvovirus, pneumonia virus of mice, transmissible murine encephalomyelitis virus, adenovirus, lymphocytic choriomeningitis virus, and *Mycoplasma pulmonis*. No endo or ectoparasites were found, nor were any respiratory or enteric bacterial pathogens cultured. Rats were housed in polycarbonate microisolator cages on corn cob bedding, (7097, Harlan Teklad, Madison, WI) with a 12:12 light cycle. Room temperature was maintained between 68 and 72°F, and humidity between 40 and 60%. Feed (Lab Diet® 5001, PMI® International, Inc., Brentwood, MO) and water (via water bottles) were provided ad libitum. Rats were out bred, they are not closely related.

3.3.2.2 Preparation of Matrigel for Injection

BD Matrigel Matrix (BD Biosciences, Franklin Lakes, NJ) is a soluble basement membrane extract of the Engelbreth-Holm-Swarm (EHS) tumor that gels at room temperature to form a genuine reconstituted basement membrane. The major components of BD Matrigel Matrix are laminin, collagen IV, entactin and heparin sulfate proteoglycan, growth factors, collagenases, and plasminogen activators. Matrigel solidifies at room temperature. Basic

fibroblast growth factor (BD Biosciences) was used at 300 ng per ml of matrigel. Two different types of matrigel were prepared for control and treatment (ceramide). Control matrigel was prepared with basic fibroblast growth factor without ceramide. Treatment matrigel included both bFGF and ceramide. Ceramide was dissolved in DMSO and mixed in matrigel at of thirty mg of ceramide was used per kilogram weight of the rat.

3.3.2.3. Subcutaneous Injection of Matrigel into the Rats

Prior to injection rats were anesthetized with isoflurane. The isoflurane was delivered at 2-3% via nose cone and 100% oxygen used as the carrier gas. After the animals were anesthetized, the flank was shaved and disinfected with ethyl alcohol. Matrigel premixed with basic fibroblast growth factor, with or without ceramide, was injected subcutaneously to both sides of flank. A total of 1 ml of matrigel with 300 ng of bFGF per each rat was used in control and ceramide for the treatment. Ceramide was used as 30 mg per kg body wt of rat. Control matrigel was injected on the left flank while the right flank contained the treatment matrigel. The same sequence was maintained for all 5 rats. Sterilized syringes and needles were used to inject the matrigel subcutaneously. Matrigel was gel at 4°C and solidified as soon as it was injected into the rat. After the injection the rats were kept in their standard animal housing with free access to food and water. Figure 3.1 depicts a rat injected with matrigel plugs in both left and right flank.

3.3.2.4. Quantification of Angiogenesis

Matrigel was removed from the rats after 7 days. Rats were euthanized through inhalation of CO_2 and the matrigel plugs were carefully removed from the underneath the skin. Matrigel plugs were gently washed with saline (0.9% NaCl), homogenized with 2 ml of distilled water on ice and centrifuged at 10,000 rpm for 6 min at 4°C to get clear solution. The supernatant was

used in duplicates to measure the hemoglobin concentration along with standards using Drabkins reagent at 540 nm in an ELISA plate reader. Hemoglobin standards were prepared and measured simultaneously at 540 nm. Hemoglobin was expressed in mg of hemoglobin per ml of plug.

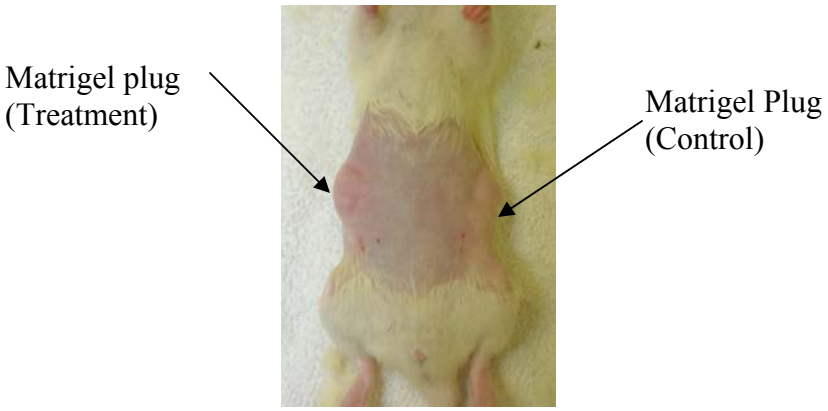

Figure 3.1: Matrigel Plugs on the Flank of Rat

3.4. Statistical Analysis

Statistical analysis was performed by paired t-test, one-way analysis of variance (ANOVA) for significance. Differences were considered to be statistically significant when $P < 0.05$.

CHAPTER: 4
RESULTS AND DISCUSSION

4.1 Ceramide Identification by Electrospray Ionization (ESI) –Mass Spectrometry (MS)

The yield of ceramide methylaminoethylphophonate was 0.16 %. Molecular species of ceramide was identified using electrospray ionization mass spectrometry (ESI-MS). The ESI-MS spectra gave one strong protonated molecular ion [M+H] (+) at m/z 413 with two other molecular ions at m/z 803 and 429 (Figure 4.1). Collision induced dissociation on ceramide produced product ions at m/z 55.01, 104.98, 203.05, 219.02. Alkaline hydrolysis of ceramide showed that the presence of a fragment at 55 and 97 and indicated the presence of phosphonate and phosphate groups, respectively. In the positive mode, the ions at m/z 140 and 138 originated from CMAEPn (Matsubara et al., 1990).

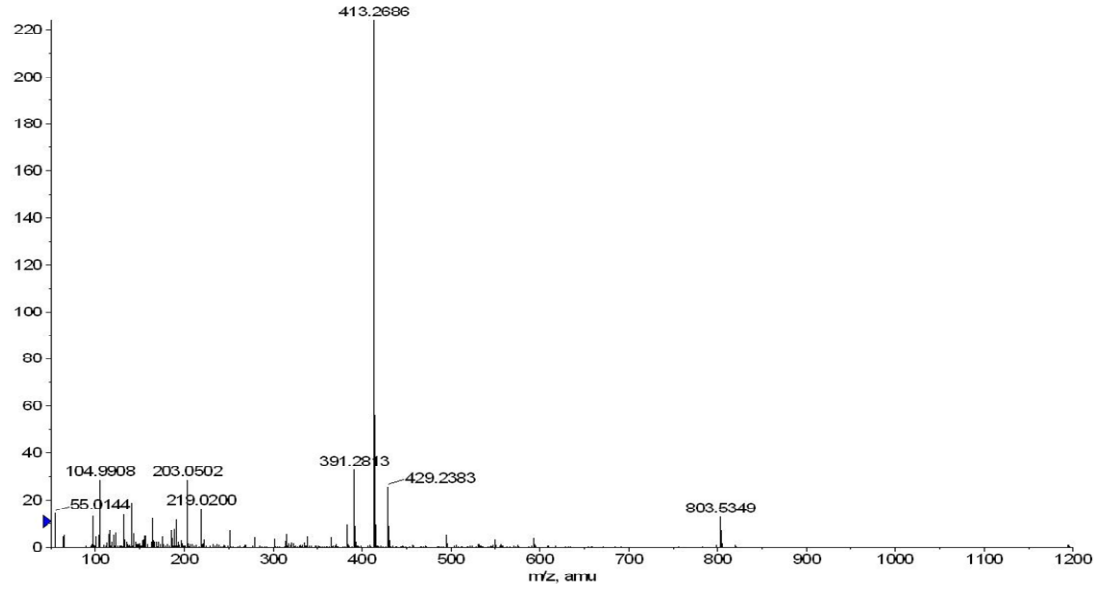

Figure 4.1: Ceramide Identification by ESI-MS

4.2. *In-Vitro* Angiogenesis Assays

4.2.1. CMAEPn Inhibits Cell Proliferation and Viability in Breast Cancer Cells

Breast cancer cells were incubated with 0-500 μM of ceramide for 0-48 h. Cell proliferation and viability determined by cell morphology under the microscope showed that cells died dose-dependently within 24 h. At concentration above 125 μM, both MCF-7 and MDA-MB 435s showed less number of viable cells after 24 h than untreated MCF-7 and MDA-MB 435s cells. At concentrations below 125 μM more viable cells were observed (Figure 4.2-4.5).

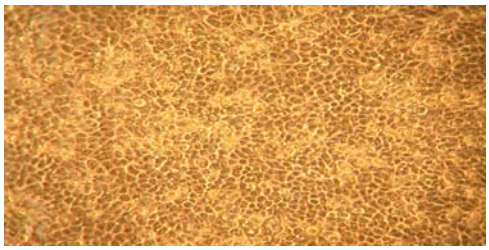

Figure 4.2: MCF-7 Control

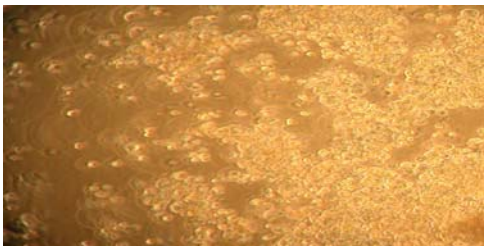

Figure 4.3: MCF-7 Treated with 100 μM Ceramide

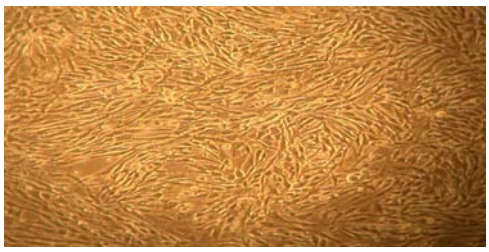

Figure 4.4: MDA-MB-435s Control

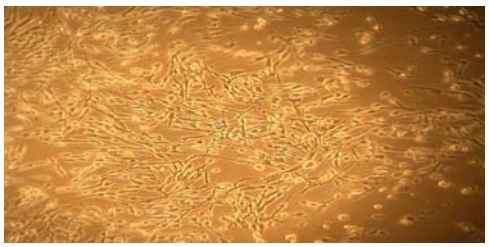

Figure 4.5: MDA-MB-435s Treated with 250 μM ceramide

Proliferation was significantly inhibited at 125 and 250 μM of ceramide treated MCF-7 and MDA-MB-435s cells, respectively. Viability was reduced to 76% and 38% with the treatment of 125 and 250 μM of ceramide respectively in MCF-7 cells (Fig 4.6). At 500 μM the number of viable cells was very low. In MDA-MB-435s cells the viability was reduced to 85% and 45% at 125 and 250 μM of ceramide for 48 h treatment. Viability was reduced to 27% at 500 μM ceramide concentration (Fig 4.7).

MCF-7

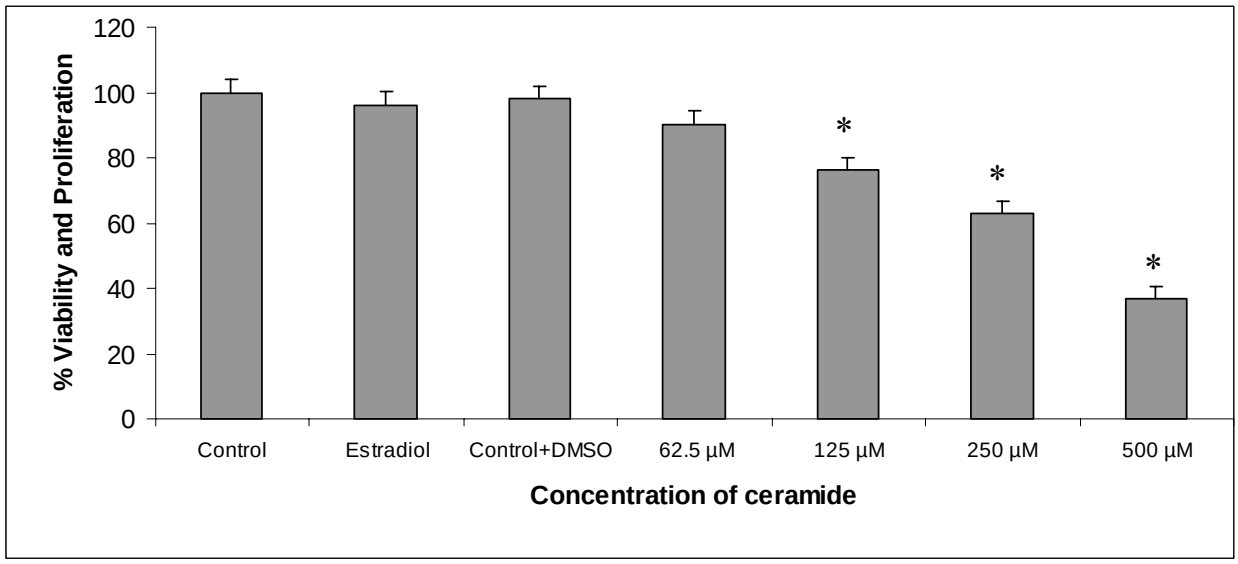

Figure 4.6: Viability and Proliferation of Control and Ceramide Treated MCF-7 Breast Cancer Cells

* Indicates the significant difference at P<0.05 compared to control

MDA-MB-435s

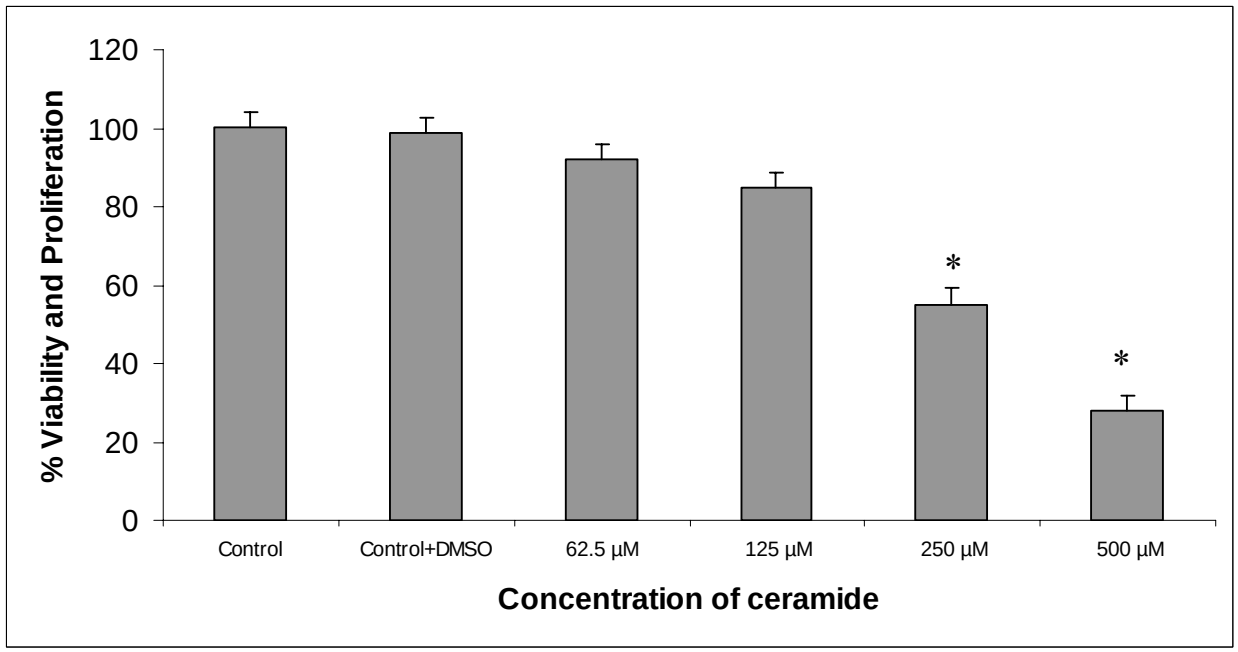

Figure 4.7: Viability and Proliferation of Control and Ceramide Treated MDA-MB-435s Breast Cancer Cells

* Indicates the significant difference at P<0.05 compared to control

Ceramides inhibit the cell proliferation and viability by the hydrolysis of ceramide to sphingosine-1- phosphate or by the formation of glucosylceramide (Radin, 2003).

The ceramide aminoethylphosphonate from oysters was effective in inhibiting the proliferation and viability of both breast cancer cell lines (MCF-7 and MDA-MB 435s). Cell proliferation is the important step in angiogenesis

4.2.2. CMAEPn Decreases the Levels of VEGF in Treated Breast Cancer Cells

The VEGF levels in the CMAEPn treated MCF-7 and MDA-MB-435s cells were lower than the controls (Fig 4.8-4.9). The concentration of ceramide used for the determination of VEGF levels ranged from 50-200 μM. The VEGF levels in the controls of MCF-7 and MDA-MB-435s cells were 246 and 291 pg/ml, respectively. The levels of VEGF in 200 μM ceramide treated MCF-7 and MDA-MB-435s were 115 and 145 pg/ml in MCF-7 and MDA-MB-435s breast cancer cells. The levels of VEGF were reduced significantly at 200 μM in both MCF-7 and MDA-MB 435s cells

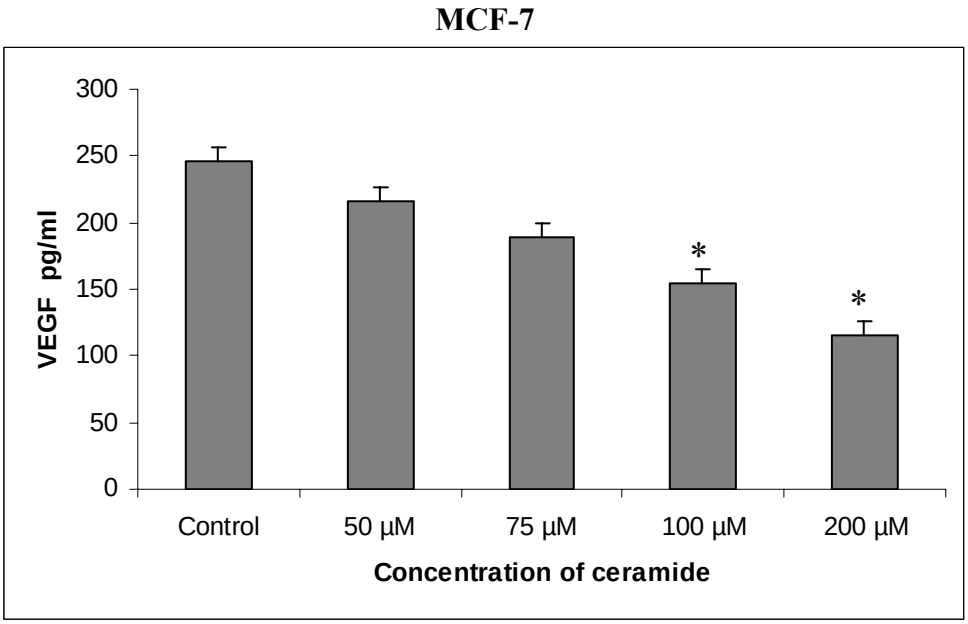

Figure 4.8: VEGF Levels in Control and Ceramide Treated MCF-7 Cells
* Indicates the significant difference at $P<0.05$ compared to control

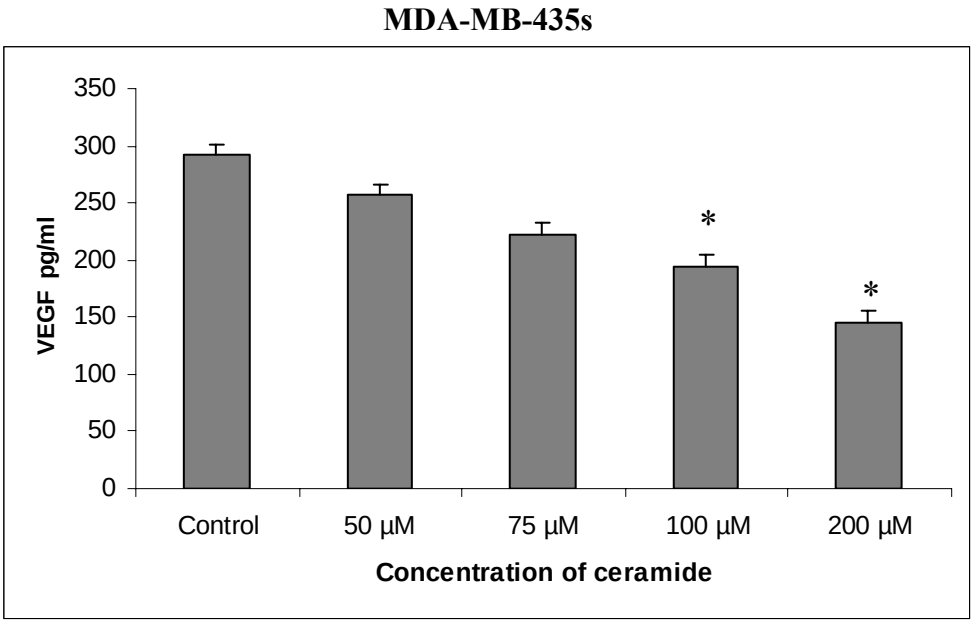

Figure 4.9: VEGF Levels in Control and Ceramide Treated MDA-MB-435s Cells
* Indicates the significant difference at $P<0.05$ compared to control

There was 49% and 46% reduction of VEGF levels in MCF-7 and MDA-MB-435s cells treated with 200 µM ceramide. VEGF is a very important angiogenic stimulator in migration and proliferation of endothelial cells. Cell proliferation and migration are very important processes in angiogenesis. CMAEPn from oyster reduced the levels of VEGF in both MCF-7 and MDA-MB-435s breast cancer cells

4.2.3 CMAEPn Inhibits the Levels of EGF in MCF-7 and MDA-MB-435s Cells

MCF-7 and MDA-MB-435s breast cancer cells were treated with 50-200 µM of ceramide concentration and incubated for 48 h. EGF levels were determined using EGF ELISA protocol. The range of EGF in MCF-7 and MDA-MB-435 breast cancer cells was determined as 106-165 pg/ml and 123-189 pg/ml, respectively (Fig 4.10-4.11).Controls MCF-7 and MDA-MB-435s contained to be 194 and 228 pg/ml EGF respectively. The concentration of EGF in 200 µM ceramide treated MCF-7 and MDA-MB-435s were 106 and 123 pg/ml, respectively.

MCF-7

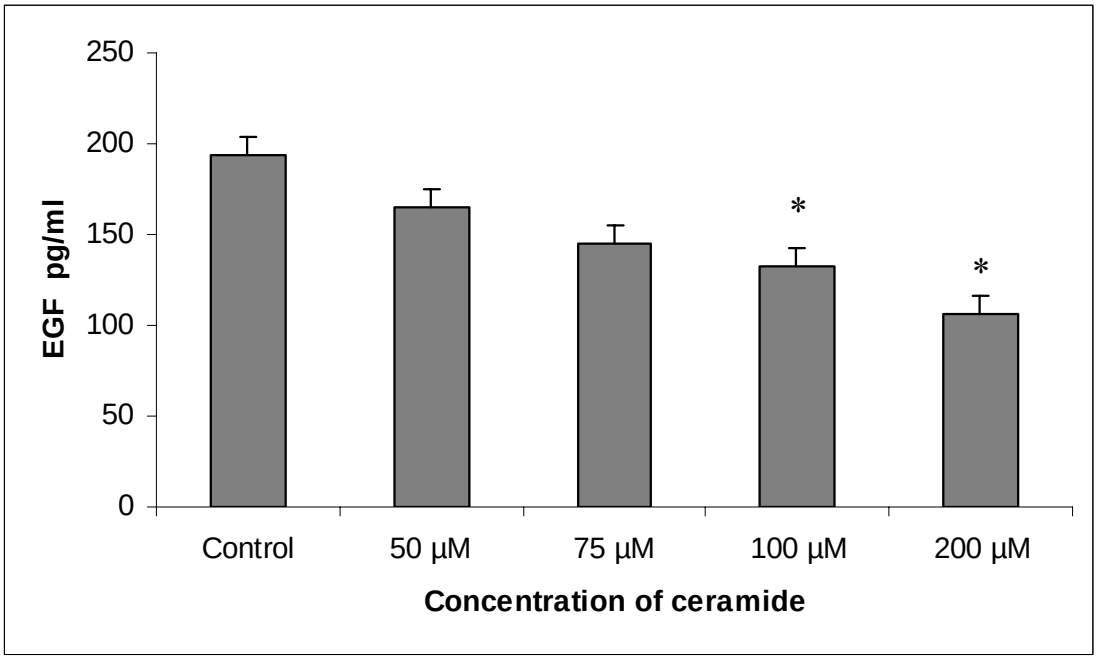

Figure 4.10: EGF Levels in Control and Ceramide Treated MCF-7 Cells

* Indicates the significant difference at P<0.05 compared to control

MDA-MB-435s

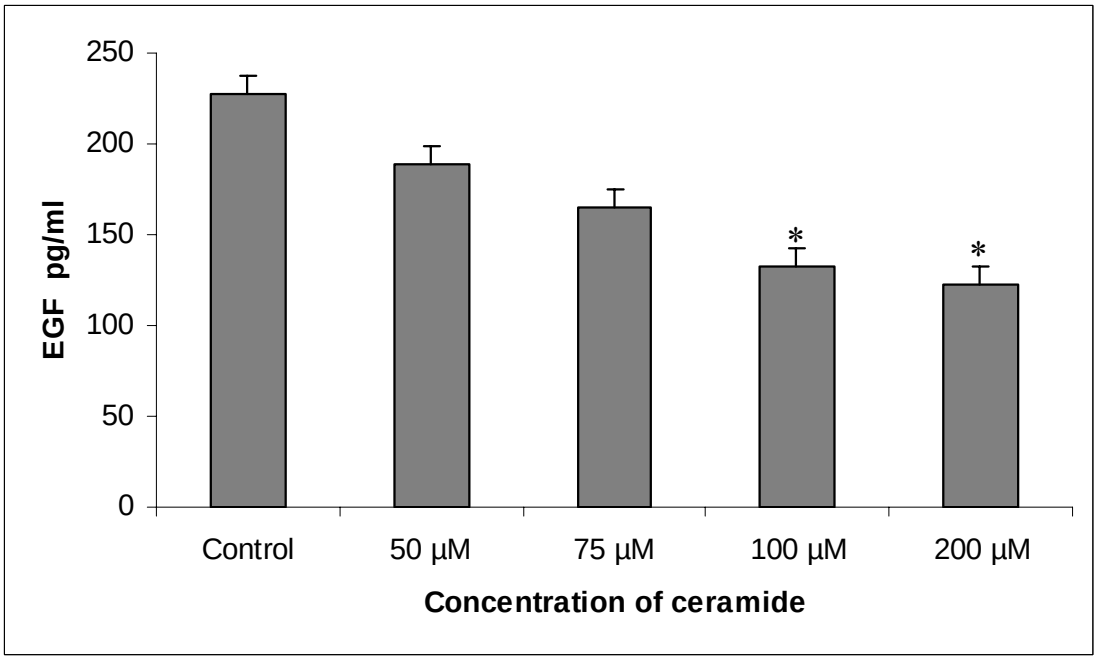

Figure 4.11: EGF levels in Control and Ceramide Treated MDA-MB-435s cells

* Indicates the significant difference at P<0.05 compared to control

The levels of EGF in MCF-7 and MDA-MB-435s breast cancer cells were reduced by 65% and 64% respectively. EGF is also an important stimulator of angiogenic processes. The results of this study show that CMAEPn is an inhibitor of EGF in MCF-7 and MDA-MB-435S breast cancer cells and may regulates the process of angiogenesis in breast cancer cells

4.2.4. CMAEPn Inhibits Breast Cancer Cell Migration

Invasive breast cancer cells MDA-MB 435s were used for the migration studies. Breast cancer cells were treated with CMAEPn from 0-125 μmole/l for 48 h followed by evaluation of cell migration in the presence of VEGF as chemoattractant. There was a significant inhibition of migration at concentration of 125 μM ceramide (Fig 4.12).

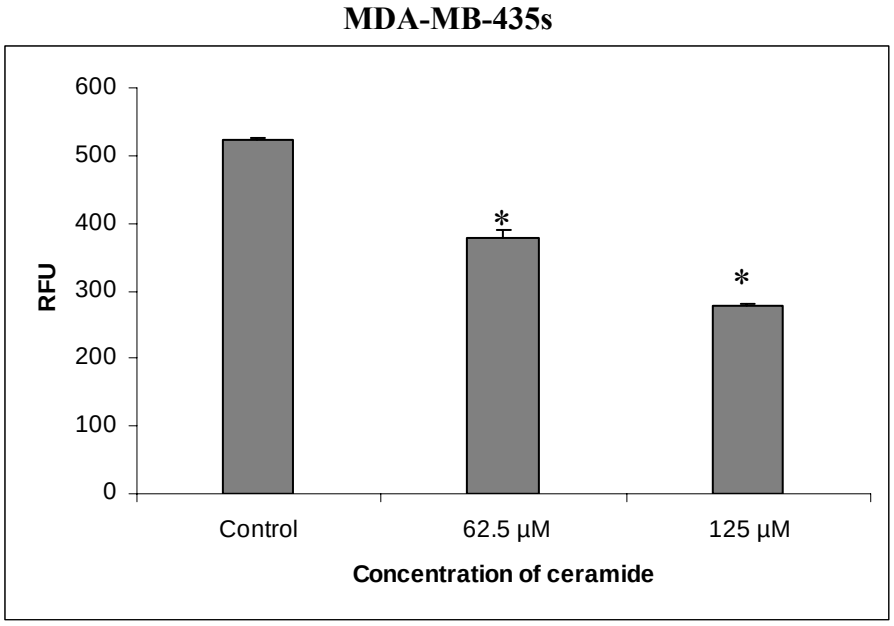

Figure 4.12: Cell Migration of Control and Ceramide Treated Breast Cancer Cells
* Indicates the significant difference at $P<0.05$ compared to control

Endothelial cells migrate by a process called chemotaxis, caused by angiogenesis-inducing growth factor such as VEGF. Inhibition of VEGF receptors reduces the migration of endothelial cells. Reduced migration of ceramide treated cell in presence of VEGF may be associated with reduced levels of VEGF in the ceramide treated cells. The levels of VEGF in the

ceramide treated cells were analyzed (4.2.2.) and the significant difference between the control and ceramide treated cells may explain the reduced migration of breast cancer cells.

4.2.5. CMAEPn Inhibits Breast Cancer Cell Invasion

MDA-MB-435s invasive breast cancer cell line was used for invasion studies and cells were treated with 0-125 μM concentration of CMAEPn.

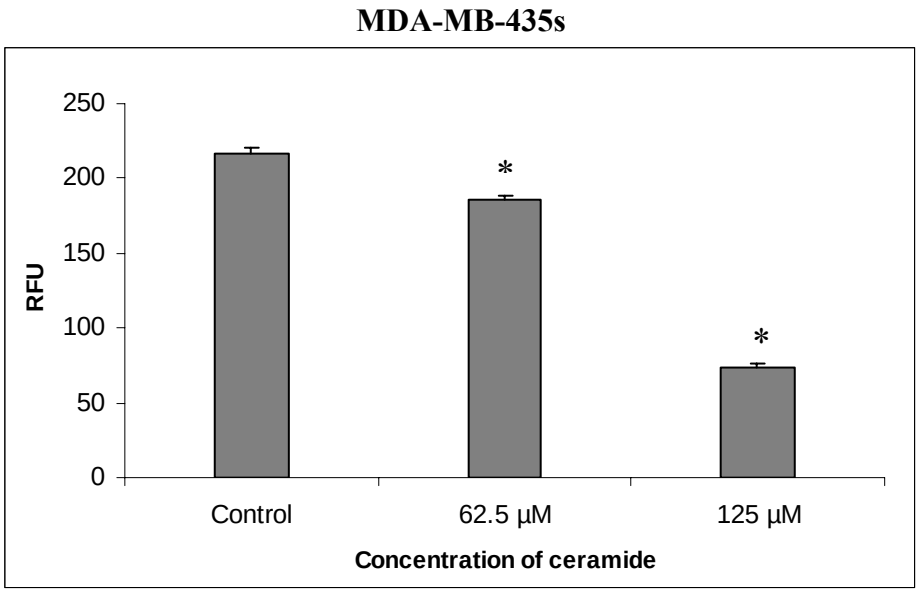

Figure 4.13: Cell Invasion of Control and Ceramide Treated Breast Cancer Cells
* Indicates the significant difference at $P<0.05$ compared to control

At 125 μM concentration cell invasion was significantly inhibited than at 62.5μM ceramide and control. The difference between the invasion of untreated cells and ceramide treated cells shows that ceramide was effective in inhibiting the invasion of breast cancer cells. Invasion of endothelial cells depend on the stability of extracellular matrix (ECM) and the stability of ECM can be imbalanced by proteases. CMAEPn from oyster may inhibit the action of proteases and stabilizes ECM, which assists in the reduction of breast cancer cell invasion.

4.2.6. CMAEPn Inhibits Endothelial Cell Tube Formation

On a synthetic basement membrane matrix, cells are capable of morphological differentiation into an extensive network of capillary-like structures composed of highly

organized three-dimensional cords. The alignment of endothelial cells in capillary-like structures is a functional trait that is crucial to angiogenesis.

In the presence of 10 ng/ml of VEGF, human umbilical vein endothelial cells (HUVEC plated on the EC matrix aligned and formed capillary-like structures within 6h (Figure 4.14). CMAEPn inhibited tube formation within 6h. At 50 µM ceramide, the capillary-like network of the ECmatrix was completely disrupted (Figure 4.15).

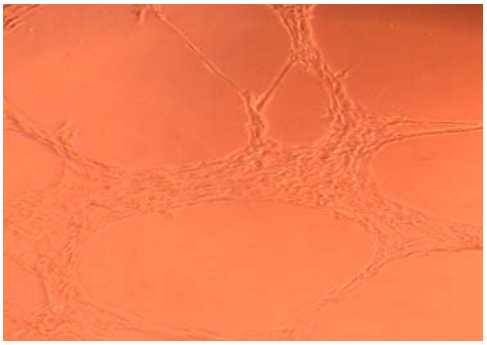

Figure 4.14: HUVEC Control

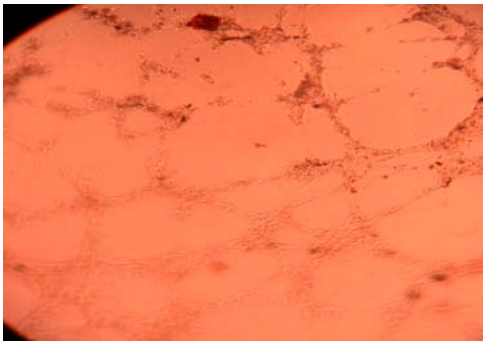

Figure 4.15: HUVEC Control Treated with 50 µM Ceramide

4.2.7. Ceramide Methylaminoethylphosphonate Mediated Cell Death

Both hormone dependent and independent breast cancer cells, MCF-7 and MDA-MB 435s were studied for the detection of apoptosis after treatment with ceramide. 125 and 250 µM of ceramide concentrations were used for MCF-7 and MDA-MB-435s cells, respectively. The concentration at which the cells died was to explain the cell death. As apoptosis is directly associated with the stimulation of caspase activity, caspase- activity was assessed following treatment of MCF-7 and MDA-MB-435s with CMAEPn. Cells that were treated with the CAEPn showed significant decrease in the activity of caspases (Fig 4.16-4.17). Untreated cells had higher levels of caspases activity for both cell lines (Fig 4.16-4.17). Caspase activity decreased in ceramide treated MCF-7 and MDA-MB-435s cells and indicated ceramide caused caspase independent cell death.

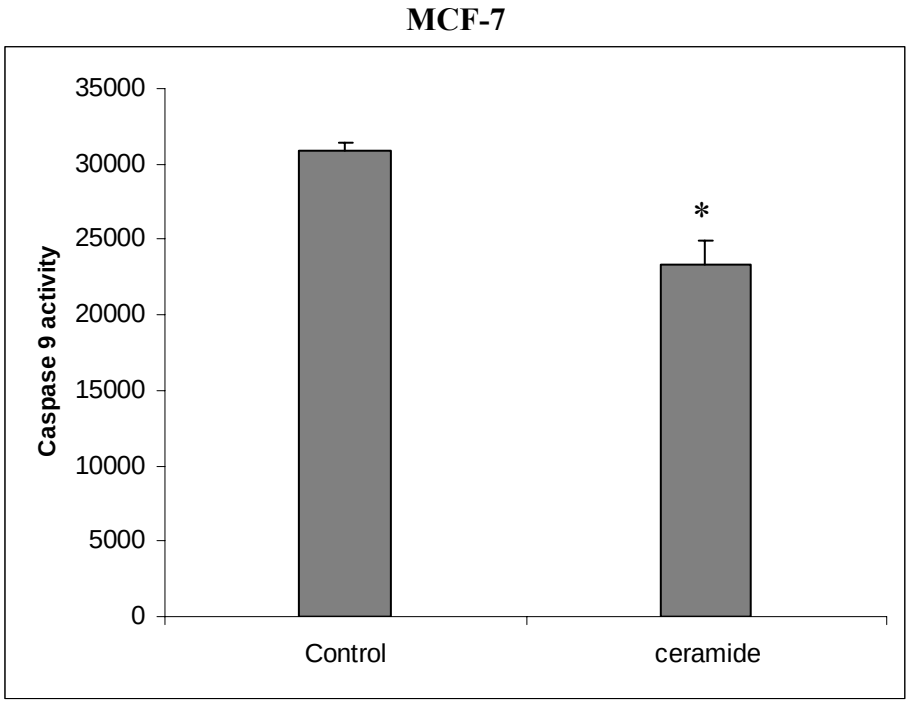

Figure 4.16: Caspase Activity of Control and Ceramide (125 μM) treated MCF-7 Cells
* Indicates the significant difference at P<0.05

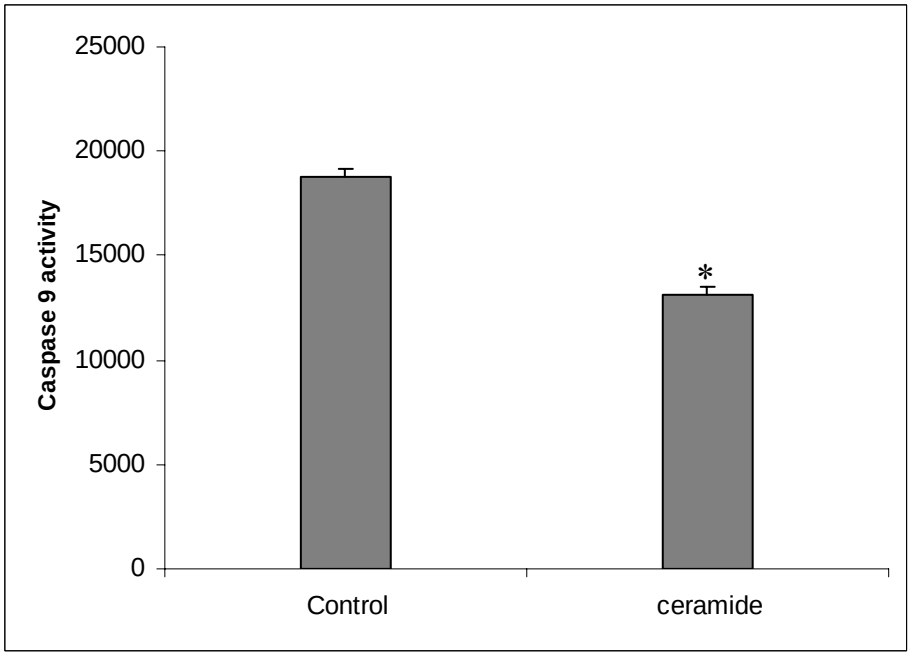

Figure 4.17 Caspase Activity of Control and Ceramide (250 μM) Treated MDA-MB-435s Cells
* Indicates the significant difference at P<0.05

The accumulation of intracellular ceramide by induction, modification of endogenous ceramide metabolism, or exogenous supply of synthetic ceramides causes cancer cells to die by apoptosis may be a useful approach to cancer treatment (Lucci et al., 1999; Shabbits and Mayer, 2003; Wang et al., 2003; Chan et al., 2006).Ceramide is the key regulator of apoptosis and activates the apoptotic proteins such as caspases, ceramide activated protein kinase (CAPK), cathepsin D, and serine/threonine protein phosphatases (Pettus et al., 2002). Elevated levels of cathepsin D was observed in MCF-7 cells treated with C6 ceramide (Bansode, 2005).It has been shown that some synthetic ceramides mediate caspase independent cell death in cancer cells (Mochizuki et al., 2002; Scaralatti et al., 2004; Thon et al., 2005). CMAEPn from oyster also showed decreased activity of caspases in MCF-7 and MDA-MB-435s, indicating that ceramide from oyster may not be effective in causing cell death by apoptosis.

4.2.8. Ceramide Methylaminoethylphosphonate Mediated Autophagy

Breast cancer cells were grown on cover slips, treated with CMAEPn and incubated for 48 h. The concentration of ceramide used for MCF-7 and MDA-MB-435s cells were 125 and 250 μM, respectively. Cell death was observed within 48 h. The cells were treated with monodansylcadaverine (MDC) and washed PBS, and observed under a fluorescent microscope for autophagic vacuoles. Both MCF-7 and MDA-MB-435s cells showed autophagic vacuoles with CMAEPn treatment (Fig 4.18-4.21).

Fig 4.18: MCF-7 Control

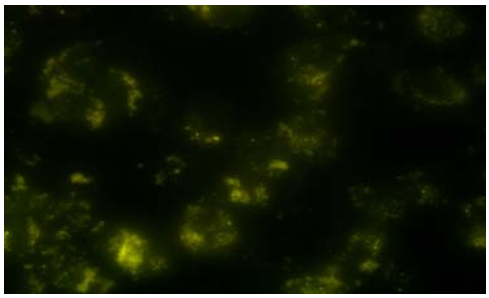

Fig 4.19: Ceramide Treated MCF-7 Cells

Figure 4.20: MDA-MB-435s Control

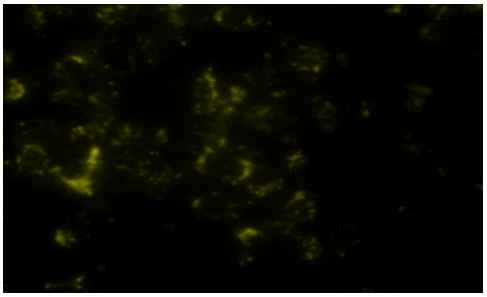

Figure 4.21: Ceramide Treated MDA-MB-435s Cells

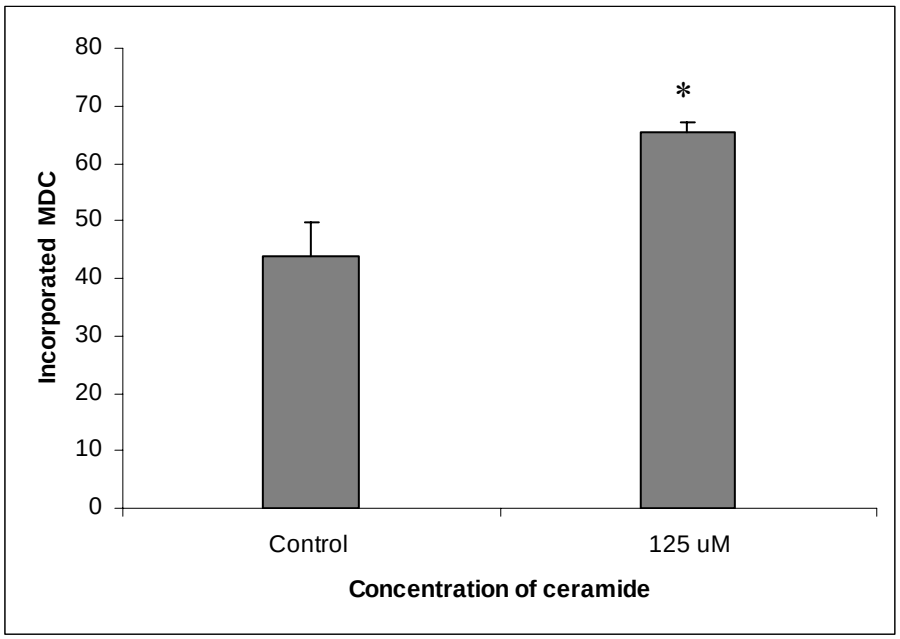

Figure 4.22: MDC Incorporation into Control and Ceramide Treated MCF-7 Cells
* Indicates the significant difference at $P<0.05$

Intracellular MDC was measured for both MCF-7 and MDA-MB-435 cells. More MDC was incorporated in ceramide treated cells than the control cells (Fig 4.22-4.23).

MDC is a specific marker for autolysosomes (Biederbick et al., 1995). MDC incorporation was assessed for the visualization of autolysosomes. Ceramide treated cells showed an increased number of autophagic vacuoles than control in both breast cancer cell lines MCF-7 and MDA-MB-435s, respectively. There were no autophagic vacuoles observed in both controls, indicating that ceramide caused cell death by autophagy (Munafo and Colombo, 2001).

It has been shown that ceramides induces cell death by non apoptotic pathway, autophagy. Ceramides are involved in regulation of autophagy by non-mutually exclusive two mechanisms. Ceramides inhibit class III PI3K signaling pathways and stimulates beclin I protein and mediates autophagy (Scaralatti et al., 2004). The protein kinases involved in the autophagic responses are increased by ceramide treatment (Pelled et al., 2002).

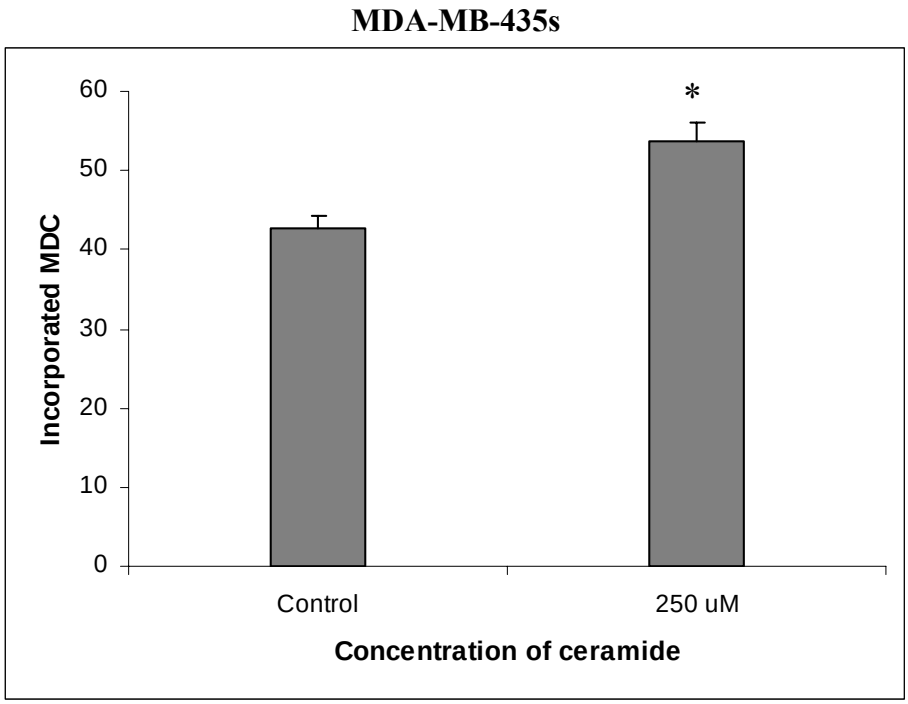

Figure 4.23: MDC Incorporation into Control and Ceramide Treated MDA-MB 435s Breast Cancer Cells.
* Indicates the significant difference at $P<0.05$

Ceramides stimulate autophagy by interfering with class I PI3K signaling pathways and increases the expression of autophagic genes. More over ceramide levels are lower in cancer cells than normal cells, suggesting that ceramide is considered to be an important factor in the regulation of autophagy (Selzner et al., 2001).The presence of PI3K was confirmed by western blot. MCF-7 & MDA-MB-435s controls showed the presence of PI3K (Fig 4.24). CMAEPn

treated MCF-7& MDA-MB-435s cells contained undetected levels of PI3K as shown by the absence of bands in ceramide treated cells.

Figure 4.24: Western blot Analysis of PI3K in Control and Ceramide Treated MCF-7 and MDA-MB-435s Cells

1- MCF-7 Control, 2- MDA-MB-435s Control, 3-MCF-7 treated with 125 μM ceramide, and 4-MDA-MB-435s treated with 250 μM ceramide.

4.2.9 CMAEPn Inhibits Nuclear Factor-kappa B (NF-κB) Activation

NF-κB inactivation is important for cancer cell death as it promotes cell proliferation and survival (Fulda et al., 1998; Toker, 1998). CMAEPn treatment inactivated NF-κB activity in MCF-7 and MDA-MB-435s breast cancer cells (Fig 4.25-4.26).

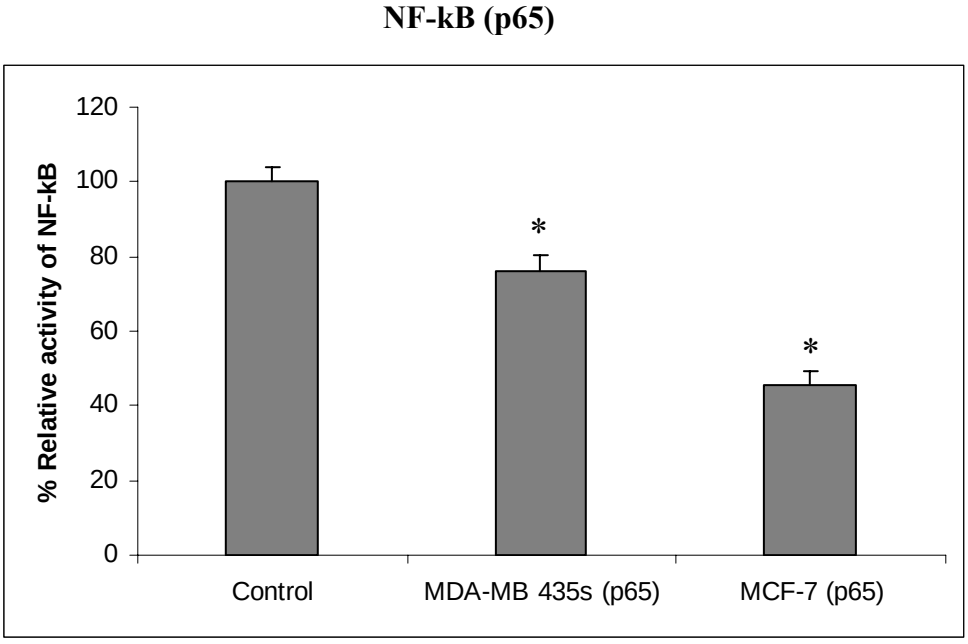

Figure 4.25: NF-κB (p65) Activity in Control and Ceramide Treated MCF-7 and MDA-MB-435s Cells

* Indicates the significant difference at $P<0.05$

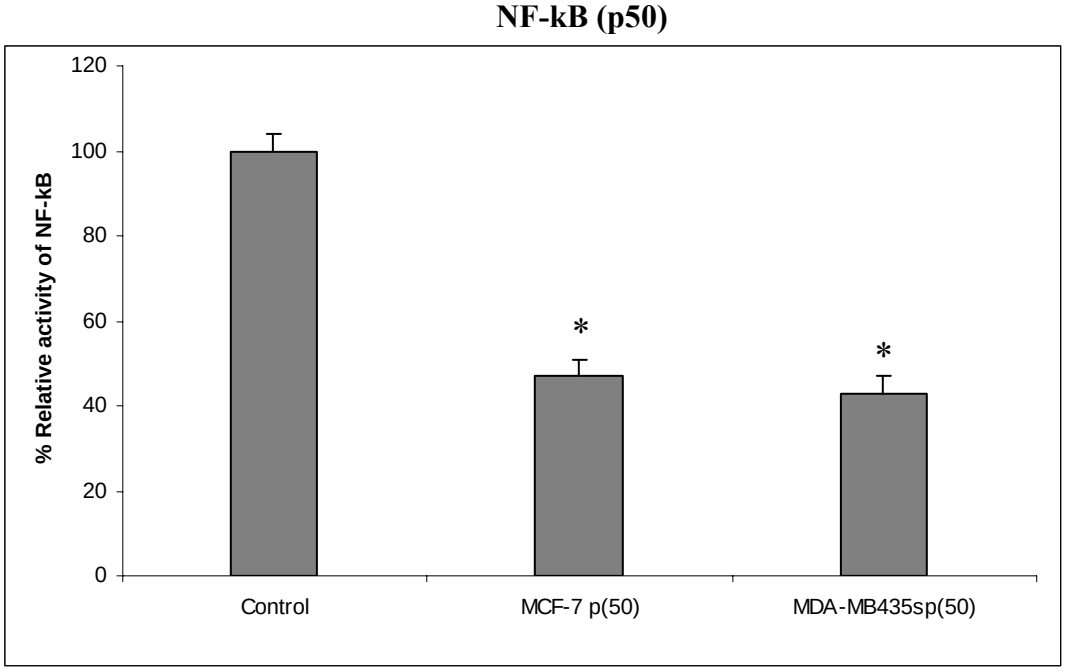

Figure 4.26: NF-κB (p50) Activity in Control and Ceramide Treated MCF-7 and MDA-MB-435s Cells

* Indicates the significant difference at $P<0.05$

NF-κB is a multisubunit nuclear transcription factor that regulates cellular functions such as cell growth, cell development, and adaptive responses to redox balance. In its inactive form, NF-κB resides in the cytoplasm and binds to the inhibitory proteins that shield the DNA binding site. It is over expressed in breast cancer cell lines and stimulates cell survival and proliferation. So the inactivation of NF-κB leads to cell death by autophagy or apoptosis (Simstein et al., 2003). CMAEPn from oyster inactivated the NF-κB subunits p50 and p65 in both MCF-7 and MDA-MB-435s breast cancer cells.

4.3. *In Vivo* Angiogenesis Assay

4.3.1. CMAEPn Inhibition of *In Vivo* Angiogenesis

After 7 days, the subcutaneously injected matrigel plugs containing bFGF with or without ceramide were quantitatively analyzed by optical density reading at 540 nm. Growth factor bFGF induced angiogenesis, which can be visually observed in the control matrigel plug (avascular

before injection) whereas CMAEPn inhibited bFGF induced angiogenesis (Fig 4.27 and Fig 4.28 respectively).

The plugs were homogenized and centrifuged to obtain a clear solution. Optical density at 540 nm was taken to measure the hemoglobin concentration. Control plugs had higher hemoglobin concentration and ceramide treated matrigel plugs had lower levels (Fig 4.29). The average hemoglobin concentration was higher in the control matrigel (37.91mg/ml) than that seen in the treatment matrigel (21.623mg/ml).Treatment of ceramide varied from rat to rat, but the results was consistent except for rat #1. All the other four rats showed increased angiogenesis in control plugs and inhibition of angiogenesis in ceramide treated Matrigel plugs

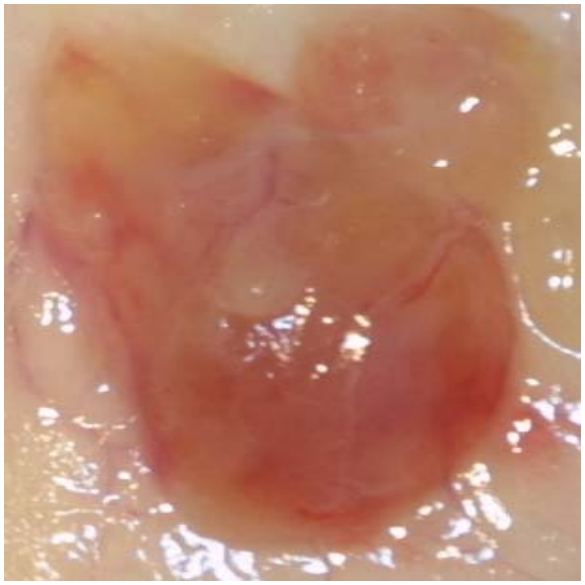

Figure 4.27: Control Matrigel Plug

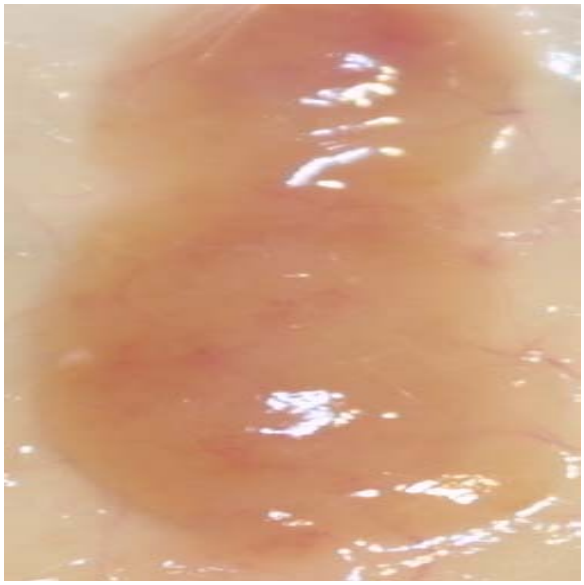

Figure 4.28: Ceramide Treated Matrigel plug

Regardless of the *in vitro* tests, *in vivo* tests are necessary for accurate evaluation of angiogenesis (Auerbach et al, 2003). There is no standard *in vivo* angiogenesis assay and the limitation to progress in angiogenesis has been the availability of reproducible, simple, and quantitative assay (Kragh et al, 2003). We used a simple quantitative *in vivo* angiogenesis assay, Matrigel plug assay, in our study. Matrigel is easy to handle and subcutaneous injection of

matrigel and growth factor exposure to the animal are same. In the present study, we tested the anti angiogenic activity of the ceramide. Ceramide was added to matrigel premixed with growth bFGF and subcutaneously injected to the rat. After 7 days matrigel plugs were removed from the rat skin. The control showed more blood vessels by visual observation indicating that bFGF induced angiogenesis and ceramide treated matrigel plugs showed less blood vessels than control , indicating the anti angiogenic activity of ceramide. These results show that bFGF induced angiogenesis in matrigel as the matrigel plug was avascular prior to injection. We found that exogenous administration of ceramide inhibits angiogenesis *in vivo.*

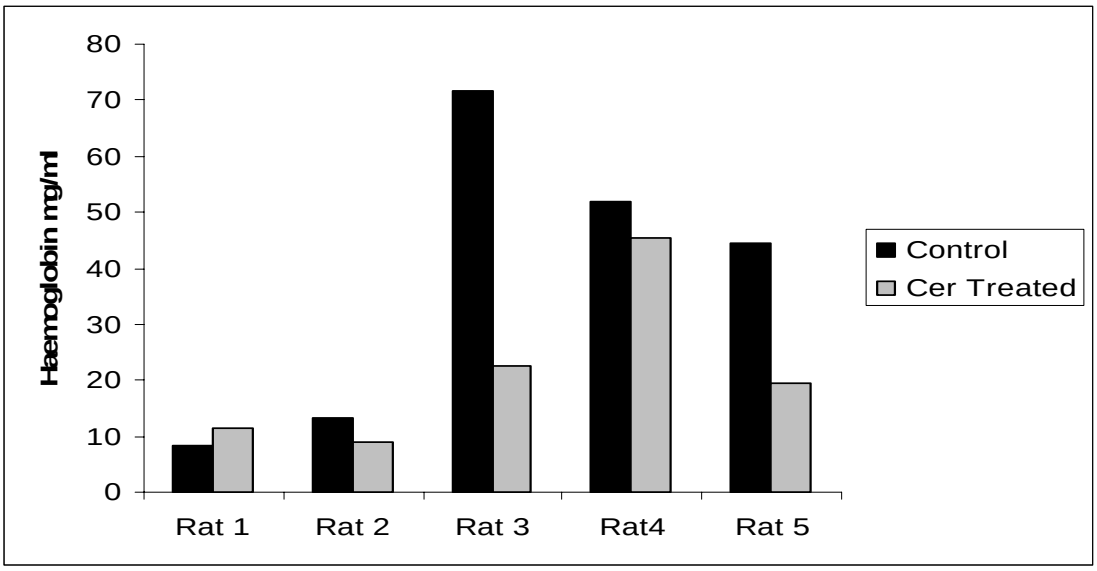

Figure 4.29 Hemoglobin Concentration of Control and Treated Matrigel Plugs of the Rats

The average hemoglobin concentration of controls was ±37.91 mg/ml and for ceramide treated was ± 21.623 mg/ml. There was 57% of inhibition of hemoglobin levels with the ceramide treatment within 7 days. The difference in hemoglobin concentration between controls and ceramide treated matrigel plugs demonstrate that ceramide inhibited bFGF induced angiogenesis *in vivo.*

CHAPTER 5
SUMMARY AND CONCLUSION

The main objective of this study was to isolate, purify ceramide aminoethylphosphonate from oyster and determine the anti angiogenic properties of the isolated ceramide using MCF-7 and MDA-MB435s breast cancer cells.

Ceramide methylaminoethylphosphonate (CMAEPn) was isolated from oysters by a series of organic solvent extractions and followed by silicic acid chromatography. CMAEPn was identified by ESI-MS. The yield of ceramide methylaminoethylphosphonate was 0.16% on dry weight basis. The purified CMAEPn was tested for its anti angiogenic properties by *in vitro* and *in vivo* angiogenesis assays.

From the *in vitro* angiogenesis assays it was found that, CMAEPn greatly inhibited viability and proliferation of MCF-7 and MDA-MB-435s breast cancer cells. The ceramide inhibited the migration and invasion of invasive MDA-MB-435s breast cancer cells. At 50 μM, ceramide inhibited the formation of tubules by endothelial cells In the presence of ceramide VEGF levels in MCF-7 and MDA-MB-435s were reduced by 49% and 46% respectively. The levels of EGF were reduced by 65 and 64% in MCF-7 and MDA-MB-435s respectively with the treatment of ceramide. CMAEPn also inactivated the NF-kB, which promotes cell survival and proliferation. Both MCF-7 and MDA-MB-435s breast cancer cells showed low caspase activity treated with the 125 and 250 μM of ceramide, respectively. Oyster CMAEPn caused cell death by autophagy in both MCF-7 and MDA-MB-435s breast cancer cells. Results of western blot indicated that CMAEPn inhibited the activity of phosphatidylinositol kinase in MCF-7 and MDA-MB-435s breast cancer cells.

In vivo angiogenesis was assessed by matrigel plug assay. CMAEPn at 30mg/kg body wt inhibited the formation of blood vessels in vivo. Treated matrigel plugs had lower values of

hemoglobin than the controls suggesting a lower number of blood vessels. Hemoglobin levels were 57% lower with the ceramide treatment.

Overall *in vitro* and *in vivo* assay results suggest that ceramide methyl amino ethylphosphonte was effective against hormone dependent MCF-7and hormone independent MDA-MB-435s breast cancer cells. The results of this study demonstrate that oyster ceramide inhibited angiogenesis in vitro and in vivo. Moreover, oyster ceramide inhibited the viability of hormone dependent and hormone independent breast cancer cell by inactivating VEGF, EGF, and PI3K, which are some of the main signaling pathways associated with the progression of breast cancer.

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