2005

In vitro and in vivo anti-angiogenic activities of milk sphingolipids

Rishipal Rastrapal Bansode
Louisiana State University and Agricultural and Mechanical College

IN VITRO AND *IN VIVO* ANTI-ANGIOGENIC ACTIVITIES OF MILK SPHINGOLIPIDS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Food Science

by
Rishipal Rastrapal Bansode
B. Tech., Osmania University, 1998
M.S., Louisiana State University, 2002
December, 2005

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Jack N. Losso; he was a great mentor and a good friend. I would also like to thank my advisory committee Dr. J. Samuel Godber, Dr. Changaram S Venugopal and Dr. Ralph J Portier for their time serving on my committee as well as their helpful advice over the years. I thank Dr. Kurt Svoboda for his generosity with his time and knowledge; all of the data presented pertaining to *in vivo* studies on zebrafish was obtained with his help. I also, thank Dr. Robert E Traux for providing the cell-lines for conducting the *in vitro* studies.

My fellow graduate students contributed immeasurably to this work. Their help came in the form of laboratory assistance, lively discussions and sharing of resources. I received spirited assistance from several undergraduate summer interns especially Ashley Graham, Brandi DeYoung, and Crystal Janani with whom I had worked during their training program at LSU in 2004. They have contributed immensely in the research work related to my dissertation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Anti-angiogenic therapies aimed at halting new blood vessel formation are now being extensively studied as inhibitors of excessive angiogenesis. Conversely, compounds with ability to stimulate angiogenesis are being considered as a therapeutic approach for insufficient angiogenesis. Food-borne bioactive compounds such as genistein, resveratrol, curcumin, the Bowman-Birk inhibitor, and catechins are being potentially established as good candidates for angioprevention.

The aim of our study was to determine the anti-or pro-angiogenic activity of milk-based sphingolipids such as C6-ceramide (Cer), Sphingomyelin (SPM) and Glucosylceramide (GluCer), *in vitro*, using breast cancer (MCF-7), colon cancer (Caco-2) and prostate cancer (DU-145) cell-lines, on angiogenic factors such as vascular endothelial growth factor (VEGF), cathepsin-D and hypoxia inducing factor-1alpha (HIF-1α) expression and cell migration under normoxia and hypoxia. Another aim was to conduct an *in vivo* study using chorioallantoic membrane (CAM) and zebrafish model system to substantiate the *in vitro* results.

Breast cancer cells (MCF-7) treated with SPM had reduced cell migration under hypoxic conditions. Cathepsin-D expression under SPM treated MCF-7 cells was significantly lower under both conditions. GlcCer had significant apoptotic activity under hypoxic MCF-7 cells. Colon cancer cells (Caco-2) treated with Cer had reduced cell growth at > 50 μM under normoxic as well as hypoxic conditions. Cathepsin-D, cell migration and HIF-1α expression were significantly reduced under hypoxic condition. SPM had low cathepsin-D levels and cell migrations in normoxic and hypoxic conditions as well as low HIF-1α at hypoxic condition. In GlcCer treated cells, the levels of cathepsin-D and cell migration were reduced under normoxic and hypoxic conditions.

Prostate cancer cells (DU-145) exposed to SPM had reduced cell viability. All the

compounds had lower levels of VEGF expression at normoxic conditions at 50 μM exposure; only GlcCer had lower VEGF expression under hypoxic condition. The cell migration was reduced under normoxic condition and also for cells exposed to Cer under hypoxic condition. *In vivo* results showed ceramide was anti-angiogenic as confirmed by both CAM assay as well as zebrafish model. SPM proved to facilitate sprouting, however, the blood vessels looked dilated. GlcCer disrupted the neovascularization in CAM model and restricted the ISV formation in zebrafish.

CHAPTER 1. INTRODUCTION

There are more than eighty chronic degenerative diseases characterized by the uncontrolled growth of new capillaries. These are known as “angiogenic diseases”. Angiogenesis is the formation of new blood vessels from preexisting ones. Unlike physiological angiogenesis which is tightly controlled and important for embryonic development and wound healing, pathological angiogenesis is uncontrolled and may be excessive or insufficient. Diseases in which it would be useful to inhibit excessive angiogenesis include chronic inflammation, solid and hematological malignancies, diabetes and age-related vision loss, diabetes-related kidney complications, HIV and AIDS, multiple sclerosis, rheumatoid arthritis, obesity, and many others. Pro-angiogenic compounds are useful for human diseases that are associated with insufficient angiogenesis such as ischemia/reperfusion, diabetic delayed wound healing, cardiovascular diseases, and many others. Anti-angiogenic therapies aimed at halting new blood vessel formation are now being extensively studied as inhibitors of excessive angiogenesis (Folkman and Kalluri, 2004; Carmeliet, 2004; Cao et al., 2005; Cline et al., 2002). Conversely, compounds with ability to stimulate angiogenesis are being considered as therapeutic approach for insufficient angiogenesis. Food-borne bioactive compounds such as genistein, resveratrol, curcumin, the Bowman-Birk inhibitor, and catechins are being potentially established as good candidates for angioprevention (Cao et al., 2002; Shao et al., 2002; Losso, 2002; Fotsis et al., 1993).

The uniqueness of milk fat lies in its content of several highly desirable biologically active compounds having considerable potential for use in the food and pharmaceutical industries, particularly relative to developments in the area of angiogenesis. Milkfat contains ceramides, sphingosine, sphingomyelin, and short chain fatty acids. These bioactive compounds are not required for growth but have been identified as health enhancing in models of

carcinogenesis and cell cultures (Vesper et al., 1999). Sphingosine-1-phosphate, a biosynthetic product of sphingosine phosphorylation may be pro-angiogenic.

We hypothesize that milkfat sphingolipids or their metabolites may modulate pathological angiogenesis. We propose to bring our experience in the screening of anti- or pro-angiogenic functional foods to the study of angiogenic differentiation of milkfat sphingolipids and sphingosine-1-phosphate in the context of the specific aims: (1) determination of the anti-or pro-angiogenic activity of sphingolipids such as C6-ceramide (Cer), Sphingomyelin (SPM) and Glucosylceramide (GluCer), *in vitro*, using breast cancer (MCF-7), colon cancer (Caco-2) and prostate cancer (DU-145) cell-lines in absence or presence of stimulators of angiogenesis, (2) conduct an *in vivo* study using chorioallantoic membrane (CAM) assay in chick embryo and a zebrafish model system to substantiate the *in vitro* results.

CHAPTER 2. LITERATURE REVIEW

2.1 Characteristics of Normal Cells

Normal cells live in complete harmony with their existing environment, undergoing communication with the neighboring cells, repair and replacement of tissues, and other aspects of cell behavior. The cells interact either directly, via cell-to-cell contact, or indirectly by exchange of messenger compounds such as hormones and growth factors. The cells communicate and respond to the neighboring cells with the help of cell adhesion molecules such as cadherins and/or through cell-to-cell portals called gap junctions. This process helps cells to respond to external stimuli and undergo either a programmed cell death called apoptosis, proliferate if new cells are needed, or stop cell-division (Hanahan and Weinberg, 2000; Boik, 2001). Cells participate in a series of events involving cell division called mitosis where cells divide and share one-half of each chromosome with the offspring. The cycle consists of four phases, namely, gap phase (G_1), synthesis phase (S), second gap phase (G_2), and mitotic phase (M). The process of cell division and cell cycle is tightly regulated in normal cells.

2.2 Characteristics of Cancer Cells

Cancer cells enter the normal cell cycle except that the stimulation is in excess. The process of over-expression of the stimuli leads to abnormal production of growth factors, the binding of growth-factors to their receptors, abnormal signal transduction, and abnormal production or activity of transcription factors. Cancer in humans can be caused by specific chemicals or mixture of chemicals (cigarette smoke, various therapeutic agents, diet, the workplace, and the general environment), radiation (both ultraviolet and ion radiation), viruses (hepatitis B, Epstei-Barr, papilloma, and certain retroviruses), and oxidative damage to DNA due to endogenous metabolic reactions. Carcinogenesis involves complex interactions between several factors, both exogenous (environmental) and endogenous (genetic, hormonal, immunologic, etc.). The stages

are mainly divided into initiation, promotion (appearance of benign tumors), and progression (the conversion of benign to malignant tumors). The common events that play a major role in induction of carcinogenesis can be clustered under six pro-cancer events as described by Hanahan and Weinberg (2000). These events serve as the hot spots for cancer treatment. The following are the events and descriptions:

2.2.1 Self-sufficiency in Growth Signals

Mitogenic growth signals (GS) are required by normal cells before they move from the quiescent state into active proliferative state. Tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from normal tissue microenvironment by acquiring GS autonomy. The common molecular strategies for achieving autonomy are the alteration of extracellular growth signals such as platelet-derived growth factor (PDGF) and tumor growth factor (TGFα); transcellular transducers of those signals are mitogenic growth factor (GF) receptors. Epidermal growth factor receptor (EGR-R/*erbB*) and extracellular matrix receptors (integrins) are some of the cell surface growth receptors that stimulate progrowth signals; and intracellular pathways that translate those signals into action.

2.2.2 Insensitivity to Anti-growth Signals

Anti-growth signals in normal cells can block proliferation by two distinct mechanisms. The cells may be forced out of the active proliferative cycle into the quiescent (G_0) state or be induced to permanently relinquish their proliferative potential by being induced to enter post-mitotic states. Cancer cells evade the anti-proliferative signals by avoiding the cytostatic antigrowth signals. Most of the anti-proliferative signals are regulated through the retinoblastoma protein (pRb), which is disrupted in cancerous cells. Disruption of the pRb pathway liberates E2Fs and allows numerous genes to activate the progression of cells from G1 into S phase.

2.2.3 Evading Apoptosis

Programmed cell death, or apoptosis, is present in latent form in all cell types throughout the body. This program triggers a series of steps under the influence of physiological signals. Apoptosis involves two components- sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular conditions of normality or abnormality that influence the survival or destruction of the cell. The intracellular sensors monitor abnormalities such as DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia. The survival signal is conveyed by IGF-1/IGF-2 through their receptors, IGF-1R, and by IL-3 and its cognate receptor (IL-3R). Death signals are conveyed by FAS ligand binding the Fas receptor and by TNFα binding TNF-R1. The effectors of apoptosis include intracellular proteases called caspases. Two important caspases, caspase -8 and -9, are activated by death receptors such as the FAS receptor or by the cytochrome C released from mitochondria, respectively. Cancer cells evade apoptosis by over expressing oncogenes such as Bcl-2 and c-Myc and through loss of proapoptotic regulating via mutations involving the p53 tumor suppressor gene.

2.2.4 Limitless Replicative Potential

Most mammalian cells can only reproduce a limited number of times in laboratory cultures before they lose the ability to divide and become what is called senescent. The senescence can be circumvented by disabling pRb and p53 tumor suppressor proteins, allowing them to continue multiplying for additional generations until they enter into a second state, termed crisis. The crisis state is characterized by massive cell death associated with end-to-end fusion of chromosomes which acquires ability to multiply without limit, termed immortalization. Most tumor cells exhibit this immortalized state of unlimited replicative potential. Also, tumor cells maintain telomeres at a length above a critical threshold, which in turn, permits unlimited

multiplication of descendant cells.

2.2.5 Induction of Angiogenesis

Angiogenesis is the growth of new blood vessels towards and within the tumor. Cancer cells trigger the production of growth factors to initiate angiogenesis, which is required for the tumor to grow. The newly formed blood vessels around the tumor provide a constant supply of oxygen and nutrients. The angiogenic signaling factors such as vascular endothelial growth factor (VEGF) and acidic fibroblast growth factors (FGF1/2) bind to transmembrane tyrosine kinase receptors expressed on endothelial cells. The role of angiogenesis in cancer is elaborated in the following section of this chapter.

2.2.6 Invasion and Metastasis

Tumor cells utilize the newly formed blood vessels and lymph circulation to migrate to distant tissues and organs. This stage is often associated with malignancy. The activation of extracellular proteases and the altered binding specificities of cadherins, cell adhesion molecules (CAMs), and integrins, are clearly central to the acquisition of invasiveness and metastatic ability. While malignant cells metastasize, they also express immunosuppressive compounds in order to evade the immune system.

Various direct and indirect therapies have been developed to inhibit the different events during carcinogenesis. Some of the well-known strategies include containing the cancer cells either by reverting them to normalcy or inducing programmed cell death. Other methods involve inhibition of angiogenesis, the process with which the tumor is deprived of oxygen and nutrients eventually forcing it to die, or controlling the metastasis and making the cancer cells sensitive to the immune system (Grothey, 2005).

2.3 Overview of Angiogenesis

Angiogenesis is a complex process in which existing mature blood vessels generate

sprouts, and these sprouts develop into complete new vessels. During angiogenesis, vascular cells proliferate at abnormally rapid rates of about 7-10 days, whereas normal process involves capillary cell division once every 7 years (Boik, 2001).

Angiogenesis in tumor development involves at least four steps: (i) cancer cells or adjacent tissues secrete angiogenic factors, (ii) the basement membrane, which is a layer of specialized connective tissue and which also provides structural support to the capillary, serves as the connection point between the extracellular matrix (ECM), the ground substance surrounding cells and tissues, holding them in place, and the capillary itself. This basement membrane dissolves and a bud begins to grow, (iii) vascular endothelial cells proliferate and migrate from the bud toward the angiogenic stimulus, and (iv) the sprout eventually forms a hollow tube called the lumen and joins its end with another sprout to form a new capillary vessel (Boik, 2001).

2.4 Physiological and Pathological Angiogenesis

2.4.1 Physiological Angiogenesis

Physiological angiogenesis is tightly regulated by a network of stimulators and inhibitors and is short in duration. It plays an important role in reproduction, development of a child in mother's womb, for connective tissue matrix formation, maintenance of vascular integrity during wound healing, repair process of damaged tissue, bone growth and bone fracture repair, and maintenance of oxygen delivery to working muscle under increased metabolic activity (Losso and Bansode, 2004).

The mechanism of physiological angiogenesis involves several steps, which include (i) the removal of pericyte from endothelium and angiopoietin-2 (Ang-2)-catalyzed transformation of endothelial cells from a stable to a proliferative phenotype; (ii) growth factors, specially VEGF and VE cadherin catalyzed vessel hyper-permeability and matrix remodeling by the action of serine and matrix metalloproteinase; (iii) VEGF, FGF, and EGF catalyzed endothelial cell

proliferation; (iv) endothelial cell migration catalyzed by $\alpha_v\beta_3$ integrin, VEGF, and FGF; (v) VE cadherin and ephrin B2/ephrin B4catalyzed cell-cell contact; (vi) FGF, PDGF, TNF-α, and Eph-2A catalyzed tube formation as blood conduits; (vii) PDGF- and Ang1/Tie2-catalyzed proliferation and migration of mesenchymal cells along the new vessels and TGF-β catalyzed pericyte differentiation into mature pericytes; and finally (viii) Ang1/Tie2, PDGF, VE cadherins and TGF-β catalyzed vessel stabilization (Losso and Bansode, 2004).

2.4.2 Pathological Angiogenesis

In a healthy body, the vasculature is quiescent, and in most healthy adult tissues, growth is almost non-existent (Hobson and Denekamp, 1984). Physiological angiogenesis occurs during embryo development where the vasculature is needed and is tightly regulated by a balance between stimulators and inhibitors. In adulthood, physiological angiogenesis occurs during wound healing and during the ovarian cycle. Pathological angiogenesis are mainly of two types: excessive and insufficient angiogenesis. Excessive angiogenesis occurs when angiogenic stimulators outbalance inhibitors and insufficient angiogenesis occurs when stimulators are deficient (Losso, 2002).

The mechanism of excessive angiogenesis involves: (i) retraction of pericytes from the abluminal surface of the capillary, (ii) release of proteases from the activated endothelial cells, (iii) protease-catalyzed dissolution of the basement membrane surrounding the pre-existing vessels, (iv) endothelial cell migration toward an angiogenic stimulus and their proliferation, (v) formation of tube-like structures, (vi) fusion of the formed vessels, and (vii) initiation of blood flow. Insufficient angiogenesis is characterized by (i) insufficient vascularization, (ii) delayed formation of granulation tissue, (iii) decreased collagen content, (iv) low breaking strength compared to normal tissue, loss of vascular tone, (v) higher content of oxidized lipids, (vi) absence of microtibular structures, (vii) impaired collateral vessel formation, and (viii) impaired

signal transduction pathways (Losso and Bansode, 2004). A list of human diseases associated with pathological angiogenesis is presented in Table 2.1.

Angiogenesis plays a critical role in the development of cancer. Solid tumors smaller than 1 to 2 cm^3 are not vascularized. They require oxygen and nutrients supplied by the blood vessels, which also remove the metabolic wastes from the tissues. Also, blood vessels serve as a medium for migration of cancerous cells to a different location in the body called metastasis. Cancer cells are capable of expressing several angiogenic factors including basic-fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), transforming growth factor-β (TGF-β), and others that cause endothelial cell recruitment and proliferation. These stimuli, when expressed by the cancer cells, trigger the differentiation of the tumor endothelium into a mature vessel. However, the vascularization is rarely complete and tumor vessels show abnormal morphology (Tosetti et al., 2002).

Additionally, oxygen and nutrients have difficulty diffusing to the cells in the center of the tumor beyond critical volume of 2 cm^3. Depletion of oxygen availability induces hypoxia in tissues and enhances tumoral angiogenesis. Therefore, neovascularization is important for tumor survival and progression. It favors the transition from hyperplasia to neoplasia i.e. the passage from a state of cellular multiplication to a state of uncontrolled proliferation, which is characteristic of tumor cells. Neovascularization also influences the dissemination of cancer cells throughout the entire body eventually leading to metastasis formation. The vascularization level of a solid tumor is thought to be an excellent indicator of its metastatic potential.

Some of the mechanisms to prevent the progression of angiogenesis in a tumor are: suppressing endogenous angiogenic factors, such as bFGF (basic Fibroblast Growth Factor) and VEGF (vascular endothelial growth factor), inhibiting enzymes (matrix metalloproteinases) responsible for the degradation of the basement membrane of blood vessels, inhibiting

endothelial cell proliferation; endothelial cell migration; and activation and differentiation of endothelial cells. The process regulating the molecular mechanism of switching angiogenesis 'on' or 'off' depends on the external and internal microenvironment, which includes metabolic stress, mechanical stress, immune/inflammatory response,, and genetic mutations (Losso and Bansode, 2004).

Table 2.1 A Partial List of Human Diseases Associated with Pathological Angiogenesis

Pathological Angiogenesis	
Insufficient	**Excessive**
• Delayed wound healing • Ischemia (myocardial, peripheral, cerebral) • Stroke • Heart disease • Scleroderma • Infertility • Diabetic neuropathy • Systemic sclerosis • Coronary heart disease • Placental insufficiency • Impaired healing of fracture • Pulmonary and systemic hypertension • Vascular dementia • Lymphoedema • Impaired collateral vessel formation • Chronic non-healing ulcer	• Rheumatoid arthritis • Multiple sclerosis • Age-related macular degeneration • Diabetes retinopathy • AIDS complications • Tumor growth and metastasis • Osteoporosis • Alzheimer's • Parkinson's • Obesity • Psoriasis • Hepatitis • Thyroid enlargement • Ocular neovascularization • Retinopathy of prematurity • Synovitis • Bone/cartilage destruction • Hematological malignancies

(adapted from Losso and Bansode, 2004)

Tumor vessels develop by sprouting from pre-existing vessels. Circulating endothelial precursors, shed from the vessel wall or mobilized from the bone marrow, can also contribute to tumor angiogenesis. Tumor cells can also grow around an existing vessel to form a perivascular cuff. Vascular endothelial growth factor (VEGF) and the angiopoietin (Ang) family have predominant roles in vasculogenesis. The angiogenic activity of VEGF is tightly regulated by gene dosage. Several molecules, including a number of angiogenesis inhibitors, seem to be

involved mainly in tumor angiogenesis. The temporal and spatial expression of these regulators is not as well coordinated in tumors as it is in physiological angiogenesis and their mechanism of action is poorly understood. In addition, tumor vessels lack protective mechanisms that normal vessels acquire during growth. Tumor vessels lack functional perivascular cells, which are needed to protect vessels against changes in oxygen or hormonal balance, provide them necessary vasoactive control to accommodate metabolic needs and induce vascular quiescence. Finally, the vessel wall is not always formed by a homogenous layer of endothelial cells. Instead, it may be lined with only cancer cells or a mosaic of cancer and endothelial cells. Tumor blood flow is chaotic and variable, and leads to hypoxic and acidic regions in the tumors. Hypoxia may select for clonal expansion of cells that have lost their apoptotic response to hypoxia (Carmeliet and Jain, 2000).

Tissues require a constant supply of oxygen in order to survive; beyond a certain size, simple diffusion of oxygen to metabolizing tissues becomes inadequate, and specialized systems of increasing complexity have evolved to meet the demands of oxygen delivery in higher animals (Pugh and Ratcliffe, 2003). The physiological response to hypoxia is aimed at maximizing oxygen intake and delivery to vital organs, increasing ventilation, heart rate, cardiac output, blood pressure, growth of capillary beds to supply vital organs, production of red blood cells, hemoglobin levels, and blood volume. Hypoxia can result in sympathoadrenal stimulation and lead to an increase in peripheral resistance. Postnatal hypoxia induces increased respiratory effort and causes a rise in cardiac performance (Huang et al., 2004).

An acute hypoxic exposure will activate both the fast and delayed responses. The fast response involves, (a) metabolic changes, with a possible switch from mitochondrial based metabolism, fuelled by glycolysis and (b) activation of various plasma membrane ionic conductance, regulated either directly or indirectly by oxygen. The main difference between the

oxygen-sensing cells and other cell types in an organism, with respect to acute responses, is in the type of ion channels. Ion channels can be activated by specialized sensor cells in response to the hypoxic exposure and thus initiate specific signaling cascades that are able to integrate the signal generated at the sensor into a larger tissue or whole body response. The delayed responses are mainly based on genomic activation triggered by the up-regulation of a number of transcription factors (Pugh and Ratcliffe, 2003).

A major advance in the understanding of the oxygen-sensing processes and of the mechanisms that regulates the cellular and tissue response to hypoxia came with the discovery of the hypoxia-inducible-factor (HIF-1). HIF-1 is ubiquitously expressed and participates in most chronic cellular responses to hypoxia. HIF-1 is a heterodimer composed of two subunits: HIF-1α and HIIF-1β. HIIF-1β, a factor that can dimerize with aryl hydrocarbon receptor (AHR) and activates the genes encoding cytochrome P450 enzymes involved in the metabolism of aryl hydrocarbons. Both subunits contain PAS (PER-ARNST-SIM) domains that mediate the heterodimer formation between the subunits and are also necessary for DNA binding. The main feature of HIF activity is that in normal (normoxic) conditions, both subunits are expressed, but only HIF-1β can be detected, because the HIF-1α subunit is rapidly degraded (Toescu, 2004).

A large number of genes involved in angiogenesis are independently responsive to hypoxia in tissue culture such as nitric acid synthase involved in vascular tone, growth factors such as VEGF, angiopoietins, fibroblast growth factors and their various receptors, genes involved in matrix metabolism (matrix metalloproteinases, plasminogen activator receptors and inhibitors), and collagen prolyl hydroxylase. Also, many of the individual phenotypic processes during angiogenesis such as cell migration or endothelial tube formation can be induced by hypoxic tissue culture (Pugh and Ratcliffe, 2003).

HIF plays an important role in tumor angiogenesis as many common cancers exhibit up-

regulation of the HIF system influenced by multiple genetic and environmental mechanisms. Activation of hypoxia by the HIF system is also induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways such as insulin, insulin-like growth factor-1, epidermal growth factor and mutant Ras and Src kinase pathways. Activation of HIF in cancer microenvironments occurs at the simplest level by physiological activation of the oxygen-sensitive pathways by hypoxia within a growing mass of cells. The prolyl hydroxylation of HIF is often incomplete in tumor cells, even in fully oxygenated tissue culture. Iron imbalance associated with rapidly growing tumor cells is due to the enhanced activity of HIF hydroxylase, which degrades HIF-1α. This implies that the reduction in cellular iron availability, as well as hypoxia, limit HIF hydroxylase activity in cancer cells and contributes to activation of HIF in tumors. The interconnection between angiogenic and hypoxia pathway suggests a prevalent condition in cancer (Figure 2.1). Cell proliferation, HIF activation, and angiogenesis are all linked by pathways that operate physiologically to preserve oxygen homeostasis (Pugh and Ratcliffe, 2003).

2.5 Inhibition of Angiogenesis

2.5.1 Biomedical Approach- Success and Failure

The anti-angiogenic drugs presently under investigation are being targeted either by specific biomarkers of angiogenesis (VEGF, bFGF, and IGF) or others that indirectly affect the endothelial cell function/response (matrix metalloproteinase breakdown). Some drugs such as thalidomide target both mechanisms simultaneously. Clinical trials are presently ongoing on direct administration of endogenous angiogenic inhibitors such as angiostatin, endostatin, and the gene transfer of DNA that encodes for angiogenesis inhibitors, including angiostatin and platelet factor IV. Synthetic angiogenic inhibitors that prevent endothelial cell division, such as synthetic derivatives of fumagillin are also underway (Rosen, 2000).

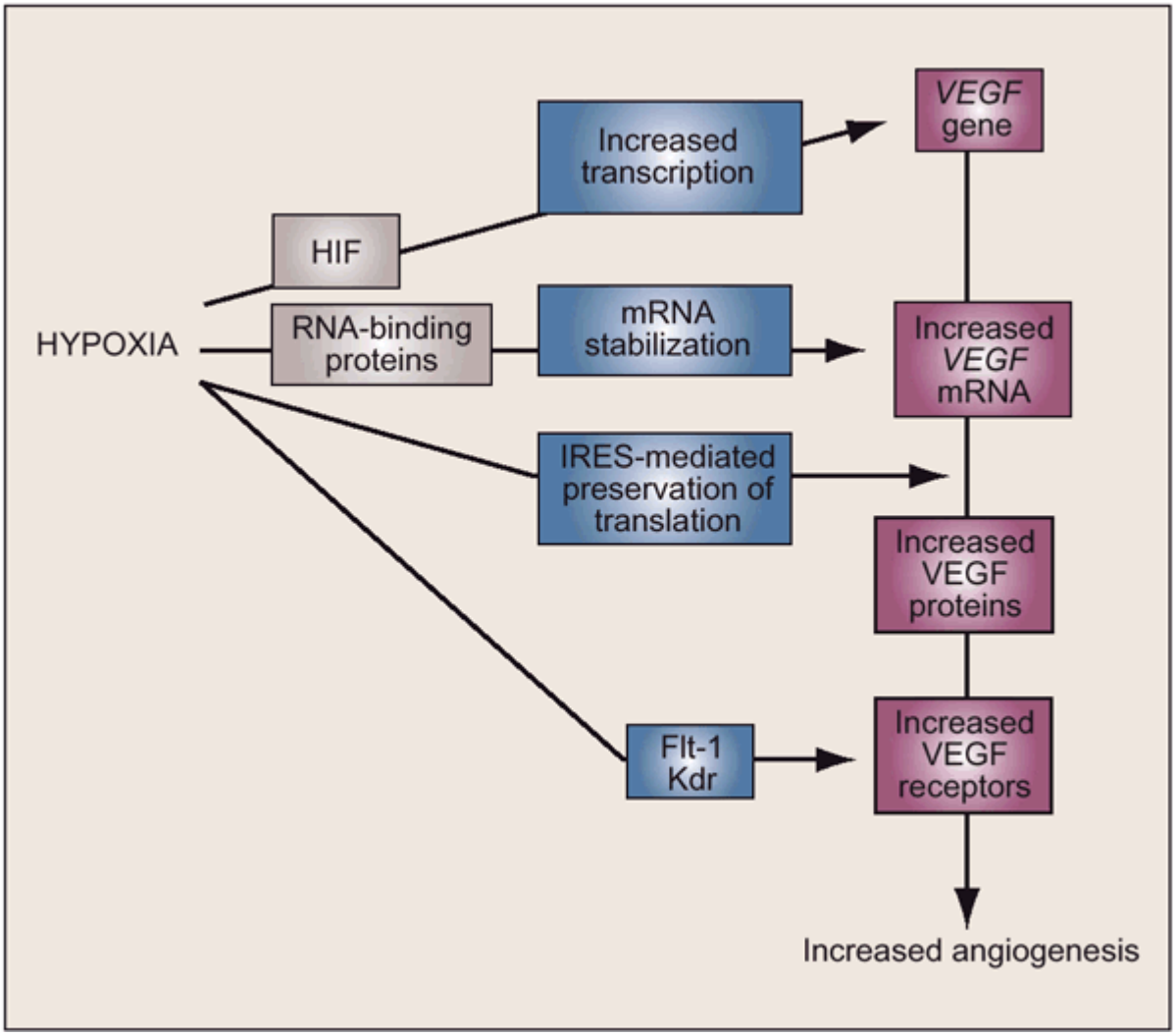


Figure 2.1 Multiple Interfaces of Hypoxia Pathways with the Angiogenic Growth Factor VEGF. (Pugh and Ratcliffe, 2003[1]).

Other strategies include counterbalancing the effects of ECM degradative enzymes (collagenase and gelatinase) with inhibitors such as TIMPs. The advantage of containing angiogenesis against tumor progression, unlike chemotherapy, is that anti-angiogenic inhibitors do not exhibit toxicity while drugs that target cell proliferation produce gastrointestinal symptoms and myelosuppression (Rosen, 2000). Drug resistance is another major problem with

[1] Reproduced with permission from Nature Medicine (Pugh and Ratcliffe, 2003) copyright (2005) Macmillan Magazines Ltd.

chemotherapy as cancer cells are genetically unstable and prone to mutations. Alternately, angiogenic compounds mainly target endothelial cells and are cytostatic rather than cytotoxic (Rosen, 2000).

2.5.2 Prevention of Cancer by Dietary Bioactive Compounds

Increasing consumer knowledge of the link between diet and health has raised the awareness and demand for functional food ingredients and nutraceuticals (FitzGerald et al., 2004). A major initiative in food product development is in discovering functional ingredients, or components possessing nutritional benefits, beyond the provision of essential nutrients and calories. Various dietary biomolecules have been associated anecdotally with functional and nutritional benefits and work is currently underway to characterize the mechanisms underlying these modes of action (Ward and German, 2004).

Sulforaphane, phenolic acids, and selenium from broccoli have been demonstrated to reduce the risk of cancer (Finley et al., 2005). Epidemiological studies focusing on tomato consumption and recent animal studies investigating lycopene and other phytochemicals in tomatoes, established its role against prostate cancer and cardiovascular diseases (Canene-Adams et al., 2005).

Herbal flavonoids (quercetin, curcumin, rutin, and silymarin) and one whole herb mixture (ginseng powder) have been shown to suppress aberrant crypt foci (ACF) in an azoxymethane (AOM)-induced rat colon cancer model. The ability of quercetin and curcumin to modulate ACFs correlates well with their ability to induce apoptosis by affecting caspase 9, Bax (proapoptotic) and Bcl-2 (antiapoptotic) proteins from the colon cells via the mitochondrial pathway (Volate et al., 2005). Phytochemicals, including phenolics and flavonoids from apples, have been shown to inhibit mammary cancer growth in a rat model due to their antioxidant and antiproliferative activities (Liu et al., 2005). Preclinical and clinical evidence indicate that

indole-3-carbinol (I3C), a key bioactive food component in cruciferous vegetables, has multiple anticarcinogenic and antitumorigenic properties. Evidence that p21, p27, cyclin-dependent kinases, retinoblastoma, Bax/Bcl-2, cytochrome P-450 1A1, and GADD153 are all targets for I3C already exists. Modification of nuclear transcription factors, including Sp1, estrogen receptor, nuclear factor kappaB, and aryl hydrocarbon receptor, may represent a common site of action to help explain downstream cellular responses to dietary I3C, and ultimately to its anticancer properties (Kim and Milner, 2005).

2.5.3 Dietary Bioactive Compounds as Anti-angiogenic Compounds against Cancer

The rate-limiting factor in angiogenesis seems to be increased vascular permeability and vascular endothelial growth factor (VEGF), one of the most potent inducers of permeability known to play a key role in angiogenesis. Reduction of VEGF production and reduced vascular permeability has been suggested as measures of pathological angiogenesis prevention (Boik, 2001). VEGF is produced in response to hypoxia (low oxygen) conditions, which are prevalent within most tumors (Hanahan and Weinberg, 2000; Kieran et al., 2003). Other growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), tumor necrosis factor (TNF), and transforming growth factor-beta (TGF-β), which are present in macrophages, also produce VEGF. The expression of VEGF can be reduced by antioxidants, PTK inhibitors, PKC inhibitors, and leukotriene inhibitors, as well as inhibitors of AP-1 (Tsai et al., 2003; Fierro et al., 2002; Matsushita et al., 2000). Reduced production of VEGF has been reported with genistein, a PTK inhibitor, EPA, a PKC inhibitor, and curcumin, an AP-1 inhibitor. Epigallo catechin gallate (EGCG), curcumin, caffeic acid phenethyl ester (CAPE), and genistein have also been reported to reduce EGF and PDGF signaling in a variety of cells (Sah et al., 2004; Smith et al., 2004; Zheng et al., 1995; Dean et al., 1989). Other flavanoids, such as luteolin and apigenin, can inhibit *in-vitro* angiogenesis by inhibition of VEGF expression during hypoxia by

suppression of HIF-1α and p53 tumor suppressor gene (Hasebe et al., 2003; Fang et al., 2005). The primary effect of VEGF is to minimize the vascular permeability, which is caused by pro-inflammatory cytokine activity of VEGF, especially VEGF-A. VEGF-A regulates enzymes such as cyclooxygenase-2 (COX-2), adhesion molecules (*e.g.* E-selectin, VCAM-1), and pro-coagulant factors (*e.g.* tissue factor) (Wilkinson-Berka, 2004). Some of the naturally derived compounds that are believed to reduce vascular permeability are: anthocyanidins, Butcher's broom, *Centella asiatica*, horsechestnut, and proanthocyanidins (Boik, 2001).

2.5.4 Role of Sphingolipids in Angiogenesis

The role of sphingolipids in angiogenesis is not well established. However, recent studies show that sphingolipids could mediate cell migration and angiogenesis by their well-known mechanism as secondary messenger (Le Stunff et al., 2004; Wang et al., 2003). Sphingosine-1-phosphate (S1P), formed by sphingosine kinase, has been proven to complex with the EDG-1 (a GPCR) and mediates cell migration and vascular maturation (Rosenfeldt et al., 2001; Morales-Ruiz et al., 2001). EDG-1 functions as an integrator linking the PDGFR to lamellipodia extensions and cell migration. S1P stimulated EDG-1, results in activation and integration of downstream signals important for directional movement toward chemoattractants, such as PDGF. PDGF, which further stimulates sphingosine kinase and leads to increased S1P levels in many cell types, also induces translocation of sphingosine kinase to membrane ruffles (Katsuma et al., 2000). The study conducted by Ryu et al., (2002) established that S1P could stimulate smooth muscle cells (SMCs) and endothelial cells, modulate vascular tone bimodally by directly constricting SMCs and stimulating production of nitric oxide, and stimulate endothelial permeability and angiogenesis. On the other hand, ceramide induced apoptotic cell death while S1P acted as a survival factor. S1P counteracts the pro-apoptotic effects of ceramide through protein kinase C and inhibits apoptosis in macrophages by depressing acidic-Sphingomyelinase

(aSmase) (Yabu et al., 2005).

A recent study conducted by Yabu et al., (2005) on zebrafish demonstrated that S1P decreases ceramide content by inhibiting neutral Sphingomyelinase (nSMase) and restores a thalidomide-induced vascular defect in zebrafish embryo by increasing the expression of neuropilin-1 and Flk-1, suggesting that the balance between ceramide and S1P-mediated signal regulates angiogenesis through VEGF receptors, Flk-1, and neuropilin-1.

2.6 *In Vitro* and *In Vivo* Angiogenesis Assay

Angiogenesis assays are used to test efficacy of both pro- and anti-angiogenic agents, which rely on both *in vitro* and *in vivo* tests. The selection of assays depends on the criteria under investigation and generally involves a combination of assays in providing the necessary information. *In vitro* assays are generally used for quantification purpose while *in vivo* assays are used to substantiate the in vitro results. Also, *in vivo* assays are essential because of the complex nature of vascular response to test reagent (Staton et al., 2004). The ideal assay would be reliable, straightforward, easily quantifiable and physiologically relevant. It is usually recommended a combination of the cellular and molecular events in angiogenesis be used to examine the full range of effects for a given test compound (Auerbach et al., 2003). This section elaborates on the most commonly used *in vitro* and *in vivo* assays for accessing the efficacy of compounds towards angiogenesis.

2.6.1 *In Vitro* Assays

2.6.1.1 Cell Proliferation

There are two major classes of proliferation assays: those that determine net cell number and those that evaluate cell-cycle kinetics. Net cell number can be established by use of a haemocytometer or by use of an electronic counter such as a Coulter counter. Other techniques involve staining, such as 3-(4,5-demethylthiazol-2-yl)-2,5-diphenylltetrazolium bromide) (MTT),

a tertrazolium salt that gives blue formazan product when cleaved by active mitochondria, and assessing DNA synthesis using DNA binding dye (thymidine) to measure the cell viability with colorimetric techniques. An alternative method for assessing proliferation is by cell-cycle analysis, using DNA binding molecules (bromodeoxyuridine, BrdU) coupled with flow cytometry analysis (Auerbach et al., 2003).

2.6.1.2 Cell Migration

Blind-well chemotaxis (modified Boyden chamber) is the most commonly used assay for measuring the migration of cells in response to a test factor. The cells are placed on the upper layer of a cell permeable filter placed in the medium below the filter. This requires cell enumeration to measure the number of cells that migrate across the filter compared with that which was retained in the upper layer. This process reflects the conditions that are operative *in vivo* and lends itself to testing concentration gradients of the compound under study (Staton et al., 2004).

2.6.1.3 Tube Formation

One of the most specific tests for angiogenesis is the measurement of the ability of endothelial cells to form three-dimensional structures (tube formation). Endothelial cells spontaneously form tubules *in vitro* by laying down approximate extracellular matrix components. Endothelial cells are usually cultured on matrices consisting of fibrin, collagen and Matrigel, which stimulates the attachment, migration and differentiation of these cells into tubules in a manner comparable to *in vivo* conditions (Staton et al., 2004).

2.6.2 *In Vivo* Assays

There are currently three commonly used *in vivo* approaches for assaying compounds with regards to vessels growth: (1) microcirculatory preparations in animals, (2) vascularization into biocompatible matrix implants, and (3) excision of vascularized tissues. Although the first

method is an *in vivo* technique, the latter two are *in vitro* assays that require subsequent *in vivo* validation. *In vivo* assays are usually expensive, are experimentally exhaustive and are accountable for the study of angiogenesis but not for drug screening. Therefore, there is an important requirement for an inexpensive and faster *in vivo* technique for screening angiogenesis (West et al., 2001).

2.6.2.1 Chorioallantoic Membrane (CAM) Assay as an Angiogenesis Model System

The study of the angiogenic process and the search for novel therapeutic agents to inhibit or stimulate angiogenesis has employed a wide range of *in vivo* 'angiogenic' assays. These differ greatly in their difficulty, quantitative nature, rapidity, and cost. The classical *in vivo* models include the rabbit ear chamber, hamster cheek pouch, dorsal skin chamber, dorsal skin and air-sac model, anterior chamber/iris and avascular corneal pocket assay, and the chick embryo chorioallantoic membrane (CAM) assay. Most recent methods involve implantation of preloaded matrigel or alginate plugs, or collagen or polyvinyl sponges, and zebrafish screening. CAM is the most widely used *in vivo* model largely due to its simplicity and low cost (Ribatti et al., 2000).

Folkman and coworkers initially developed the CAM assay to examine the angiogenic activity of tumor tissues. There are two different methods commonly employed. The most widely used method is performed with CAM assay *in situ*, the samples (up to two per egg) are applied through a window made in the shell. The other technique involves transfer of the early, 3- or 4-days-old, embryo and its extra-embryonic membranes to a glass Petridish for further development. This gives a wider surface area, allowing multiple sample applications on the same egg, and provides easier viewing and photography, but suffers from poor embryo viability (Ribatti et al., 2000).

However, CAM has several limitations for large scale screening: (1) the CAM assay takes a minimum of 10 days, compared with 1-3 days for the zebrafish assay (2) the number of

compounds and/or dilutions that can be tested at one time is limited by availability and cost of egg production compared with inexpensive zebrafish assay embryo production, and (3) the assay is more difficult to quantitate than the zebrafish assay, which impedes the generation of dose-response relationships (Serbedzija et al., 1999).

2.6.2.2 Zebrafish Assay as Angiogenesis Model System

The zebrafish (*Danio rerio*) is a small tropical fish, which develops *ex utero* and is optically transparent during early development. Between 1 to 6 days post-fertilization (dpf), zebrafish develop discrete organs and tissues, including brain, heart, liver, pancreas, kidney, intestines, bones, muscles, nervous systems, and sensory organs. These organs and tissues have been demonstrated to be similar to their mammalian counterparts at the anatomical, physiological, and molecular levels. Historically, zebrafish have provided a powerful model for studying the genetics and developmental biology of vertebrates, as well as for assessing the aquatic toxicity of environmental pollutants, but in more recent years the value of the zebrafish has been increasingly recognized as a model organism for drug screening, target validation, and toxicological studies (Parng, 2005).

Zebrafish have 50 chromosomes that contain orthologs or homologs of most human genes. The conservation in the proteins' functional domains, including substrate binding regions, ligand binding regions, enzyme catalytic domains, DNA binding motifs, and nucleus translocation signal sequences is considerably higher between zebrafish and humans. Since these domains are highly conserved or even structurally identical in zebrafish and humans, drugs designed to interact with these functional domains in a protein of interest elicit comparable pharmacological effects (Parng, 2005).

The major advantages of a zebrafish-based assay compared with existing assays are: (1) hundreds of compounds can be tested simultaneously using a microplate format, (2) the assay is

fast, approximately 1-3 days from addition of compounds to assay results, (3) embryo maintenance is easy during assay procedures, and (4) compound addition and embryo assessment is straight-forward due to ease of access (Serbedzija et al., 1999).

Zebrafish have several inherent advantages for drug screening as they are small, inexpensive to maintain, and easily bred in large numbers (a single spawning produces 100 to 200 eggs). Development during the segmentation period is shown in Figure 2.2. Adult zebrafish are 3-cm-long. The larvae, which are only 1-to 4-mm-long, can live for 7 days in single well of standard 96 or 386-well plates supported by nutrients stored in their yolk sacs. Administration of drugs to zebrafish is straight forward; zebrafish larvae can absorb small molecules diluted in the surrounding water through their skin. Since zebrafish begin to swallow at 72 h post-fertilization (hpf), drugs can be delivered via the oral route for assays performed after this time. Highly hydrophobic compounds can be injected into the yolk sac, the sinus venous, or the veins. Compared to testing in other animal models, statistically significant numbers of zebrafish can be used for each assay and only a small amount (milligram scale) of drug is required. In addition, the transparency of the embryo provides easy accessibility for *in vivo* observations in live or whole mount-fixed specimens, including visualization of vital dyes, fluorescent tracers, antibodies, and riboprobes. Therefore, the zebrafish enables highly efficient phenotypical analysis and rapid assay for primary drug screening (Parng, 2005).

2.6.2.3 Angiogenesis in Zebrafish

The study of zebrafish, a leading model organism for developmental biology is rapidly expanding to include human disease and mechanisms that have been developed in several therapeutic areas, including blood diseases, diabetes, muscular dystrophy, neurodegenerative disease, angiogenesis and lipid metabolism. Recent literature suggests that the zebrafish has served as a novel *in vivo* model for drug discovery through the identification of novel drug

targets, validation of these targets, and screening for new therapeutic compounds (Serbedzija et al., 1999). Target identification refers to the process of identifying gene products that, when modulated by a drug, can have a positive impact on disease state progression (Rubinstein, 2003). Vasculature patterning in zebrafish embryo has been recently studied and used for drug screening to determine the anti-angiogenic properties of these compounds. The embryo vessels in the vertebrate trunk are a single large artery and vein, generated by the differentiation and coalescence of angioblasts from the lateral mesoderm, which is called vasculogenesis.

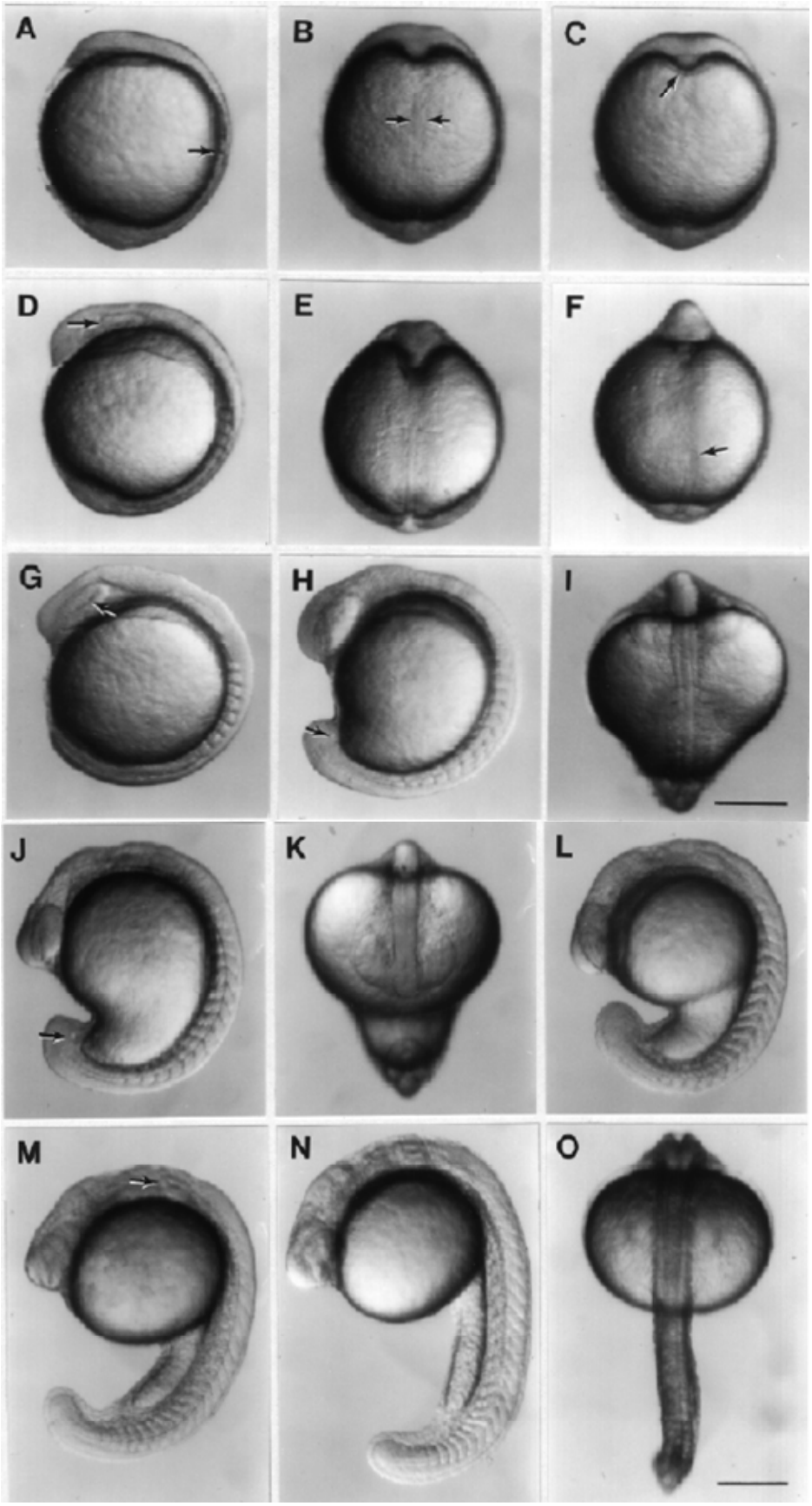
The sprouting of new blood vessels from the pre-existing vessels is termed angiogenesis. Angiogenesis at a cellular level involves localized endothelial cell proliferation and migration, followed by remodeling of the nascent vessel, the latter including the removal, growth or subdivision of the new channels. As discussed earlier several growth factors have been shown to be crucial for normal angiogenesis in the embryo. Vascular endothelial growth factor, VEGF, is expressed in the vicinity of sprouting vessels, its receptor (VEGF-R2/Flk-1/kdr) is located on the angioblast and new vessels are required for vasculogenesis and angiogenesis. Angiopoietin 1 and angiopoietin 2, and their receptor Tie 2, are reciprocally expressed in surrounding mesenchyme and early vessels. They appear to function in vascular remodeling and stabilization. Several EphB receptors and ephirnB ligands are expressed, some with arterial versus venous specificity, both on vessels and in surrounding tissues, and are required for remodeling of angiogenesis.

In zebrafish, cells in the lateral posterior mesoderm express endothelial and hematopoietic markers, suggesting that they include bipotential precursors for both the hematopoietic and angioblastic lineages, termed hemangioblasts (Crosier et al., 2002). Childs et al., 2002 determined that the angiogenic precursor cells arise in the lateral posterior mesoderm, at an axial level that corresponds to the eventual sprout, and migrate medially to the aorta. There they then sprout dorsally to connect to aorta with dorsal longitudinal anastomotic vessel

Figure 2.2 Development of Zebrafish Embryo during the Segmentation Period.

Left side views, except where noted, with anterior up and dorsal to the left. A: 2-somite stage (10.7 h). Somite 2 is the only one entirely pinched off at this time, the arrow indicates its posterior boundary; somite 1 is just developing a clear anterior boundary at this stage. B: 2-somite stage, dorsal view. The notochord rudiment shows between the arrows, just anterior to the level of somite 1. C: 2-somite stage, ventral view. The arrow indicates the polster. D: 4-somite stage (11.3 h). Somite 1 now has an anterior boundary. The optic primordium begins to show (arrow). E: 4-somite stage, dorsal view, focus is on the notochord at the level of the boundary between somites 2 and 3. Note at the top how the brain rudiment and underlying axial mesoderm prominently indent the yolk cell in the midline. F: 5-somite stage (11.7 h), ventral view, focus is on the newly forming Kupffer's vesicle (arrow). G: 8-somite stage (13 h). The optic primordium has a prominent horizontal crease (arrow). The midbrain rudiment lies just dorsal and posterior to optic primordium. The segmental plate, developing paraxial mesoderm posterior to the somite row, is clearly delineated. H: 13-somite stage (15.5 h). Somites begin to take on a chevron shape. The yolk cell begins to look like a kidney-bean, heralding formation of the yolk extension. The tail bud becomes more prominent and Kupffer's vesicle shows from the side (arrow). I: 14-somite stage (16 h), dorsal view, and positioned so that the first somite pair is at the center. Note at the top the shape of the brain primordium, at the level of the midbrain. J: 15-somite stage (16.5 h). The arrow shows Kupffer's vesicle. K: 15-somite stage from a dorsal-view to show the optic primordia. Kupffer's vesicle is also nearly in focus. L: 17-somite stage (17.5 h). The otic placode begins to hollow. The yolk extension is now clearly delimited from the yolk ball as the tail straightens out. M: 20-somite stage (19 h). The arrow indicates the otic vesicle. N: 25-somite stage (21.5 h). The telencephalon is prominent dorsally, at the anterior end of the neuraxis. O: 25-somite stage, dorsal view. The hindbrain's fourth ventricle shows at the top. Scale bars: 250 μm (Sprague et al. 2001[2]).

[2] Images for this dissertation were retrieved from the Zebrafish Information Network (ZFIN), the Zebrafish International Resource Center, University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org/; [September, 2005].


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

(DLAV). Three cell types constitute the sprout. Two resemble T-joints, one pointing dorsally off the aorta, and one pointing ventrally off the DLAV. The third constitutes a connecting tube in between as shown in Figure 4.2.

Vasculogenesis in zebrafish involves the differentiation of hemangioblasts from mesoderm, with a subsequent differentiation of angioblasts and endothelial cells. By the 12 somite stage (approximately 12 h post-fertilization, h pf), cells of the lateral mesoderm start expressing hemangioblast markers such as SCL/Tal-1 and Flk-1. These mesodermal bands converge until they lie within the embryo between the somites and the yolk sac, and form the intermediate cell mass (ICM). By 24 hpf, a simple circulatory loop consists of the dorsal aorta (DA) and axial vein (AV); blood cells subsequently differentiate from cells within the ICM. By 72 hpf, a functioning vasculature has developed, including the major trunk vessels and angiogenic sprouts, such as the subintestinal vessels (SIVs). Vessels in the posterior tail further differentiate and express SCL/Tal-1 at 72 hpf (Serbedzija et al., 1999).

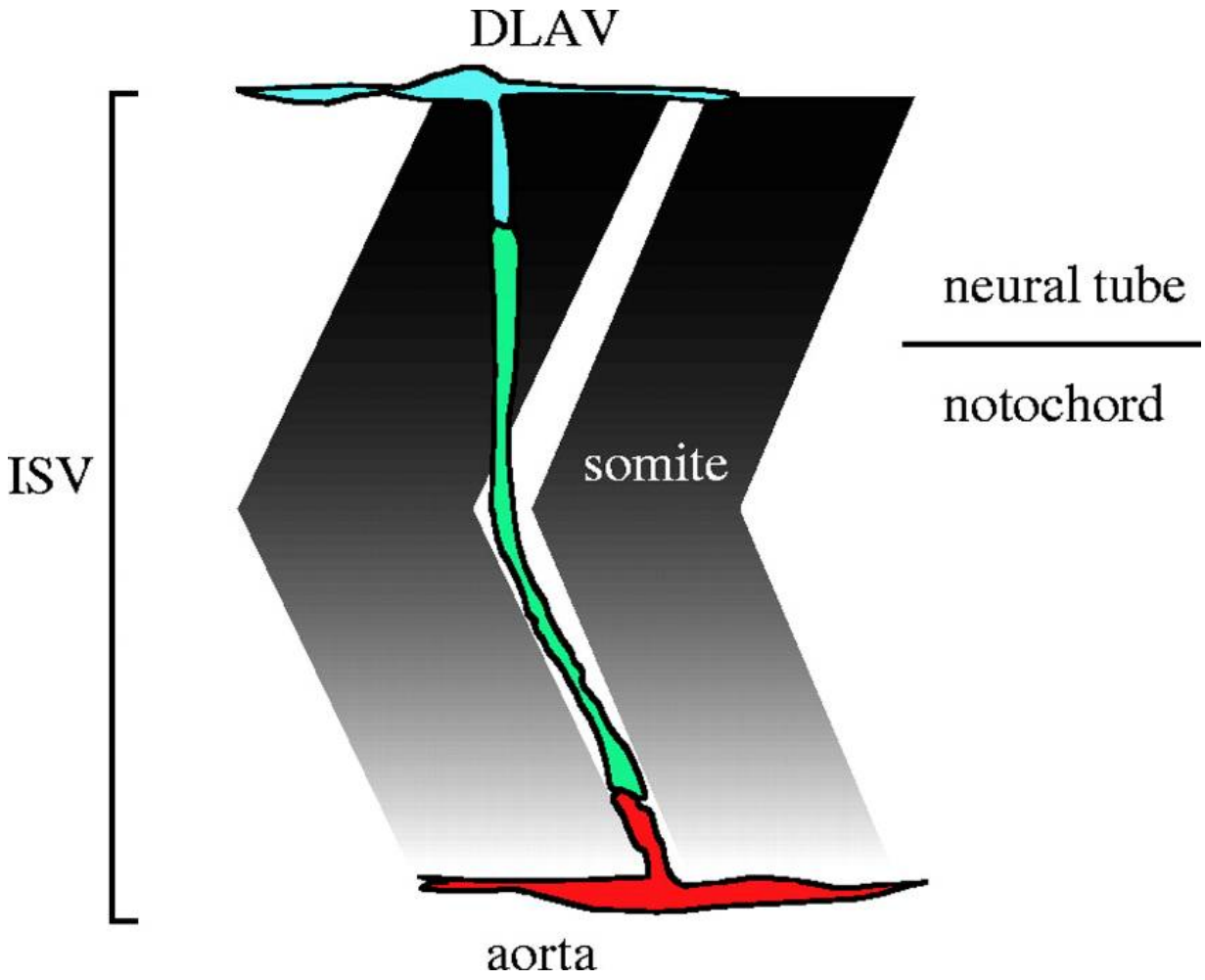
2.7 Overview of Sphingolipids

Sphingolipids are a class of complex lipids containing an amide-linked fatty acid and a long-chain (sphingoid) base that are important structural components of cell membranes, and are well recognized in both vertebrate and invertebrate cells. Different combinations of sphingoid long-chain bases, fatty acids, and head group moieties lead to a large number of sphingolipids and sphingolipids as illustrated in Figure 2.4. They are also involved in cellular processes such as cell proliferation, growth, migration differentiation, senescence, and apoptosis. Recent studies have shown that sphingolipids play an important role in signaling and therefore cell fate (Testi, 1996). Intercellular effects of a particular sphingolipid metabolite depend on cell type, the stage of cell development, and the ratio between different metabolites on the specific subcellular compartment of generation. Sphingolipids contribute to cellular signaling either by acting as first

Figure 2.3 Model of the Construction of a Zebrafish ISV.

An intersegmental vessel (ISV) is composed of three types of endothelial cells, distinguished by their morphologies. The dorsal connection to the DLAV is a T-shaped cell (blue); the ventral connection to the aorta is an inverted 'T' (red). The connecting cell (green) courses between the somites ventral to the notochord-neural tube interface, and appears not to follow the somite boundary dorsally, where it runs directly to the DLAV. (Childs et al., 2002[3]).

[3] Reproduced with permission, Childs et al., 2002 and The Company of Biologists Ltd, Copyright (2005).


DLAV
ISV
neural tube
notochord
somite
aorta

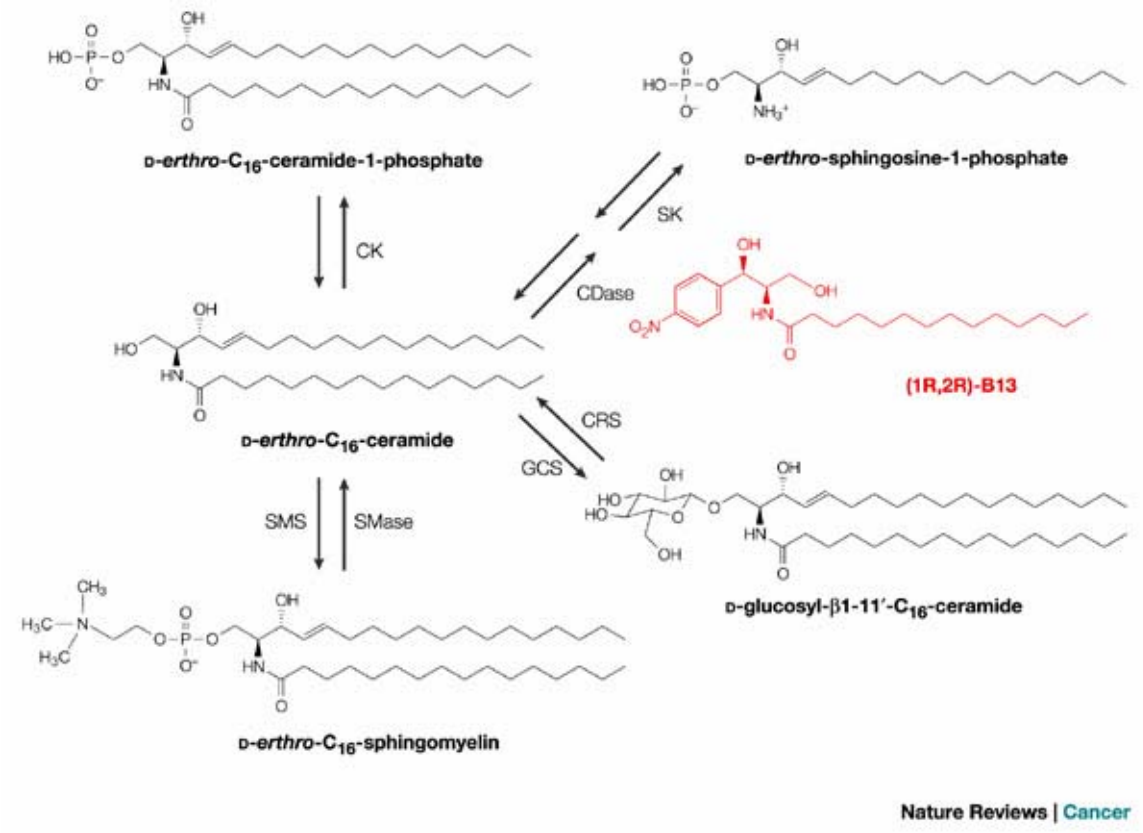


Figure 2.4 Structures of Sphingolipids.

The pro-apoptotic action of ceramide can be mimicked by small molecules such as C_2 and C_6 ceramides, or by analogues that inhibit clearance of ceramide, such as B13 (shown in red), an inhibitor of acid CDase (Ogretmen and Hannun, 2004[4]).

[4] Reproduced with permission from Nature Reviews Cancer (Ogretmen and Hannun, 2004)copyright (2005) Macmillan Magazines Ltd.

messengers binding to a seven-spanning, G protein-coulped receptor subfamily, or as intracellular second messengers, capable of interacting with a multiplicity of targets (Colombaioni and Garcia-Gil, 2004).

2.7.1 Synthesis of Sphingolipids

Metabolism of sphingolipids utilizes a diverse group of enzymes and *de novo* synthesis of sphingomyelin, sphingomyelinases, ceramidases, and sphingosine kinases (Figure 2.5). All are regulated by stimuli such as growth factors and stress, as well as some pathological conditions. *De novo* synthesis of sphingolipids (shown in Figure 2.6), occurs at the cytosolic face of the endoplasmic reticulum and is initiated by condensation of serine and palmitoyl-CoA via serine palmitoyl-transferase. The resulting keto-sphinganine is reduced to form sphinganine and N-acylated by ceramide synthase to dihydroceramide, which then desaturates to yield ceramide (N-acylsphingosine). Ceramide can be phosphorylated by ceramide kinase to ceramide-1-phosphate, or can serve as a substrate for the synthesis of sphingomyelin by addition of phosphocholine, or glycolipids by addition of oligosaccharides. Ceramide can also be metabolized by ceramidase to release sphingoid bases that can either be reutilized for complex sphingolipid biosynthesis or phosphorylated. Sphingosine is phosphorylated to yield sphingosine-1-phosphate, which is then dephosphorylated by lipid phosphate phosphatase or cleaved by the sphingosine-1-phosphate lyase (Colombaioni and Garcia-Gil, 2004).

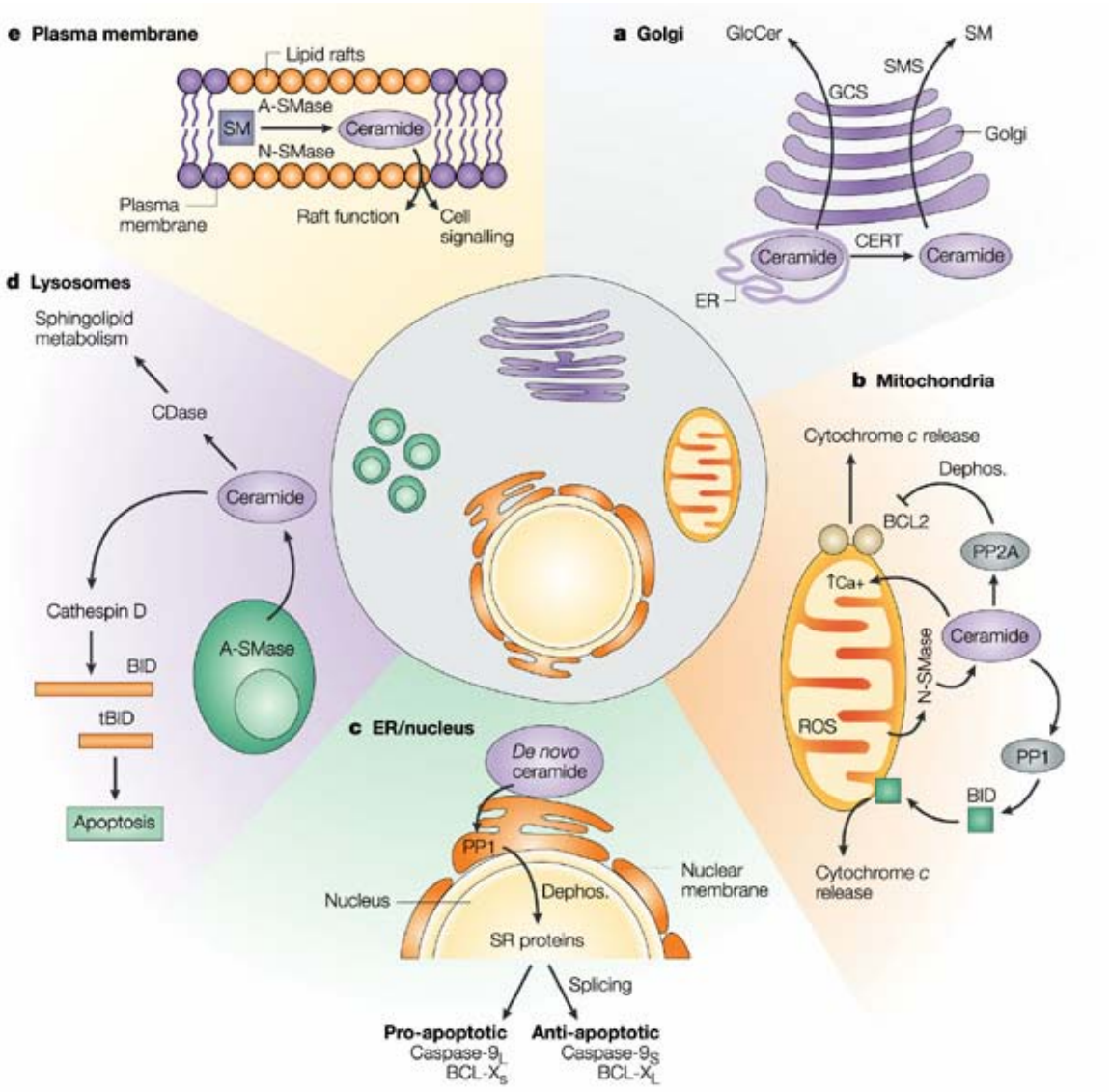
2.7.2 Sphingolipids as Extracellular Receptors

Recent studies have identified sphingosine-1-phosphate (S1P) and lysophosphotidic acid (LPA) as extracellular ligands for a family of G protein-coupled receptors (GPCRs) (Radeff-Huang et. al., 2004). This family of receptors is also known as endothelial differentiation gene (EDG)-regulated family. These receptors have a distinct coupling pattern to various G proteins and therefore are capable of activating multiple intracellular signaling pathways. The binding of

Figure 2.5 *De novo* Synthesis and Metabolism of Sphingolipids.

De-novo-generated ceramide in the endoplasmic reticulum (ER) is transported by CERT to the Golgi membranes (a) for the synthesis of sphingomyelin (SM). Ceramide can also be converted to glucosylceramide (GlcCer) by the action of glucosylceramide synthase (GCS) in the Golgi, but this is not CERT-dependent. In mitochondria (b), ceramide is generated by neutral sphingomyelinase (N-SMase), which can be activated by reactive oxygen species (ROS) generated in mitochondria. Ceramide generated here can activate the ceramide-activated protein phosphatases protein phosphatase 1 (PP1) and PP2A. PP2A can then dephosphorylate and inactivate anti-apoptotic proteins such as BCL2 and AKT, leading to apoptosis. PP1 also acts on the pro-apoptotic protein BID. In the ER/nucleus (*c*), *de-novo*-generated ceramide can activate PP1, which leads to dephosphorylation of SR proteins that mediate the alternative splicing of *BCL-X*. In lysosomes (d), ceramide is generated by the action of acid SMase (A-SMase). Ceramide can activate cathepsin D and mediate activation of the pro-apoptotic protein BID, cleaving it to truncated BID (tBID) leading to activation of caspase-9 and caspase-3, resulting in apoptosis. Ceramide generated in lysosomes can also be used as a substrate for acid ceramidase for the generation of sphingosine and, from this, the generation of sphingolipids in other compartments. The generation of ceramide in the plasma membrane (e) can occur within specific sub-compartments of the membrane known as lipid rafts. Ceramide produced here can affect specific signaling pathways generated by receptors aggregated in the rafts, such as FAS. CDase, ceramidase. SMS, sphingomyelin synthase (Ogretmen and Hannun, 2004[5]).

[5] Reproduced with permission from Nature Reviews Cancer (Ogretmen and Hannun, 2004) copyright (2005) Macmillan Magazines Ltd.


e Plasma membrane
Lipid rafts
A-SMase
SM
Ceramide
N-SMase
Plasma membrane
Raft function
Cell signalling
a Golgi
GlcCer
SM
SMS
GCS
Golgi
Ceramide
CERT
Ceramide
ER
d Lysosomes
Sphingolipid metabolism
CDase
Ceramide
Cathespin D
BID
tBID
Apoptosis
A-SMase
b Mitochondria
Cytochrome c release
Dephos.
BCL2
PP2A
↑Ca+
Ceramide
N-SMase
ROS
PP1
BID
Cytochrome c release
c ER/nucleus
De novo ceramide
PP1
Nucleus
Dephos.
Nuclear membrane
SR proteins
Splicing
Pro-apoptotic
Caspase-9L
BCL-XS
Anti-apoptotic
Caspase-9S
BCL-XL
Nature Reviews | Cancer

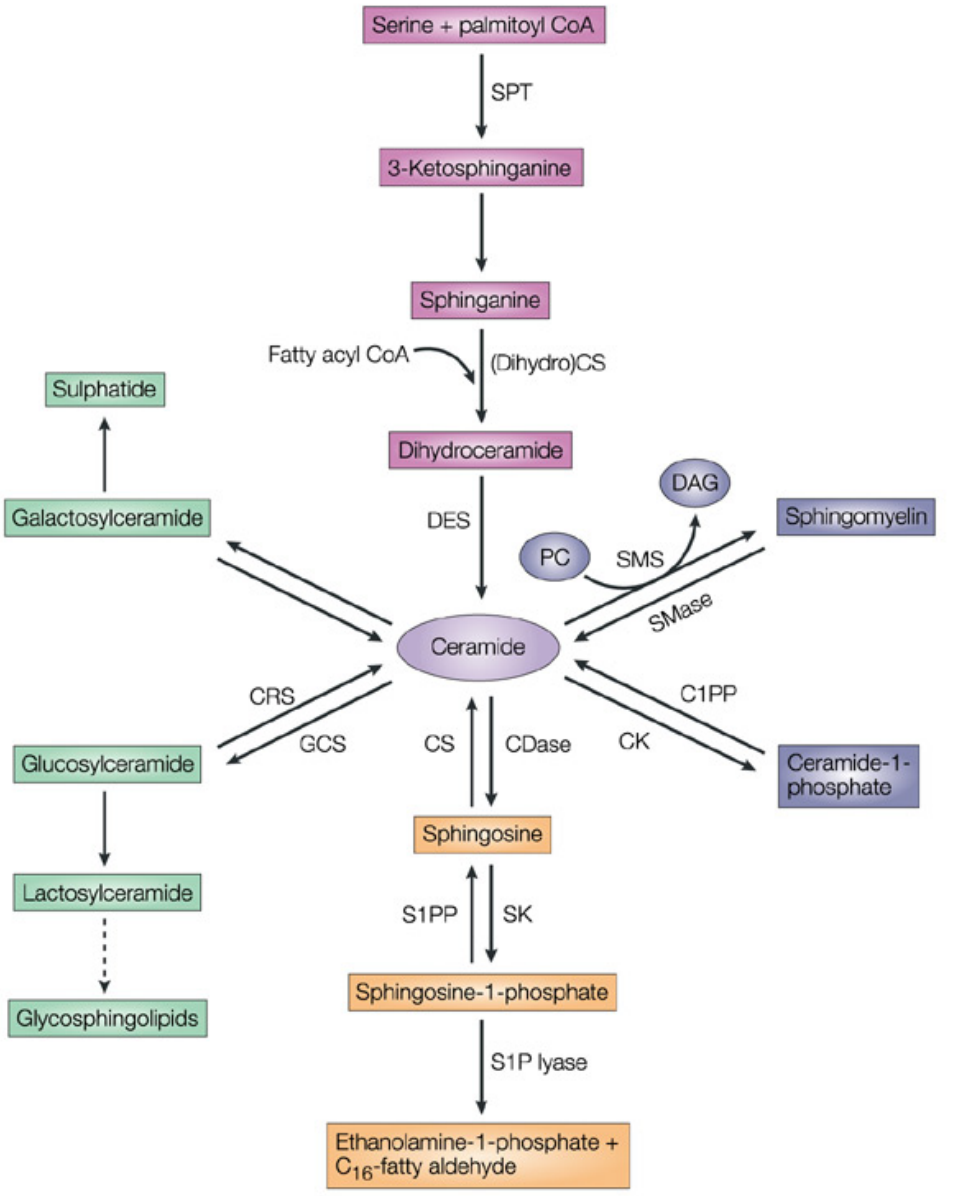
the extracellular receptors activates different signaling pathways involving trimeric G proteins, which in turn, control adenylate cyclase, phospholipases C and D (PLC, PLD), PI3K, ERK, JNK, p38, small GTPases such as Ras and Rho, and non-receptor activated protein-tyrosine kinases and tyrosine phosphatases (Servitja et al., 2003). The responses elicited by S1P and LPA include effects on cell proliferation, survival, morphology, adherence, chemotaxis, and activation of ionic conductance.

2.7.3 Sphingolipids as Secondary Messengers

Sphingolipids and their derived products, including ceramide, sphingosine, and sphinganine, are potent suppressors of growth (Yang et al., 2004). Sphingolipids can modify the activity of a number of proteins, including receptors, ion channels, and enzymes as well as intracellular calcium levels. The interaction of sphingolipids, especially SM and cholesterol, drives the formation of plasma membrane rafts (also known as glycosphingolipid-enriched microdomains [GEMs] or detergent-insoluble glycosphingolipid-enriched microdomains [DIGs]).These rafts, formed in the Golgi apparatus, are targeted to the plasma membrane. SM hydrolysis to ceramide, usually via acidic-Sphingomylinase (aSMase), occurs within the rafts. Ceramide generated within rafts appears to alter the raft structure. These microdomains rapidly fuse into larger domains, termed ‘platforms’ and form caps on the cell surface to facilitate oligomerization. FAS trimers localize to these domains to form higher order structure for the oligomerization of the downstream adaptors of FADD/MORT-1 and caspase-8, which activates the FAS apoptotic signal (Kolesnick, 2002). Sphingomyelin hydrolysis/ceramide accumulation involves extracellular/ biological inducers such as ionizing and ultraviolet radiation, Fas ligand, tumor necrosis factor, oxidative stress, 1, 25-dihydroxyvitamin D_3, interferon gamma, endotoxin, chemotherapeutic agents, nerve growth factor, heat, CD28, IL-1, progesterone, retinoic acid, serum withdrawal, as well as human immunodeficiency virus (Lee and Amoscato, 2004).

Figure 2.6 Pathways of Sphingolipid Metabolism.
Ceramide can be formed *de novo* (pink) or from hydrolysis of sphingomyelin (blue) or cerebrosides (green). Conversely, ceramide can be phosphorylated by ceramide kinase to yield ceramide-1-phosphate, or can serve as a substrate for the synthesis of sphingomyelin or glycolipids. Ceramide can be metabolized (orange) by ceramidases (CDases) to yield sphingosine, which in turn is phosphorylated by sphingosine kinases (SKs) to generate sphingosine-1-phosphate (S1P). S1P can be cleared by the action of specific phosphatases that regenerate sphingosine or by the action of a lyase that cleaves S1P into ethanolamine-1-phosphate and a C_{16}-fatty-aldehyde. C1PP, ceramide-1-phosphate phosphatase; CRS, cerebrosidase; CK, ceramide kinase; CS, ceramide synthase; DAG, diacylglycerol; DES, dihydroceramide desaturase; GCS, glucosylceramide synthase; PC, phosphatidylcholine; S1PP, S1P phosphatase; SMS, sphingomyelin synthase; SMase, sphingomyelinase; SPT, serine palmitoyl transferase(Ogretmen and Hannun, 2004[6]).

[6] Reproduced with permission from Nature Reviews Cancer (Ogretmen and Hannun, 2004) copyright (2005) Macmillan Magazines Ltd.



Nature Reviews | Cancer

2.8 Role of Sphingolipids in Apoptosis, Cell Proliferation, and Differentiation

2.8.1 Sphingosine

Sphingosine-induced apoptosis requires the release of cytochrome C from mitochondria, the activation of caspase-3, and the inhibition of anti-apoptotic pathways such as that mediated by AKT kinase, a serine/threonine kinase that protects cells from apoptosis induced by a variety of extracellular stresses (Yang et al., 2004). Other possible mechanisms include relocation of lysosomal proteases to the cytosol leading to the release of cathepsin-D and one or more cysteine proteases resulting in caspase activation and a change in the mitochondria membrane potential. Sphingosine is also implicated in mitochondria-dependent, Fas-mediated apoptosis (Cuvillier et al., 2000).

2.8.2 Ceramide

Ceramide can signal apoptosis by a pathway involving a kinase suppressor of Ras/Ras/raf1/MEK1. Ceramide can bind to and regulate PKC ζ activity, which further interacts with Ras and activates MEK/ERK (Ruvolo, 2003; Willaime et al., 2001). Cathepsin-D, an endosomal acidic aspartate protease involved in apoptosis, can also be bound by ceramide (Heinrich et al., 2000). Similarly, ceramide activated protein phosphatases (PP1 and PP2A), its substrates and protein kinases are involved in apoptosis (Yang et al., 2004).

An increase in ceramide levels has been observed in response to a number of cancer chemotherapeutic agents, including etoposide, vincristine, daunorubicin, fludarabine, paclitaxel, PSC 833, fenretinide, and irinotecan, or after radiation treatment (Reynolds et al., 2004). Strategies that elevate ceramide levels are being used for therapies aimed to arrest growth or promote apoptosis (Kolesnick, 2002). Contrary to its apoptotic activity, ceramide also forms products that stimulate cell growth and proliferation. For example, sialo-glucosphingolipid (gangliosides), a metabolite of ceramide, inhibits the immune system's dendritic cells, which are

vital for the complete destruction of cancer cells (Radin, 2004). Therefore, ceramide metabolism plays a vital role in normal cell function and stability due to its interacting effect in growth/death or differentiation of normal cells.

2.8.3 Sphingosine-1-Phosphate (S1P)

S1P has been implicated as a second messenger that promotes cellular proliferation and survival by protection against ceramide-mediated cell death. The cell surface S1P receptors (also known as the EDG receptors) play an important role in intercellular signaling (Reynolds et al., 2004). The balance between ceramide and S1P as messengers may be an important factor determining survival or death of mammalian cells. Studies suggest that S1P antagonizes ceramide-mediated biologic responses, while ceramide activates various protein kinase C (PKC) isoforms, which may contribute to the S1P rheostat by activating the pro-life transcription factor NFkappa-β (Reynolds et al., 2004).

2.9 Sphingolipids in Food

2.9.1 Sphingolipid Content in Plant and Animal Sources

Sphingolipids in food are found in varying amount from plant and animal sources. The amount of sphingolipids in food commodities and its yearly consumption are listed in Table 2.2. The amounts vary considerably, from the low micromoles per kilogram in fruits and some vegetables to ~2 mmol/kg (1–2 g/kg) in dairy products, egg and soybeans. Except for milk (Jensen, 1995; Keenen and Patton, 1995), little is known about variation in sphingolipid amounts over season (day of lactation, in the case of milk), losses during food preparation, and other aspects of food chemistry.

2.9.2 Sphingolipids from Milk as Dietary Bioactive Compounds

Milk has afforded compelling examples of a food material designed by selective pressure to provide optimal health to healthy mammalian offspring. Milk contains components that are

more than assembled essential amino acid and that provide biological activities that improve the competitive success of offspring who consume them (Ward and German et al., 2004).

Milk contains (per L) 39–119 mg of sphingomyelin, 6–11 mg of glucosylceramide, 6.5–15 mg of lactosylceramide and ~11 mg of gangliosides (~9–13 mg G_{D3}, 1.2 mg G_{D1b1}, 0.7 mg G_{M2}, 0.3 mg G_{M3} and 0.001 mg G_{M1}) (Jensen, 1995); the lipid backbones of milk sphingomyelin have mainly sphingosine (d18:1^{t4}, with smaller amounts of sphinganine and other chain length homologs) and 16:0, 22:0, 23:0 and 24:0 as the major fatty acids (Vesper et al., 1999; Morrison, 1969; Schmelz et al., 1996; Zeisel et al., 1986).

Table 2.2 Sphingolipids in Food and Yearly Sphingolipid Consumption in US

Food Source	Sphingolipid content µmol/kg	Food consumed per capita kg/y	Sphingolipids consumed per capita µmol/y
Dairy Products			38,464
Meat products and fish			34,270
Eggs	2250	14	31,500
Cereals			
Wheat flour	576	66	38,016
Total sphingolipids intake (µmol/y)			153,551
Total sphingolipids intake (g, calculated as sphingomyelin)			116

(adapted from Vesper et al., 1999)

2.9.3 Structural Variation of Sphingolipids in Food

The sphingolipid backbones, fatty acids, and headgroups vary considerably with the type of food. Most foods of mammalian origin (beef, milk) or poultry (Chicken, Turkey, eggs) have a wide spectrum of complex sphingolipids (sphingomyelins, cerebrosides, globosides, gangliosides or sulfatides) that are comprised of many different headgroup components (phosphocholine, glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid,

fucose and other carbohydrates) and ceramide backbones (d18:1^{4}, d18:0 and t18:0, with amide-linked fatty acids of 16–30 carbon atoms in length, some of which have an hydroxyl group) (Merrill and Sweeley, 1996). In contrast, the complex sphingolipids of plants are mainly cerebrosides (mono- and oligohexosylceramides) with glucose (Glc, the most common hexose), galactose (Gal), mannose (Man), and inositol.

Wheat grain has glycosphingolipids with primarily Glc, but also, Man-Glc, $[Man]_2$-Glc and $[Man]_3$-Glc headgroups, and has the sphingoid base backbones d18:1^{4}, d18:1^{8}, d18:2 ^{4,8}, t18:0 and t18:1^{8} with 14:0–26:0 fatty acids (most as -hydroxy fatty acids) (Fujino et al., 1985); rice grain has Glc, Man-Glc, Glc-Glc, $[Man]_2$-Glc, Glc-Man-, $[Man]_3$-Glc with d18:0, d18:1^{4} and d18:2^{4,8} sphingoid bases and 16:0–24:0 fatty acids (including some -hydroxy fatty acids) (Fujino et al., 1985) Spinach leaf sphingolipids are comprised of Glc, Cellobiose and Glc-$[Man]_2$-Glc with d18:0, d18:1^{8}, d18:2^{4,8}, t18:0, t18:1^{8} with 16:0–24:0 fatty acids (Ohnishi et al. 1983); soybean has a single cerebroside, Glc ceramide (GlcCer), with d18:0, d18:1^{4}, d18:1^{8}, d18:2^{4,8}, t18:0, t18:1^{8} and 16:0–26:0 fatty acids (including -hydroxy and ,ß-dihydroxy fatty acids) (Ohnishi and Fujino, 1982); bell pepper and tomato also have mainly GlcCer with d18:2^{4,8}, d18:1^{8}, t18:1^{8} sphingoid bases and 16:0–24:0 (including -hydroxy-) fatty acids (Whitaker, 1996). Recent developments in the analysis of sphingolipids by GC/HPLC/mass spectroscopy (MS) and tandem mass spectrometry are making it feasible to accurately identify and quantify complex sphingolipids (Vesper et al., 1999).

2.9.4 Dietary Intake of Sphingolipids

From Table 2.2 it is evident that dairy products appear to be major sources of sphingolipids, followed by meat and fish, eggs, and vegetables; with the contribution from vegetables was the most difficult to estimate from available data (Vesper et al., 1999). Yearly per capita intake of sphingolipids from the foods in Table 2.2 average 154 mmol, which is equivalent

to ~116 g. Estimating fruits and vegetables that contribute as described in footnotes 7 and 8 of Table 2.2, add up to 28 mmol, for a total of 181 mmol (139 g) consumed per year. Sphingolipids constitute between 0.01 to 0.02% of the diet (by weight). This amount (0.3–0.4 g/d) provides few "fat calories" but is comparable with lipids such as cholesterol and tocopherols (Ensminger et al., 1994). Consumption could vary considerably among individuals who consume foods that are particularly rich in sphingolipids (Vesper et al., 1999).

2.10 Bioavailability of Sphingolipids

2.10.1 Hydrolysis of Sphingolipids in the Gastrointestinal Tract

Sphingomyelin and cerebrosides undergo little cleavage in the stomach, but are hydrolyzed in all subsequent regions of the small intestine and colon of rats and mice (Schmelz et al., 1994; Nilsson, 1968 and 1969b). The luminal contents of rat small (and large) intestine contain substantial sphingomyelinase, glucoceramidase, and ceramidase activities (Nilsson, 1969a and 1969b). Not all of the ingested sphingolipids are hydrolyzed and absorbed (Vesper et al., 1999). Nilsson (1968) reported that ~25% of an administered dose of sphingomyelin was excreted in feces, of which 10% was the intact molecule, 80–90% was ceramide, and 3–6% was free sphingosine. There is a direct correlation between the amounts of sphingomyelin that is fed vs. the amount found in the colon (Nyberg et al., 1998). Germ-free mice show a drastically reduced hydrolysis of sphingomyelin, which suggests that intestinal microflora are major contributors to sphingolipid turnover in the lower bowel (Duan et al., 1995 and 1996). Similar studies with cerebrosides (Nilsson, 1968) found that 43% was excreted, with 40–70% being intact molecule, and 25–60% as ceramide. Less it is known about human metabolism of sphingolipids, but human pancreatic juices contain a taurocholate-dependent neutral sphingomyelinase (Chen et al., 1992) and an alkaline sphingomyelinase has also been detected in human bile (Nyberg et al., 1996).

2.10.2 Uptake of Sphingolipids

Much of the sphingosine (and ceramide) that is derived from hydrolysis of complex sphingolipids is rapidly taken up by intestinal cells and degraded to fatty acids (via fatty aldehydes) or reincorporated into complex sphingolipids that remain associated primarily with the intestine (Nilsson, 1968; Schmelz et al., 1994). When sphingoid-base–labeled sphingolipids were fed to rats, a small amount of the radiolabeled sphingoid base was found in lymph, blood and liver, which implies that some component(s) of dietary sphingolipids were transported through the mucosa to the systemic circulation (Nilsson, 1968; Schmelz et al., 1994). Chylomicrons may be involved in sphingolipid transport because intestinal lymph contains ~1 nmol/mL of sphingolipid of which about 40% is ceramide (Merrill et al., 1995).

2.10.3 Transport of Sphingolipids via Serum Lipoproteins

Sphingolipids are components of serum lipoproteins. The greatest amounts appear in LDL followed by VLDL and then HDL (Merrill et al., 1995). Sphingomyelin is the major sphingolipid of LDL and HDL, whereas VLDL contains mainly ceramide. Small amounts of free sphingoid bases are present in the blood (Wang et al., 1992), associated with albumin and circulating cells (both erythrocytes and leukocytes) (Wilson et al., 1988); sphingosine 1-phosphate is also found in plasma and serum, but appears to be derived from platelets (Yatomi et al., 1995) because of its high affinity for Edg-1 receptors (Van Brocklyn et al., 1998).

2.10.4 Cellular Metabolism of Sphingolipids and Regulation of Sphingolipid Biosynthesis by Diet

All organs appear to be capable of *de novo* sphingolipid biosynthesis (Merrill et al., 1985, Nagiec et al., 1996) and there is no evidence that consumption of dietary sphingolipids is required for growth under normal conditions. Nonetheless, exogenous sphingolipids are required for the growth of mammalian cells with defects in serine palmitoyltransferase (Hanada et al.,

1992), the initial enzyme of sphingolipid biosynthesis, which establishes that sphingolipids are necessary for normal cell function (Vesper et al., 1999).

De novo sphingolipid synthesis is subject to some degree of feedback regulation. Studies using radiolabeled sphingolipids confirmed that sphingoid base backbones that are recovered from dietary sphingolipids affect tissue sphingolipid biosynthesis (Mandon et al., 1991; van Echten-Deckert et al., 1997).

2.11 Perspective of Chemoprevention through Diet

Although targeted therapy is yielding promising results in the treatment of specific cancers, drug resistance poses a problem. Acquired drug resistance to chemotherapy occurs after primary surgical intervention and subsequent chemotherapy treatments. The majority of patients develop disease recurrence, which is due to tumor cell heterogeneity and genetic instability. However, endothelial cells show genetic stability and proliferate normally. As endothelial cells participate in tumor angiogenesis, developing anti-angiogenic strategies serves as a greater benefit in containing tumor development and metastasis (Benouchan and Colombo, 2005).

Furthermore, recent studies have implicated chemoprevention through diet as a viable approach against cancer. There is strong evidence that many daily-consumed dietary compounds possess cancer-protective properties that might interrupt the carcinogenesis process (Brenner and Gescher, 2005; Chen and Kong, 2005). These properties include the induction of cellular defense detoxifying and antioxidant enzymes, which can protect against cellular damage, caused by environmental carcinogens or endogenously generated reactive oxygen species. These compounds can also affect cell-death signaling pathways, which could prevent the proliferation of tumor cells (Chen and Kong, 2005).

Therefore, focus would be on the response of nutraceuticals or dietary components on cancer and tumor progression. It would greatly benefit efforts of supplementing diet as a

chemopreventive measure not only against cancer but various degenerative diseases. Our approach was in determining the potential of sphingolipids against selected cancers. Our hypothesis was based on the literary evidence establishing the role of dietary sphingolipids in inhibiting colon cancer and that of sphingosine and ceramide as apoptosis inducing agents. It was evident that sphingolipids participated in the sphingomyelin/ceramide pathway. Changes of sphingomyelin metabolism have been found to be associated with tumorigenesis in various tissues and a particular link between sphingomyelin metabolism and colon cancer has been indicated (Duan, 2005). A study conducted by Lemonnier et al., (2003) on mice fed with sphingomyelin before and after tumor initiation determined that sphingolipids were as effective in the chemoprevention of tumors as in early intervention. Our goal was to determine the role of sphingolipids in the process of angiogenesis that could prove to be an important characterization of sphingolipids in diet and therapeutics.

CHAPTER 3. *IN VITRO* ANALYSIS OF SPHINGOLIPIDS AS ANTI-ANGIOGENIC COMPOUNDS

3.1 Introduction

Epidemiological studies suggest that about 35% of cancer related deaths are attributed to diet with a range of 20 to 60% for various sites (Parodi, 1997). Conversely, there are many bioactive compounds readily available in food that can be used as preventive measures towards diseases. The evaluation of natural compounds with cancer preventive properties in food is now an important element in overall cancer prevention strategies. Sphingolipids are found in many foods including milk, cheese, eggs, and soybeans. One liter of milk contains 39-119 mg sphingomyelin, 6-11 mg glucosylceramide, 6.5-15 mg lactosylceramide, and ~ 11 mg of ganglioside (Vesper et al., 1999). Glycosphingolipids belong to a group of membrane components in eukaryotes known as sphingolipids. Sphingolipids are one of the most structurally diverse categories of lipid in nature (Berra et al., 2002). There is indirect evidence suggesting a role for dietary sphingolipids in combating colon cancer in humans (Symolon et al., 2004; Vesper et al., 1999; Merrill et al., 1997).

Several studies have demonstrated that the intake of dairy sphingolipids, such as cerebroside (glucosylceramide), sphingomyelin, and ganglioside significantly reduce incidence of colonic aberrant crypt foci and adenocarcinoma in CF1 mice treated with 1,2-dimethylhydrazine, and of intestinal adenoma in Min mice (Berra et al., 2002). It was also noted that dietary sphingolipids that caused these effects on colon cancer had no deleterious effects on the animals. Although no human clinical trials or epidemiological studies have been undertaken to elucidate the effects of dietary intake of sphingolipids, it is believed that sphingolipid consumption would not cause any adverse toxicological effects (Berra et al., 2002). The role of sphingolipids, especially ceramide has been extensively studied as a pro-apoptotic agent in

MCF-7 breast cancer cells (Ameyar et al., 1998), and LNCaP and PC3 prostate cancer cells (Sumitomo et al., 2002; Kimura et al., 2003).

The role of sphingolipids in angiogenesis is poorly understood. Kim et al. (2002), identified sphingomyelin as the active component for vesicle-induced endothelial cell migration, tube formation, and neovascularization. Igarashi et al. (2003), explored the role of VEGF in sensitizing the vascular endothelium to the effect of lipid mediators by promoting the induction of S1P1 receptors, representing a potentially important point of cross-talk between the receptor-regulated eNOS signaling pathways in the vasculature. Yabu et al. (2005), determined in an *in vivo* zebrafish model system that thalidomide-induced antiangiogenic action is regulated by the balance between ceramide and S1P signal. However the potential role of sphingolipid group with respect to the expression of angiogenic factors in various cancer cells needs to be studied to determine a possible mechanism with which these compounds would participate in neovascularization of the tumor under the influence of normal and depleted oxygen environment (hypoxia). Hypoxia plays an important role in tumor angiogenesis. Hypoxia initiates expression of hypoxia inducing factors such as HIF-1α subunit. Pugh and Ratcliffe (2003), suggested that HIF-1α induces upregulation of several angiogenic factors including VEGF. It is therefore imperative to study the effect of sphingolipids on HIF-1α translocation.

To examine the effect of the sphingolipids on angiogenesis, we selected biomarkers that play an important role in initiation of angiogenesis in pathological as well as physiological conditions. The biomarkers chosen for the study of angiogenesis were VEGF, Cathepsin-D and HIF-1α. The expression of these biomarkers were examined under conditions of normoxia as well as hypoxia. Vascular endothelial growth factor (VEGF) is the major endothelial-cell specific stimulatory factor and is regarded as the most important positive regulator of angiogenesis and vascular permeability. It participates in crucial biological processes, including

wound repair and tumorigenesis. The elevated expression of VEGF and its receptors has been found in tumor malignancies and has being associated with metastasis formation and poor prognosis (Chodorowska et al., 2004). Cathepsin-D is an aspartic protease enzyme, which is synthesized in the endoplasmic reticulum, and belongs to the group of lysozymal hydrolases (Kokkonen et al., 2004). Many cancer cells have increased amounts of cathepsin B and D in the extracellular space, which influences matrix degradation and tumor cell invasion through proteolytic cascades. Individual proteases have distinct roles in tumor growth, invasion, migration and angiogenesis (Koblinski et al., 2000). Hypoxia-inducible factor-1 (HIF-1) is a dimeric transcriptional complex that has been recognized primarily for its role in the maintenance of oxygen and energy homoeostasis (Heather et al., 1998). HIF-1 increases the transcription of several genes for proteins that promote blood flow and inflammation, including vascular endothelial growth factor (VEGF), heme oxygenase-1, endothelial and inducible nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2). Both the hypoxic and the cytokine-induced activation of HIF-1 involve the phosphatidylinositol- 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) signaling pathways (Hellwig-Burgel, 2005). The study on the effects of sphingolipids on the chosen biomarkers of angiogenesis would therefore lead us to establish the role of sphingolipids as anti-angiogenic compounds. Thus implying the consumption of milk would be a preventive measure against progression of cancer. Further, the presence of sphingolipids in dietary sources especially in milk would substantiate them as nutraceuticals with health benefits.

3.2 Materials and Methods

3.2.1 Cell Harvesting

HEL 299 normal human lung fibroblast cell, MCF-7 breast, Caco-2 colon, and DU-145 prostate cancer cells were obtained from American Type Culture Collection (Rockville, MD) and

harvested in a continuous culture as per the ATCC procedure. The growing medium consisted of MEM (EBSS) + 10% FBS + 1 mM Na^+ pyruvate. The cells were passaged once per week at a split of 1:2 and reffed once per week. The cells were then grown as a monolayer. They were attained at 80% confluence. The cells were raised under both hypoxic and normoxic conditions. Hypoxia was maintained by addition of 20 μM $CoCl_2$ to deplete the cell of oxygen availability (Lee et al., 2003). The cells were also serum starved for 24 h before treatment.

3.2.2 Compound Treatment

N-Hexanoyl-D-*erythro*-Sphingosine (C6-Ceramide, Cer); (2S,3R,4E)-2-acylaminooctadec-4-ene-3-hydroxy-1-Phosphocholine (Sphingomyelin, SPM); and D-Glucosyl-ß1-1'-N-Dodecanoyl-D-*erythro*-Sphingosine (C12 ß-D-Glucosyl Ceramide, GlcCer) were purchased from Avanti Polar Lipids (Alabaster, AL). DMSO was used as a carrier solvent for C6-ceramide and C12 ß-D-glucosylceramide, whereas ethanol was used to solubilize sphingomyelin. The compounds, at a dosage concentration of 0-100 μM, were prepared in appropriate solvents for the cell proliferation assay. The cells were treated at a concentration of 50 μM and incubated for 48 h at 37° C and 5% CO_2 for determining VEGF, cathepsin-D and HIF-1α expression. After compound exposure and incubation, the media was sampled to determine the level of VEGF. The cells were then trypsinized and subsequently lysed. The fractions were then centrifuged at 14000 x *g*, to separate cytoplasmic and nuclear factor. The protein concentrations in each fraction were normalized and cytoplasmic factor was used for examining the cathepsin-D expression while nuclear factor was used to determine if HIF-1α translocated.

3.2.3 Cell Proliferation Assay

After compound treatment and incubation, the viability of cells was measured using ViaLight ™ HS (BioWhittaker Lab, Rockland, ME). The cells were treated with nucleotide

releasing reagents and subsequently treated with nucleotide monitoring reagent as described in the manufacturer's protocol. The luminescent signal generated by the enzyme luciferase, which catalyzes the formation of light from ATP and luciferin, was measured using a Perkin Elmer LS 50B luminometer. The result was derived as an ATP concentration, which was used to determine the amount of apoptosis.

3.2.4 Cell Migration Assay

In this assay, the principle of the Boyden chamber was utilized. The cells were trypsinized and resuspended in a serum free media. They were then added into a migration chamber consisting of a gelatin membrane obtained from Chemicon Quantitative Cell Migration (QCM ^{TM}, catalog number ECM 510). The migration chamber was inserted into the feeder tray consisting of 10% FBS as chemoattractant. The cells in the microplate were treated with the compound and incubated for 24 hours at 37° C and 5% CO_2. The migration chamber was then removed and lysis/dye solution was added to the feeder tray. The fluorescence was then measured at 480/520 nm.

3.2.5 Cathepsin-D Expression

The cytoplasmic factor and biotinylated detector antibody (rabbit polyclonal) were pipetted into the wells and allowed to incubate simultaneously for 4 hours at 37° C. This allowed binding of the antigen by the capture-antibody as described by the Cathepsin D assay kit (EMD Biosciences, Inc. CA). The amount of detector antibody bound to the antigen was then measured by binding it with a streptavidin/horseradish peroxidase conjugate, which catalyzed the conversion of the chromogenic substrate TMB from a colorless solution to a blue solution. The color was quantified by spectrophotometry. This indicates the amount of Cathepsin-D protein in the sample when compared to the standards.

3.2.6 VEGF Expression

Pre-coated mouse monoclonal antibodies generated against human VEGF were used to capture human VEGF using ChemKine ^{TM} ELISA protocol in a media from the treated cells. Biotinylated VEGF specific rabbit polyclonal antibodies was used to detect VEGF in the sample. The VEGF standard provided by the manufacturer was used to plot a standard curve. The assay was visualized using a streptavidin alkaline phosphatase conjugate and a chromogenic substrate reaction. The amount of VEGF detected in each sample was compared to a VEGF standard curve, which demonstrated a direct relationship between optical density (OD) and cytokine concentration. The higher the OD, the higher the cytokine concentration in the sample, and thus higher VEGF.

3.2.7 HIF-1α Expression

The extracted nuclear factor from the treated cells was analyzed for HIF-1α levels using sandwich immunoassay TransAM HIF-1 Transcription factor assay kit (Active Motif, CA) by capturing HIF-1α with mouse monoclonal antibody as per the manufacturers guidelines and quantitated using streptavidin coated-HRP conjugate and diaminobenzidin as substrate. The reaction was visualized spectrophotometrically at 450 nm.

3.2.8 Statistical Analysis

Statistical analysis was performed by Student's *t*-test or, where appropriate, by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for significance. Differences were considered to be statistically significant when $P<0.05$. Within group comparison were determined by LSD post-hoc analysis.

3.3 Results and Discussion

3.3.1 Cell Viability Assay

Under normoxic conditions the effect of C6-ceramide (Cer) on the HEL 299 (normal lung

cell lines) had a proliferative effect whereas sphingomyelin (SPM) and C12 ß-D-Glucosylceramide (GlcCer) caused a reduced proliferation in a dose dependent manner (Figure 3.1 A-B). MCF-7 cell lines showed an increased proliferation when exposed to compound (Figure 3.1 C-D). Colon cancer cells (Caco-2) showed significant reduction in the cell viability with SPM exposure under 1 μM and gradually increased to 93 % at 50 μM and reduced to (72 %) at 100 μM (Figure 3.1 E-F). C6-ceramide and GlcCer caused lower proliferation at concentrations ranging from 0.05-0.1 μM as against higher proliferation at concentrations ranging from 0.2-25 μM. In DU-145 cells, SPM had an anti-proliferative effect at the concentration tested, while Cer and GlcCer increased the cell viability (Figure 3.1 G-H).

Under HIF induced conditions for MCF-7 breast cancer cells, GlcCer showed reduced cell viability at concentrations ranging from 10-100 μM, with the lowest being at a concentration of 10 μM (Figure 3.2 A-B). C6-ceramide caused higher proliferation from 0-50 μM but was significantly reduced at 100 μM to 75 % of cell survival. Colon cancer cells (Caco-2) treated with SPM had reduced cell viability when compared to Cer and GlcCer. C6-ceramide treated cancer cells showed significantly higher cell proliferation than GlcCer (Figure 3.2 C-D). Prostate cancer cells (DU-145) had very low cell viability when exposed to Cer, SPM and GlcCer at the concentration of 2 μM. The reduction in cell viability was 48%, 84% and 45%, respectively, with increased cell proliferation at higher concentrations (Figure 3.2 E-F).

The cell proliferation observed for Cer treated cells can be caused by hydrolysis of Cer to sphingosine 1-phosphate or by glucosylation of Cer, which are known to induce cell proliferation (Radin, 2003). In a study conducted by Cuvillier and Levade 2001, on human leukemia cells, they proposed that S1-P likely exerts its inhibitory effect on apoptosis through a mechanism upstream of the mitochondrion.

Figure 3.1 Cell Viability of Compounds Treated at Concentrations of 0-100μM Under Normoxic Conditions.

A-B. HEL 299 Normal Lung Fibroblast Cells; C-D. MCF-7 Breast Cancer Cells; E-F. Caco-2 Colon Cancer Cells; G-H. DU-145 Prostate Cancer Cells.

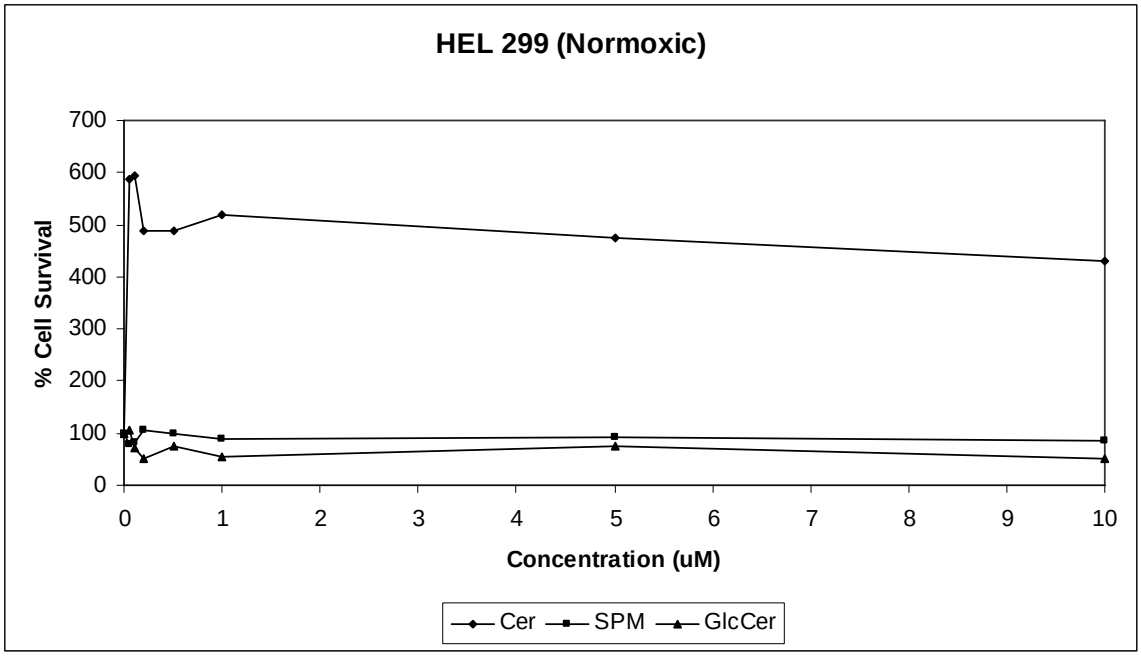


Fig. 3.1-A

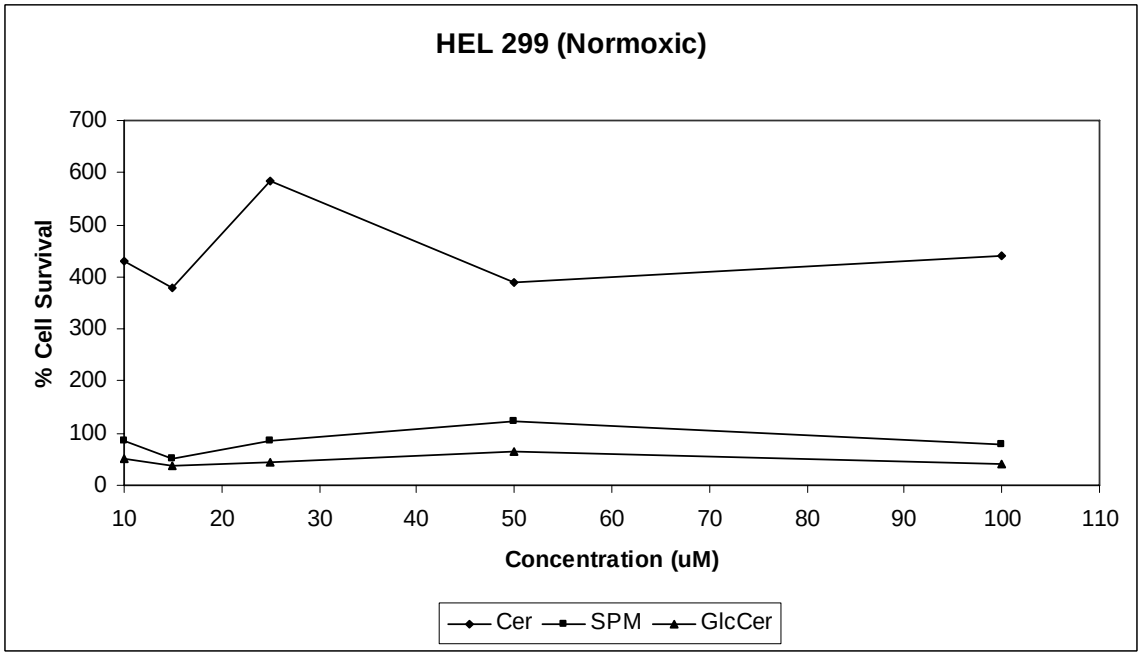


Fig. 3.1-B

Figure Contd…

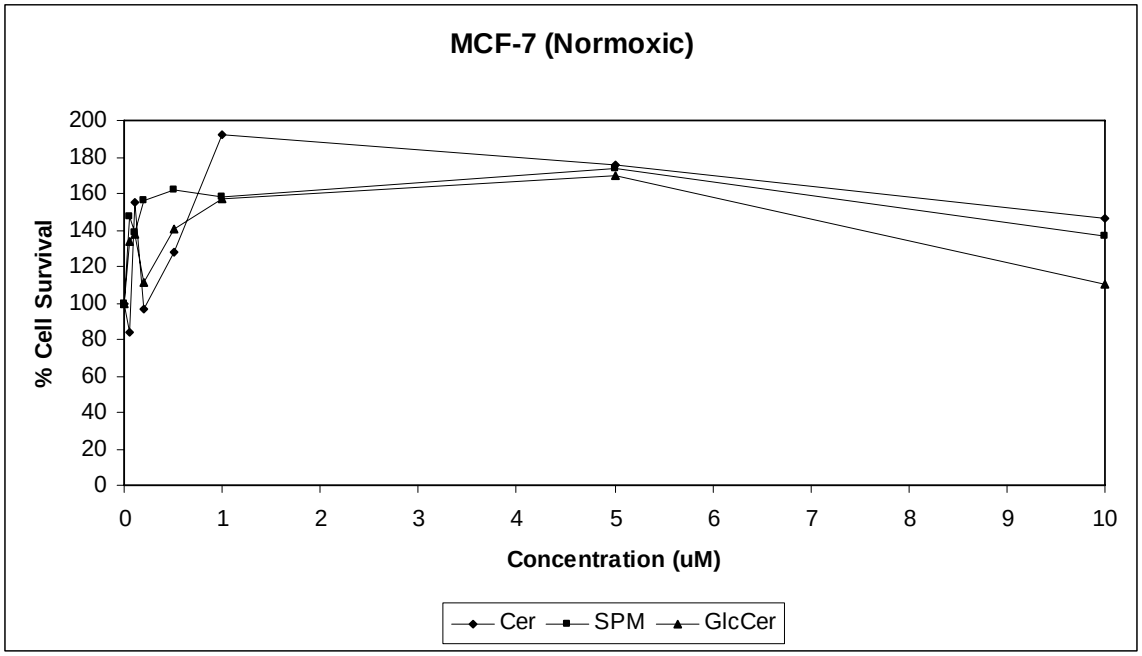


Fig 3.1-C

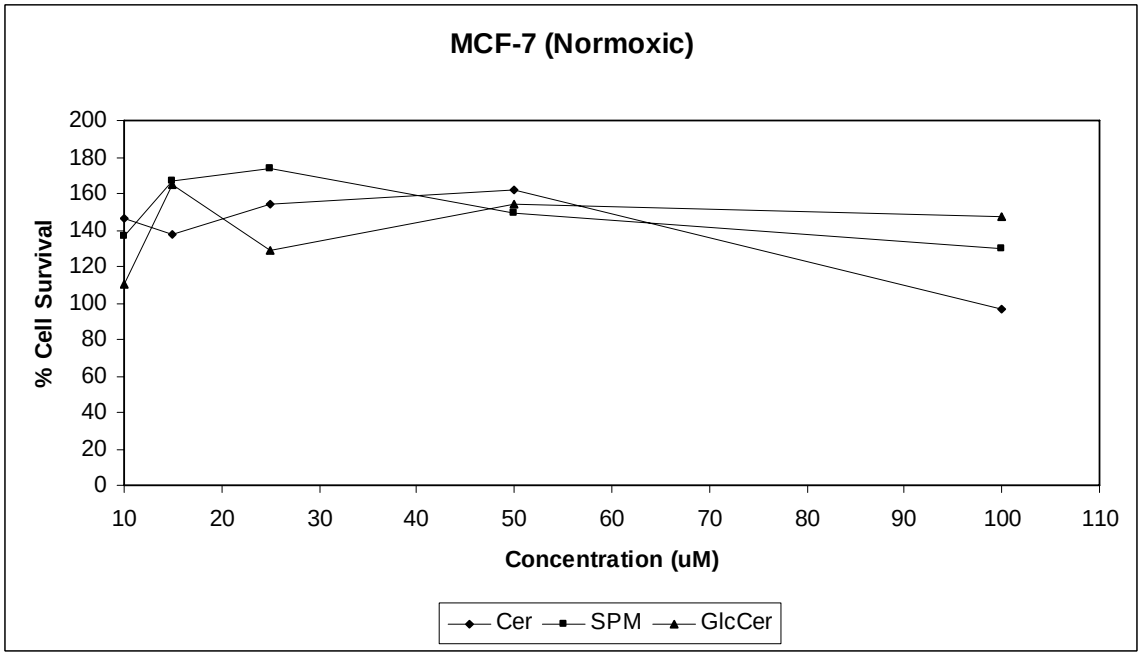


Fig 3.1-D

Figure Contd…

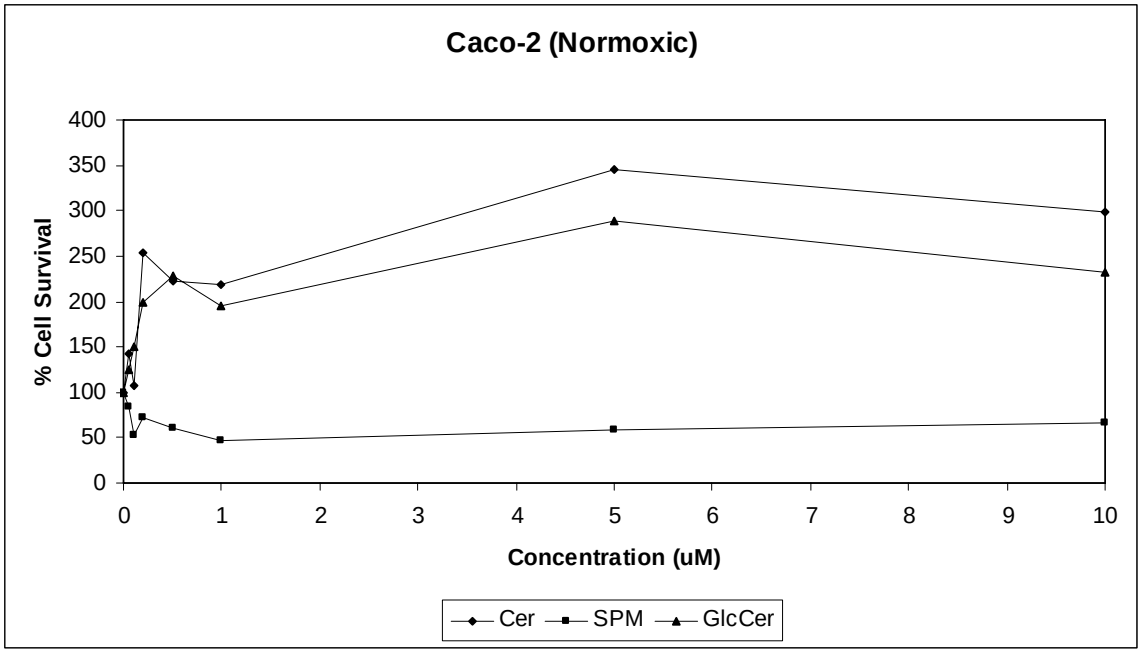


Fig. 3.1-E

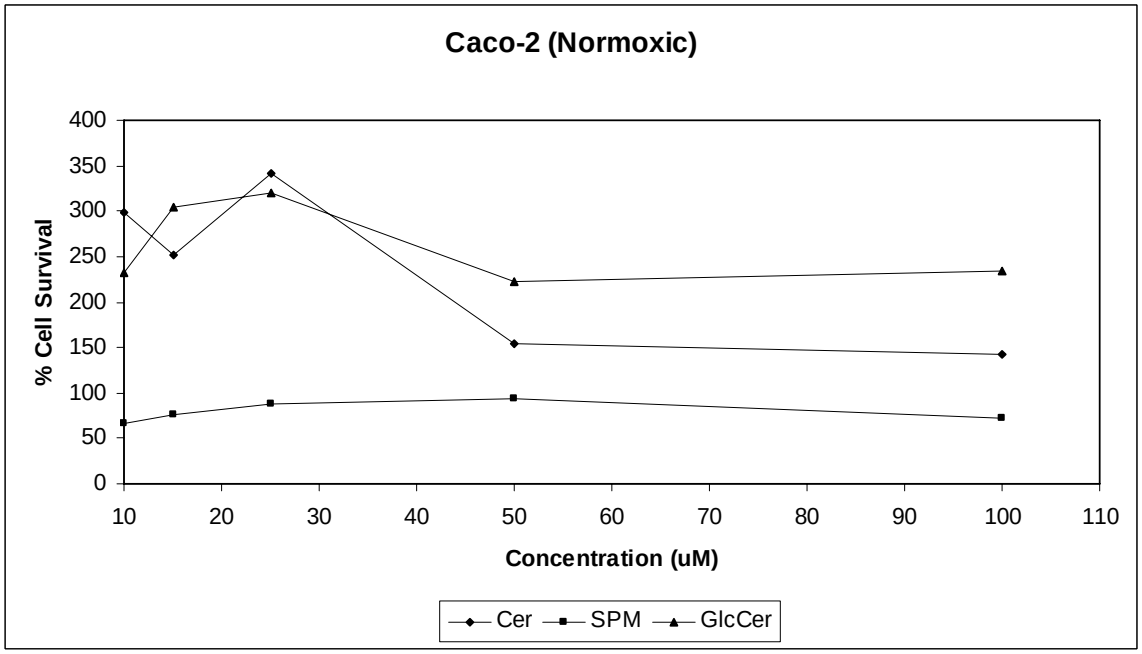


Fig. 3.1-F

Figure Contd…

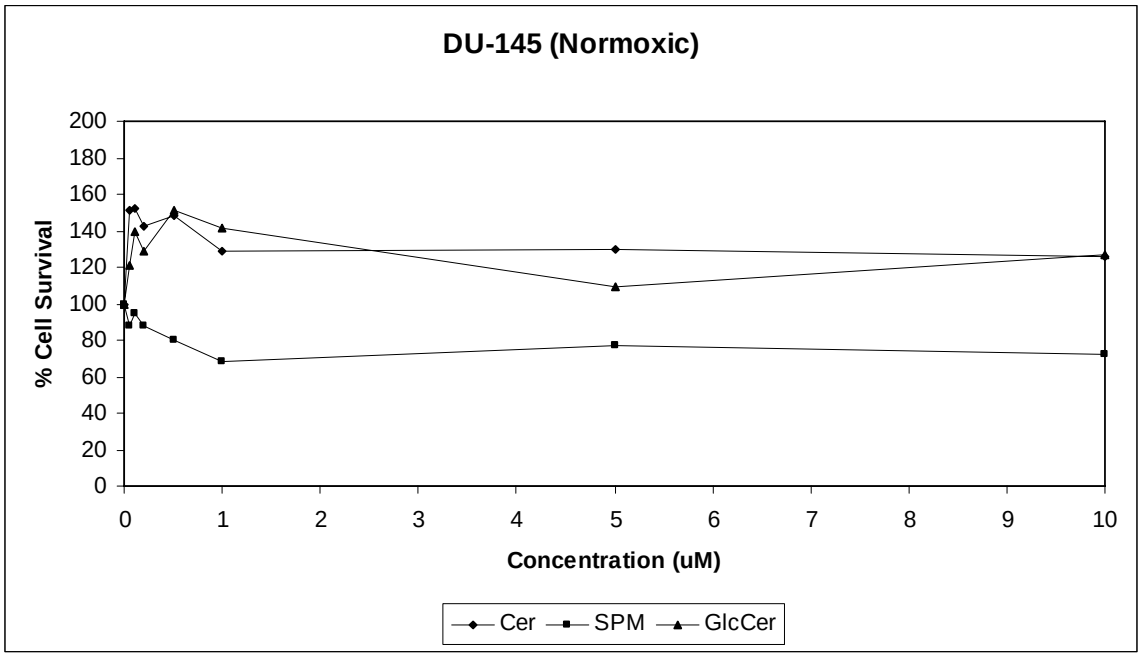


Fig 3.1-G

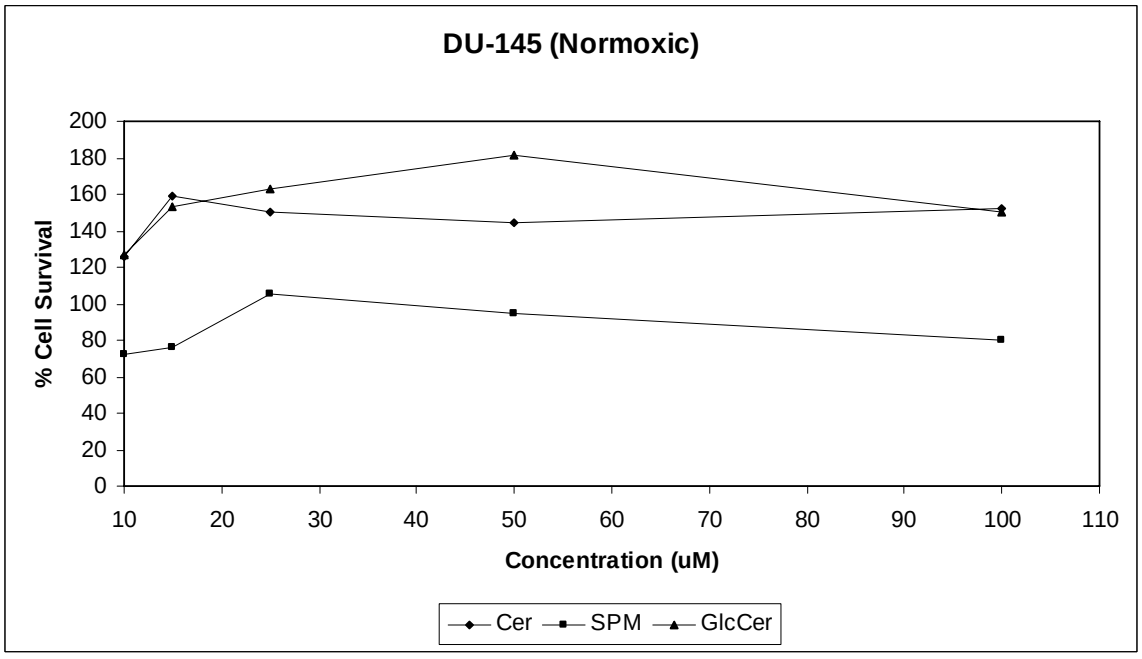


Fig 3.1-H

Figure 3.2 Cell Viability of Compounds Treated at Concentrations of 0-100μM Under HIF-1α Induced Conditions.

A-B. MCF-7 Breast Cancer Cells; C-D. Caco-2 Colon Cancer Cells; E-F. DU-145 Prostate Cancer Cells.

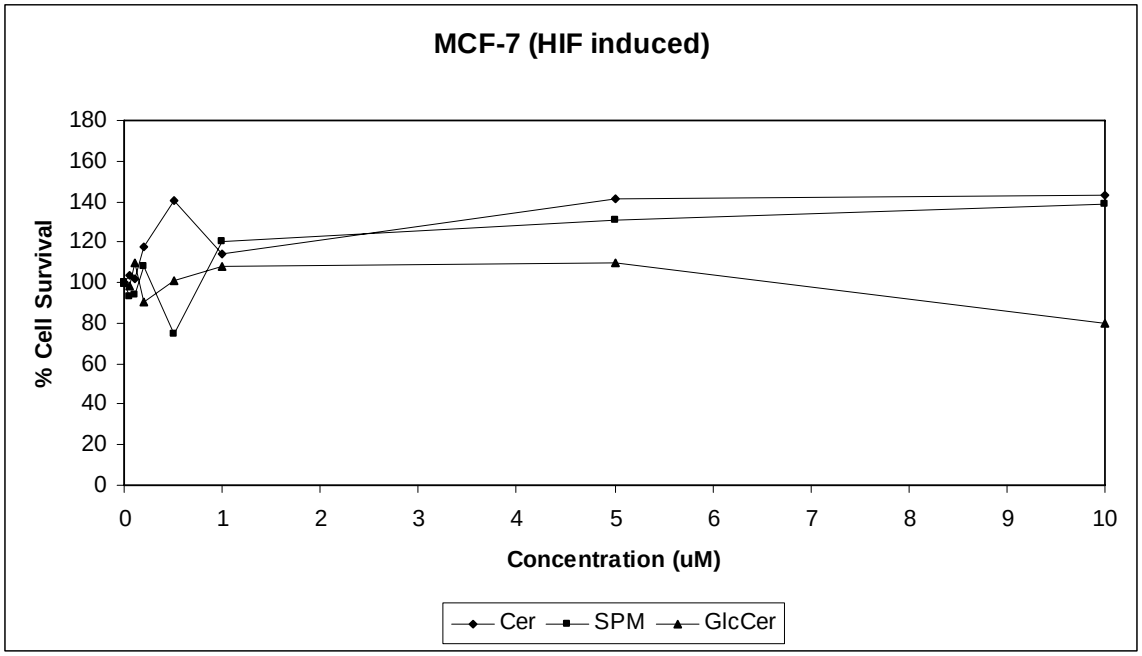


Fig. 3.2-A

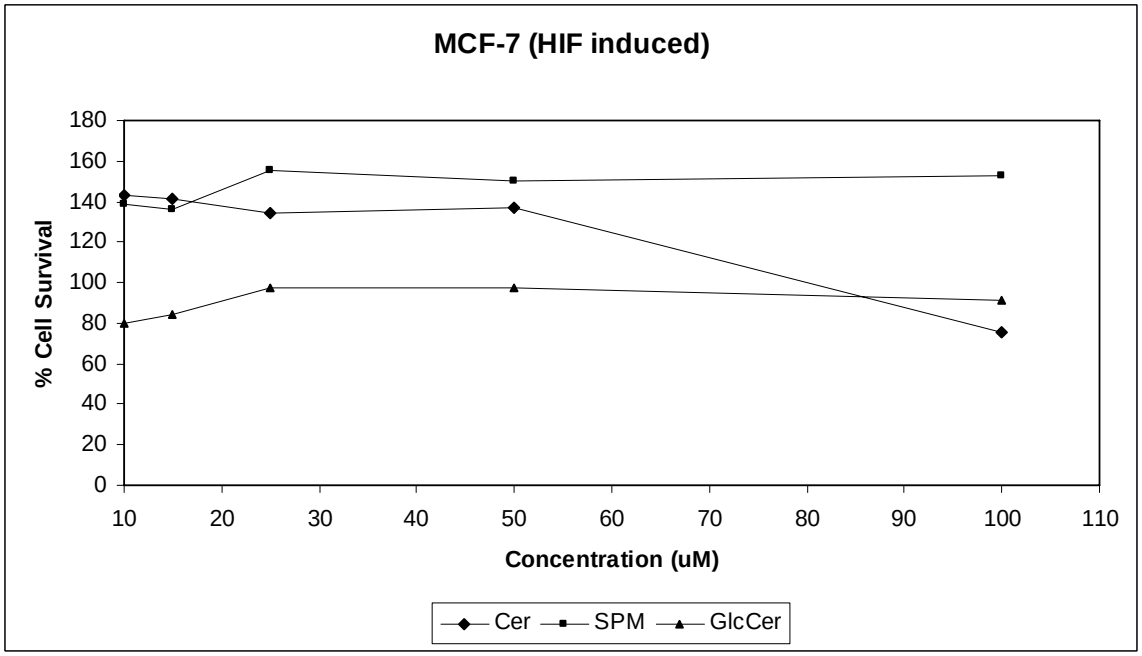


Fig. 3.2-B

Figure Contd….

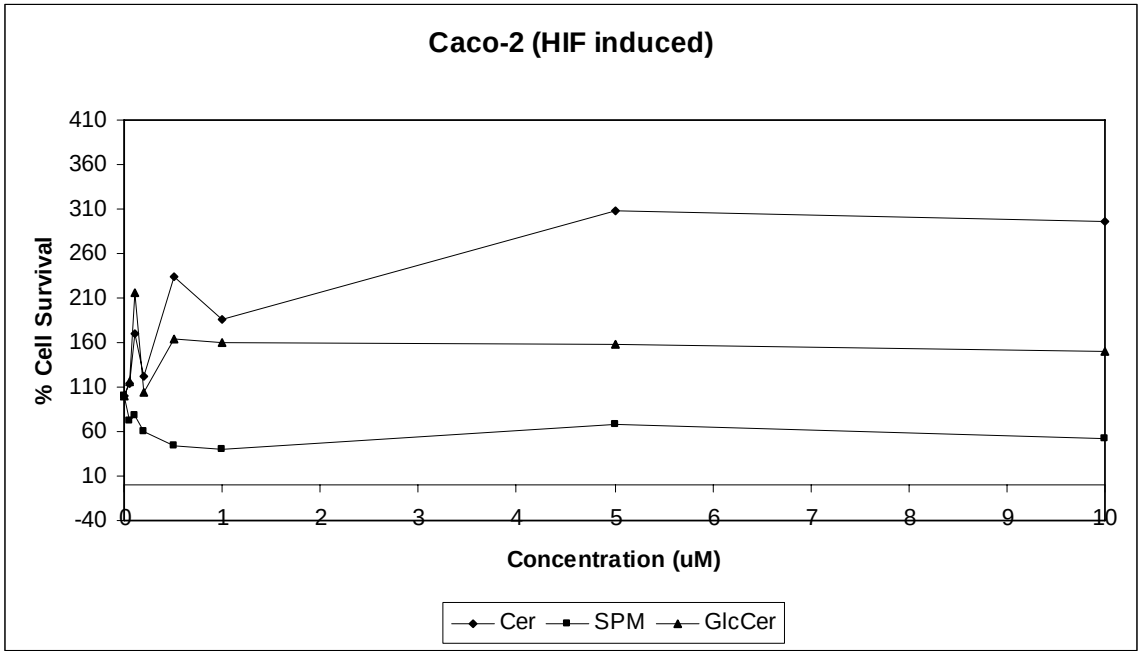


Fig. 3.2-C

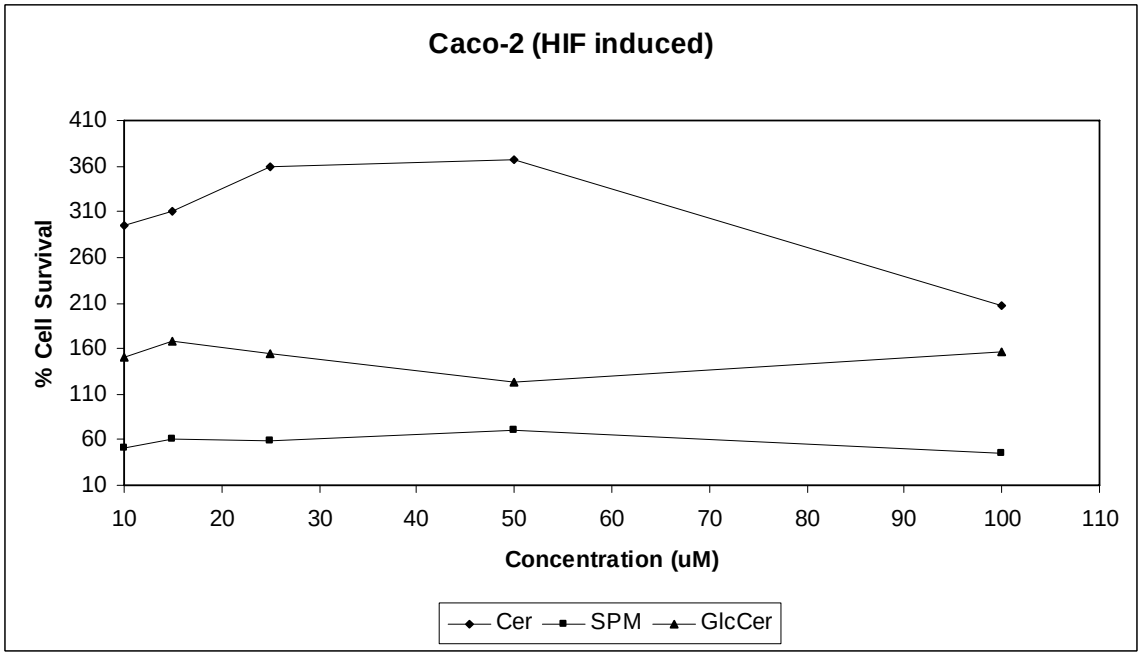


Fig 3.2-D

Figure Contd….

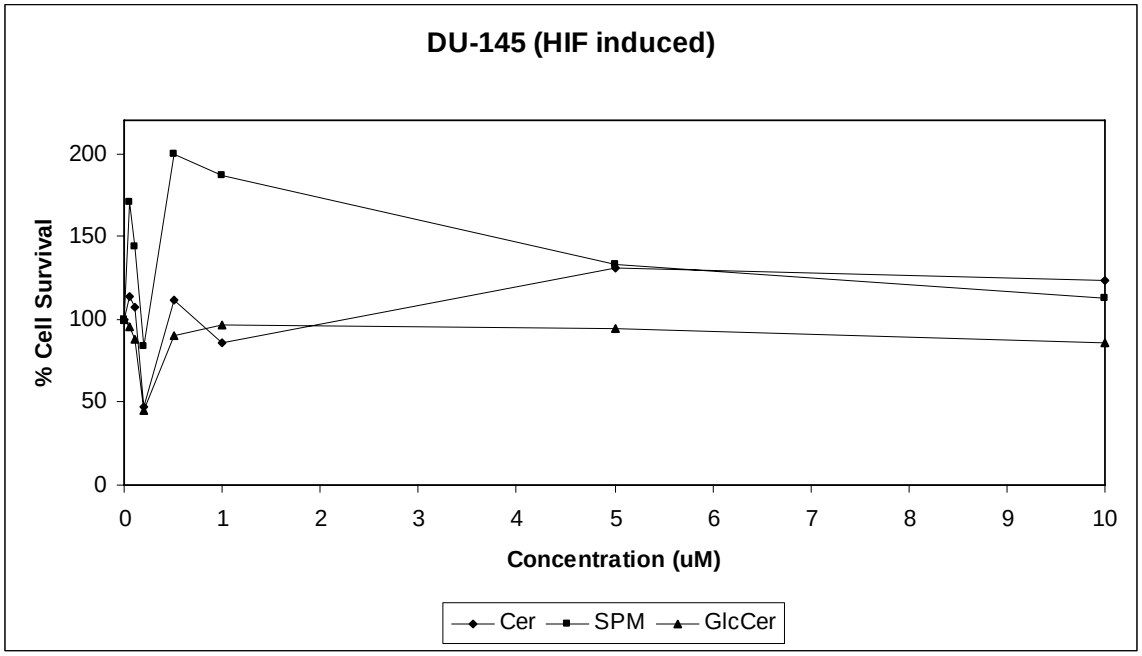


Fig 3.2-E

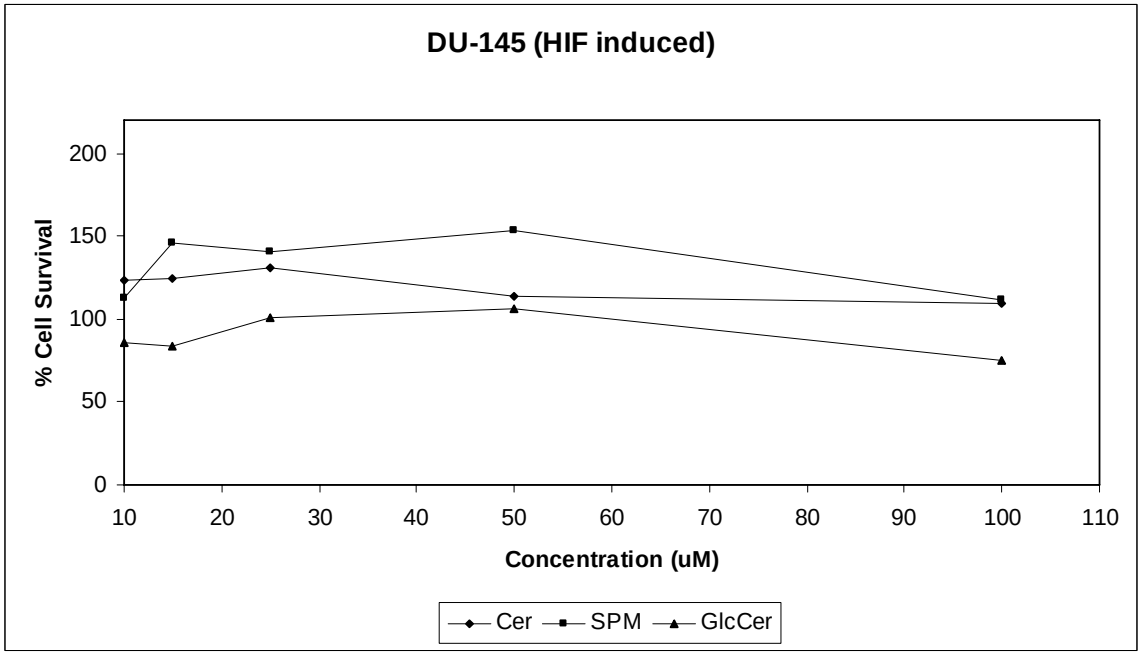


Fig 3.2-F

S1-P co-treatment inhibited the translocation of the mitochondrial key proteins cyt *c* and Smac/DIABLO to the cytoplasm. Thus promoting the induction of apoptosis by stimulation of the cell surface Fas and TNF-α death receptors, by serum deprivation. This was also achieved by addition of exogenous ceramides in human acute leukemia cells. They hypothesized that sphingosine kinase (SphK) activity is likely to be involved in clearing the cell of sphingosine and ceramide, both of which have proapoptotic functions, thus tilting the balance toward survival. Another study established that Bcl-2 inhibited ceramide-induced death, but not ceramide generation. In contrast, Cytokine response modifier A (CrmA), a potent inhibitor of Interleukin-1β converting enzyme and related proteases, inhibited ceramide generation and prevented TNF-α-induced death. Exogenous ceramide could overcome the CrmA block to cell death, but not the Bcl-2 block (Dbaibo et al., 1997). Conversely, exogenous SPM can stimulate SMase to elevate Cer. GlcCer on the other hand, rapidly hyrolyzes to Cer by the action of GlcCer glucosidase, which helps cause the cell to undergo apoptosis.

Overall, it can be implied that the effect of Cer on cancer cells was cytostatic whereas SPM showed inhibitory activity in most of the cancer cells. The effect of Cer can be attributed to its effect on the cell cycle rather than having a direct impact on the death-signaling pathway of the cancer cell. More research is needed to confirm the role of Cer on the cell cycle.

3.3.2 VEGF Levels

The VEGF expression for normal lung cancer cells did not show any major increase with the exposure of the compounds under study (Figure 3.3-A). This is reasonably anticipated as normal cells do not show substantial levels of VEGF and therefore the exposure of compounds do not result in altered VEGF levels. However cancer cells, due to its abnormal expression of growth factors may exhibit increased response to compound exposure. The C6-ceramide treated

cells, however had a significantly higher VEGF expression than the control. MCF-7 cells under normoxic conditions had lower expression under the influence of Cer but had no significant effect under GlcCer or SPM, whereas, hypoxic MCF-7 cells had lower VEGF expression under C6-ceramide and GlcCer, while SPM did not affect the VEGF expression (Figure 3.3-B). Caco-2 normoxic cells did not upregulate the VEGF expression with respect to Cer and GlcCer exposure but showed an increased level of VEGF with SPM (Figure 3.3-C). DU-145 cells under normoxic conditions had very low expression of VEGF with respect to all the compounds. Hypoxia induced DU-145 cells had VEGF expressions under the influence of GlcCer and SPM, but Cer did not have any effect on the VEGF expression (Figure 3.3-D). It is noteworthy that DU-145 hypoxia treated cells had comparatively lower VEGF expression than hypoxia induced cells. Also, contrary to the Caco-2 results, normoxic DU-145 cells showed lower expression of VEGF when exposed to all the compounds.

3.3.3 Cathepsin-D Expression

SPM and GlcCer exposed normal cells (HEL 299), MCF-7, and Caco-2 (normoxic and hypoxic) had significantly lower Cathepsin-D expression while Cer increased the levels of cathepsin-D (Figure 3.4-A). This is in agreement with the established role of ceramide, which specifically binds to and activates the endosomal acidic aspartate protease cathepsin D. Direct interaction of ceramide with cathepsin D results in autocatalytic proteolysis of the 52 kDa pre-pro cathepsin D to form the enzymatically active 48/32 kDa isoforms of cathepsin D (Heinrich et al., 2000). The best result of lower cathepsin-D expression was observed with GlcCer in MCF-7, Caco-2 and DU-145 (Figure 3.4-B, 3.4-C, 3.4-D). SPM had lower expression of cathepsin-D in hypoxic conditions. Cer only proved effective on the DU-145 treated cells under both normoxic as well as hypoxia induced conditions.

Figure 3.3 VEGF Expression in Cells Exposed to Sphingolipids at a Concentration of 50 μM Under Normoxic and Hypoxic Conditions.

A. HEL 299 Normal Lung Fibroblast Cells; B. MCF-7 Breast Cancer Cells; C. Caco-2 Colon Cancer Cells; D. DU-145 Prostate Cancer Cells.
[a] Significantly different from Control under normoxic conditions.
[b] Significantly different from Control under HIF-1α induced conditions.

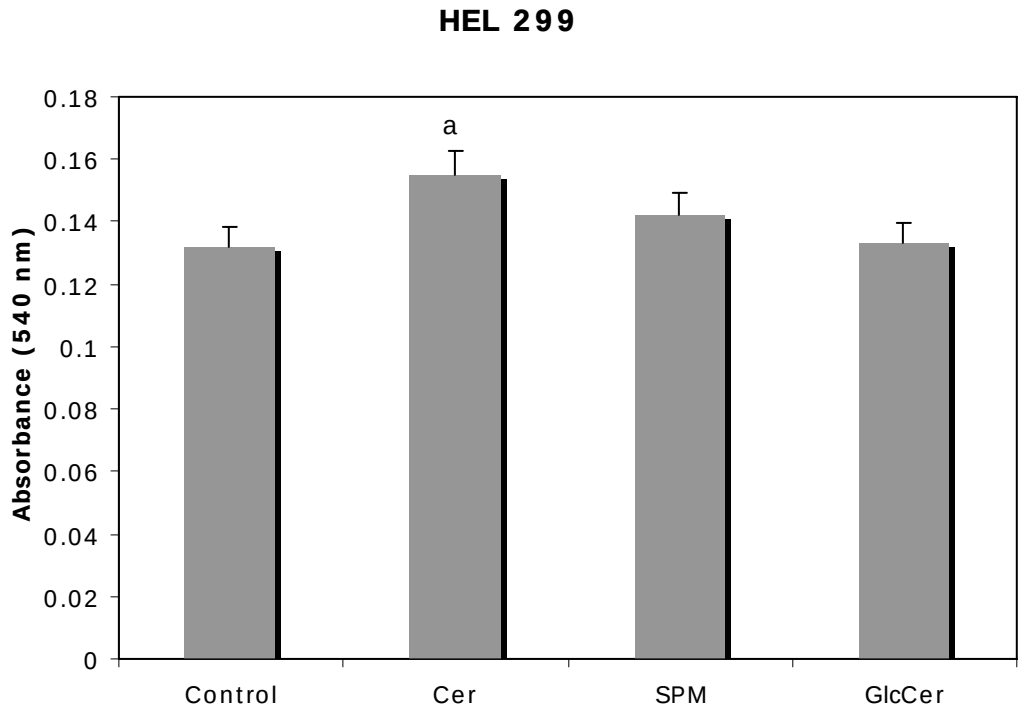


Fig. 3.3-A

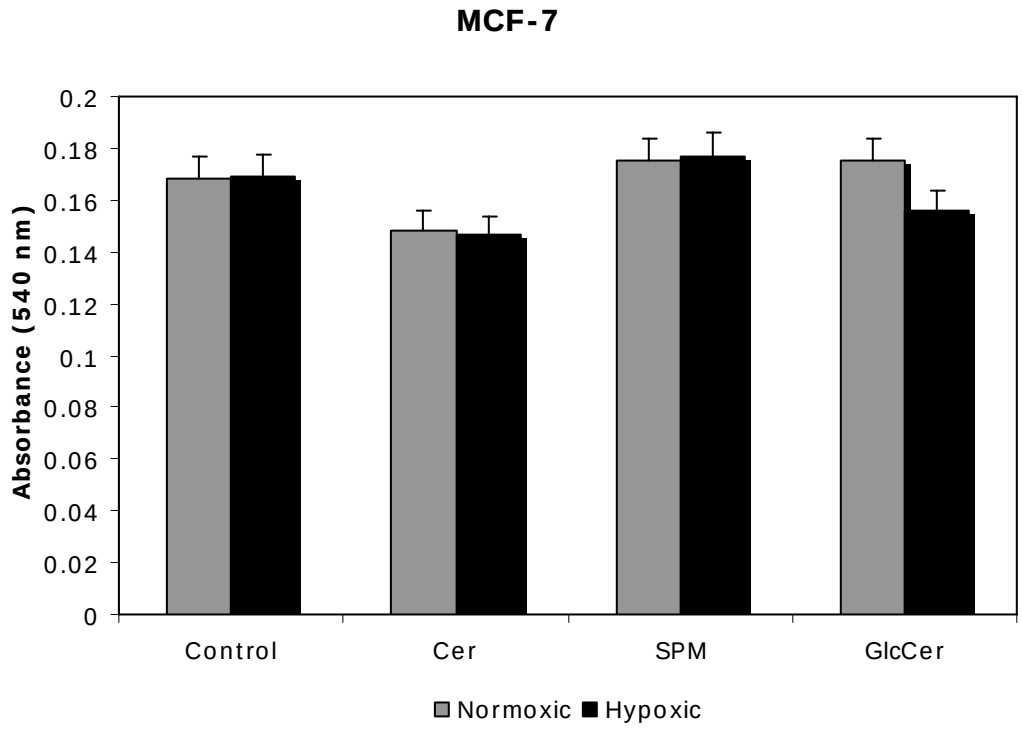


Fig. 3.3-B

Figure Contd…

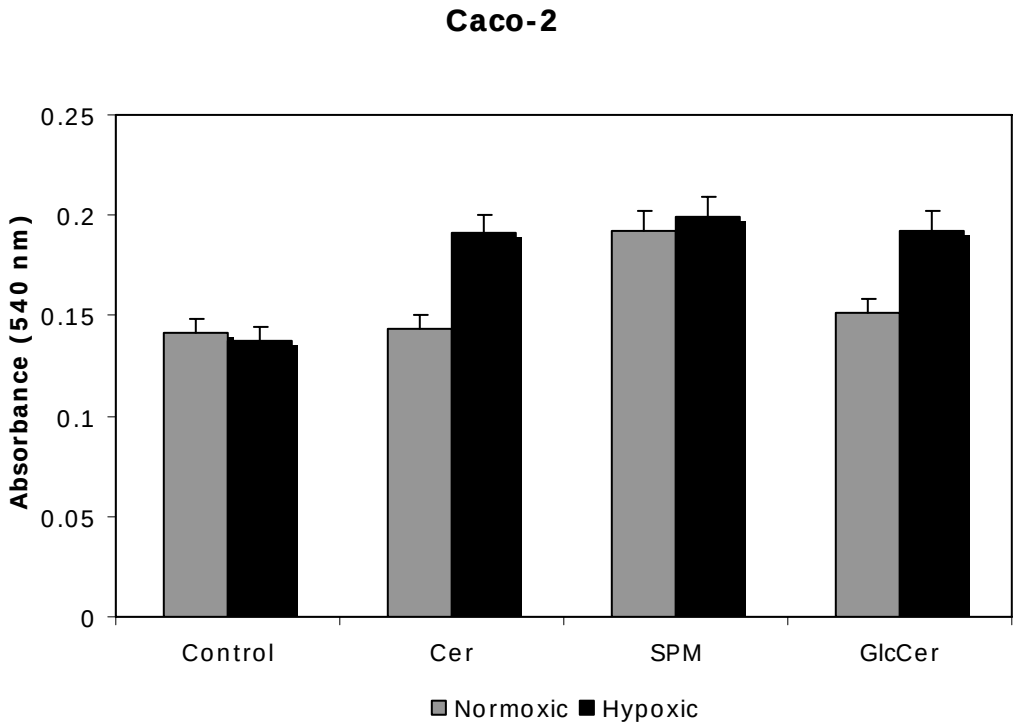


Fig. 3.3-C

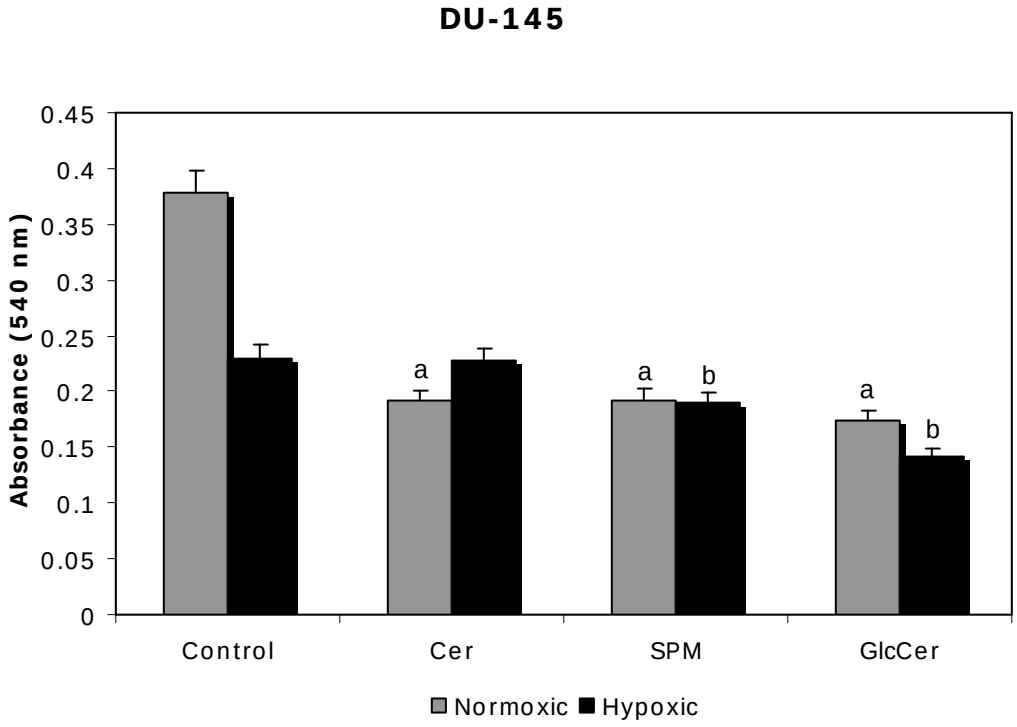


Fig. 3.3-D

Figure 3.4 Cathepsin-D Expression in Cells and Cancer Cells Exposed to Sphingolipids at a Concentration of 50 μM Under Normoxic and Hypoxic Conditions.

A. HEL 299 Normal Lung Fibroblast Cells; B. MCF-7 Breast Cancer Cells; C. Caco-2 Colon Cancer Cells; D. DU-145 Prostate Cancer Cells.

[a] Significantly different from Control under normoxic conditions.
[b] Significantly different from Control under HIF-1α induced conditions.

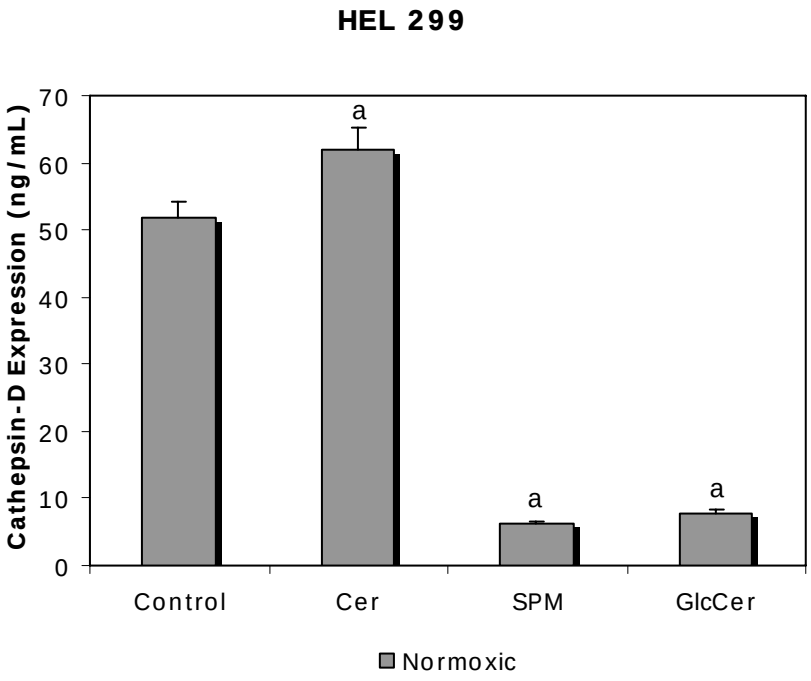


Fig. 3.4-A

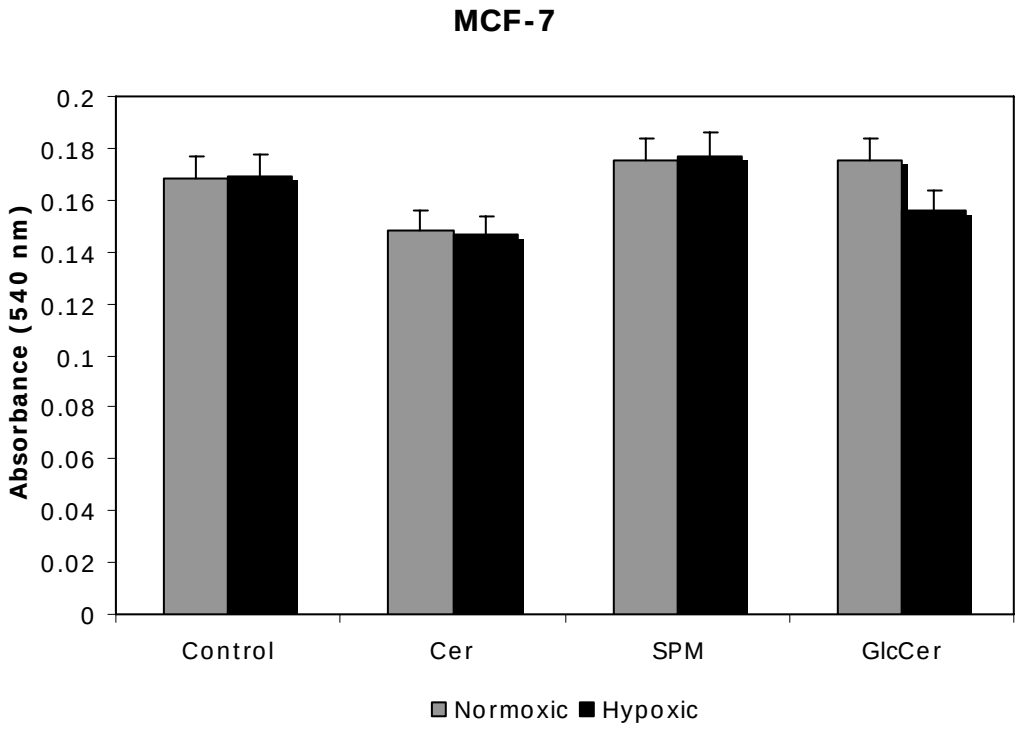


Fig. 3.4-B

Figure Contd…

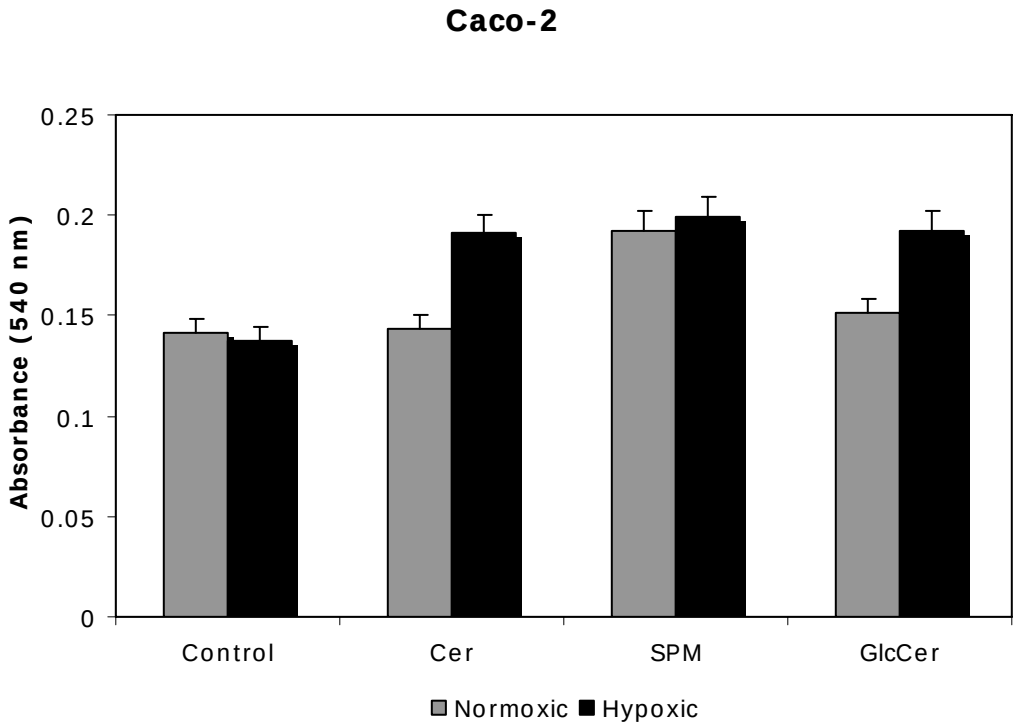


Fig. 3.4-C

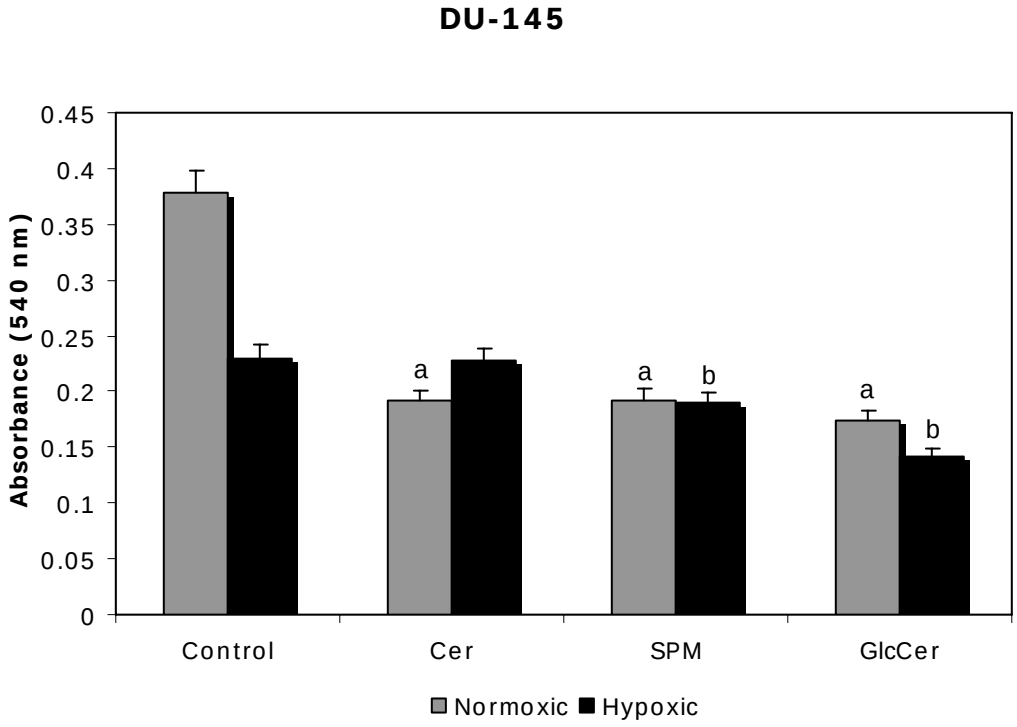


Fig. 3.4-D

Figure Contd…

3.3.4 Cell Migration Assay

All compounds induced cell migration in HEL 299 (Control) cells (Figure 3.5-A). Although this result could be specific for cell culture study, normal cells existing in tissues do not exhibit migration under external stimuli. In the case of cancer cell lines, C6-ceramide induced cell migration in Caco-2 cells under both conditions, whereas it did not affect cell migration in MCF-7 (normoxic) cells. C6-Ceramide significantly reduced migration under MCF-7 cells in hypoxic conditions and DU-145 under normoxic and hypoxic conditions (Figure 3.5-B, 3.5-C, 3.5-D). Sphingomyelin had no significant effect on MCF-7 cell migration but reduced the migration of Caco-2 under both conditions and DU-145 cells under normoxic conditions. Glucosylceramide did not affect the MCF-7 cell migration but affected the migration of Caco-2 (normoxic and hypoxic) and DU-145 under normoxic conditions. It is noteworthy that with MCF-7 cells, which are highly metastatic, the effect seemed to be less with treatment of all the compounds, except for ceramide treated to hypoxic MCF-7 cell, which reduced the cell migration.

3.3.5 HIF-1α Expression

As HIF-1α is predominantly expressed in response to hypoxia, our results clearly demonstrated significantly high amounts of HIF-1α in hypoxic treated cells (Figure 3.6-A, 3.6-B, 3.6-C, 3.6-D). Except for Cer exposure of Caco-2 cell, all the compounds under study exhibited significant reduction in HIF-1α expression when treated in hypoxic medium.This is desirable since HIF-1α regulates the transcription of mitogenic growth factors associated with angiogenesis under hypoxia. The suppression of hypoxia by sphingolipids would cause cells to activate pro-angiogenic factors and subsequently restrict the availability of nutrients and oxygen to sustain the survival and induce apoptosis.

Figure 3.5 Cell Migration in Cancer Cells Exposed to Sphingolipids at a Concentration of 50 μM Under Normoxic and Hypoxic conditions.

A. HEL 299 Normal Lung Fibroblast Cells; B. MCF-7 Breast Cancer Cells; C. Caco-2 Colon Cancer Cells; D. DU-145 Prostate Cancer Cells.

[a] Significantly different from Control under normoxic conditions.
[b] Significantly different from Control under HIF-1α induced conditions.

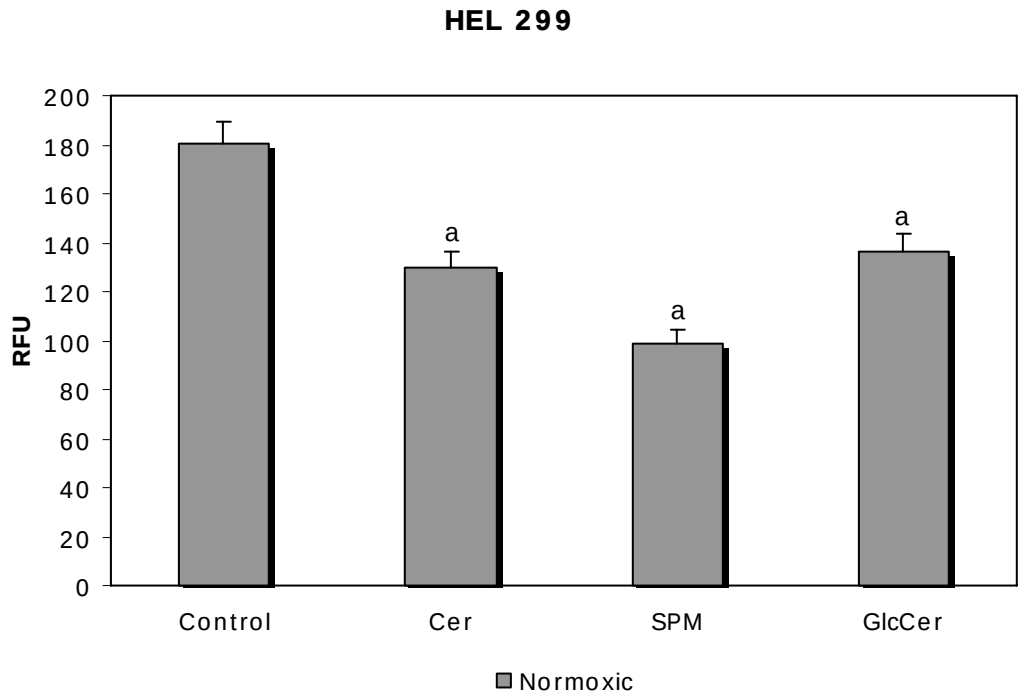


Fig. 3.5-A

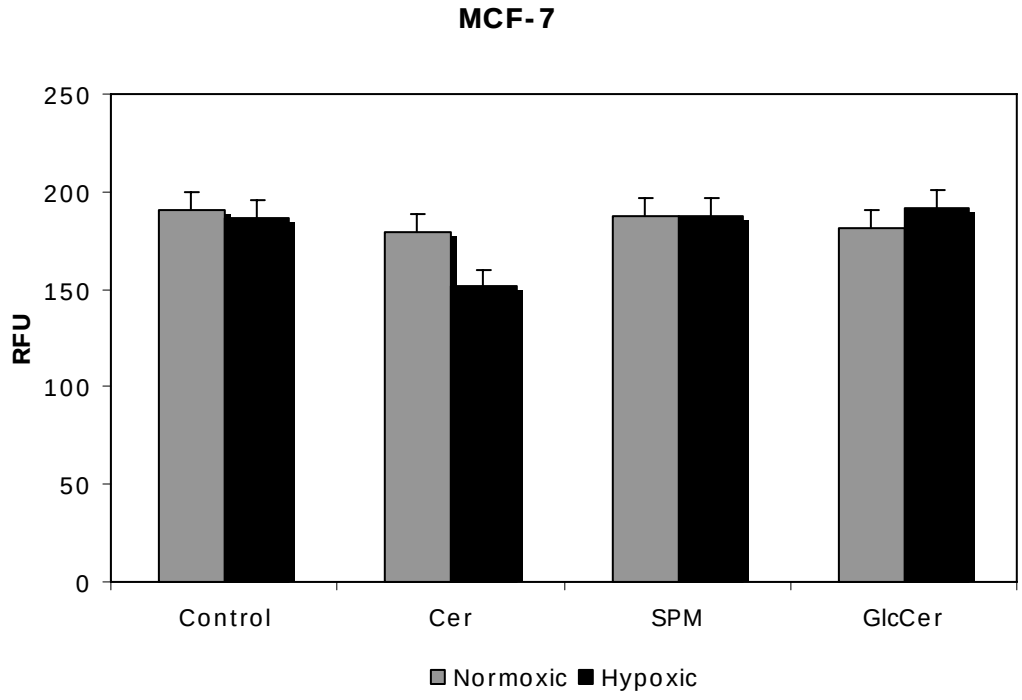


Fig. 3.5-B

Figure Contd…

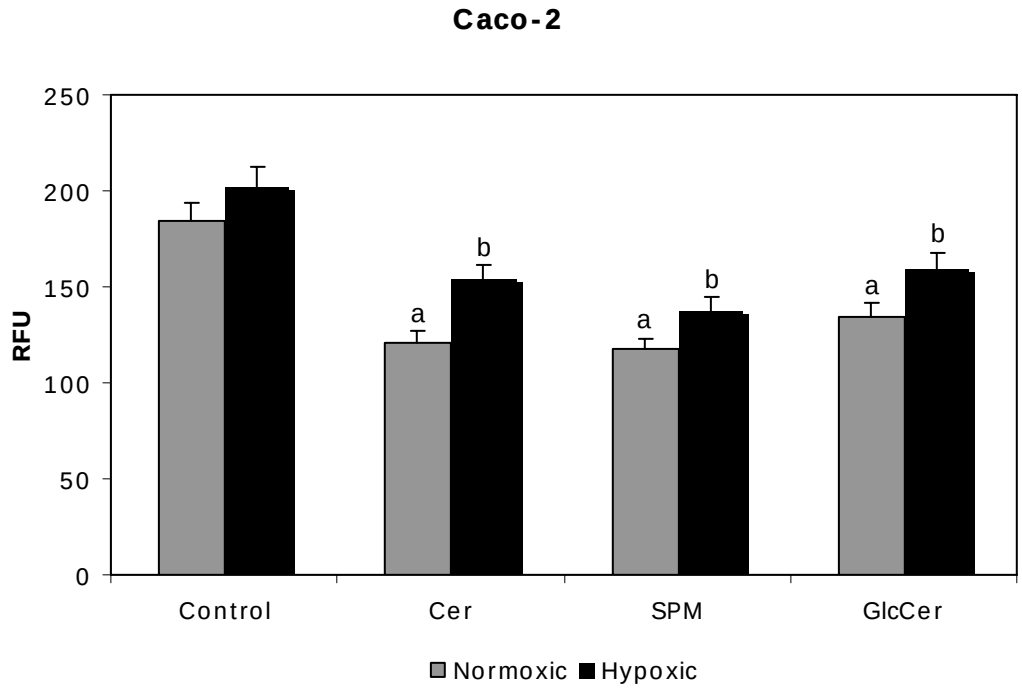


Fig. 3.5-C

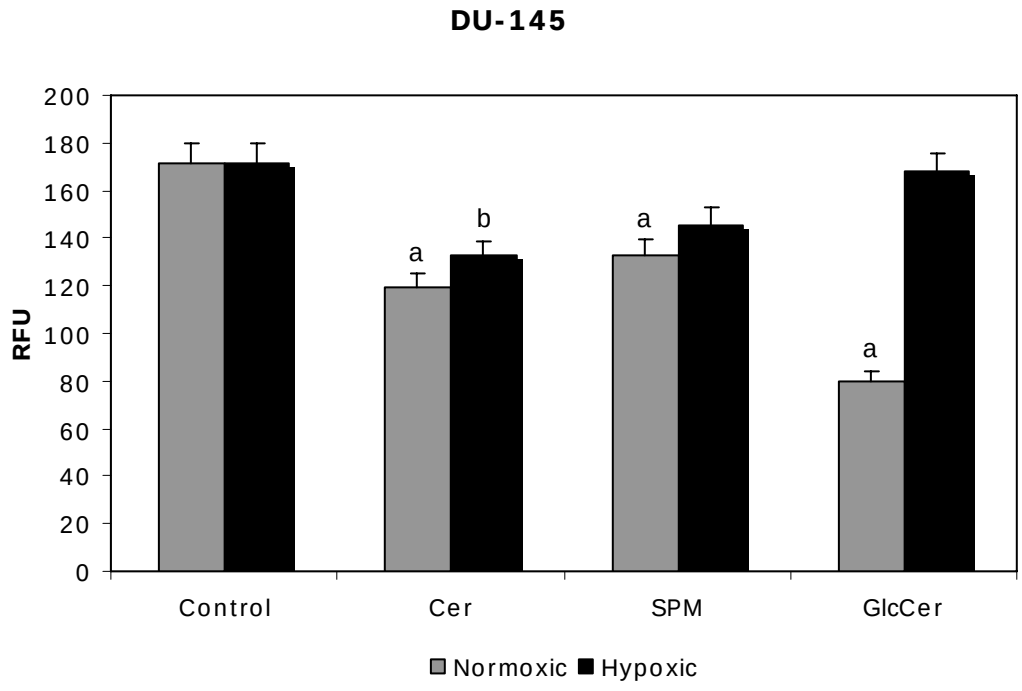


Fig. 3.5-D

Figure 3.6 HIF-1α Expression in Cancer Cells Exposed to Sphingolipids at a Concentration of 50 μM Under Normoxic and Hypoxic Conditions.

A. MCF-7 Breast Cancer Cells; B. Caco-2 Colon Cancer Cells; C. DU-145 Prostate Cancer Cells.

[a] Significantly different from control under normoxic condition.
[b] Significantly different from control under HIF-1α induced condition.

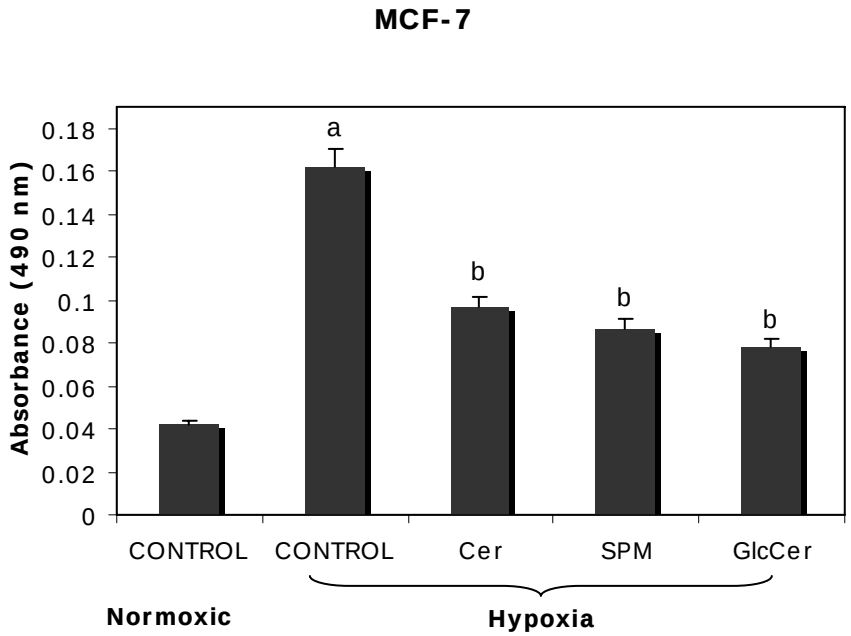


Fig. 3.6-A

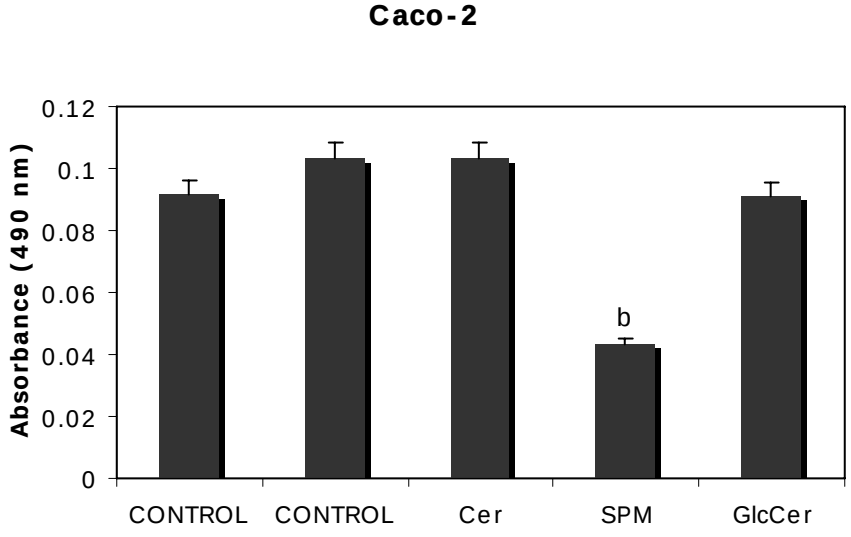


Fig. 3.6-B

Figure Contd…

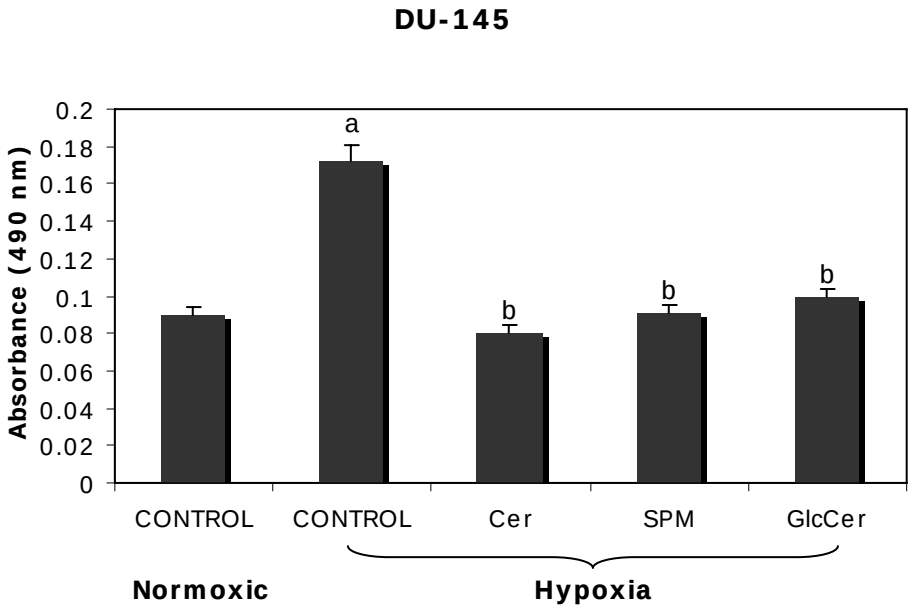


Fig. 3.6-C

3.4 Conclusion

Breast cancer MCF-7 cells treated with Cer had significant effect on the cell death at higher concentrations with lower VEGF expression compared to GlcCer and SPM. The levels of cathepsin-D were higher in Cer treated cell, which explains the higher cell viability compared to the rest of the compounds. The cell migration was similar under normoxic conditions but reduced under hypoxic conditions. Cathepsin-D expression under SPM treated MCF-7 cells was significantly lower under both conditions than control. GlcCer had significant apoptotic activity on hypoxic MCF-7 cells. Also, the cathepsin-D and HIF-1α were significantly reduced under hypoxic conditions.

Colon cancer cells (Caco-2) treated with Cer had reduced cell growth at > 50 μM under normoxic as well as hypoxic conditions. Cathepsin-D, cell migration and HIF-1α expression

were significantly reduced under hypoxic condition. SPM had low cathepsin-D levels, cell migration in normoxic and hypoxic conditions as well as low HIF-1α at hypoxic condition. In GlcCer treated cells, the levels of cathepsin-D expression, and cell migration were reduced under normoxic as well as hypoxic conditions.

Prostate cancer cells (DU-145) exposed to SPM had reduced cell viability. All the compounds had lower levels of VEGF expression under normoxic conditions at 50 μM exposure; only GlcCer had lower VEGF expression under hypoxic condition. Cathepsin-D levels were significantly reduced under both conditions for all the compounds. Cell migration was reduced under normoxic condition and also for cells exposed to Cer under hypoxic condition. The HIF-1α was significantly reduced in all the compounds treated.

It can be interpreted from the results that different cell-lines under different conditions responded distinctly to the three compounds under investigation. Various cancer cells have been known to have abnormal activities of the enzymes involved in the ceramide *de novo* pathway. Knowing the activity of each enzyme in specific cancer cell-lines would shed light on designing specific/combination of sphingolipids in diet composition to facilitate homeostasis or induce apoptosis in those relevant cancer cells or to act as a chemopreventive bioactive agent in a normal diet.

The results show the importance of diet based sphingolipids role in preventing cancer progression by inhibiting angiogenesis. It has been demonstrated that all the compounds under investigation had distinctive effect on different cancer cell-lines and the process with which they inhibit angiogenesis. This implies that, appropriate diet can be recommended or formulated to prevent cancer and other chronic diseases.

CHAPTER 4. *IN VIVO* ANALYSIS OF SPHINGOLIPIDS AS ANTI-ANGIOGENIC COMPOUNDS

4.1 Introduction

The study of angiogenic process and the development of therapeutic agents to inhibit, or stimulate angiogenesis involves a wide range of *in vivo* assays. These assays vary depending upon their quantitative nature, difficulty, rapidity and cost (West et al., 2001). The most commonly used *in vivo* angiogenesis assay is the chorioallantoic membrane (CAM) assay. The main advantages of the CAM assay is its low cost, simplicity, reliability, and large scale screening capabilities. However, the disadvantages of CAM is that it already contains a well-developed vascular network and the vasodilation may be hard to distinguish from the effects on vascularization by the test substance to that caused due to its manipulation during experimentation. Also, it undergoes nonspecific inflammatory reactions due to hyperplastic reactions of the chorion epithelium (Ribatti et al., 2000). Another *in vivo* model that has gained acceptance recently is the zebrafish assay. Zebrafish has long been an accepted model for studies of vertebrate developmental biology. The zebrafish vasculature system is highly conserved and blood vessel formation by angiogenic sprouting requires the same proteins that are necessary for blood vessel growth in mammals (Isogai et al., 2001; Vogel and Weinstein, 2000). The advantages of the zebrafish assay are rapid screening, use of large number of embryos for quantitative measurement, and ease of maintenance and replication (Serbedzija et al., 1999).

Based on the *in vitro* results, our aim was to perform the *in vivo* assessment to qualitatively establish the role of sphingolipids under physiological conditions. We examined the effect of the selected compounds namely Cer, SPM and GlcCer in the *in vivo* angiogenesis models such as CAM and zebrafish.

4.2 Materials and Methods

4.2.1 Chorioallantoic Membrane (CAM) Assay

Fertilized white broiler eggs were obtained from Spafas (Roanoke, IL) and the outside of the shells were washed with 70% ethanol. The eggs were then incubated at 37°C for 3 days and rotated 180 degrees three times daily. After incubation, the eggs were then cleaned with 70% ethanol and cracked, and the embryos were explanted into sterile Petri dishes and incubated for two days at 37°C and 5% CO2.

4.2.1.1 Compound Delivery

DMSO, Cer, SPM, and GlcCer at a concentration of 50 μM was applied to 5mm nylon mesh and freeze dried for 24 h. The mesh was then placed on an outer third of the CAM and the Petri dish was returned to the humidified chamber and incubated for 24h of growth before examination by image analysis.

4.2.2 Zebrafish Assay

4.2.2.1 Embryo Collection

Embryos were generated by natural pair-wise matings, as described in the zebrafish handbook (Sprague et al., 2001). For each mating, 4-5 pairs were set up and an average of 100-150 embryos per pair were generated. Embryos were maintained in embryo medium (0.2 g/l of Instant Ocean Salt in distilled water) at 27-28 °C for approximately 10h before sorting for viability, using both morphology and developmental stage as criteria. Healthy embryos were then dechorinated by enzymatic digestion with 1.4 mg/ml protease (Sigma, St. Louis, Missouri, USA) for 7-8 min at room temperature. The dechorinated embryos were then washed at least ten times in embryo water. Because the fish receives nourishment from an attached yolk sac for the duration of the experiment, no additional nutritional maintenance was required.

4.2.2.2 Compound Addition

DMSO, Cer, SPM, and GluCer were added directly to the embryonic media in which the fish swim. The chemical was added to the media at 10-14 hours post fertilization (hpf), before angiogenic vessels express Flk-1 (~19 hpf). The number of embryos per treatment is included in Table 4.1 and Table 4.2.

4.2.2.3 Visual Screen

After compound addition, the embryos were maintained in individual Petri dishes at 27-28°C until 48-72 hpf. Embryos were visually inspected for viability, gross morphological defects, heart rate and circulation. Circulation was assayed by visually comparing the movement of blood cells in treated and control embryos to assess the relative flow rate. The embryos were visually inspected every six hours following compound treatment until 36 hpf and then monitored every hour until 56 hpf to notice the ISV blood circulation which usually starts between 36-48 hpf and is complete by 56 hpf. Each embryo in a Petri dish was videotaped using a camera mounted on a Brightfield dissecting microscope.

4.2.2.4 Morphological Techniques: Nervous System-Staining

Whole mount immunohistochemistry was carried out using a modified version of a previously published protocol; Svoboda et al., 2001 & 2002. Embryos processed for immunohistochemistry were first fixed in 4% paraformaldehyde overnight at 4^{o}C and then stored in PBS. After permeablization, they were incubated in a primary antibody overnight at 4^{o}C. The primary antibody znp1 was used at a dilution of 1:250. The following day, the embryos were washed for 60 minutes in PBS buffer and then incubated in a fluorescent secondary antibody, Alexa 546 (1:1000 dilution) for 90 minutes. They were then rinsed in PBST for another 60 minutes and prepared for image analysis. Images of stained embryos were digitally captured with the aid of a Hammamatsu digital camera mounted to a Zeiss (Mot 2) inverted microscope.

4.2.2.4 GFP-labeled transgenic zebrafish

Intersegmental vessels (ISVs) patterning was observed using Fli-GFP-labeled transgenic zebrafish. Fli-1 is an endothelial marker and GFP labeling on Fli-1 promoter aids in the observation of vasculature structure formation of zebrafish during embryogenesis. For examining primary motor neurons formation, NBT-GFP labeled transgenic zebrafish were used. In NBT transgenic fish, GFP can easily be detected in both CaP and MiP motor neuron axons. Both, DMSO and ceramide were exposed to the transgenic fish between 10-12 hpf and images were acquired at 48 hpf. The GFP filter cube on the microscope was used to acquire the GFP fluorescent signal.

4.3 Results and Discussion

4.3.1 Chorioallantoic Membrane (CAM) Assay

DMSO, which was used as a carrier, when exposed on the chicken CAM, had a modest effect on the vasculature. It attenuated blood vessel formation within 24 h of exposure. However the observation of the effect of DMSO to that of the other compounds in this study were distinct (Figure 4.1 A-B). The C6-ceramide tested on chicken CAM affected blood vessels within 24 h (Figure 4.1 C-D). Longer exposure was deleterious to the embryo. The chicken embryo died within 36-48 h of age. SPM had significant vascular growth, but the vessels looked dilated and newly formed vessels were seen sprouting from the blood vessel within 24h of exposure.

It is likely that SPM affected the vascular smooth-muscle vessels or made the basement membrane more permeable (Figure 4.1 E-F). GlcCer regressed the formation of a large number of vessels (Figure 4.1 G-H). There was a complete loss of sprouting in 24 h treated area of the embryo as against the untreated area where vasculature was normal. SPM and GlcCer were non-toxic and the embryo survived during the exposure time.

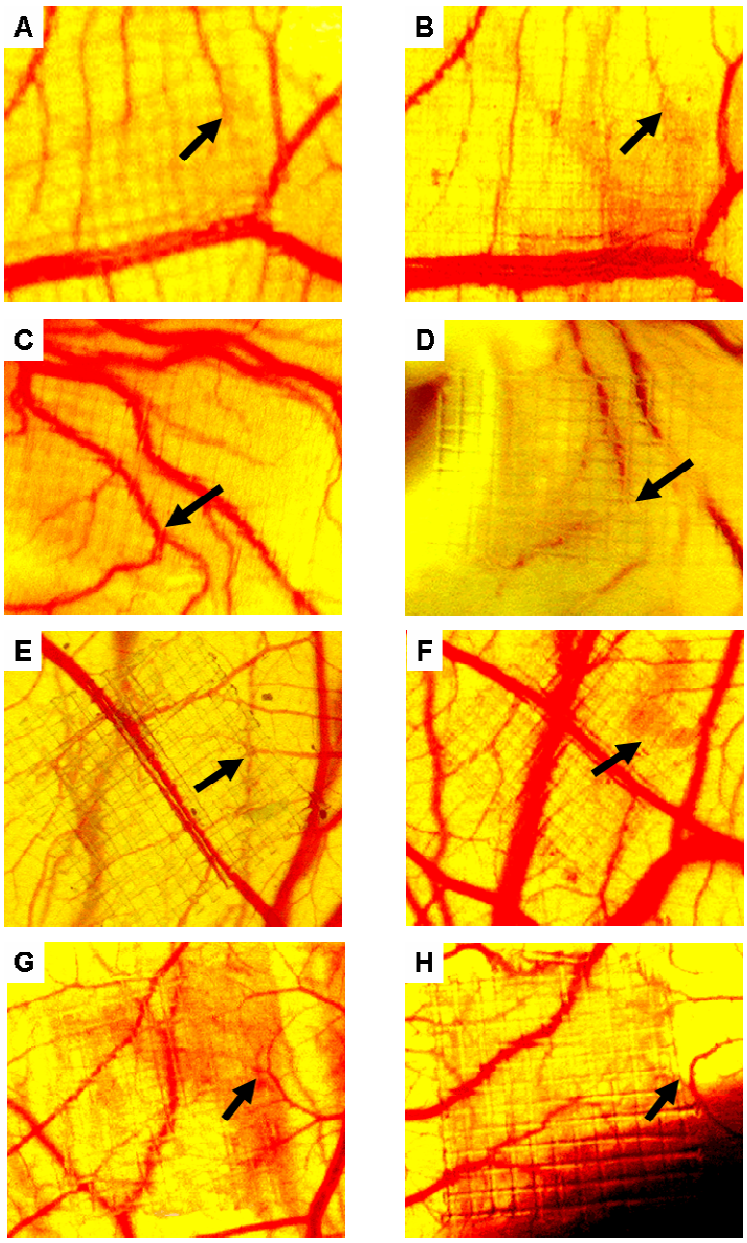


Figure 4.1 Chorioallantoic Membrane (CAM) Assay for Angiogenesis

A-B: DMSO treated region, A-0 h and B-24 h; C-D: Cer treated region, C- 0h and D-24 h; E-F: SPM treated region, E-0h and F-24h; G-H: GlcCer treated region, G-0h and H-24h.

Table 4.1 Analysis of 2-day Old Larvae in Trunk Region for Intersegmental Vessel (ISV) Patterning for Embryos Treated with DMSO and Cer

Compound	Dosage	Zebrafish type	Exposure Stage (hpf)	48-54 hpf					Blood circulation affected (%)
				Survival (N)	Observations			Abnormal	
					normal	no circulation	disrupted circulation		
DMSO	0.8%	HuC	10-14	21	18	1	1	1	14
		NBT	14	12	11	0	1	0	8
		MTAB	10-12	35	31	2	2	0	11
		FLI	10-12	4	4	0	0	0	0
DMSO		Total	10-14	72	64	3	4	1	11
Cer	400 μM	HuC	10-14	22	8	0	14	0	64
		NBT	14	7	0	1	6	0	100
		MTAB	10-12	68	25	0	43	0	63
		FLI	10-12	24	4	0	18	0	
Cer		Total	10-14	121	37	1	81	0	69

Table 4.2 Analysis of 2-day Old Larvae in Trunk Region for Intersegmental Vessel (ISV) Patterning for Embryos Treated with Ethanol, SPM and GlcCer

Compound	Dosage	Zebrafish type	Exposure Stage (hpf)	48-54 hpf					Blood circulation affected (%)
				Survival (N)	Observations			Abnormal	
					normal	no circulation	disrupted circulation		
EtOH	0.8%	MTAB	12	13	4	0	9	0	69
SPM	60 μM	MTAB	12	31	6	0	25	0	81
GlcCer	100 μM	MTAB	12	4	1	0	3	0	75

4.3.2 Zebrafish Angiogenesis Assay

The intersegmental vessels (ISVs) in zebrafish are arranged in an extremely regular pattern. In the medial-lateral plane, the ISVs run between the notochord and somite ventrally, while they are restricted between the neural tube and somite boundaries in the dorsally (Childs et al., 2002). Intersegmental artery and intergemental veins connect ventrally to either dorsal aorta or posterior cardinal vein, respectively (Figure 4.2-A) and run dorsally between and adjacent to the notochord and neural tube and the somites (Isogai et al., 2003).

Three primary motor neurons develop in the ventral spinal cord and have been individually identified as the caudal primary (CaP), middle primary (MiP), and rostral primary (RoP) neurons. Additionally, a fourth motor neuron called variable primary neuron (VaP), also develops adjacent to the CaP neuron, but only in some segments (Eisen et al., 1989). Ultimately, the VaP motor neurons die round 24hpf.

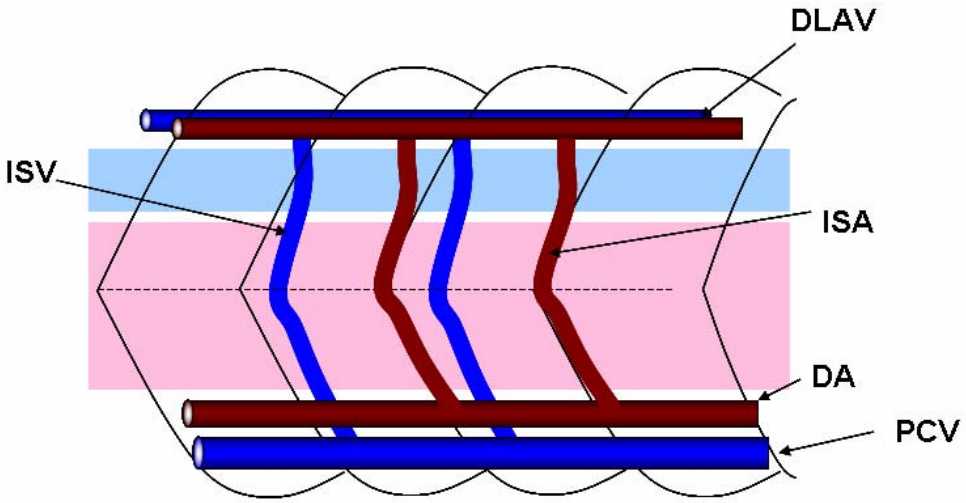
The axons of the primary motor neurons have been shown to follow specific trajectories to the periphery (Hjorth and Key, 2002; Lewis and Eisen, 2003). The pioneer motor neurons of zebrafish spinal cord develop at 15 hpf and extend axons at approximately 17 hpf. The CaP axon pioneers the common pathway of motor axons to the horizontal myoseptum separating the dorsal and ventral myotomes. CaP axons extend into ventral somite after contacting the muscle pioneer cells (Figure 4.2-B). The most ventral extension of CaP axons is believed to be dependent on restricted expression of *semaZ1b,* a ligand within the posterior portion of the somite. It is repulsive to CaP axon growth cones (Hjorth and Key, 2002). The MiP axons project dorsally into the peripheral muscle mass. The ability of zebrafish to tolerate the solvent was considered and no morphological changes were observed at 1% DMSO. Based on the solubility criteria and effective dose concentration of Cer, a working dose concentration of 0.8% DMSO as a carrier solvent was finalized.

Figure 4.2 Illustrations of Zebrafish Mid-trunk Region

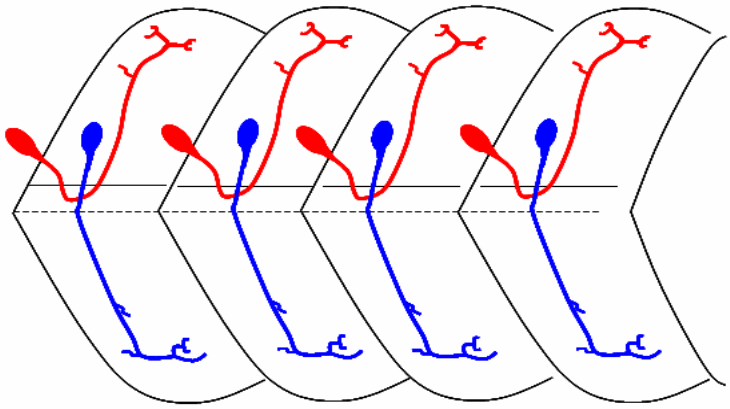
(A) Schematic diagram of ISVs (ISA: Intersegmental artery; ISV: Intersegmental vein; DLAV: dorsal longitudinal anastomotic vessel). Intersegmental vessels are indicated by arrows. ISVs are represented with white arrows and blood cells are represented with black arrows; (B) Schematic diagram of a lateral view showing ventral CaP (blue) and dorsal MiP (red) axon trajectories. Anterior is to the left, dorsal is up.

Anterior is to the left, dorsal is up. Blood flow caudally (to the right) via the dorsal aorta (DA), and returns rostrally (to the left) via post cardinal vein (PCV).

A



B



Earlier studies with labrasol as solvent at concentrations of 1.0 and 0.1% had a paralytic effect on the embryo. Preliminary studies were conducted with Cer dose ranging from 100 μM to 400 μM. The intended phenotype was observed on exposure concentration of 400 μM.

4.3.3 Morphological Assessment of Zebrafish

Zebrafish embryos at the 10-14 hpf stage were incubated with Cer at a concentration of 400 μM. Treatment of Cer up to 80 hpf caused loss of blood flow in 69% of zebrafish (Table 4.1). During treatment, embryos were mobile and responsive to external stimuli. Except disrupted blood flow through ISVs no other deleterious effects were observed. There were no observable morphological changes observed in the embryos treated under two conditions (Figure 4.3). However, there was a distinctive loss of blood flow due to constricted ISVs (Figure 4.4-B, vessel b) in Cer treated embryos at 48 hpf compared to the controls treated with 0.8% DMSO (Figure 4.4-A). When the Cer exposure was removed and replaced with embryonic media after 56 hpf, the blood flow was distinctively different than the controls (Figure 4.4-C) when observed between 72-80 hpf (Figure 4.4-D). Blood flow through the ISVs in ethanol and SPM treated embryos did not start until 54 hpf when compared to DMSO treated sample (usually between 48-52 hpf).

SPM treated zebrafish embryo showed aberrant blood flow when observed between 48-72 hpf and had broadened vessels at the choicepoint in the mid-trunk regions as seen in Figure 4.4-E (vessel *b*). The effect on ISV blood vessel disruption by ethanol and SPM treated embryos were 69% and 81%, respectively (Table 4.2). GlcCer affected the survival of the embryos as only 20% of the treated embryos survived until 80 hpf (Table 4.1). The vasculature of the ISVs was constricted (Figure 4.4-F) and aberrant with GlcCer having 75% embryos affected with abnormal ISVs (Table 4.2). The GFP-labeled ISVs showed aberrant ISV formation in Cer treated

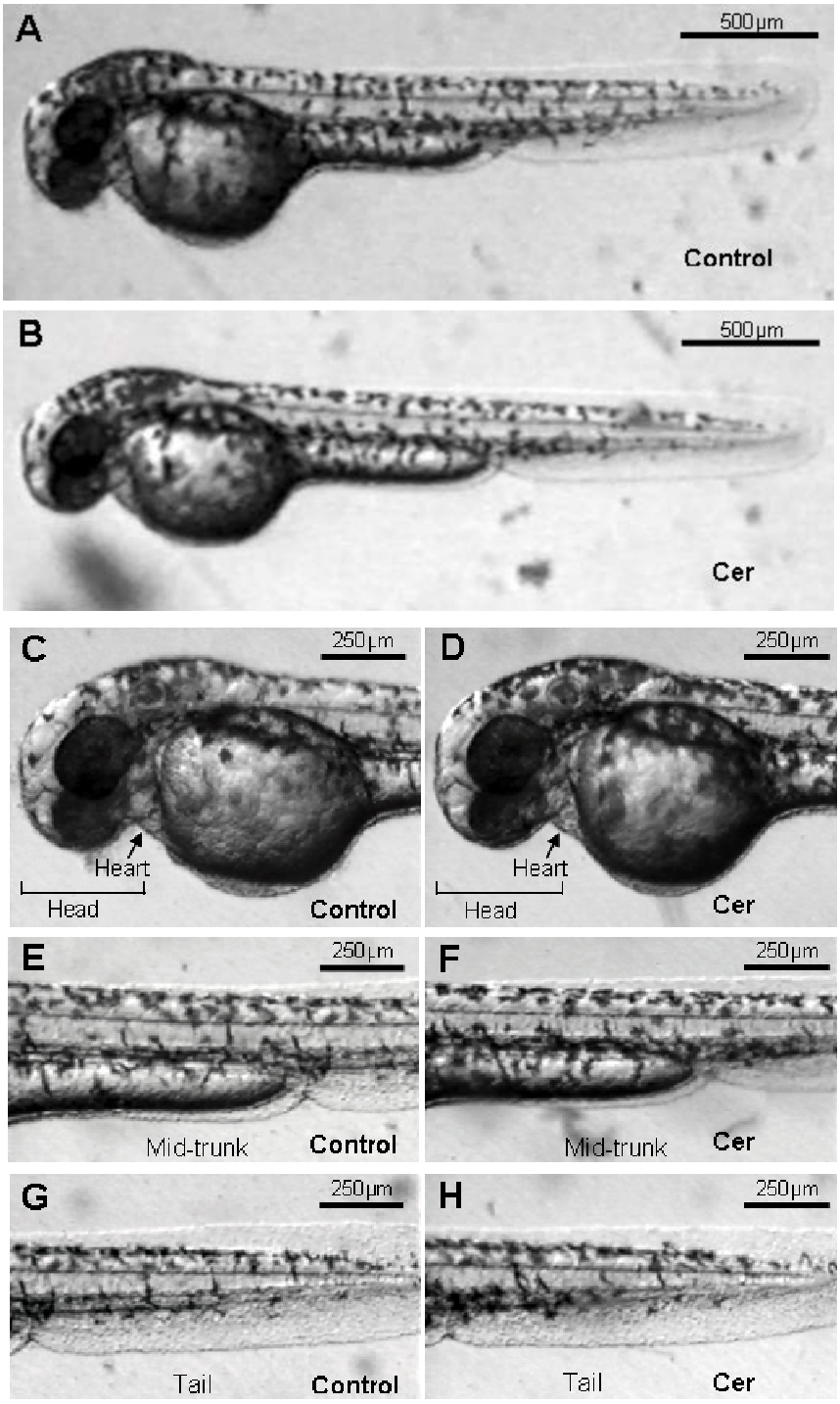


Figure 4.3 Morphological Observation of Zebrafish Embryo at 40 hpf

DMSO treated embryos are represented in A, C, E, and G; Cer treated embryos are represented in B, D, F, and H. A-B: whole embryo; C-D: anterior (forebrain, midbrain, hindbrain, eye and heart (represented with arrow)); E-F: mid-trunk region; G-H: posterior (tail-region).

Figure 4.4 Intersegmental Vessel (ISV) Patterning in the Mid-trunk of Zebrafish Embryo.

(A) DMSO treated embryo at 48 hpf; (B) Cer treated embryo at 48 hpf; (C) DMSO treated embryo at 72 hpf; (D) Cer treated embryo at 72 hpf (E) SPM treated embryo at 72 hpf; (F) GlcCer treated embryo at 72 hpf.

Anterior is to the left, dorsal is up. Blood flow caudally (to the right) via the dorsal aorta (DA), and returns rostrally (to the left) via post cardinal vein (PCV).

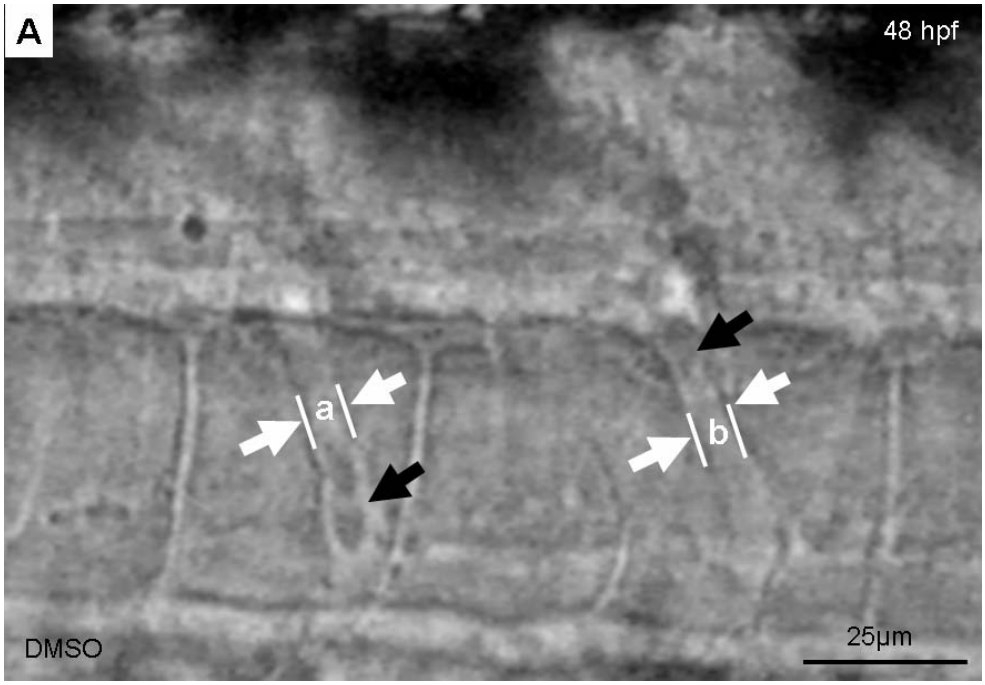


Fig. 4.4-A

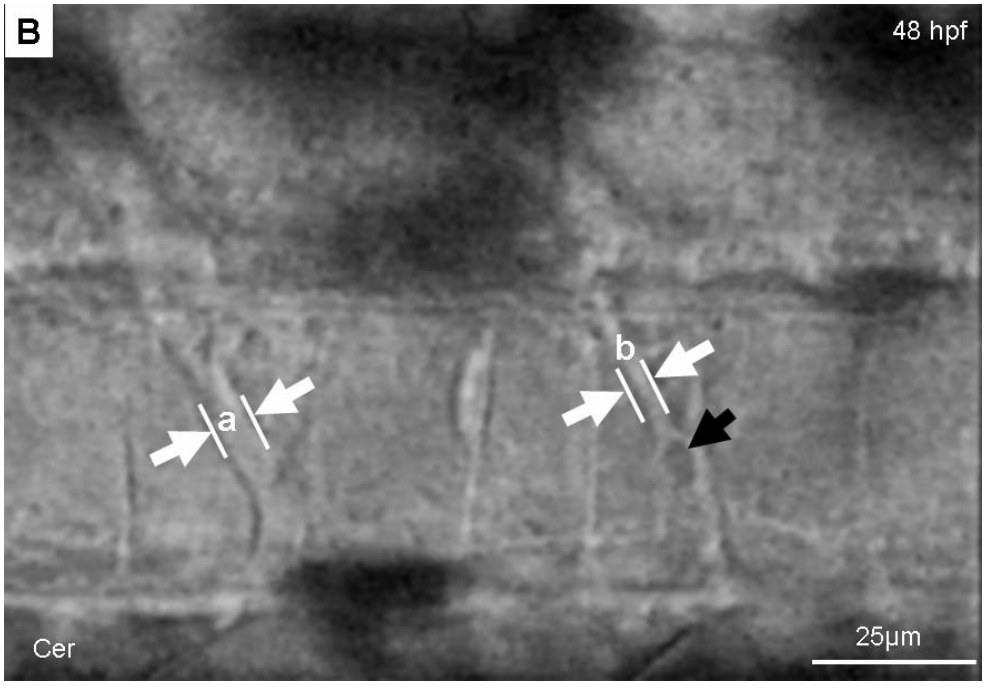


Fig. 4.4-B

Figure Contd….

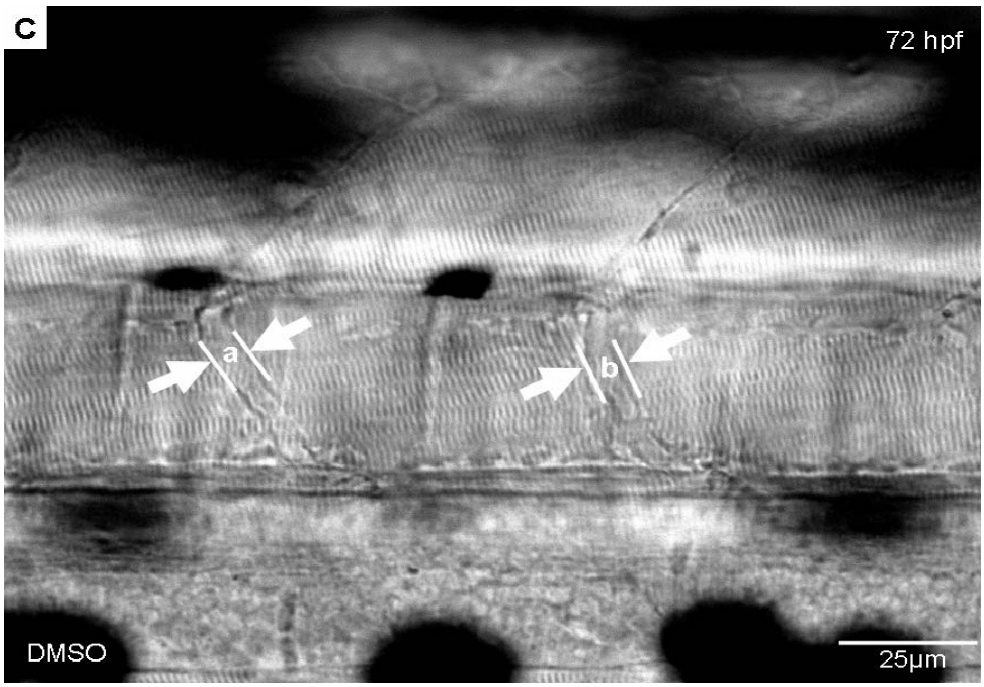


Fig. 4.4-C

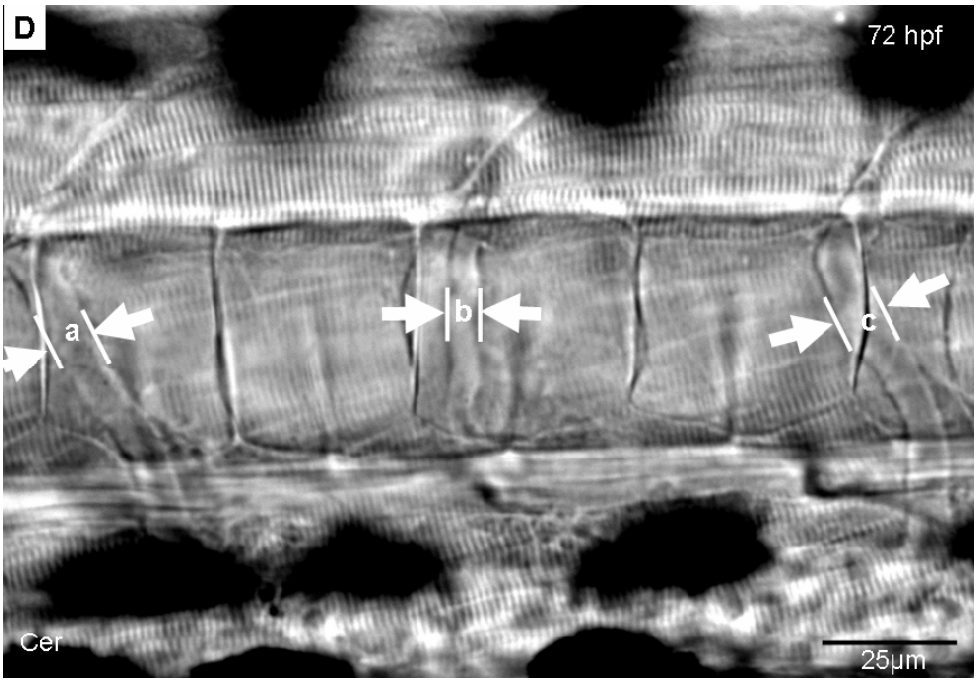


Fig. 4.4-D

Figure Contd…

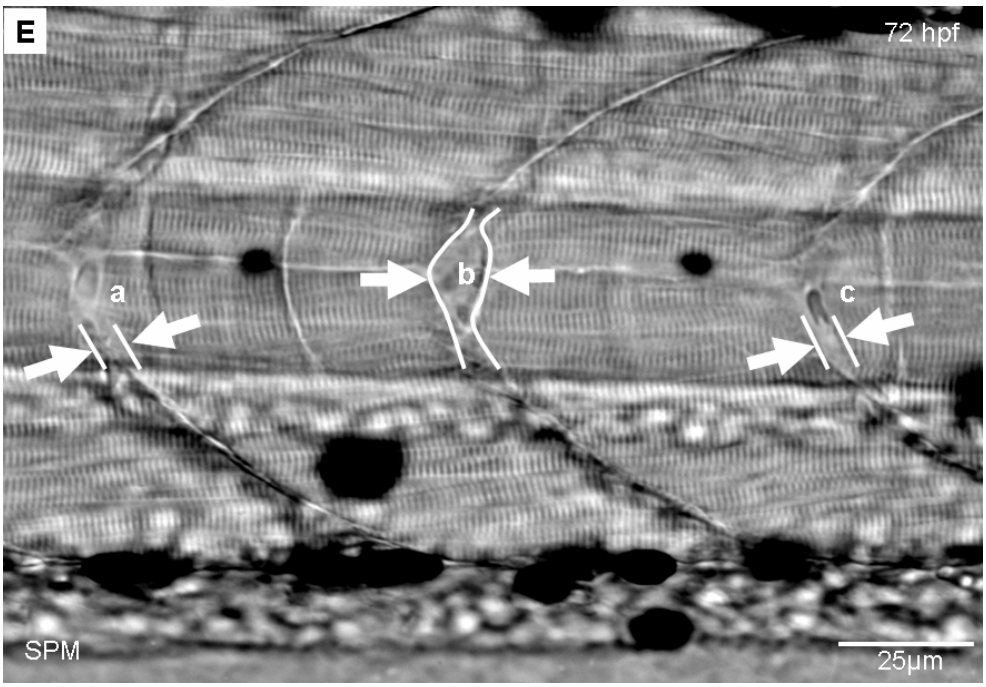


Fig. 4.4-E

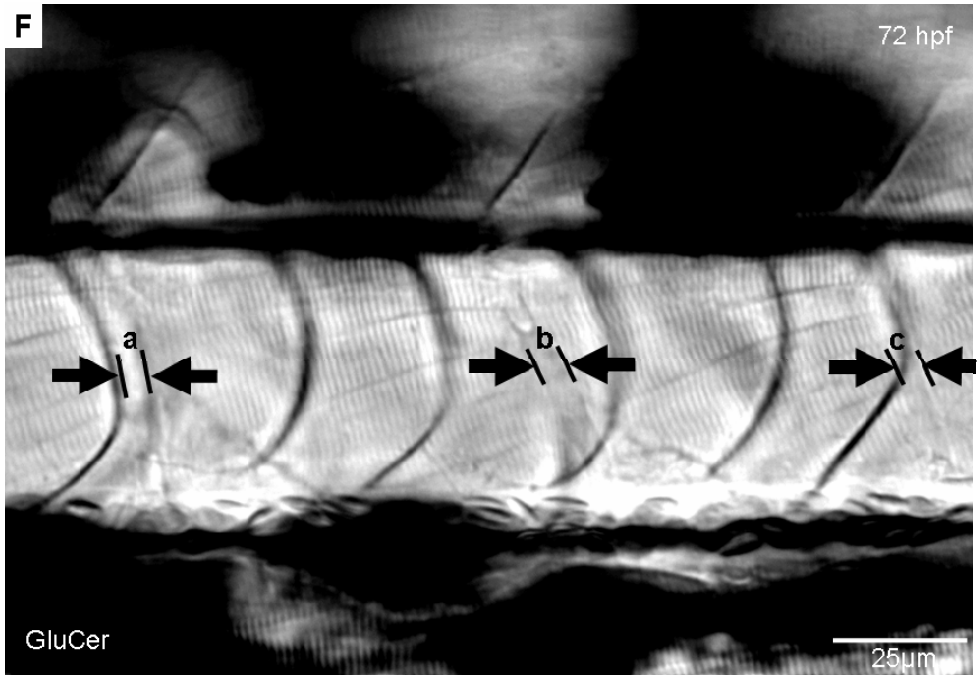


Fig. 4.4-F

Figure Contd…

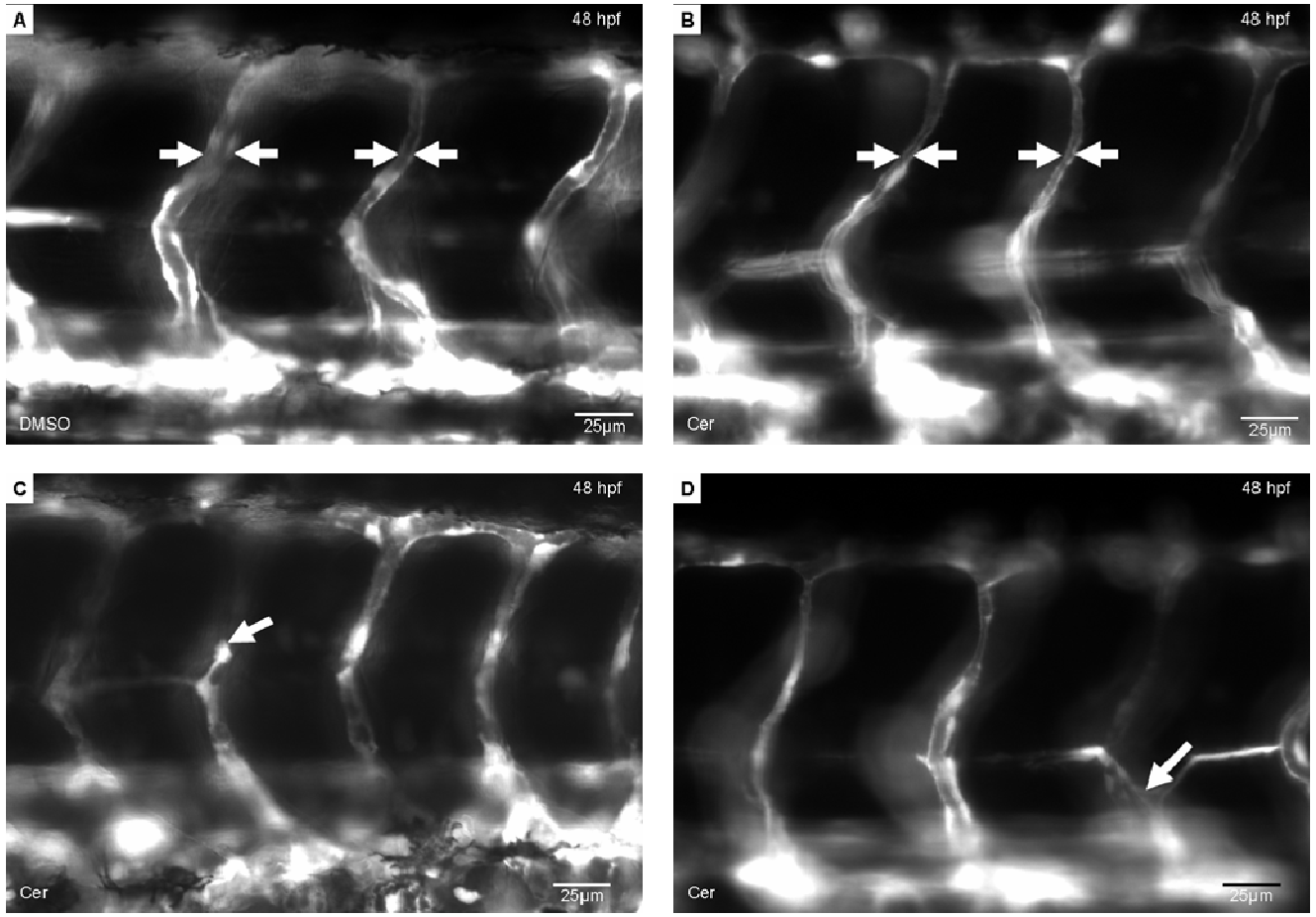


Figure 4.5 GFP-labeled Intersegmental Vessels (ISV) Cross-section of Transgenic Zebrafish.

A. DMSO treated zebrafish; B-D. Cer treated zebrafish

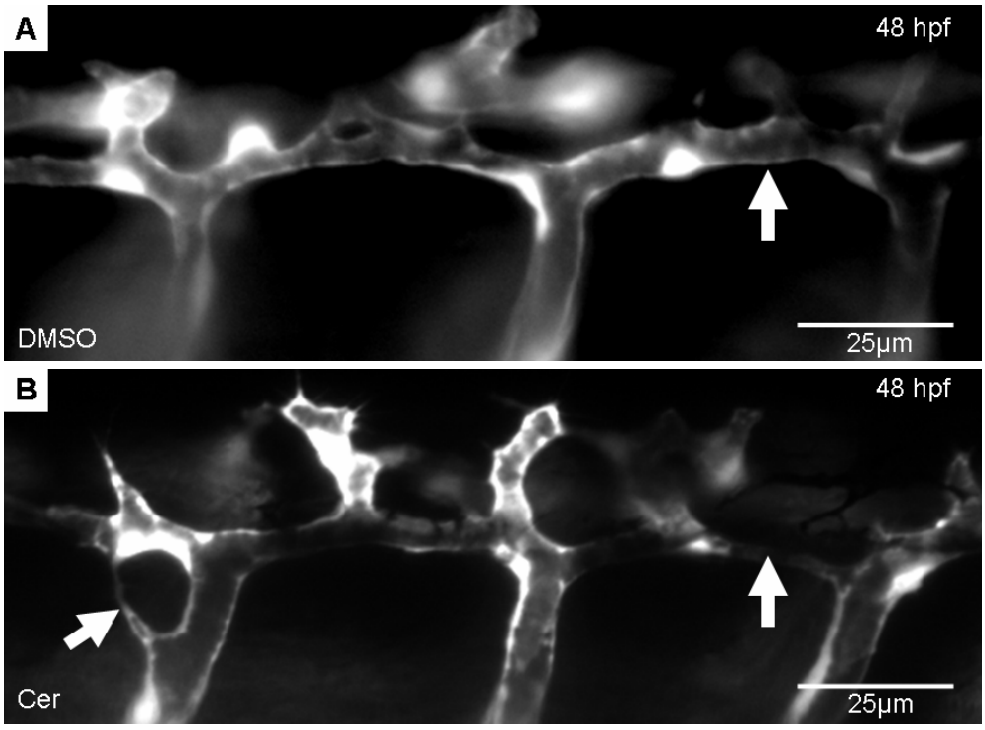


Figure 4.6 GFP-labeled Dorsal Longitudinal Anastomatic Vessels (DLAV) Cross-section of Transgenic Zebrafish.

A. DMSO treated Zebrafish; B-D. Cer treated zebrafish

transgenic zebrafish. Compared to DMSO treated transgenic zebrafish (Figure 4.5-A), Cer treated transgenic zebrafish ISVs were observed to be attenuated (Figure 4.5-B), some were stunted (Figure 4.5-C) and others were observed to have patterning defects (Figure 4.6-D). Also, The DLAV in Cer treated zebrafish was observed to be constricted as shown in figure 4.6-B.

4.3.4 Znp-1 Staining

We conducted antibody staining with znp-1 to mark MiP and CaP primary motor neuron axons in zebrafish. The primary motor neuron axonal trajectories were affected in Cer exposed embryos as shown in Figure 4.7-B compared to DMSO treated embryos (Figure 4.7-A) C6-Ceramide exposed embryos displayed aberrant primary motor neuron and axonal trajectories. In Cer treated embryos, CaP axon guidance was impaired in the ventral somite region as seen in Figure 4.7-D. Also, MiP motor axons developed ectopic branches (Figure 4.7-F, 4.7-G), which

Figure 4.7 Znp-1 Antibody Staining of DMSO and Cer Treated Zebrafish Embryo.

(A) DMSO treated embryo at 32X magnification; (B) Cer treated embryo at 32X magnification; (C) DMSO treated embryo at 20X magnification with traced trajectories at the bottom; (D) Cer treated embryo at 20X magnification with traced trajectories at the bottom; (E) DMSO treated embryo at 40X showing CaP trajectory; (E, F) Cer treated embryos with branched CaP axon, trajectory traced at the bottom of the figure; (G) DMSO treated GFP-labeled NBT mutant zebrafish showing primary axon pathway; (H) Cer treated GFP-labeled NBT mutant zebrafish showing primary axon pathway.

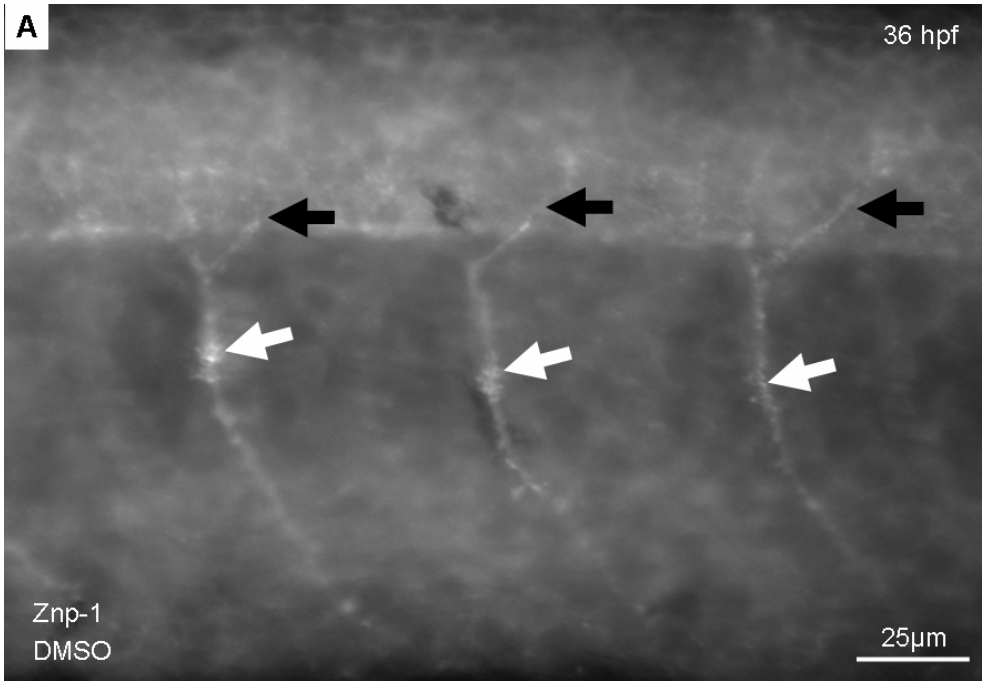


Fig. 4.7-A

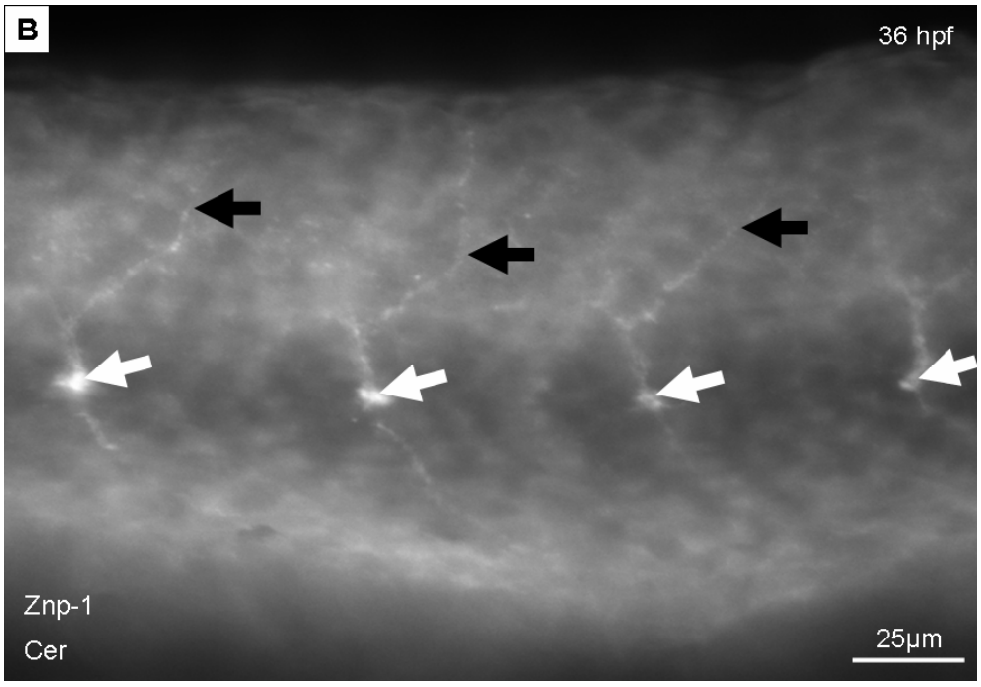


Fig. 4.7-B

Figure Contd…

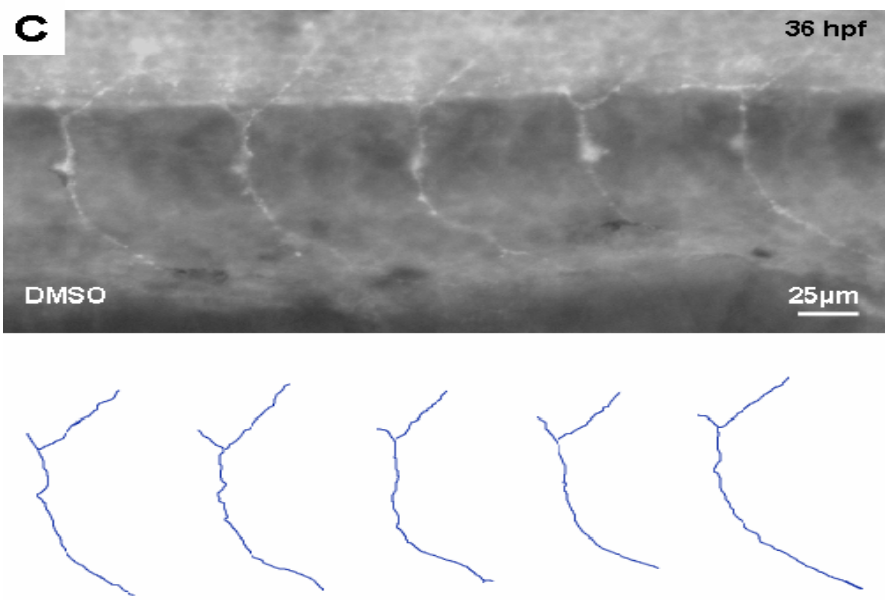


Fig. 4.7-C

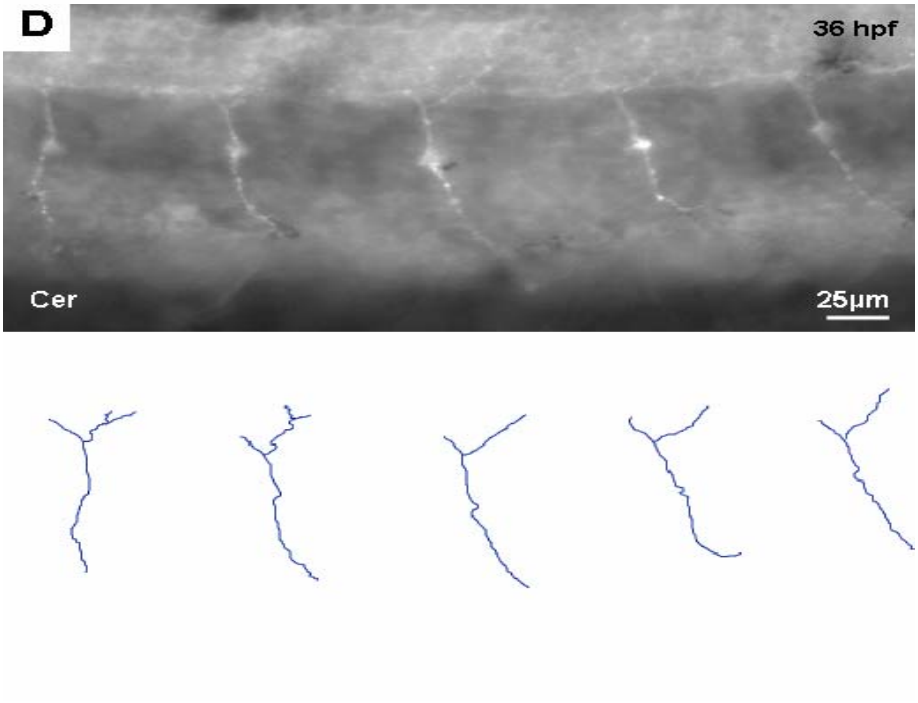


Fig. 4.7-D

Figure Contd…

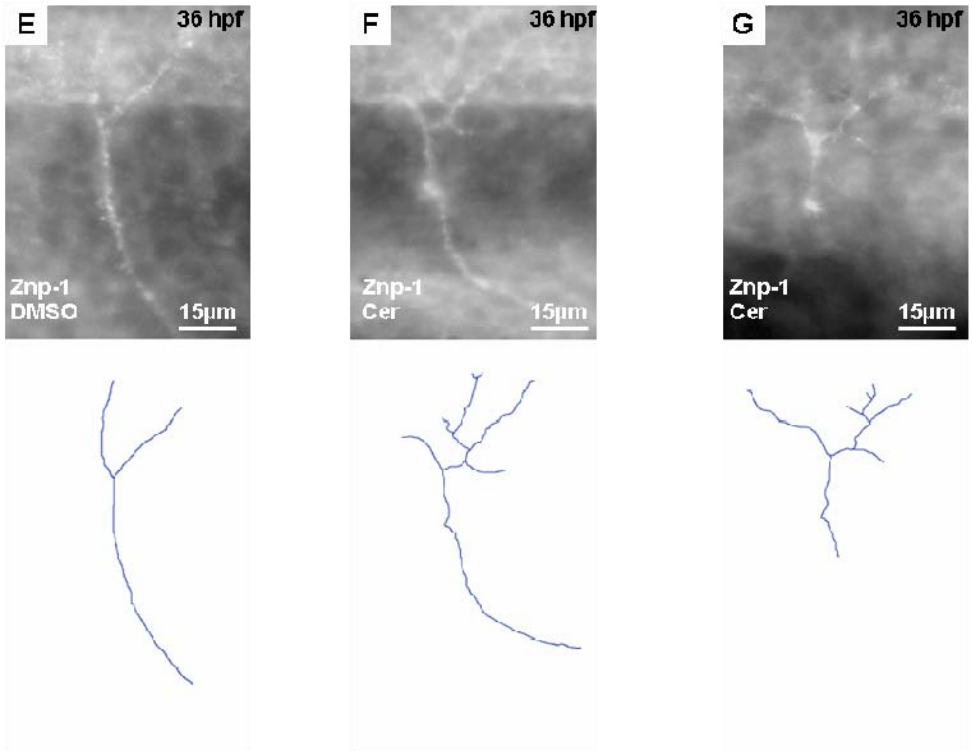


Fig. 4.7-E,F,G

was also confirmed using GFP labeled NBT transgenic fish showing an early branching in the mid-somite region (Figure 4.8-B) compared to DMSO treated transgenic fish (Figure 4.8-A).

4.4 Conclusion

Our results show that ceramide was anti-angiogenic as confirmed by both the CAM assay as well as zebrafish model. SPM proved to facilitate sprouting, however, the blood vessels looked dilated. GlcCer, disrupted the neovascularization in the CAM model and restricted the ISV formation in zebrafish. These results, based on the repeatability of the experiment and the number of embryos treated during our study, establishes the role of Cer as an anti-angiogenic bioactive agent. However, the role of SPM and GlcCer in angiogenesis needs to be further investigated to confirm these initial observations. Taken together with its already proven role as a pro-apoptotic agent, Cer could serve as an extremely potent chemopreventive bioactive

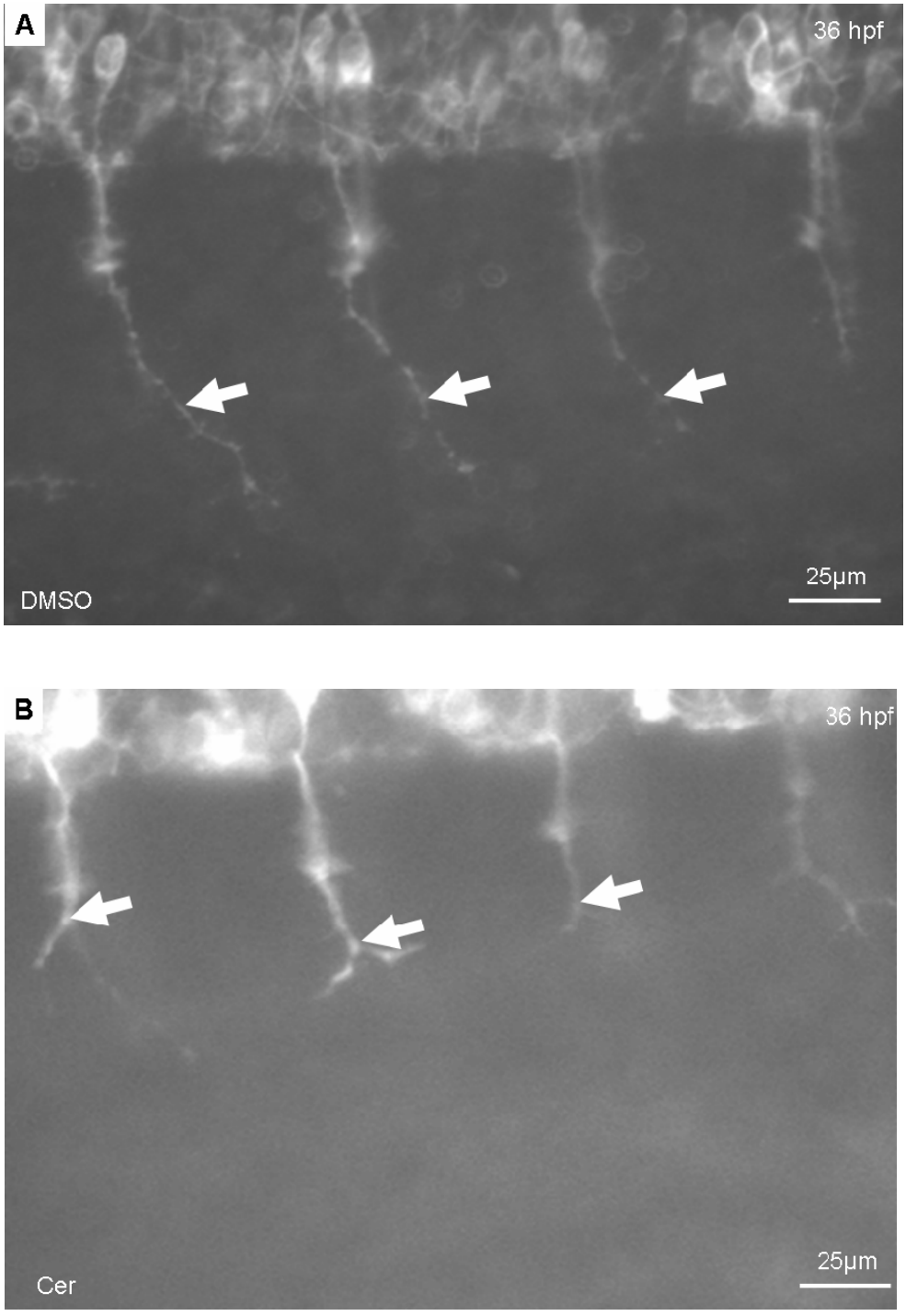


Figure 4.8 GFP-labeled Primary Motor Neurons of Transgenic Zebrafish.
A. DMSO treated zebrafish; B. Cer treated zebrafish

compound from a dietary source. The role of ceramide in axonal genesis and pathfinding is also worth consideration as our experiments have demonstrated its effect on the axon guidance. It can be readily interpreted that Cer participates in vasculature as well as neuronal growth and/or axon guidance.

Our results are in agreement with the recent studies on the factors that guide growth cone extensions that can also regulate migration of putative angioblasts. It has been demonstrated that VEGF, which regulates migration of angioblast, is also involved in axonal pathfinding (Shoji et al., 2003). Recent studies on glycoproteins such as neuropilin-1 (NRP1) and neuropilin-2 (NRP2) have been implicated in regulating both semaphorins in neuronal guidance and vascular endothelial growth factor (VEGF) in angiogenesis (Negishi et al., 2005). It would be interesting to investigate the role of Cer in regulating the levels of guidance factors such as the semaphorins, ephrins and nitrins and growth factors such as VEGF, EGF, bFGF.

CHAPTER 5. SUMMARY AND CONCLUSIONS

Functional foods have gained prominence in recent years. The ability to prevent chronic diseases through healthy diet has been of main research focus in the field of food science. Many bioactive compounds from food sources have been proven to be beneficial to humans. Prevention of degenerative diseases through diet now is considered to be a viable strategy. In keeping with the research focus in our mind, we set forth to assess the bioactive compounds of milk source against cancer. Our hypothesis was to test the efficacy of a group of compounds called sphingolipids found in many foods of plant and animal origin. Milk being the highest source of sphingolipids, based on the consumption per capita in US, we used the sphingolipids of milk source as our compounds of interest. The effect of these compounds on cancer proliferation and its growth assisted by angiogenesis was accessed. Angiogenesis plays an important role in tumor growth initiated by growth factors expressed by tumor which leads to formation if new blood vessels to the tumor. Tumors are generally under the influence of hypoxia and require oxygen and nutrients in order to grow. Hypoxic environment leads to the expression of Hypoxia inducing factors (HIF) which further promotes expression of angiogenic growth factors such as VEGF which initiates new blood vessel sprouting. The newly formed blood vessels supply oxygen and nutrient to the tumor which aids in its proliferation and subsequently helps in localization of cancer to other parts of the body, called as metastasis. Therefore, preventing the formation of new blood vessels can serve as an approach to inhibit tumor from proliferation. Angiogenesis can be inhibited by various anti-angiogenic compounds which can inhibit growth factors such as VEGF, bFGF and EFG etc.

We investigated the effect of sphingolipids using *in vitro* techniques using cancer cell-lines. We also examined the anti-angiogenic activity of sphingolipids on the expression of angiogenic biomarkers such as VEGF. The ability of the cancer cells to migrate under the

influence of sphingolipids was also studied by conducting cells migration assays. Further, the effect of sphingolipids on cancer metastasis was accessed by testing the levels of cathepsin-D in the cancer cells exposed to the compounds. Response of the cancer cell to sphingolipids under the influence of hypoxia was determined by measuring the levels of hypoxia inducing factors such as HIF-1α subunit.

The *in vivo* study was conducted using chic chorioallantoic membrane vessel (CAM) assay and zebrafish model system. As zebrafish serves as a models system for developmental biology and is a novel system for studying the motor neuron and vasculature patterning during embryo genesis, we investigated how sphingolipids would simultaneously affect the process of angiogenesis as well as motor neuron axon guidance.

For normal lung cells (HEL 299), Cer seemed to be proliferative with high VEGF expression and also high cathepsin-D, but low cell migration. SPM had static growth with no significant difference in VEGF expression and low levels of cathepsin-D expression and low cell migration. GlcCer had lower proliferation with no significant VEGF expression. However, there was lower cathepsin-D and reduced cell migration. Breast cancer MCF-7 cells treated with Cer had a significant effect on the cell death at higher concentrations with lower VEGF expression compared to GlcCer and SPM. The levels of cathepsin-D were higher. The cell migration was similar under normoxic conditions but was reduced under hypoxic conditions. SPM had a proliferative effect on MCF-7 cell lines under normoxic conditions but there was no significant difference under hypoxic conditions. Cathepsin-D expression was significantly lower under both conditions. However, cell migration remained unchanged. GlcCer had a proliferative effect under normoxic conditions on MCF-7 cells but had significant apoptotic activity under hypoxic conditions. Also, the cathepsin-D and HIF-1α were significantly reduced under hypoxic

conditions. The levels of VEGF and cell migration remained unchanged when compared to control.

Colon cancer cells (Caco-2) treated with Cer had increased proliferation under concentrations < 50 μM but had reduced cell growth at > 50 μM under normoxic as well as hypoxic conditions. VEGF expression was greatly increased under the influence of Cer in hypoxic condition. However, cathepsin-D, cell migration and HIF-1α were significantly reduced under hypoxic conditions. SPM resulted in arrested cell growth under the concentrations studied in normoxic and hypoxic conditions with low cathepsin-D levels, cell migrations in normoxic and hypoxic conditions as well as low HIF-1α at hypoxic condition. GlcCer increased the cell viability with increased VEGF expression under both conditions and caused no significant difference in HIF-1α expression under hypoxic conditions. The levels of cathepsin-D expression and cell migration were reduced under normoxic as well as hypoxic conditions.

Prostate cancer cells (DU-145) exposed to Cer and GlcCer had greater cell viability at high concentration (> 30 μM) while SPM reduced cell viability. All the compounds caused lower levels of VEGF expression at normoxic conditions with 50 μM exposure; only GlcCer had lower VEGF expression under hypoxic condition. Cathepsin-D exposure was significantly reduced under both conditions for SPM and GlcCer treatments. The cell migration was reduced under normoxic condition and also for cells exposed to Cer under hypoxic condition. The HIF-1α was significantly reduced in all the compounds treated.

The C6-ceramide tested in the chicken CAM affected blood vessels within 24 h and SPM caused significant vasculature growth, but the vessels looked dilated and newly formed vessels were seen sprouting from the Aorta within 24h of exposure. GlcCer regressed the formation of a large number of vessels. Zebrafish treated with Cer had a distinctive loss of blood flow at 48 hpf

compared to the controls treated with 0.8% DMSO. SPM proved to facilitate aberrant blood flow through ISVs; however, the blood vessels looked dilated at choicepoint in the mid-trunk region. The survival rate in SPM treated embryos was 83%. GlcCer constricted the ISV formation in zebrafish when observed between 48-80 hpf with a mortality of 80%. C6-Ceramide treated embryos had aberrant motor neuron axon trajectories with MiP and CaP motor axons developing ectopic branches at the region of the choice point dorsally and ventral somite region respectively.

The overall *in vitro* results suggests that ceramide was effective against MCF-7 cell lines under normoxic as well as hypoxia induced conditions. Sphingomyelin was effective against Caco-2 and DU-145 cancer cell-lines treated under normoxic conditions. Sphingomyelin was also effective in reducing the levels of VEGF, Cathepsin D and HIF-1α under hypoxia induced environment. Glucosylceramide was specifically effective on DU-145 cell-lines under both normoxic as well as hypoxia induced conditions. *In vivo* studies conclude the activity of ceramide and glucosylceramide in inhibiting the vasculature patterning both by CAM assay and Zebrafish analysis. Also, ceramide was proven to affect the motor neuron axon guidance simultaneous as determined using zebrafish model system. The results therefore validate the potential of sphingolipids from milk-source as anti-angiogenic compounds.

REFERENCES

Ameyar M, Atfi A, Cai Z, Stancou R, Shatrov V, Bettaieb A, Chouaib S. 1998. Analysis of human breast adenocarcinoma MCF7 resistance to tumor necrosis factor-induced cell death. Lack of correlation between JNK activation and ceramide pathway. J Biol Chem. 273(44):29002-8.

Auerbach R, Lewis R, Shinners B, Kubai L, Akhtar N. 2003. Angiogenesis assays: a critical overview. Clin Chem. 49(1): 32-40.

Benouchan M, Colombo BM. 2005. Anti-angiogenic strategies for cancer therapy (Review). Int J Oncol. 27(2):563-71.

Berra B, Colombo I, Sottocornola E, Giacosa A. 2002. Dietary sphingolipids in colorectal cancer prevention. Eur J Cancer Prev. 11(2):193-7.

Boik J. 2001. Natural compounds in cancer therapy. First Edition. Oregon Medical Press. OR.

Brenner DE, Gescher AJ. 2005. Cancer chemoprevention: lessons learned and future directions. Br J Cancer. 2005 Sep 6; [Epub ahead of print]

Canene-Adams K, Campbell JK, Zaripheh S, Jeffery EH, Erdman JW Jr. 2005. The tomato as a functional food. J Nutr.135(5):1226-30.

Cao Y, Cao R, Brakenhielm E. 2002. Antiangiogenic mechanisms of diet-derived polyphenols. J Nutr Biochem. 13(7):380-390.

Cao Y, Hong A, Schulten H, Post MJ. 2005. Update on therapeutic neovascularization. Cardiovasc Res. 65(3):639-48.

Carmeliet P, Jain RK. 2000. Angiogenesis in cancer and other diseases. Nature. 407(6801):249-57.

Carmeliet P. 2004. Manipulating angiogenesis in medicine. J Intern Med. 255(5):538-61.

Chen C, Kong AN. 2005. Dietary cancer-chemopreventive compounds: from signaling and gene expression to pharmacological effects. Trends Pharmacol Sci. 26(6):318-26.

Chen H, Born E, Mathur SN, Johlin FC, Field FJ. 1992. Sphingomyelin content of intestinal cell membranes regulates cholesterol absorption. Biochem. J. 286: 771-777.

Childs S, Chen JN, Garrity DM, Fishman MC. 2002. Patterning of angiogenesis in the zebrafish embryo. Development. 2002 Feb;129(4):973-82.

Chodorowska G, Chodorowski J, Wysokinski A. 2004. Vascular endothelial growth factor (VEGF) in physiological and pathological conditions. Ann Univ Mariae Curie Sklodowska. 59(2):8-14.

Cline EI, Bicciato S, DiBello C, Lingen MW. 2002. Prediction of *in vivo* synergistic activity of

anti-angiogenic compounds by gene expression profiling. Cancer Research. 62 (24):7143-8.

Colombaioni L, Garcia-Gil M. 2004. Sphingolipids metabolites in neural signaling and function. Brain Res Brain Res Rev. 46(3): 328-55.

Crosier PS, Kalev-Zylinska ML, Hall CJ, Flores MV, Horsfield JA, Crosier KE. 2002. Pathways in blood and vessel development revealed through zebrafish genetics. Int J Dev Biol. 46(4): 493-502.

Cuvillier O, Edsall L, Spiegel S. 2000. Involvement of sphingosine in mitochondria-dependent Fas-induced apoptosis of type II Jurkat T cells. J Biol Chem. 275(21):15691-700.

Cuvillier O, Levade T. 2001. Sphingosine 1-phosphate antagonizes apoptosis of human leukemia cells by inhibiting release of cytochrome c and Smac/DIABLO from mitochondria. Blood. 98(9):2828-36.

Dbaibo GS, Perry DK, Gamard CJ, Platt R, Poirier GG, Obeid LM, Hannun YA. Cytokine response modifier A (CrmA) inhibits ceramide formation in response to tumor necrosis factor (TNF)-alpha: CrmA and Bcl-2 target distinct components in the apoptotic pathway. J Exp Med. 185(3): 481-90.

Dean NM, Kanemitsu M, Boynton AL. 1989. Effects of the tyrosine-kinase inhibitor genistein on DNA synthesis and phospholipid-derived second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells. Biochem Biophys Res Commun. 165(2): 795-801.

Duan RD, Hertervig E, Nyberg L, Hauge T, Sternby B, Lillienau J, Farooqi A, Nilsson A. 1996. Distribution of alkaline sphingomyelinase activity in human beings and animals. Tissue and species differences. Dig. Dis. Sci. 41:1801-1806.

Duan RD, Nyberg L, Nilsson A. 1995. Alkaline sphingomyelinase in rat gastrointestinal tract: distribution and characteristics. Biochim. Biophys. Acta. 1259: 49-55.

Duan RD. 2005. Anticancer compounds and sphingolipid metabolism in the colon. In Vivo. 19(1):293-300.

Eisen JS, Pike SH, Debu B. 1989. The growth cones of identified motoneurons in embryonic zebrafish select appropriate pathways in the absence of specific cellular interactions. Neuron. 2(1):1097-104.

Ensminger AH, Ensminger ME, Konlade JE, Robson JRK. 1994. The Concise Encyclopedia of Food and Nutrition. CRC Press Boca Raton, FL. p 384-469.

Fang J, Xia C, Cao Z, Zheng JZ, Reed E, Jiang BH. 2005. Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. FASEB J. 19(3):342-53.

Fierro IM, Kutok JL, Serhan CN. 2002. Novel lipid mediator regulators of endothelial cell proliferation and migration: aspirin-triggered-15R-lipoxin A(4) and lipoxin A(4). J Pharmacol Exp Ther. 300(2):385-92.

Finley JW, Sigrid-Keck A, Robbins RJ, Hintze KJ. 2005. Selenium enrichment of broccoli: interactions between selenium and secondary plant compounds. J Nutr. 135(5):1236-8.

FitzGerald RJ, Murray BA, Walsh DJ. 2004. Hypotensive peptides from milk proteins. J. Nutr. 134(4): 980S-8S.

Folkman J, Kalluri R. 2004. Cancer without disease. Nature. 427(6977):787.

Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L. 1993. Genistein, a dietary-derived inhibitor of in vitro angiogenesis. Proc Natl Acad Sci U S A. 90(7):2690-4.

Fujino Y, Ohnishi M, Ito S. 1985. Molecular species of ceramide and mono-, di-, tri- and tetraglycosylceramide in bran and endosperm of rice grains. Agric. Biol. Chem. 49(9):2753-2762

Grothey A. 2005. Antiangiogenic therapy in cancer: a new era has begun. Oncology. 19(4 Suppl 3): 5-6.

Hanada K, Nishijima M, Kiso M, Hasegawa A, Fujita S, Ogawa T, Akamatsu Y. 1992. Sphingolipids are essential for the growth of Chinese hamster ovary cells. Restoration of the growth of a mutant defective in sphingoid base biosynthesis by exogenous sphingolipids. J. Biol. Chem. 267: 23527-23533.

Hanahan D, Weinberg R A. 2000. The hallmarks of cancer. Cell. 100(1):57-70.

Hasebe Y, Egawa K, Yamazaki Y, Kunimoto S, Hirai Y, Ida Y, Nose K. 2003. Specific Inhibition of Hypoxia-inducible Factor (HIF)-1α Activation and of Vascular Endothelial Growth Factor (VEGF) Production by Flavonoids. Biological & Pharmaceutical Bulletin. 26(10): 1379-1383.

Heather ER, Lo J, Randhall SJ. 1998. HIF-1α is required for solid tumor formation and embryonic vascularization. J EMBO. 17(11): 3005-3015.

Heinrich M, Wickel M, Schneider-Brachert W, Sandberg C, Gahr J, Schwandner R, Weber T, Saftig P, Peters C, Brunner J, Kronke M, Schutze S. 2000. Cathepsin D targeted by acid sphingomyelinase-derived ceramide. EMBO J. 17;19(2):315.

Hellwig-Burgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann W. 2005. Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions. J Interferon Cytokine Res. 25(6):297-310.

Hjorth J, Key B. 2002. Development of axon pathways in the zebrafish central nervous system. Int J Dev Biol. 46(4):609-19.

Hobson B, Denekamp J. 1984. Endothelial proliferation in tumors and normal tissues: continous labeling studies. Br. J. Cancer. 49(4): 405-413.

Huang Y, Hickey RP, Yeh JL, Liu D, Dadak A, Young LH, Johnson RS, Giordano FJ. 2004. Cardiac myocyte-specific HIF-1alpha deletion alters vascularization, energy availability, calcium

flux, and contractility in the normoxic heart. FASEB J. 18(10):1138-40.

Igarashi J, Erwin PA, Dantas AP, Chen H, Michel T. 2003. VEGF induces S1P1 receptors in endothelial cells: Implications for cross-talk between sphingolipid and growth factor receptors. Proc Natl Acad Sci U S A. 100(19):10664-9.

Isogai S, Horiguchi M, Weinstein BM. 2001. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. Dev Biol. 230(2): 278-301

Isogai S, Lawson ND, Torrealday S, Horiguchi M, Weinstein BM. 2003. Angiogenic network formation in the developing vertebrate trunk. Development. 130(21):5281-90.

Jensen RG. 1995. eds. Handbook of milk composition. Academic Press New York, NY.

Katsuma S, Hada Y, Ueda T, Shiojima S, Hirasawa A, Tanoue A, Takagaki K, Ohgi T, Yano J, Tsujimoto G. 2000. Signalling mechanisms in sphingosine 1-phosphate-promoted mesangial cell proliferation. Genes Cells. 7(12):1217-30.

Keenen TW, Patton S. 1995. The structure of milk: implications for sampling and storage. Jensen R. G. eds. Handbook of Milk Composition. Academic Press New York, NY. P 5-50.

Kieran MW, Folkman J, Heymach J. 2003. Angiogenesis inhibitors and hypoxia. Nat Med. 9(7): 822-3.

Kim CW, Lee HM, Lee TH, Kang C, Kleinman HK, Gho YS. 2002. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. Cancer Res. 62(21):6312-7.

Kim YS, Milner JA. 2005. Targets for indole-3-carbinol in cancer prevention. J Nutr Biochem. 16(2):65-73.

Kimura K, Markowski M, Edsall LC, Spiegel S, Gelmann EP. 2003. Role of ceramide in mediating apoptosis of irradiated LNCaP prostate cancer cells. Cell Death Differ. 2003 Feb;10(2):240-8.

Koblinski JE, Ahram M, Sloane BF. 2000. Unraveling the role of proteases in cancer. Clin Chim Acta. 291(2):113-35.

Kokkonen N, Rivinoja A, Kauppila A, Suokas M, Kellokumpu I, Kellokumpu S. 2004. Defective acidification of intracellular organelles results in aberrant secretion of cathepsin D in cancer cells. J Biol Chem. 279(38): 39982-8 .

Kolesnick R. 2002. The therapeutic potential of modulating the ceramide/sphingomyelin pathway. J Clin Invest. 110(1):3-8.

Le Stunff H, Mikami A, Giussani P, Hobson JP, Jolly PS, Milstien S, Spiegel S. 2004. Role of sphingosine-1-phosphate phosphatase 1 in epidermal growth factor-induced chemotaxis. J Biol Chem. 279(33):34290-7.

Lee M, Hwang JT, Lee HJ, Jung SN, Kang J, Chi SG, Kim SS, Ha J. 2003. AMP-activated

protein kinase is critical for hypoxia-induced factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU-145 cells. J Biol Chem. 278(41): 39653-39661.

Lee YJ, Amoscato AA. 2004. TRAIL and ceramide. Vitam Horm. 67: 229-55.

Lemonnier LA, Dillehay DL, Vespremi MJ, Abrams J, Brody E, Schmelz EM. 2003. Sphingomyelin in the suppression of colon tumors: prevention versus intervention. Arch Biochem Biophys. 419(2):129-38.

Lewis KE, Eisen JS. 2003. From cells to circuits: development of the zebrafish spinal cord. Prog Neurobiol. 69(6):419-49.

Liu RH, Liu J, Chen B. 2005. Apples prevent mammary tumors in rats. J Agric Food Chem. 53(6):2341-3.

Losso JN, Bansode RR. 2004. Anti-angiogenic functional food, degenerative disease and cancer. In: Remacle, C, and Reusens, B. Functional foods, ageing and degenerative disease. Woodhead publishing limited, Cambreidge: England. p 485-523.

Losso, J.N. 2002. Preventing degenerative by anti-angiogenic functional foods. Food Technol. 56(6): 78-88.

Mandon EC, van Echten G, Birk R, Schmidt RR, Sandhoff K. 1991. Sphingolipid biosynthesis in cultured neurons. Down-regulation of serine palmitoyltransferase by sphingoid bases. Eur. J. Biochem. 198: 667-674.

Matsushita K, Motani R, Sakuta T, Yamaguchi N, Koga T, Matsuo K, Nagaoka S, Abeyama K, Maruyama I, Torii M. 2000. The role of vascular endothelial growth factor in human dental pulp cells: induction of chemotaxis, proliferation, and differentiation and activation of the AP-1 dependent signaling pathway. J Dent Res. 79(8):1596-603.

Merrill AH Jr, Schemlz EM, Wang E, Dillehay DL, DL, Rice LG, Meredith F, Riley RT. 1997. Importance of sphingolipids and inhibitors of sphingolipid metabolism as components of animal diets. J Nutr. 127(5 Suppl): 830S-833S.

Merrill AH Jr, Sweeley CC. 1996. Sphingolipids: metabolism and cell signaling. Vance D. E. Vance J. E. eds. Biochemistry of lipids, lipoproteins and membranes. Elsevier New York, NY. p 43-73.

Merrill AH, Jr, Lingrell S, Wang E, Nikolova-Karakashian M, Vales TR, Vance DE. 1995. Sphingolipid biosynthesis de novo by rat hepatocytes in culture. Ceramide and sphingomyelin are associated with, but not required, for very low density lipoprotein secretion. J. Biol. Chem. 270: 13834-13841.

Merrill AH, Jr, Nixon DW, Williams RD. 1985. Activities of serine palmitoyl-transferase (3-ketosphinganine synthase) in microsomes from different rat tissues. J. Lipid Res. 26: 617-622.

Morales-Ruiz M, Lee MJ, Zollner S, Gratton JP, Scotland R, Shiojima I, Walsh K, Hla T, Sessa WC. 2001. Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis

through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. J Biol Chem. 276(22): 19672-7.

Morrison, WR. 1969. Polar lipids in bovine milk. I. Long-chain bases in sphingomyelin. Biochim. Biophys. Acta. 176: 537-546.

Nagiec MM, Lester RL, Dickson RC. 1996. Sphingolipid synthesis: identification and characterization of mammalian cDNAs encoding the Lcb2 subunit of serine palmitoyltransferase. Gene. 177: 237-241.

Negishi M, Oinuma I, Katoh H. 2005. Plexins: axon guidance and signal transduction. Cell Mol Life Sci. 62(12): 1363-1371.

Nilsson Å. 1968. Metabolism of sphingomyelin in the intestinal tract of the rat. Biochim. Biophys. Acta. 164: 575-584.

Nilsson Å. 1969a. Metabolism of cerebrosides in the intestinal tract of the rat. Biochim. Biophys. Acta. 187:113-121

Nilsson, Å. 1969b. The presence of sphingomyelin and ceramide-cleaving enzymes in the intestinal tract of the rat. Biochim. Biophys. Acta. 176:339-347

Nyberg L, Duan RD, Axelson J, Nilsson A. 1996. Identification of an alkaline sphingomyelinase activity in human bile. Biochim Biophys Acta. 1300(1): 42-8.

Nyberg L, Duan RD, Nilsson A. 2000. A mutual inhibitory effect on absorption of sphingomyelin and cholesterol. J Nutr Biochem. 11(5):244-9.

Nyberg L, Nilsson Å, Lundgren P, Duan R-D. 1998. Localization and capacity of sphingomyelin digestion in the rat intestinal tract. J. Nutr. Biochem. 8(3):112-118.

Ogretmen B, Hannun YA. 2004. Biologically active sphingolipids in cancer pathogenesis and treatment. Nat Rev Cancer. 4(8):604-16.

Ohnishi M, Fujino Y. 1982. Sphingolipids in immature and mature soybeans. Lipids. 17: 803-810.

Ohnishi M, Ito S, Fujino Y. 1983. Characterization of sphingolipids in spinach leaves. Biochim. Biophys. Acta. 752: 416-422.

Parng C. 2005. *In vivo* zebrafish assays for toxicity testing. Curr Opin Drug Discov Devel. 8(1):100-6.

Parodi PW. 1997. Cows' milk fat components as potential anticarcinogenic agents. The J Nutr. 127(6): 1055-1060.

Pugh CW, Ratcliffe PJ. 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med. 9(6):677-84.

Radeff-Huang J, Seasholtz TM, Matteo RG, Brown JH. 2004. G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. J Cell Biochem. 92(5):949-66.

Radin NS. 2003. Killing tumours by ceramide-induced apoptosis: a critique of available drugs. Biochem J. 371(Pt 2): 243-56.

Radin NS. 2004. Poly-drug cancer therapy based on ceramide. Eksp Onkol. 26(1): 3-10.

Reynolds CP, Maurer BJ, Kolesnick RN. 2004. Cancer Lett. 206(2):169-80.

Ribatti D, Vacca A, Roncali L, Dammacco F. 2000. The chick embryo chorioallantoic membrane as a model for in vivo research on anti-angiogenesis. Curr Pharm Biotechnol. 1(1):73-82.

Rosen L. 2000. Antiangiogenic stratergies and agents in clinical trials. Oncologist. 5 suppl 1:20-27.

Rosenfeldt HM, Hobson JP, Maceyka M, Olivera A, Nava VE, Milstien S, Spiegel S. 2001. EDG-1 links the PDGF receptor to Src and focal adhesion kinase activation leading to lamellipodia formation and cell migration. FASEB J. 15(14): 2649-59.

Rubinstein AL. 2003. Zebrafish: from disease modeling to drug discovery. Curr Opin Drug Disc. 6(2):218-223.

Ruvolo PP. 2003. Intracellular signal transduction pathways activated by ceramide and its metabolites. Pharmacol Res. 47(5):383-92.

Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O, Takuwa Y. 2002. Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. Circ Res. 90(3):325-32.

Sah JF, Balasubramanian S, Eckert RL, Rorke EA. 2004. Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases. J Biol Chem. 279(13):12755-62.

Schmelz E-M, Crall K L, LaRocque R, Dillehay DL, Merrill AH, Jr. 1994. Uptake and metabolism of sphingolipids in isolated intestinal loops of mice. J. Nutr. 124(5):702-712.

Schmelz E-M, Dillehay DL, Webb SK, Reiter A, Adams J, Merrill AH, Jr. 1996. Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with1, 2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. Cancer Res. 56: 4936-4941

Serbedzija GN, Flynn E, Willett CE. 1999. Zebrafish angiogenesis: a new model for drug screening. Angiogenesis. 3(4):353-9.

Servitja JM, Masgrau R, Pardo R, Sarri E, von Eichel-Streiber C, Gutkind JS, Picatoste F. 2003. Metabotropic glutamate receptors activate phospholipase D in astrocytes through a protein kinase C-dependent and Rho-independent pathway. Neuropharmacology. 44(2):171-80.

Shao ZM, Shen ZZ, Liu CH, Sartippour MR, Go VL, Heber D, Nguyen M. 2002. Curcumin exerts multiple suppressive effects on human breast carcinoma cells. Int J Cancer. 98(2):234-40.

Shoji W, Isogai S, Sato-Maeda M, Obinata M, Kuwada JY. 2003. Semaphorin3a1 regulates angioblast migration and vascular development in zebrafish embryos. Development. 130(14):3227-36.

Smith PC, Santibanez JF, Morales JP, Martinez J. 2004. Epidermal growth factor stimulates urokinase-type plasminogen activator expression in human gingival fibroblasts. Possible modulation by genistein and curcumin. J Periodontal Res. 39(6):380-7.

Sprague J, Doerry E, Douglas S, Westerfield M. 2001. The Zebrafish Information Network (ZFIN): a resource for genetic, genomic and developmental research. Nucleic Acids Res. 29: 87-90.

Staton CA, Stribbling SM, Tazzyman S, Hughes R, Brown NJ, Lewis CE. 2004. Current methods for assaying angiogenesis in vitro and in vivo. Int J Exp Pathol. 85(5):233-248.

Sumitomo M, Ohba M, Asakuma J, Asano T, Kuroki T, Asano T, Hayakawa M. 2002. Protein kinase Cdelta amplifies ceramide formation via mitochondrial signaling in prostate cancer cells. J Clin Invest. 109(6):827-36.

Svoboda KR, Linares AE, Ribera AB. 2001. Activity regulates programmed cell death of zebrafish Rohon-Beard neurons. Development. 128(18): 3511-20.

Svoboda KR, Vijayaraghavan S, Tanguay RL. 2002. Nicotinic receptors mediate changes in spinal motoneuron development and axonal pathfinding in embryonic zebrafish exposed to nicotine. J Neurosci. 22(24): 10731-41.

Symolon H, Schmelz EM, Dillehay DL, Merrill AH Jr. 2004. Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1,2-dimethylhydrazine-treated CF1 mice and ApcMin/+ mice. J Nutr. 134(5): 1157-1161.

Testi R. 1996. Sphingomyelin breakdown and cell fate. Trends Biochem Sci. 21(2): 468-471.

Toescu EC. 2004. Hypoxia response elements. Cell Calcium. 2004 36(3-4):181-5.

Tosetti F, Frrari N, Flora SD, Albini A. 2002. Angioprevention: angiogenesis is a common and key target for cancer chemopreventive agents. The FASEB J. 4(16): 2-14.

Tsai JC, Teng LJ, Chen CT, Hong TM, Goldman CK, Gillespie GY. 2003. Protein kinase C mediates induced secretion of vascular endothelial growth factor by human glioma cells. Biochem Biophys Res Commun. 309(4):952-60.

Van Brocklyn JR, Lee MJ, Menzeleev R, Olivera A, Edsall L, Cuvillier O, Thomas DM, Coopman PJP, Thangada S, Liu CH, Hla T, Spiegel S. 1998. Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. J. Cell Biol. 142: 229-240.

van Echten-Deckert G, Zschoche A, Bär T, Schmidt RR, Raths A, Heinemann T, Sandhoff K. 1997. cis-4-Methylsphingosine decreases sphingolipid biosynthesis by specifically interfering with serine palmitoyltransferase activity in primary cultured neurons. J. Biol. Chem. 272: 15825-15833.

Vesper H, Schmelz EM, Nikolova-Karakashian MN, Dillehay DL, Lynch DV, Merrill AH Jr. 1999. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. J Nutr. 129(7):1239-50.

Vogel AM, Weinstein BM. 2000. Studying vascular development in the zebrafish. Trends Cardiovasc Med. 10(8):352-360

Volate SR, Davenport DM, Muga SJ, Wargovich MJ. 2005. Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng and rutin). Carcinogenesis. 26(8):1450-6.

Wang E, Ross PF, Wilson TM, Riley RT, Merrill AH, Jr. 1992. Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. J. Nutr. 122:1706-1716.

Wang J, Hu XS, Shi JP. 2003. Sphingolipid and apoptosis. Sheng Li Ke Xue Jin Zhan. 34(3):217-21.

Ward RE, German JB. 2004. Understanding milk's bioactive component: A goal foe the genomic toolbox. J Nutr. 134(4): 962S-7S.

West DC, Thompson WD, Sells PG, Burbridge MF. 2001. Angiogenesis assays using chick chorioallantoic membran. (ed): Murray CJ, in Methods in molecular medicine. vol. 46. Angiogenesis Protocol. Human Press Inc. Totowa, NJ.

Whitaker BD. 1996. Cerebrosides in mature-green and red-ripe bell pepper and tomato fruits. Phytochemistry. 42: 627-632.

Wilkinson-Berka JL. 2004. Vasoactive factors and diabetic retinopathy: vascular endothelial growth factor, cycoloxygenase-2 and nitric oxide. Curr Pharm Des. 10(27):3331-48.

Willaime S, Vanhoutte P, Caboche J, Lemaigre-Dubreuil Y, Mariani J, Brugg B. 2001. Ceramide-induced apoptosis in cortical neurons is mediated by an increase in p38 phosphorylation and not by the decrease in ERK phosphorylation. Eur J Neurosci. 13(11):2037-46.

Wilson E, Wang E, Mullins RE, Uhlinger DJ, Liotta DC, Lambeth JD, Merrill AH, Jr. 1988. Modulation of free sphingosine levels in human neutrophils by phorbol esters and other factors. J. Biol. Chem. 263: 9304-9309.

Yabu T, Tomimoto H, Taguchi Y, Yamaoka S, Igarashi Y, Okazaki T. 2005. Thalidomide-induced anti-angiogenic action is mediated by ceramide through depletion of VEGF receptors, and antagonized by sphingosine-1-phosphate. Blood. Prepublished online March 1, 2005; DOI 10.1182/blood-2004-09-3679.

Yang J, Yu Y, Sun S, Duerksen-Hughes PJ. 2004. Ceramide and other sphingolipids in cellular responses. Cell Biochem Biophys. 40(3): 323-50.

Yatomi Y, Ruan F, Hakomori S, Igarashi Y. 1995. Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood. 86: 193-202.

Zeisel SH, Char D, Sheard NF. 1986. Choline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. J. Nutr. 116:50-58

Zheng ZS, Xue GZ, Grunberger D, Prystowsky JH. 1995. Caffeic acid phenethyl ester inhibits proliferation of human keratinocytes and interferes with the EGF regulation of ornithine decarboxylase. Oncol Res. 7(9):445-52.

APPENDIX A: SAS POST-HOC ANALYSIS

Cell Migration

HEL 299

Testing the effect of compounds on cell migration of cancer cell lines 10

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	97.1764
Critical Value of t	2.77645
Least Significant Difference	27.37

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
control - glu	43.525	16.155	70.895	***
control - cer	50.180	22.810	77.550	***
control - spm	80.910	53.540	108.280	***
glu - control	-43.525	-70.895	-16.155	***
glu - cer	6.655	-20.715	34.025	
glu - spm	37.385	10.015	64.755	***
cer - control	-50.180	-77.550	-22.810	***
cer - glu	-6.655	-34.025	20.715	
cer - spm	30.730	3.360	58.100	***
spm - control	-80.910	-108.280	-53.540	***
spm - glu	-37.385	-64.755	-10.015	***
spm - cer	-30.730	-58.100	-3.360	***

MCF-7 Normoxic

Testing the effect of compounds on cell migration of cancer cell lines 11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

```
Alpha                           0.05
Error Degrees of Freedom           4
Error Mean Square             170.8516
Critical Value of t            2.77645
Least Significant Difference    36.291


Comparisons significant at the 0.05 level are indicated by ***.


                    Difference
       trt             Between     95% Confidence
   Comparison            Means         Limits

control - spm             2.89     -33.41   39.18
control - glu             9.41     -26.88   45.70
control - cer            11.39     -24.90   47.68
spm     - control        -2.89     -39.18   33.41
spm     - glu             6.52     -29.77   42.82
spm     - cer             8.50     -27.79   44.80
glu     - control        -9.41     -45.70   26.88
glu     - spm            -6.52     -42.82   29.77
glu     - cer             1.98     -34.31   38.27
cer     - control       -11.39     -47.68   24.90
cer     - spm            -8.50     -44.80   27.79
cer     - glu            -1.98     -38.27   34.31
```

MCF-7 Hypoxic

```
Testing the effect of compounds on cell migration of cancer cell lines      11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.


Alpha                           0.05
Error Degrees of Freedom           4
Error Mean Square             162.4459
Critical Value of t            2.77645
Least Significant Difference    35.387


Comparisons significant at the 0.05 level are indicated by ***.


                    Difference
       trt             Between     95% Confidence
   Comparison            Means         Limits

glu     - spm             3.80     -31.59   39.18
glu     - control         5.06     -30.33   40.44
```

glu	- cer	39.48	4.09	74.87	***
spm	- glu	-3.80	-39.18	31.59	
spm	- control	1.26	-34.13	36.65	
spm	- cer	35.69	0.30	71.07	***
control	- glu	-5.06	-40.44	30.33	
control	- spm	-1.26	-36.65	34.13	
control	- cer	34.43	-0.96	69.81	
cer	- glu	-39.48	-74.87	-4.09	***
cer	- spm	-35.69	-71.07	-0.30	***
cer	- control	-34.43	-69.81	0.96	

Caco-2 Normoxic

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	100.8373
Critical Value of t	2.77645
Least Significant Difference	27.88

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison		Difference Between Means	95% Confidence Limits		
control	- glu	50.16	22.28	78.04	***
control	- cer	63.25	35.37	91.13	***
control	- spm	67.11	39.23	94.99	***
glu	- control	-50.16	-78.04	-22.28	***
glu	- cer	13.09	-14.79	40.97	
glu	- spm	16.95	-10.93	44.83	
cer	- control	-63.25	-91.13	-35.37	***
cer	- glu	-13.09	-40.97	14.79	
cer	- spm	3.86	-24.02	31.74	
spm	- control	-67.11	-94.99	-39.23	***
spm	- glu	-16.95	-44.83	10.93	
spm	- cer	-3.86	-31.74	24.02	

Caco-2 Hypoxic

```
Testing the effect of compounds on cell migration of cancer cell lines      11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.


Alpha                           0.05
Error Degrees of Freedom           4
Error Mean Square           136.3099
Critical Value of t          2.77645
Least Significant Difference  32.416


Comparisons significant at the 0.05 level are indicated by ***.


                    Difference
       trt             Between     95% Confidence
   Comparison           Means         Limits

control - glu            42.24       9.82   74.65  ***
control - cer            48.14      15.72   80.56  ***
control - spm            64.58      32.17   97.00  ***
glu     - control       -42.24     -74.65   -9.82  ***
glu     - cer             5.90     -26.51   38.32
glu     - spm            22.35     -10.07   54.77
cer     - control       -48.14     -80.56  -15.72  ***
cer     - glu            -5.90     -38.32   26.51
cer     - spm            16.44     -15.97   48.86
spm     - control       -64.58     -97.00  -32.17  ***
spm     - glu           -22.35     -54.77   10.07
spm     - cer           -16.44     -48.86   15.97
```

DU-145 Normoxic

```
Testing the effect of compounds on cell migration of cancer cell lines      11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value


NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.
```

```
Alpha                           0.05
Error Degrees of Freedom           4
Error Mean Square            84.58778
Critical Value of t           2.77645
Least Significant Difference   25.535


Comparisons significant at the 0.05 level are indicated by ***.


                    Difference
       trt             Between      95% Confidence
   Comparison           Means           Limits

control - spm           38.955      13.420   64.490  ***
control - cer           52.285      26.750   77.820  ***
control - glu           91.550      66.015  117.085  ***
spm     - control      -38.955     -64.490  -13.420  ***
spm     - cer           13.330     -12.205   38.865
spm     - glu           52.595      27.060   78.130  ***
cer     - control      -52.285     -77.820  -26.750  ***
cer     - spm          -13.330     -38.865   12.205
cer     - glu           39.265      13.730   64.800  ***
glu     - control      -91.550    -117.085  -66.015  ***
glu     - spm          -52.595     -78.130  -27.060  ***
glu     - cer          -39.265     -64.800  -13.730  ***
```

DU-145 Hypoxic

```
Testing the effect of compounds on cell migration of cancer cell lines      11


ANOVA with SAS PROC GLM with post hoc and HOV tests


The GLM Procedure


t Tests (LSD) for value


NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.


Alpha                           0.05
Error Degrees of Freedom           4
Error Mean Square           120.2951
Critical Value of t           2.77645
Least Significant Difference   30.452
```

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
control - glu	3.93	-26.53	34.38	
control - spm	26.11	-4.34	56.56	
control - cer	39.22	8.76	69.67	***
glu - control	-3.93	-34.38	26.53	
glu - spm	22.19	-8.27	52.64	
glu - cer	35.29	4.84	65.74	***
spm - control	-26.11	-56.56	4.34	
spm - glu	-22.19	-52.64	8.27	
spm - cer	13.10	-17.35	43.56	
cer - control	-39.22	-69.67	-8.76	***
cer - glu	-35.29	-65.74	-4.84	***
cer - spm	-13.10	-43.56	17.35	

VEGF Levels
HEL 299

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000017
Critical Value of t	2.77645
Least Significant Difference	0.0114

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
cer - spm	0.013000	0.001552	0.024448	***
cer - glu	0.022000	0.010552	0.033448	***
cer - control	0.023000	0.011552	0.034448	***
spm - cer	-0.013000	-0.024448	-0.001552	***
spm - glu	0.009000	-0.002448	0.020448	
spm - control	0.010000	-0.001448	0.021448	
glu - cer	-0.022000	-0.033448	-0.010552	***
glu - spm	-0.009000	-0.020448	0.002448	
glu - control	0.001000	-0.010448	0.012448	
control - cer	-0.023000	-0.034448	-0.011552	***
control - spm	-0.010000	-0.021448	0.001448	
control - glu	-0.001000	-0.012448	0.010448	

MCF-7 Normoxic

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000089
Critical Value of t	2.77645
Least Significant Difference	0.0262

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
glu - spm	0.000000	-0.026230	0.026230	
glu - control	0.006500	-0.019730	0.032730	
glu - cer	0.026500	0.000270	0.052730	***
spm - glu	0.000000	-0.026230	0.026230	
spm - control	0.006500	-0.019730	0.032730	
spm - cer	0.026500	0.000270	0.052730	***
control - glu	-0.006500	-0.032730	0.019730	
control - spm	-0.006500	-0.032730	0.019730	
control - cer	0.020000	-0.006230	0.046230	
cer - glu	-0.026500	-0.052730	-0.000270	***
cer - spm	-0.026500	-0.052730	-0.000270	***
cer - control	-0.020000	-0.046230	0.006230	

MCF-7 Hypoxic

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000413
Critical Value of t	2.77645
Least Significant Difference	0.0564

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
spm - control	0.00800	-0.04843	0.06443	
spm - glu	0.02100	-0.03543	0.07743	
spm - cer	0.03050	-0.02593	0.08693	
control - spm	-0.00800	-0.06443	0.04843	
control - glu	0.01300	-0.04343	0.06943	
control - cer	0.02250	-0.03393	0.07893	
glu - spm	-0.02100	-0.07743	0.03543	
glu - control	-0.01300	-0.06943	0.04343	
glu - cer	0.00950	-0.04693	0.06593	
cer - spm	-0.03050	-0.08693	0.02593	
cer - control	-0.02250	-0.07893	0.03393	
cer - glu	-0.00950	-0.06593	0.04693	

Caco-2 Normoxic

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.00052
Critical Value of t	2.77645
Least Significant Difference	0.0633

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits	
spm - glu	0.04150	-0.02182	0.10482
spm - cer	0.04950	-0.01382	0.11282
spm - control	0.05150	-0.01182	0.11482
glu - spm	-0.04150	-0.10482	0.02182
glu - cer	0.00800	-0.05532	0.07132
glu - control	0.01000	-0.05332	0.07332
cer - spm	-0.04950	-0.11282	0.01382
cer - glu	-0.00800	-0.07132	0.05532
cer - control	0.00200	-0.06132	0.06532
control - spm	-0.05150	-0.11482	0.01182
control - glu	-0.01000	-0.07332	0.05332
control - cer	-0.00200	-0.06532	0.06132

Caco-2 Hypoxic

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.000529
Critical Value of t	2.77645
Least Significant Difference	0.0639

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits	
spm - glu	0.00700	-0.05688	0.07088
spm - cer	0.00850	-0.05538	0.07238
spm - control	0.06200	-0.00188	0.12588
glu - spm	-0.00700	-0.07088	0.05688
glu - cer	0.00150	-0.06238	0.06538
glu - control	0.05500	-0.00888	0.11888
cer - spm	-0.00850	-0.07238	0.05538
cer - glu	-0.00150	-0.06538	0.06238
cer - control	0.05350	-0.01038	0.11738
control - spm	-0.06200	-0.12588	0.00188
control - glu	-0.05500	-0.11888	0.00888
control - cer	-0.05350	-0.11738	0.01038

DU-145 Normoxic

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.004069
Critical Value of t	2.77645
Least Significant Difference	0.1771

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
control - spm	0.18650	0.00940	0.36360	***
control - cer	0.18750	0.01040	0.36460	***
control - glu	0.20500	0.02790	0.38210	***
spm - control	-0.18650	-0.36360	-0.00940	***
spm - cer	0.00100	-0.17610	0.17810	
spm - glu	0.01850	-0.15860	0.19560	
cer - control	-0.18750	-0.36460	-0.01040	***
cer - spm	-0.00100	-0.17810	0.17610	
cer - glu	0.01750	-0.15960	0.19460	
glu - control	-0.20500	-0.38210	-0.02790	***
glu - spm	-0.01850	-0.19560	0.15860	
glu - cer	-0.01750	-0.19460	0.15960	

DU-145 Hypoxic

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.001583
Critical Value of t	2.77645
Least Significant Difference	0.1105

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits	
control - cer	0.00250	-0.10797	0.11297
control - spm	0.04000	-0.07047	0.15047
control - glu	0.08800	-0.02247	0.19847
cer - control	-0.00250	-0.11297	0.10797
cer - spm	0.03750	-0.07297	0.14797
cer - glu	0.08550	-0.02497	0.19597
spm - control	-0.04000	-0.15047	0.07047
spm - cer	-0.03750	-0.14797	0.07297
spm - glu	0.04800	-0.06247	0.15847
glu - control	-0.08800	-0.19847	0.02247
glu - cer	-0.08550	-0.19597	0.02497
glu - spm	-0.04800	-0.15847	0.06247

Cathepsin D

HEL 299

```
Testing the effect of compounds on VEGF expression on cancer cell lines     11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.


Alpha                            0.05
Error Degrees of Freedom            4
Error Mean Square            8.294888
Critical Value of t           2.77645
Least Significant Difference   7.9964


Comparisons significant at the 0.05 level are indicated by ***.


                     Difference
       trt              Between      95% Confidence
   Comparison             Means          Limits

cer     - control        10.339       2.343   18.335  ***
cer     - spm            54.237      46.241   62.234  ***
cer     - glu            55.932      47.936   63.929  ***
control - cer           -10.339     -18.335   -2.343  ***
control - spm            43.898      35.902   51.895  ***
control - glu            45.593      37.597   53.590  ***
spm     - cer           -54.237     -62.234  -46.241  ***
spm     - control       -43.898     -51.895  -35.902  ***
spm     - glu             1.695      -6.301    9.691
glu     - cer           -55.932     -63.929  -47.936  ***
glu     - control       -45.593     -53.590  -37.597  ***
glu     - spm            -1.695      -9.691    6.301
```

MCF-7 Normoxic

```
Testing the effect of compounds on VEGF expression on cancer cell lines     11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.

Alpha                            0.05
Error Degrees of Freedom            4
Error Mean Square            217.9845
```

```
Critical Value of t           2.77645
Least Significant Difference   40.992


Comparisons significant at the 0.05 level are indicated by ***.


                    Difference
       trt             Between      95% Confidence
   Comparison            Means          Limits

cer     - control         42.37        1.38    83.37  ***
cer     - glu             79.66       38.67   120.65  ***
cer     - spm            106.78       65.79   147.77  ***
control - cer            -42.37      -83.37    -1.38  ***
control - glu             37.29       -3.70    78.28
control - spm             64.41       23.41   105.40  ***
glu     - cer            -79.66     -120.65   -38.67  ***
glu     - control        -37.29      -78.28     3.70
glu     - spm             27.12      -13.87    68.11
spm     - cer           -106.78     -147.77   -65.79  ***
spm     - control        -64.41     -105.40   -23.41  ***
spm     - glu            -27.12      -68.11    13.87
```

MCF-7 Hypoxic

```
Testing the effect of compounds on VEGF expression on cancer cell lines     10

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.


Alpha                           0.05
Error Degrees of Freedom           4
Error Mean Square            174.585
Critical Value of t          2.77645
Least Significant Difference  36.685


Comparisons significant at the 0.05 level are indicated by ***.


                    Difference
       trt             Between      95% Confidence
   Comparison            Means          Limits

cer     - control         50.85       14.16    87.53  ***
cer     - glu             57.63       20.94    94.31  ***
cer     - spm            179.66      142.98   216.35  ***
control - cer            -50.85      -87.53   -14.16  ***
control - glu              6.78      -29.91    43.47
```

```
control - spm            128.81       92.13   165.50  ***
glu     - cer            -57.63      -94.31   -20.94  ***
glu     - control         -6.78      -43.47    29.91
glu     - spm            122.03       85.35   158.72  ***
spm     - cer           -179.66     -216.35  -142.98  ***
spm     - control       -128.81     -165.50   -92.13  ***
spm     - glu           -122.03     -158.72   -85.35  ***
```

Caco-2 Normoxic

```
Testing the effect of compounds on VEGF expression on cancer cell lines     11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the
experimentwise error rate.


Alpha                            0.05
Error Degrees of Freedom            4
Error Mean Square           58.89442
Critical Value of t          2.77645
Least Significant Difference   21.307


Comparisons significant at the 0.05 level are indicated by ***.


                    Difference
       trt             Between       95% Confidence
   Comparison           Means           Limits

cer     - control        28.814        7.506    50.121  ***
cer     - glu           100.000       78.693   121.307  ***
cer     - spm           118.644       97.337   139.951  ***
control - cer           -28.814      -50.121    -7.506  ***
control - glu            71.186       49.879    92.494  ***
control - spm            89.831       68.523   111.138  ***
glu     - cer          -100.000     -121.307   -78.693  ***
glu     - control       -71.186      -92.494   -49.879  ***
glu     - spm            18.644       -2.663    39.951
spm     - cer          -118.644     -139.951   -97.337  ***
spm     - control       -89.831     -111.138   -68.523  ***
spm     - glu           -18.644      -39.951     2.663
```

Caco-2 Hypoxic

```
Testing the effect of compounds on VEGF expression on cancer cell lines     11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value
```

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	14.88106
Critical Value of t	2.77645
Least Significant Difference	10.71

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
cer - spm	25.424	14.713	36.134	***
cer - glu	28.814	18.103	39.524	***
cer - control	28.814	18.103	39.524	***
spm - cer	-25.424	-36.134	-14.713	***
spm - glu	3.390	-7.321	14.100	
spm - control	3.390	-7.321	14.100	
glu - cer	-28.814	-39.524	-18.103	***
glu - spm	-3.390	-14.100	7.321	
glu - control	0.000	-10.710	10.710	
control - cer	-28.814	-39.524	-18.103	***
control - spm	-3.390	-14.100	7.321	
control - glu	0.000	-10.710	10.710	

DU-145 Normoxic

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	10.31086
Critical Value of t	2.77645
Least Significant Difference	8.9153

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
control - glu	27.119	18.203	36.034	***
control - cer	62.712	53.797	71.627	***
control - spm	75.424	66.508	84.339	***
glu - control	-27.119	-36.034	-18.203	***
glu - cer	35.593	26.678	44.509	***
glu - spm	48.305	39.390	57.220	***
cer - control	-62.712	-71.627	-53.797	***
cer - glu	-35.593	-44.509	-26.678	***
cer - spm	12.712	3.797	21.627	***
spm - control	-75.424	-84.339	-66.508	***
spm - glu	-48.305	-57.220	-39.390	***
spm - cer	-12.712	-21.627	-3.797	***

DU-145 Hypoxic

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	6.988647
Critical Value of t	2.77645
Least Significant Difference	7.3398

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
control - spm	66.102	58.762	73.442	***
control - cer	67.797	60.457	75.136	***
control - glu	69.492	62.152	76.831	***
spm - control	-66.102	-73.442	-58.762	***
spm - cer	1.695	-5.645	9.035	
spm - glu	3.390	-3.950	10.730	
cer - control	-67.797	-75.136	-60.457	***
cer - spm	-1.695	-9.035	5.645	
cer - glu	1.695	-5.645	9.035	
glu - control	-69.492	-76.831	-62.152	***
glu - spm	-3.390	-10.730	3.950	
glu - cer	-1.695	-9.035	5.645	

HIF Levels

MCF-7 Hypoxic

Testing the effect of compounds on HIF expression on cancer cell lines 11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	5
Error Mean Square	0.000051
Critical Value of t	2.57058
Least Significant Difference	0.0183

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
cntrH - cerH	0.065500	0.047163	0.083837	***
cntrH - spmH	0.075500	0.057163	0.093837	***
cntrH - gluH	0.084000	0.065663	0.102337	***
cntrH - cntrN	0.120000	0.101663	0.138337	***
cerH - cntrH	-0.065500	-0.083837	-0.047163	***
cerH - spmH	0.010000	-0.008337	0.028337	
cerH - gluH	0.018500	0.000163	0.036837	***
cerH - cntrN	0.054500	0.036163	0.072837	***
spmH - cntrH	-0.075500	-0.093837	-0.057163	***
spmH - cerH	-0.010000	-0.028337	0.008337	
spmH - gluH	0.008500	-0.009837	0.026837	
spmH - cntrN	0.044500	0.026163	0.062837	***
gluH - cntrH	-0.084000	-0.102337	-0.065663	***
gluH - cerH	-0.018500	-0.036837	-0.000163	***
gluH - spmH	-0.008500	-0.026837	0.009837	
gluH - cntrN	0.036000	0.017663	0.054337	***
cntrN - cntrH	-0.120000	-0.138337	-0.101663	***
cntrN - cerH	-0.054500	-0.072837	-0.036163	***
cntrN - spmH	-0.044500	-0.062837	-0.026163	***
cntrN - gluH	-0.036000	-0.054337	-0.017663	***

Caco-2 Hypoxic

Testing the effect of compounds on HIF expression on cancer cell lines 11

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	5
Error Mean Square	0.00004
Critical Value of t	2.57058
Least Significant Difference	0.0162

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	95% Confidence Limits		
cerH - cntrH	0.000000	-0.016243	0.016243	
cerH - cntrN	0.012000	-0.004243	0.028243	
cerH - gluH	0.012500	-0.003743	0.028743	
cerH - spmH	0.060500	0.044257	0.076743	***
cntrH - cerH	0.000000	-0.016243	0.016243	
cntrH - cntrN	0.012000	-0.004243	0.028243	
cntrH - gluH	0.012500	-0.003743	0.028743	
cntrH - spmH	0.060500	0.044257	0.076743	***
cntrN - cerH	-0.012000	-0.028243	0.004243	
cntrN - cntrH	-0.012000	-0.028243	0.004243	
cntrN - gluH	0.000500	-0.015743	0.016743	
cntrN - spmH	0.048500	0.032257	0.064743	***
gluH - cerH	-0.012500	-0.028743	0.003743	
gluH - cntrH	-0.012500	-0.028743	0.003743	
gluH - cntrN	-0.000500	-0.016743	0.015743	
gluH - spmH	0.048000	0.031757	0.064243	***
spmH - cerH	-0.060500	-0.076743	-0.044257	***
spmH - cntrH	-0.060500	-0.076743	-0.044257	***
spmH - cntrN	-0.048500	-0.064743	-0.032257	***
spmH - gluH	-0.048000	-0.064243	-0.031757	***

DU-145 Hypoxic

ANOVA with SAS PROC GLM with post hoc and HOV tests

The GLM Procedure

t Tests (LSD) for value

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	5

```
Error Mean Square            0.000062
Critical Value of t           2.57058
Least Significant Difference   0.0203


Comparisons significant at the 0.05 level are indicated by ***.


                Difference
     trt           Between       95% Confidence
 Comparison          Means           Limits

cntrH - gluH       0.073000     0.052748  0.093252  ***
cntrH - spmH       0.081500     0.061248  0.101752  ***
cntrH - cntrN      0.082500     0.062248  0.102752  ***
cntrH - cerH       0.091500     0.071248  0.111752  ***
gluH  - cntrH     -0.073000    -0.093252 -0.052748  ***
gluH  - spmH       0.008500    -0.011752  0.028752
gluH  - cntrN      0.009500    -0.010752  0.029752
gluH  - cerH       0.018500    -0.001752  0.038752
spmH  - cntrH     -0.081500    -0.101752 -0.061248  ***
spmH  - gluH      -0.008500    -0.028752  0.011752
spmH  - cntrN      0.001000    -0.019252  0.021252
spmH  - cerH       0.010000    -0.010252  0.030252
cntrN - cntrH     -0.082500    -0.102752 -0.062248  ***
cntrN - gluH      -0.009500    -0.029752  0.010752
cntrN - spmH      -0.001000    -0.021252  0.019252
cntrN - cerH       0.009000    -0.011252  0.029252
cerH  - cntrH     -0.091500    -0.111752 -0.071248  ***
cerH  - gluH      -0.018500    -0.038752  0.001752
cerH  - spmH      -0.010000    -0.030252  0.010252
cerH  - cntrN     -0.009000    -0.029252  0.011252
```

APPENDIX B: COPYRIGHT PERMISSION LETTER

biology
cob
The Company of Biologists Ltd
Bidder Building, 140 Cowley Road, Cambridge CB4 0DL, UK,
Tel: (01223) 425 525 Fax: (01223) 423 520
e-mail: Cathy@biologists.com

Rishipal R. Bansode
Affiliation: Louisiana State University
111 Food Science Building
Louisiana State University
Baton Rouge,
LA 70802
USA

27 October 2005

Fax: number 1-225-578-5300

Dear Rishipal

Please find below the signed permissions form that you requested.
Yours sincerely,

P.P. Mandy Knowles

Cathy Walters
Permissions Administrator

Journal Reference: Development 2002 129: 973-982

Title: **Patterning of angiogenesis in the zebrafish embryo**

Authors:

Sarah Childs, Jau-Nian Chen, Deborah M. Garrity, and Mark C. Fishman

PERMISSION GRANTED
PLEASE CREDIT JOURNAL REFERENCE
AND COMPANY OF BIOLOGISTS LTD.

For The Company of Biologists Ltd

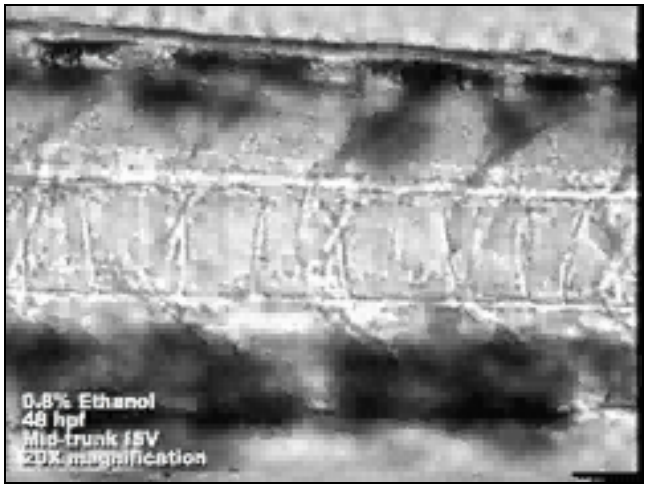
Mandy Knowles
Permissions Administrator

APPENDIX C: SUPPLEMENT DATA

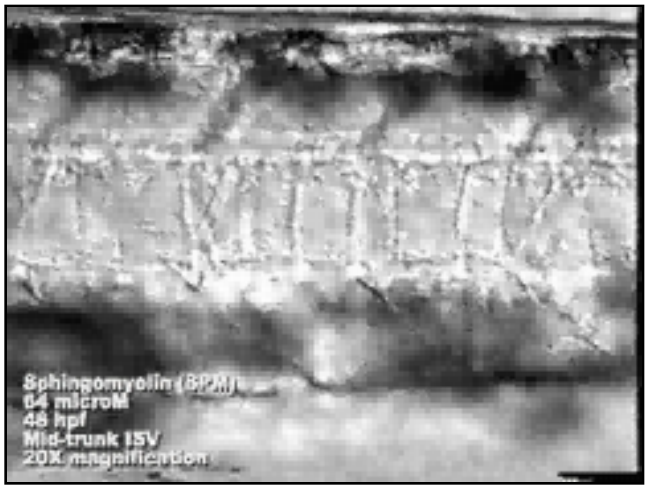
Supplemental Movies of Zebrafish Sphingolipid Exposure during Embryogenesis

Movie 1: Videomicrograph of Circulation of the Mid-trunk of a 48 hpf DMSO Treated Zebrafish Embryo.
Anterior is to the right, dorsal is up. Blood flows caudally (to the left) via the dorsal aorta (DA) and return rostrally (to the right) via posterior cardinal vein (PCV) aligned near intersomitic boundaries carry blood dorsally through intersegmental arteries and ventrally through intersegmental veins.

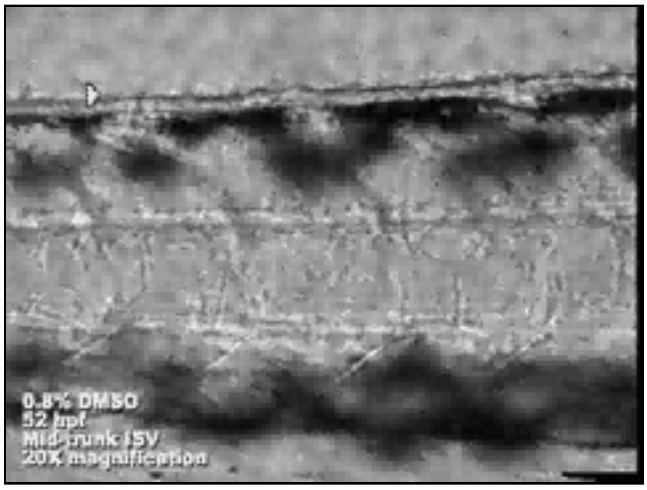
Movie 2: Videomicrograph of Circulation of the Mid-trunk of a 48 hpf Ceramide Treated Zebrafish Embryo.
Anterior is to the right, dorsal is up. Blood flows caudally (to the left) via the dorsal aorta (DA) and return rostrally (to the right) via posterior cardinal vein (PCV) aligned near intersomitic boundaries carry blood dorsally through intersegmental arteries and ventrally through intersegmental veins.



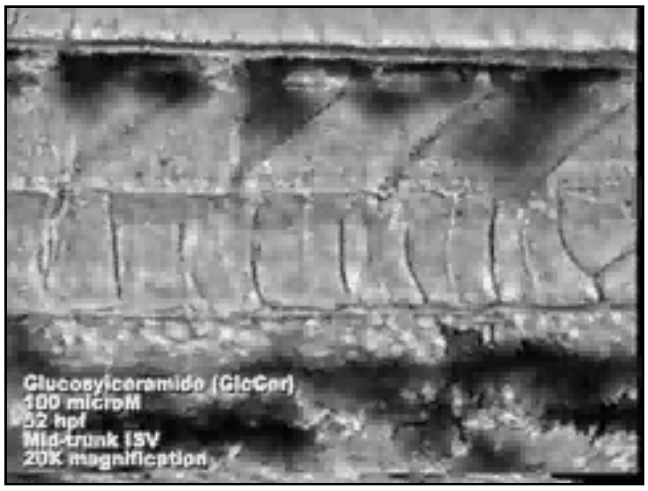
Movie 3: Videomicrograph of Circulation of the Mid-trunk of a 48 hpf Ethanol Treated Zebrafish Embryo.
Anterior is to the left, dorsal is up. Blood flows caudally (to the right) via the dorsal aorta (DA) and return rostrally (to the left) via posterior cardinal vein (PCV) aligned near intersomitic boundaries carry blood dorsally through intersegmental arteries and ventrally through intersegmental veins.



Movie 4: Videomicrograph of Circulation of the Mid-trunk of a 48 hpf Sphingomyelin Treated Zebrafish Embryo.
Anterior is to the left, dorsal is up. Blood flows caudally (to the right) via the dorsal aorta (DA) and return rostrally (to the left) via posterior cardinal vein (PCV) aligned near intersomitic boundaries carry blood dorsally through intersegmental arteries and ventrally through intersegmental veins.



Movie 5: Videomicrograph of Circulation of the Mid-trunk of a 52 hpf DMSO Treated Zebrafish Embryo.
Anterior is to the right, dorsal is up. Blood flows caudally (to the left) via the dorsal aorta (DA) and return rostrally (to the right) via posterior cardinal vein (PCV) aligned near intersomitic boundaries carry blood dorsally through intersegmental arteries and ventrally through intersegmental veins.



Movie 6: Videomicrograph of Circulation of the Mid-trunk of a 52 hpf Glucosylceramide Treated Zebrafish Embryo.
Anterior is to the left, dorsal is up. Blood flows caudally (to the right) via the dorsal aorta (DA) and return rostrally (to the left) via posterior cardinal vein (PCV) aligned near intersomitic boundaries carry blood dorsally through intersegmental arteries and ventrally through intersegmental veins.

VITA

Mr. Bansode is pursuing a doctoral program in food science at Louisiana State University, Baton Rouge. He holds a master's in food science from Louisiana State University, Baton Rouge, and a Bachelor of Technology in food processing and preservation technology, Osmania University, India. He has worked as a quality assurance officer in Concept Foods PVT. LTD, India (an ISO 9001 certified company), and as a quality control and R&D officer in Bambino Agro Industries (India)-convenient food division.

His primary expertise is in identifying the anti-angiogenic properties of bioactive components in food. He has co-authored a book chapter along with his supervising professor in 'Functional foods, ageing and degenerative disease' published by Woodhead Publishing Ltd., Cambridge, England. He was awarded the Barkate scholarship for outstanding graduate student in 2004 by the Department of Food Science. He is a certified trainee in hazard analysis and critical control points (HACCP) awarded by Association of Food and Drug Officials (AFDO) and in retort operations, processing system operations, aseptic processing and packaging operations course prescribed by U.S.F.D.A. at Louisiana State University A & M, Baton Rouge, Louisiana. He is an accredited member of Institute of Food Technologists (IFT), American Oil Chemists Society (AOCS) and American Association for the Advancement in Science (AAAS).