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Cellular and Biochemical Effects of Sparstolonin B on Endothelial Cells to Inhibit Angiogenesis

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Cellular and Biochemical Effects of Sparstolonin B on Endothelial Cells to Inhibit Angiogenesis

by

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DEDICATION

This thesis is dedicated to my lovely husband, Suliman, who has been a constant source of support and encouragement during the challenges of graduate school and life. I am truly thankful for having you in my life, and for helping me figuring out my path for bright future. This work is also dedicated to my parents who have always loved me, supported me and who were role models to work hard for the things that I aspire to achieve. They were always paying for my safety and success.

I would also to thank my daughters and sons who I really faced with them the hardest time that we may face far away from our home country. They were patient enough to postpone all of their fun plans in order for me to complete my mission; we have always being passionate to support each other with love and caring. It is my pleasure also to thank my sister for her support and love, and I thank my brothers, nieces and nephews for their care and love.

I dedicate this work to the memory of my beloved nephew, Feras Belhaj, who was a brother and a best friend of mine; may God bless his soul.

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ABSTRACT

Angiogenesis is the process of new blood vessel development by endothelial cells from pre-existing vasculature; however, abnormal angiogenesis contributes to the pathogenesis of many disorders such as cardiovascular diseases, cancer, and chronic inflammation. Angiogenesis in atherosclerotic plaques contributes to their instability and therefore increases the risk of plaque rupture and thrombus formation. Sparstolonin B (SsnB) is a novel bioactive compound isolated from *Sparganium stoloniferum*, an herb that has been used historically in the Chinese herbal medicine "SanLeng" as an herbal remedy for the treatment of several inflammatory diseases. In this study, we have explored the anti-angiogenic properties of SsnB *in vitro*. In cell culture, SsnB induced rapid changes in the morphology of human umbilical vein endothelial cells (HUVECs). After 6 hours, SsnB induced endothelial cell actin stress fibers, increased cell perimeter/area ratio, and enhanced formation of focal adhesions. These effects occurred in a dose-dependent manner in which the maximum effect was at 100μM SsnB. In addition, we have also analyzed early response gene expression in response to 100 μM SsnB. Our data show that SsnB blocked the up-regulation c-Myc and c-Fos, which occurred in response to addition of vehicle control (DMSO). These results imply that alteration of endothelial cell morphology and change of early response gene regulation may play a role in the anti-angiogenic effects induced by SsnB.

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LIST OF ABBREVIATIONS

AP-1 ..... Activator Protein-1

CAM ..... Chorioallantoic Membrane

CCNB1 ..... Cyclin B1

CCNE2 ..... Cyclin E2

CDC2 ..... Cell Dependent Kinase 1

CDC6 ..... Cell Division Cycle 6

CRP.....C-Reactive Protein

CT.....Computed Tomography

DMSO ..... Dimethyl Sulfoxide

FBS .....Fetal Bovine Serum

Figf ..... c-Fos-induced growth factor

GAPDH ..... Glyceraldehyde-3-Phosphate Dehydrogenase

HASMCs ..... Human Aortic Smooth Muscle Cells

HUVEC ..... Human Umbilical Vein Endothelial Cells

LDL .....Low Density Lipoprotein

IL..... Interleukin

LPS.....Lipopolysacharide

MAPK.....Mitogen-activated Protein Kinase

NF-κB.....Nuclear Factor κB

NMR.....Nuclear Magnetic Resonance

ROS .....Reactive Oxygen Species

SsnB ..... Sparstolonin B

THP-1 ..... Human Monocytic Leukemia Cell line

TIR.....Toll/Interleukin-1 Receptor

TLR ..... Toll-like Receptor

TSP-1..... Thrombospondin-1

VC ..... Vehicle Control

VEGF ..... Vascular Endothelial Growth Factor

VEGF-D ..... Vascular Endothelial Growth Factor D

VEGFR..... Vascular Endothelial Growth Factor Receptor

VSMC.....Vascular Smooth Muscle Cells

CHAPTER 1

INTRODUCTION

1.1 *Atherosclerosis*

Atherosclerotic cardiovascular disease is the first leading cause of morbidity and mortality in the United States, where cardiovascular risk assessment accounts for <50% of the variability in risk. Approximately 85% of middle-aged susceptible subjects have significant coronary atherosclerosis, which remains silent for decades until it finally presents with plaque rupture and thrombosis. In fact, myocardial infarction (MI) occurs in 62% of men and 42% of women with coronary atherosclerosis regardless of race or ethnicity (Weintraub et al. 2008). Many risk factors have been determined to be associated with progressive atherosclerotic disease, including hypertension, obesity, diabetes, smoking, and hyperlipidemia. Hyperlipidemia is defined as an elevation of low-density lipoprotein (LDL) cholesterol level, which is directly related to plaque formation (Victor et al. 2009).

In the normal physiological state, many of the large human arteries possess a microvasculature in their adventitial layer called the vasa vasorum. Large blood vessels are composed of three layers: tunica media, tunica intima, and tunica adventia. The tunica intima is the inner layer of the blood vessel wall and consists of endothelium. The middle layer of the vessel wall, tunica media, contains circularly arranged smooth muscle cells. The outer layer is the tunica adventia, which is made of collagen fibers mixed with nerve

fibers, lymphatic vessels, and microvasculature. Normal vasa vasorum originate from coronary artery branch points at regular intervals and run longitudinally along the vessel wall as is illustrated in (Figure 1.1).

Detection of early development of atherosclerosis can be accomplished using a variety of screening and diagnostic tests such as electrocardiogram, stress test, CT scan or arteriography. Moreover, biomarkers levels within the plasma can be helpful to diagnose atherosclerosis; for example elevated plasma concentrations of both fibrinogen and C-reactive protein (CRP), which are acute phase proteins released in response to inflammation (Kaperonis et al. 2006).

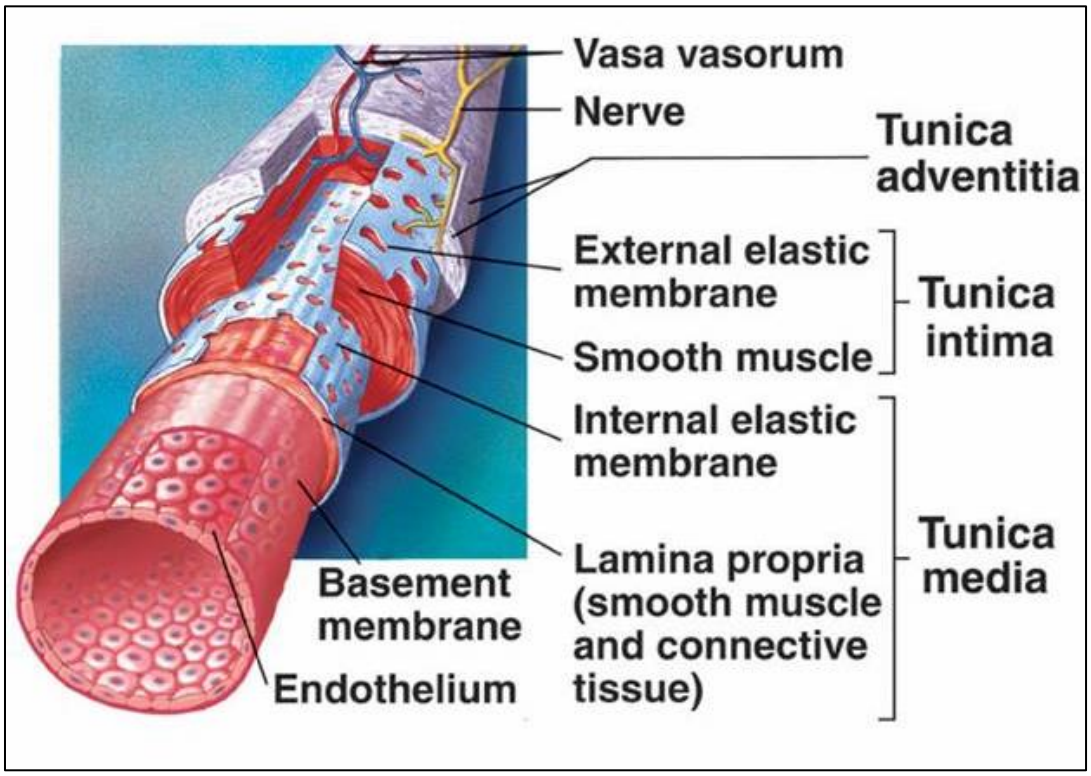

Figure 1.1 Illustration of Vasa Vasorum in Large Blood Vessel (Seeley et al. 2007)

1.2 *Plaque Formation*

Atherosclerosis mainly affects wall thickness of medium and large arterial blood vessels. Endothelial dysfunction has been thought to have a significant role in pathogenesis of atherosclerosis. Also, overproduction of reactive oxygen species (ROS) has been determined to be related to the development of atherosclerosis (Davignon and Ganz 2004).

Endothelial damage or dysfunction will increase the permeability of the endothelium, known as endothelial activation. The infiltration of LDL into the inner wall of blood vessels, the arterial intima, will increase due to impaired endothelial function. Over time, substances such as LDL, cholesterol and fatty materials will accumulate at damaged areas as shown schematically in (Figure 1.2). LDL will be oxidized by reactive oxygen species to form oxidized-LDL (ox-LDL). Then, ECs from the damaged sites will initiate immune responses and release chemoattractants to attract monocytes from the blood and induce an inflammatory reaction. Monocytes will differentiate into macrophages in response to oxidized-LDL stimulation. The first immune response will trigger a further cycle of immune responses. Macrophages will continue to take up and digest the modified cholesterol molecules. Macrophages are not able to clear the oxidized-LDL from the intima, although these white blood cells are intended to play a protective role. This process ends with formation of foam cells and eventual generation of fatty streaks, which are found in human aorta during the first decade of life (Lusis 2000).

Vascular smooth muscle cells (VSMC) begin to proliferate and migrate from the media to the intima. The initial lesion progresses into an advanced plaque with a fibrous cap, overlying a necrotic lipid core. The fibrous cap acts as a protective layer between the

lesion and the lumen of the blood vessel. The media and neo-intima will be subjected to a restriction of oxygen supply and nutrients, and the hypoxic environment may lead to expression of Vascular Endothelial Growth Factor (VEGF) and other angiogenic regulators that stimulate angiogenesis, thereby promoting plaque growth. Various research studies have identified intra-plaque hemorrhage as a critical factor in atherosclerotic plaque growth and destabilization (Sluimer etal.2009; Khurana et al. 2005; Di Stefano et al. 2009; Virmani et al. 2005; Seeley et al. 2007).

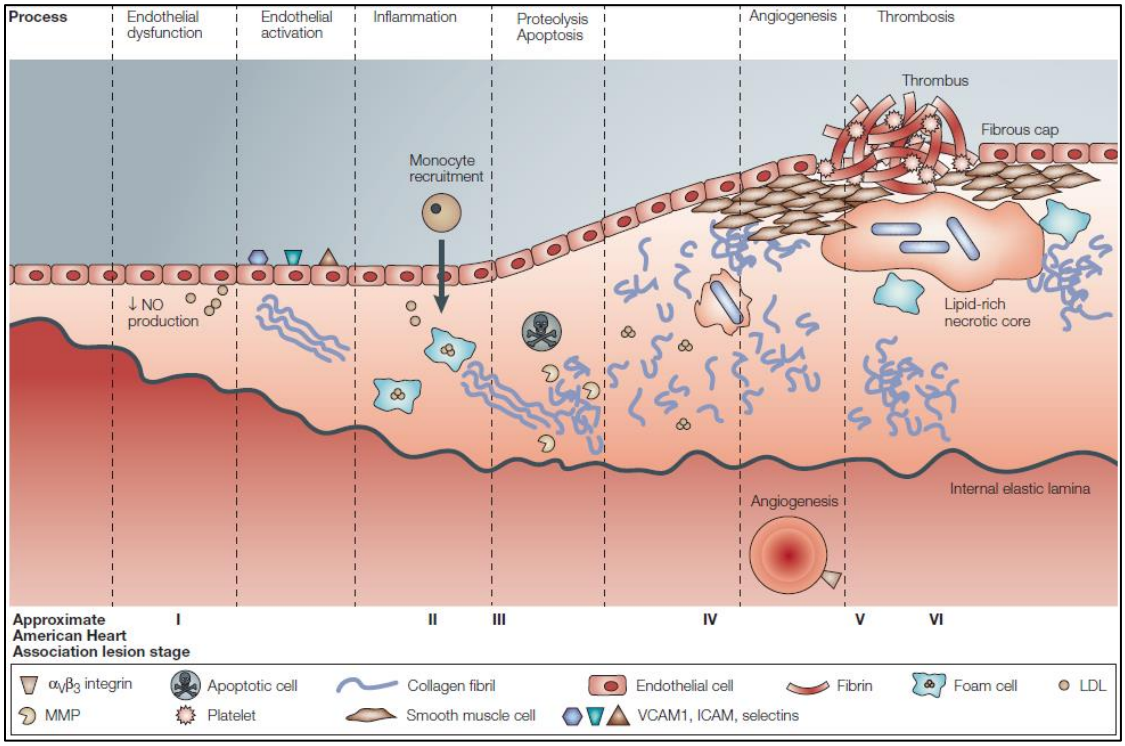

Figure 1.2 Illustration of atherosclerotic plaque formation (Choudhury, Fuster and Fayad 2004)

The composition of a plaque largely determines the risk of rupture for fibrous caps, which may cause an acute ischemic event, for example a myocardial infarction or stroke (Owens et al. 2004). Thrombus formation is a significant clinical complication of atherosclerosis resulting from rupture of an unstable atherosclerotic plaque, in which

formation of microvessels (angiogenesis) contributes to the development of plaques and increases the risk of plaque rupture (Sluimer et al. 2009).

1.3 *Endothelial Cell Function*

Endothelial cells play a wide variety of critical roles in controlling vascular function. It has been proposed that hemangioblasts, as common progenitors of endothelial, hematopoietic cells, and angioblasts, differentiate from the mesoderm in the embryo and VEGF receptor is expressed early in these progenitors (Hirashima 2009). VEGF is the most critical driver of vascular formation, as it is required to initiate the formation of immature vessels by vasculogenesis or angiogenic sprouting. Endothelial cells interact with circulating cells and cells present in the vascular wall including smooth muscle cells, during homeostasis. Being at the interface between blood and tissue, endothelial cells are most susceptible to changes in blood composition and in blood flow. Endothelial dysfunction could dispose specific areas of the vasculature to coagulation, inflammation and vasoconstriction. Endothelial dysfunction is certainly a key initiating event in several pathological conditions, including atherosclerosis (Michiels 2003).

VEGF plays a major role in all aspects of vascular development, including endothelial cell proliferation, migration, and survival. Endothelial cells are the main regulators of vascular development. The biological effects of VEGF are mediated by two receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2. It has been determined that VEGF is a survival factor for endothelial cells, both in vitro and in vivo. In vitro,VEGF prevents apoptosis induced by serum starvation where this activity is mediated by the phosphatidylinositol (PI)-3 kinase–Akt pathway. VEGF also induces expression of the anti-apoptotic proteins Bcl-2 and A1 in endothelial cells. In vivo, the prosurvival effects

of VEGF are developmentally regulated. It has also been shown that inhibition of VEGF results in extensive apoptotic changes in the vasculature of neonatal but not adult mice (Ferrara,Gerber and LeCouter 2003).

1.4 *Physiological and Pathological Angiogenesis*

Angiogenesis is the process of new blood vessel development from pre-existing vasculature. This process involves endothelial cell proliferation, degradation of the basement membrane and surrounding extracellular matrix, cell migration, and tubulogenesis. Angiogenesis consists of four stages: initiation, progression, differentiation, and maturation. It is controlled by a complex regulatory system composed of pro-angiogenic and anti-angiogenic factors, among which the VEGF family plays a key role through its pro-angiogenic activity, in addition to other factors and enzymes (Hoeben et al. 2004; Bussolino et al. 1997; Carmeliet et al. 2003). De novo blood vessel formation, or vasculogenesis, is an important process for the survival of living organisms in the embryonic state. In the adult state, physiological angiogenesis plays an essential role in wound healing, growth of the uterine lining, and the female reproductive cycle. However, excessive, insufficient or abnormal angiogenesis contributes to the pathogenesis of many disorders such as cardiovascular diseases, cancer, arthritis, psoriasis, retinopathy, and inflammation (Khurana et al. 2005; Carmeliet et al. 2003; Kaiser et al. 1999).

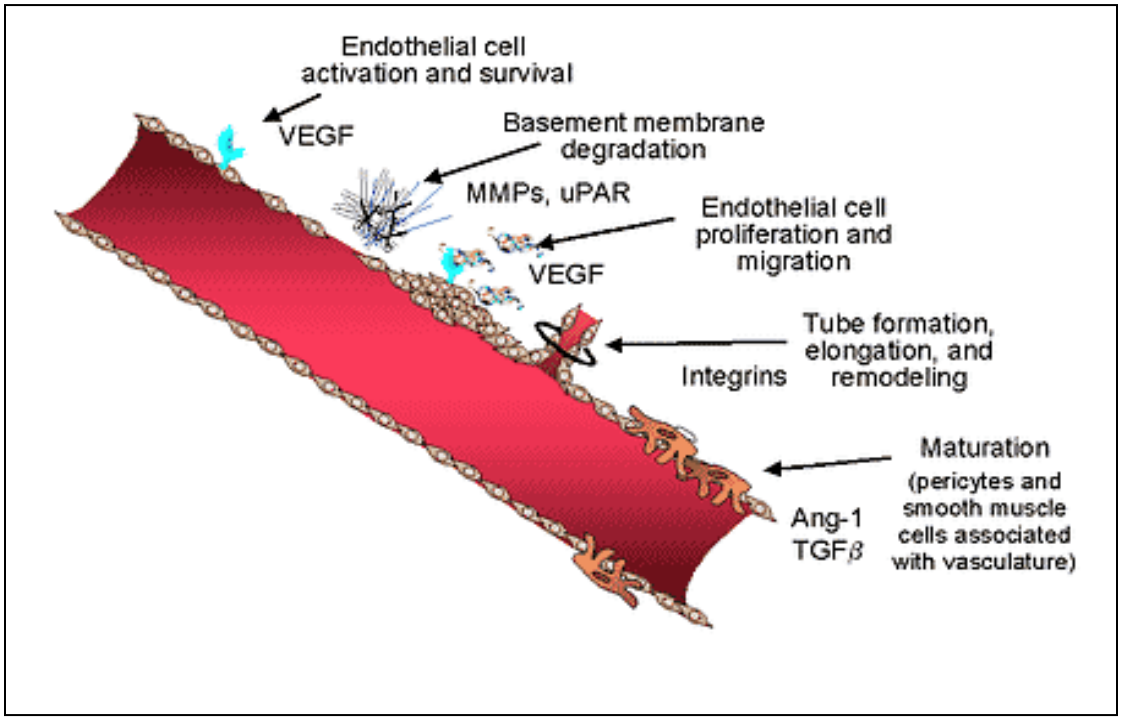

Figure 1.3 Illustration of angiogenesis process (Griffioen and Molema 2000).

1.5 *Anti-angiogenic Therapy and Sparstolonin B Effect*

Anti-angiogenic therapy has been recognized as a promising approach recently particularly in cancer treatment. This approach has advantages over conventional anticancer therapy because the therapy does not directly target the tumor but inhibits the development of blood vessels by targeting the endothelial cells. Thus, anti-angiogenic therapy is indirectly cytotoxic to the tumor cells (Hoeben et al. 2004; Somani et al. 2013). This new approach is still questionable as a treatment for atherosclerotic disease because angiogenesis in atherosclerosis is more complex and depends on disease severity (Khurana et al. 2005); however, some previous work has demonstrated effective treatment procedures to inhibit neovascularization in atherosclerotic plaques that will increase their stability and thus, decrease thrombosis (Stefanadis et al. 2007).

Most chemotherapeutic drugs are considered have some anti-angiogenic activity. This activity was demonstrated at a lower dose of chemotherapeutic drugs, including cyclophosphamide, paclitaxel, doxorubicin, and vincristine, than would be required to target tumor cells. In addition, many anti-angiogenic agents showed a successful suppression of angiogenesis especially within tumors such as anti-VEGF monoclonal antibody and tyrosine kinase inhibitors. Moreover, EGF receptor inhibitors have been shown to suppress VEGF expression through blocking the VEGF-R2 receptor signaling (Scappaticci 2002). Angiogenesis gene therapy is another promising approach that is directed to tumor endothelial cells and their microenvironment. The use of gene therapy that delivers anti-angiogenesis genes has shown promise in preclinical models in mice. The expression of EC specific cell surface molecules like vascular endothelial growth factor receptor (VEGFR), E selectin or angiogenic growth factors (VEGF, FGF, PDGF) produced by tumor cells can be inhibited by specific antibodies, antisense RNA, or gene specific siRNA. The targeted gene therapy can be achieved by using either viral vectors or nanoparticles to target anti-angiogenic genes to tumor vasculature (Tandle, Blazer, and Libutti 2002).

The *Sparganium stoloniferum* plant has been used traditionally in Chinese medicine for the treatment of cancer. Extracts and other isolated chemical compounds from this herb, including a sucrose ester, a phenylpropanoid glycerol, carboxylic acid esters, and a phenylpropanoid glycoside, possess potent anti-cancer effects. It is already known that anti-tumor agents often show the ability to inhibit angiogenesis, and Sparstolonin B (SsnB) is one of the active compounds of this plant that has anti-tumor

activity. Also, SsnB has been identified as a compound that possesses anti-angiogenic activity in addition to its anti-inflammatory properties (Bateman et al. 2013).

SsnB is a newly described polyphenolic compound isolated from the tubers of *Sparganium stoloniferum*. It has been identified as a compound containing the core structures of both xanthone and isocoumarin using NMR spectroscopy and X-ray crystallography (Liang et al. 2011) as shown in (Figure 1.5.1). The naturally occurring xanthones and isocoumarins possess anti-inflammatory, antioxidant, anti-atherosclerotic, and antitumor activities (El-Seedi et al. 2010; Fylaktakidou et al. 2004). *Sparganium stoloniferum* has been used historically in the Chinese herbal medicine "SanLeng" as an herbal remedy for the treatment of several inflammatory diseases.

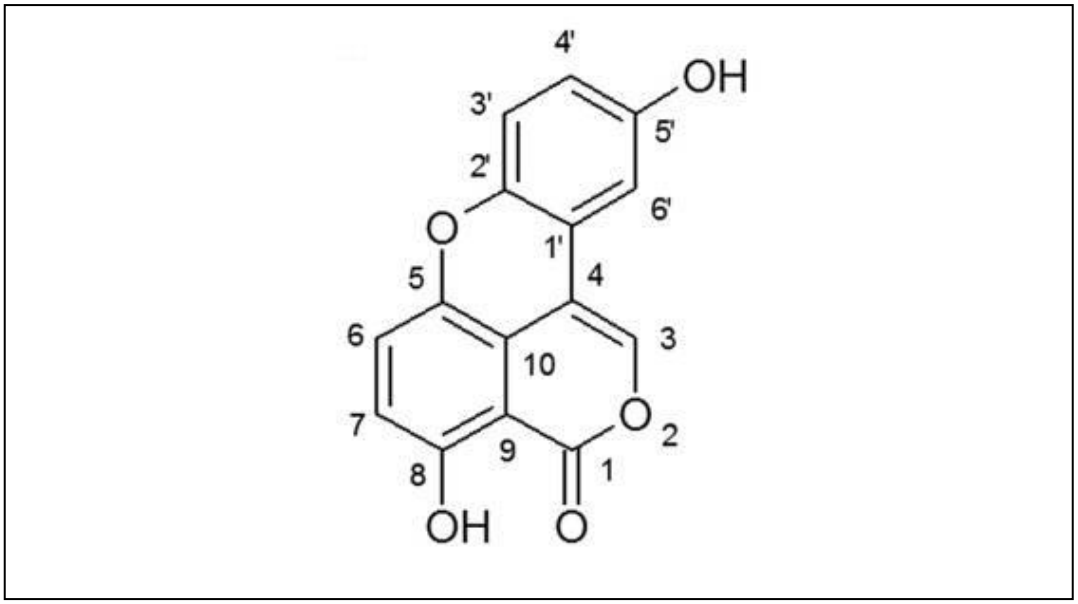

Figure 1.4 Structure of SsnB (Liang et al. 2011)

Cytotoxicity measurements of SsnB show that the compound is safe to use in concentrations up to 100μM in different cell types, which include mouse peritoneal

macrophages, human monocytic THP-1 cells, HUVECs, and human aortic smooth muscle cells (HASMCs).

Recently, SsnB has been successfully synthesized based upon its well- known xanthone core structure through development of a practical method consisting of sequential chemical reactions to yield the final SsnB product. It has been shown that the synthetic SsnB shares identical structure with the plant-derived SsnB using HPLC and NMR analysis. The final product has purity greater than 99%, which has been achieved using HPLC and mass spectrometry techniques to maintain reproducibility and to control the purity (Hu et al. 2012).

1.6 *Published Research Studies*

In previous research, it has been demonstrated that SsnB is a selective Toll-like receptor antagonist (TLR2 and TLR4); it blocks TLR2- and TLR4-triggered inflammatory signaling in macrophages by inhibiting the recruitment of MyD88 to TIR domains of TLR2 and TLR4 but does not block signaling in response to TLR3 and TLR9 ligands (Liang et al. 2011). Toll-like receptors are expressed by macrophages, dendritic cells, and many other cell types. TLRs are known for their activity as the first line of defense against invading pathogens such as bacteria and viruses (Kumar, Kawai and Akira 2011). The therapeutic consequences of blockage of excessive TLR signaling are being demonstrated for many inflammatory diseases including inflammatory cardiovascular diseases. In addition, it has been shown that SsnB suppressed multiple signaling pathways downstream of TLR2 and TLR4 activation including MAPK and NF κB pathways (Liang et al. 2011; Liang et al. 2013).

According to a previously published paper, it has been demonstrated that SsnB possesses anti-inflammatory effects on vascular endothelial cells. SsnB showed a suppressive effect on lipopolysaccharide (LPS)-induced expression of interleukin (IL)-1b and monocyte chemoattractant protein 1 at the transcriptional and translational levels in HUVECs (Liang et al. 2013). LPS is considered the major portion of the outer membrane of Gram-negative bacteria, which is an important pathogenic stimulus, in addition to being a ligand for Toll-like receptor (TLR4) (Wiedermann et al. 1999). Moreover, LPS in the blood directly promotes vascular inflammation via the activation of resident cells such as monocytes and endothelial cells (Roth et al.).

Published data from a previous study suggests that SsnB targets endothelial cells and inhibits angiogenesis by interfering with crucial steps in the process (Bateman et al. 2013). The data shows that SsnB is an inhibitor of endothelial cell morphogenesis and cell migration. In addition, the results suggest that SsnB is able to inhibit cell proliferation through arresting the cell cycle in the G0/G1 phase. Microarray data showed differential expression of important genes involved in angiogenesis in response to SsnB treatment, including genes in pathways associated with the cytoskeleton, cell proliferation, and cell cycle. Using the chick chorioallantoic membrane (CAM) assay, an *ex vivo* angiogenesis assay, it was demonstrated that SsnB causes an inhibition of blood vessel formation in the chick embryos, where the vessels in SsnB-treated embryos are shorter in length and show reduced branch formation compared to control groups (Bateman et al. 2013).

1.7 *Specific Aims*

We hypothesize that SsnB will exert its anti-angiogenic effects by changing the cell cytoskeleton, enhancing focal adhesion formation, and interfering with early response gene up-regulation.

Specific Aim 1

To test the hypothesis that SsnB influences endothelial cell morphology and cytoskeletal organization. For this purpose, we will use immunofluorescence analysis of actin filaments and microtubules.

Specific Aim 2

To test the hypothesis that SsnB promotes focal adhesion formation in HUVECs. Immunofluorescence analysis for focal adhesions will be used to investigate the anti-angiogenic effect of SsnB.

Specific Aim 3

To test the hypothesis that SsnB interferes with early response gene up-regulation. Immunoblot analysis and real time qRT-PCR will be used to examine the changes in protein and mRNA expression of c-Myc, c-Fos, and c-Jun due to the effect of SsnB treatment.

CHAPTER 2

THE EFFECTS OF SPARSTOLONIN B ON CYTOCKELETON ARRANGEMENT AND MORPHOLOGY OF ENDOTHELIAL CELLS

2.1 Introduction

Since inhibition of angiogenesis is associated with changes in cell motility and cell proliferation, we investigated in this work the effect of SsnB on the cytoskeleton including actin filaments, microtubules, and focal adhesions. The actin cytoskeleton has a vital role in various cellular processes such as migration and cytokinesis. In addition, abnormalities in actin dynamics are associated with many pathological disorders such as cancer. Actin filaments form stress fibers that contribute to cell migration; however, other studies observed that stress fibers are more prominent in stationary cells suggesting that stress fibers may inhibit cell migration under specific conditions (Tojkander et al 2012; Burridge 1981). Microtubules are structural components of the mitotic spindle, and microtubule dynamics play an important role in several cellular processes including intracellular trafficking, cell migration and cell division. Inhibition of microtubules results in blocking of cell cycle progression in mitosis, whereas prolonged mitotic arrest triggers various apoptotic pathways (Rai et al. 2012). In addition, focal adhesions, dynamic protein complexes that connect the cytoskeleton of a cell to the extracellular matrix, play an essential role in vital biological processes including cell motility, cell proliferation and differentiation. Anti-angiogenic agents have been shown to diminish

cell migration and to increase attachment of cells to their substrate (Petit and Thiery 2000).

2.2 *Materials and Methods*

Compounds—Sparstolonin B (SsnB) was purified from the plant *Sparganium stoloniferum* and its structure identified with isocoumarin core using NMR spectroscopy and X-ray crystallography (Liang et al. 2011). Recently, the compound has been synthesized and tested for efficacy (Hu et al. 2012). For the *in vitro* experiments, SsnB was dissolved in DMSO (74.5 mM and 100 mM stock solutions) and freshly diluted in HUVEC medium before use.

Cells—Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Hopkinton, MA) and cultured on petri plates (100 X 20 mm) coated with 0.1% gelatin. HUVECs were cultured in endothelial cell medium supplemented with 10% fetal bovine serum (FBS) and endothelial cell mitogen / growth supplement (Biomedical Technologies, Stoughton, MA). The endothelial cell medium was replaced every 2-3 days, and the cells were passaged after complete confluence was reached. Cells were used between third and fifth passage.

Immunofluorescence analysis of the cytoskeleton—HUVECs were trypsinized from confluent plates and then grown overnight on 0.1% gelatin-coated multi-well chamber slides with initial seeding density of 5,000 cells per well. Next, various concentrations of SsnB (1, 10, and 100 µM) or vehicle control (0.1% DMSO) were added to the cells in assigned wells. After 6 hours of treatment, cells were fixed with 2% formaldehyde in, cytoskeleton buffer with sucrose (CBS) for 20 minutes and permeabilized with PBS- 0.1%Triton X-100 for 10 minutes. Fixed cells were washed and

incubated at room temperature for 30 minutes with rabbit anti- α/β-Tubulin antibody (Cell Signaling Technologies), 1:50. After washes, cells were incubated with 1:100 Rhodamine Red X (RRX)-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) together with the F-actin- binding Alexa Fluor® 488 phalloidin (Invitrogen/ Life Technologies). Coverslips were then mounted with Dako fluorescent mounting medium. Samples were analyzed on a laser scanning confocal microscope (Zeiss LSM 510 META) in the Instrumentation Resource Facility (IRF) at the USC School of Medicine. Cells were observed with a 40X objective lens, using a FITC filter to visualize F-actin and a CY3 filter for tubulin. Results were observed in four separate experiments.

Immunofluorescence analysis of focal adhesions—HUVECs were trypsinized from confluent plates and then grown overnight on 0.1% gelatin-coated multi-well chamber slides with initial seeding of 5,000 cells per well. SsnB treatment of various concentrations (1, 10, and 100 μM) or vehicle control (0.1% DMSO) was added to the cells in assigned wells. After 6 hours of SsnB treatment, the cells were fixed with 4% formaldehyde/CBS for 20 minutes and permeabilized with PBS- 0.5% Triton X-100 for 10 minutes. After several washings, cells were incubated with mouse anti-vinculin antibody (hVIN-1; Novus Biologicals), 1:400, at room temperature for 30 minutes to stain focal adhesions. Then, cells were incubated with 1:100 RRX-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in addition to actin staining as described previously. The slide was then cover slipped using Dako fluorescent mounting medium, and the cells were observed on a laser scanning confocal microscope (Zeiss LSM 510 META) with a 40X objective lens using the FITC

filter for F-actin and the CY3 filter for vinculin. Image-Pro Plus software was used to analyze the images. First, the brightness and contrast of images were uniformly adjusted. Images were thresholded by histogram-based segmentation to identify focal adhesion pixels, the bright objects. The size of objects counted as focal adhesions was between 20 and 250 pixels in area, and the total area of focal adhesions was quantified in approximately 100 cells for each treatment. Also, the same software was used to quantify cell area and cell perimeter. The threshold for images was identified to separate cell body and background region. After thresholding the image, the regions less than 10 pixels in area and perimeter are discarded. The brightness and contrast were maintained constant for all images for each measurement (focal adhesion area or cell perimeter/cell area). Results are representative of those obtained in three separate experiments.

Statistical Analysis—Data were represented as mean ± SD for each group. Comparisons among mean values of the treatment groups and control group were done using one-way and two-way ANOVA. Holm-Sidak test was used for comparisons between groups for the data that showed significance by ANOVA ($p<0.05$).

2.3 *Results*

SsnB influences endothelial cell morphology and cytoskeleton organization—SsnB has been shown to interfere with endothelial cell migration and proliferation (Bateman et al 2013). Since cytoskeleton is directly related to migration and proliferation, we investigated the effect of SsnB on endothelial cell cytoskeleton. In addition, since changes in cell shape are often associated with changes in motility, the effects of SsnB on endothelial cell morphology were investigated. We also attempted to examine microtubule organization, but our staining procedure was unsuccessful as presented in

[Figure 2.1]. Both control cells and SsnB treated cells showed disconnected microtubules (B) whereas cells used for α/β tubulin antibody titration showed well organized microtubules (A). SsnB-treated cells had more actin stress fibers across the cell body than control cells, and this effect was more intense at 100μM concentration as can be observed qualitatively in [Figure 2.2]. Also, SsnB treatment resulted in a significant dose dependent increase in cell perimeter/cell area at concentrations 1, 10, 100 μM ($p < 0.05$ ANOVA, Sidak's test) [Figure 2.3].

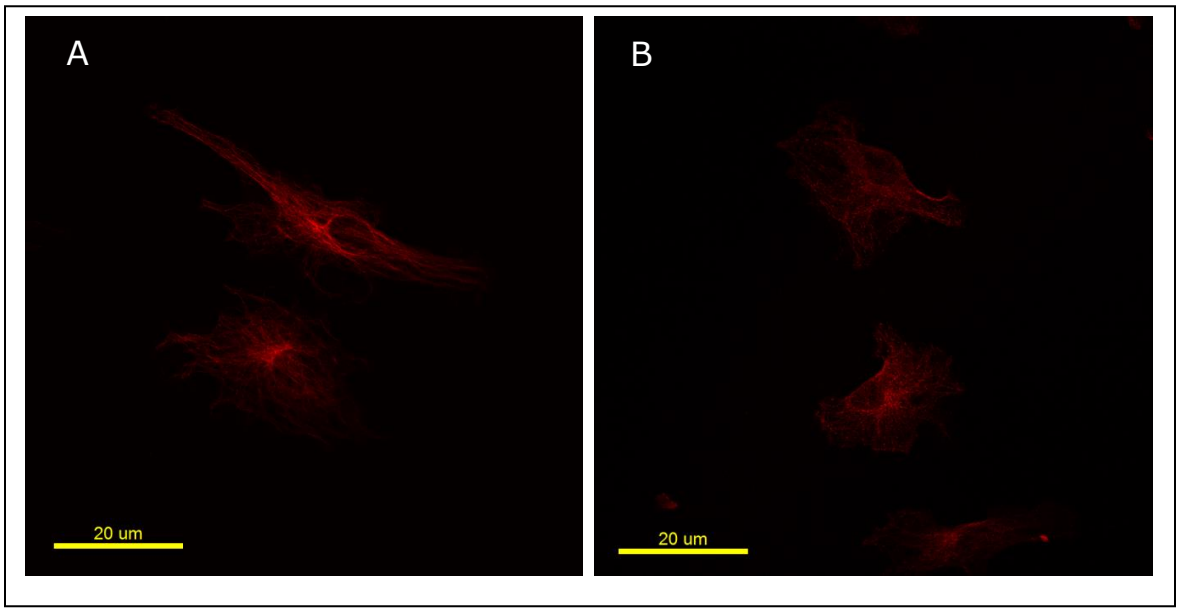

Figure 2.1 Microtubules in HUVECs. (A) Cells were stained for microtubules in red using α/β tubulin antibody for the purpose of antibody titration, to determine the ideal concentration for the experiment. (B) SsnB treated cells and control cells were stained for microtubules using 1:50 α/β tubulin antibody. Cells in (B) show fragmented microtubules in both control and treated groups.

In addition, we investigated cell shape and found that SsnB-treated cells tended to be more irregular in shape at high concentrations than control cells [Figure 2.4]. Cell shape was defined using the equation (4 x cell area/ (cell perimeter) ^{2}), where 1 indicates

a perfect circle and smaller values indicate a more irregular shape. Our data suggest that SsnB may affect endothelial cell migration by altering cytoskeletal organization.

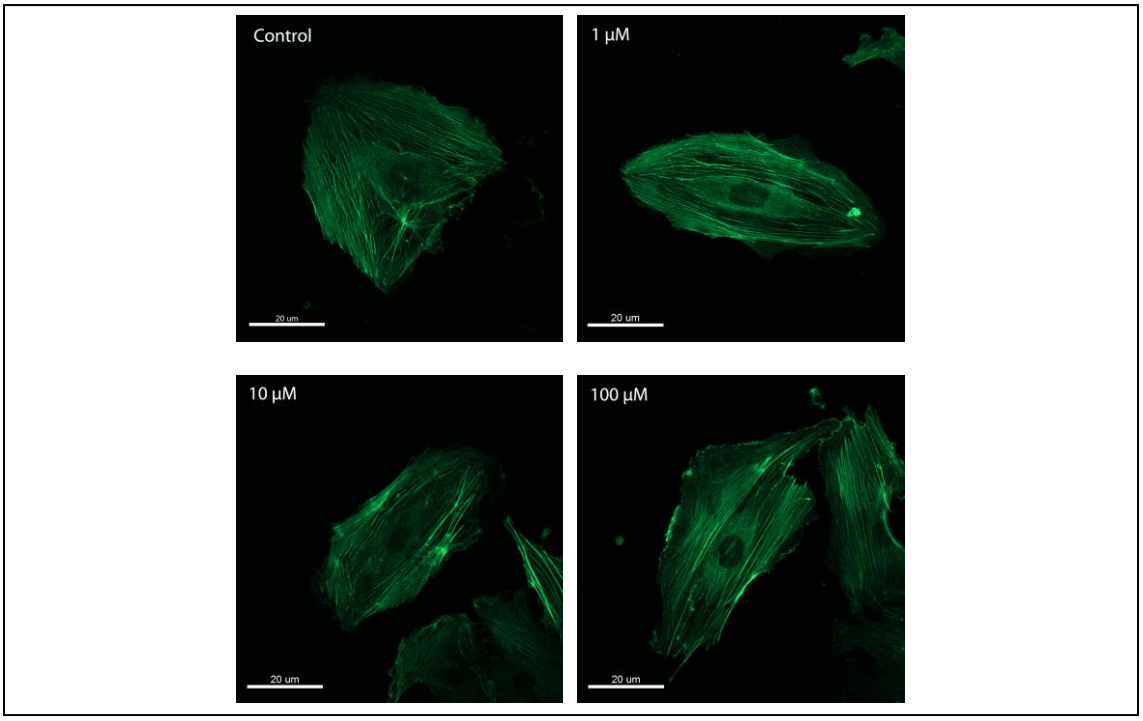

Figure 2.2 Representative example of SsnB effect on cytoskeletal organization of endothelial cells. HUVECs adherent to gelatin were exposed to 1 µM, 10 µM, and 100 µM concentrations of SsnB for 6 hours. SsnB induces stress fibers in treated cells, and the highest concentration induces the maximum effect. Green stained for F-Actin filaments, and scale bar = 20µm. n = 100 cells per group.

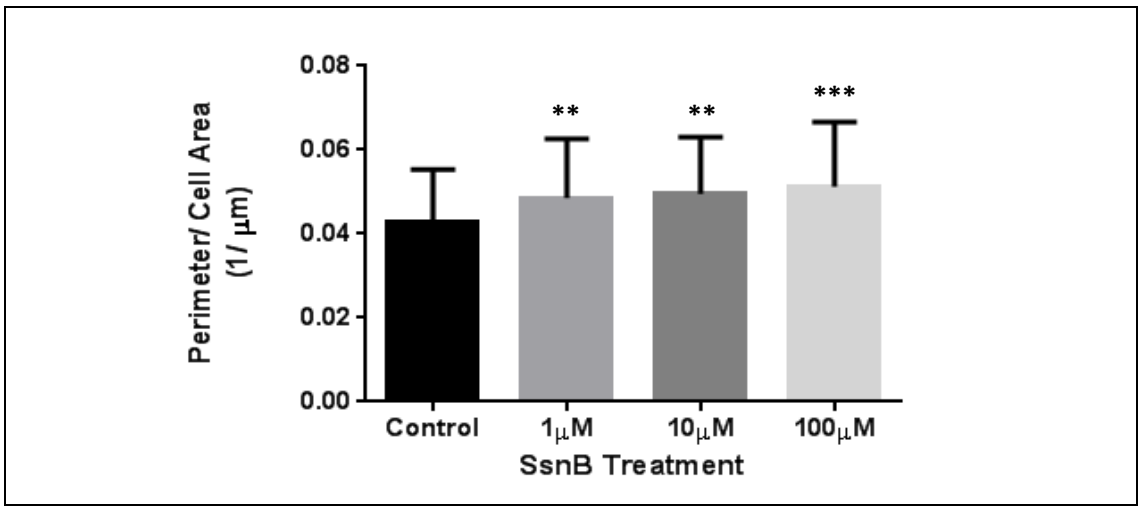

Figure 2.3 SsnB effect on HUVEC morphology. Treated cells exhibit a significant increase in cell perimeter to area ratio compared to control. **p<0.05 vs. vehicle control, Sidak's test. *** p<0.001 vs. vehicle control, Sidak's test. n = 100 cells per group, error bars correspond to +1 SD.

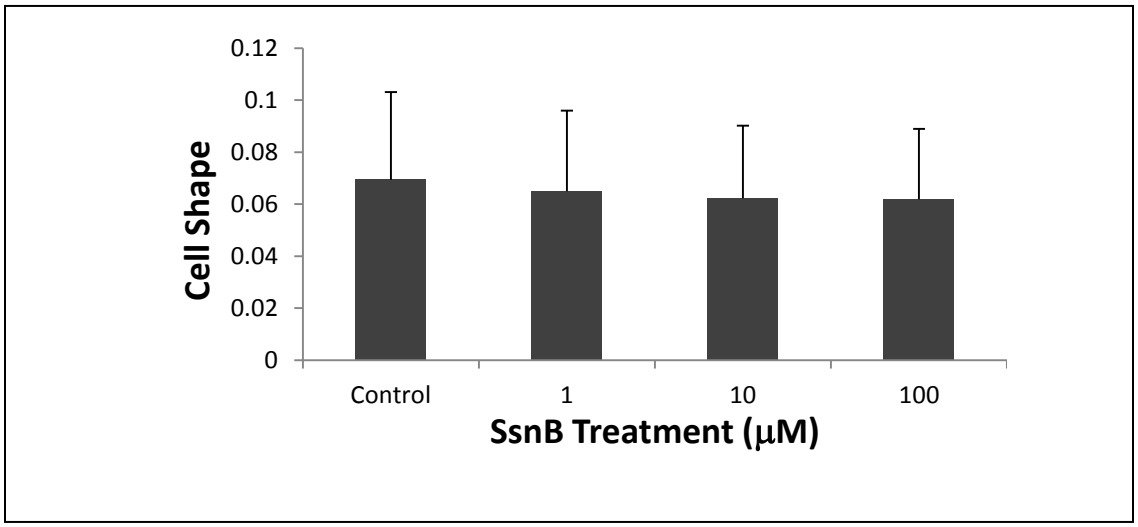

Figure 2.4 SsnB effect on HUVEC shape. Treated cells demonstrate insignificant increase in shape irregularity compared to control group where the equation (4 x cell area/ (cell perimeter)^2) was used to calculate cell shape. The closest values to 1 indicate more rounded cells. Data are calculated from more than 100 cells for each group. Error bars indicate standard deviation.

SsnB promotes focal adhesion formation—We investigated the effect of SsnB on focal adhesions due to their important role in cell migration. As presented in [Figure 2.5], endothelial cells show staining for focal adhesions in addition to actin filaments. Figure 2.6 shows representative results from focal adhesion staining of HUVECs. Cells treated with SsnB showed an enhanced formation of focal adhesions in a dose-dependent manner at concentrations of 1, 10, and 100μM. The results are statistically significant ($p<0.05$ ANOVA) even though they show high variability. These data suggest that SsnB treatment may increase attachment of endothelial cells to their substrate by increased formation of focal adhesions, consistent with previously published observations of impaired cell migration in SsnB treated HUVECs after 6 hours of treatment (Bateman et al. 2013).

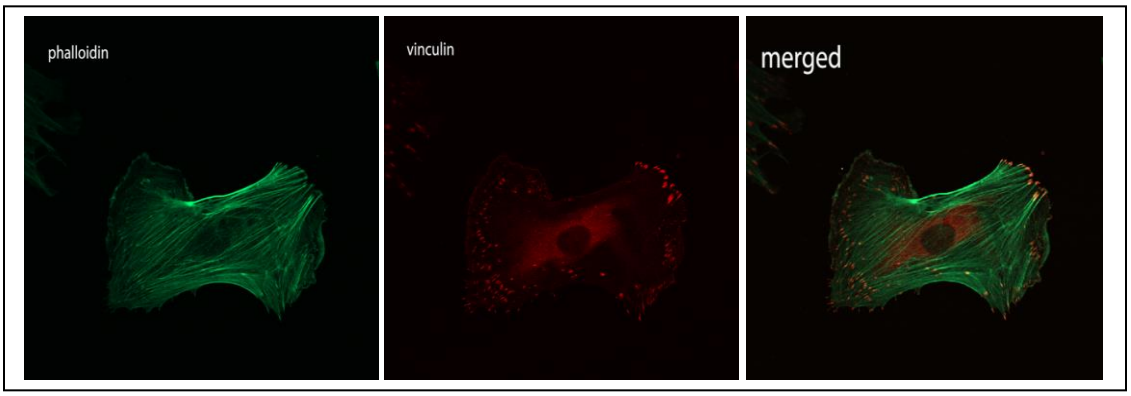

Figure 2.5 Focal adhesions in HUVECs. Control and SsnB treated HUVECs were stained with Alexa Fluor® 488 phalloidin (Green) for actin filaments, and stained with anti-vinculin antibody and RRX-labeled secondary antibody (Red) for focal adhesions.

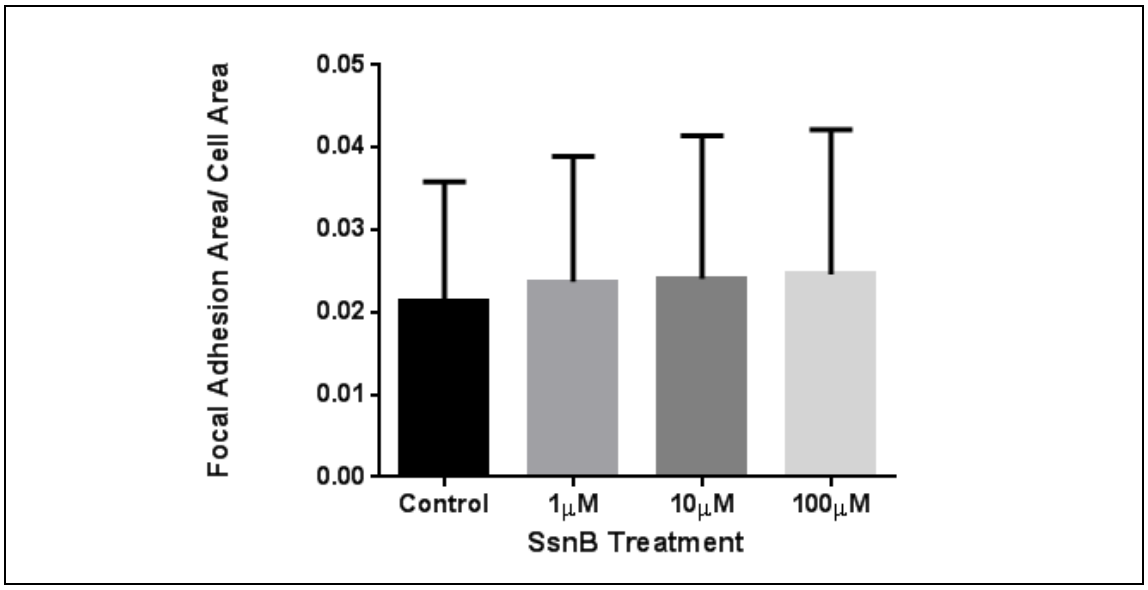

Figure 2.6 SsnB promotes focal adhesion formation. Treated cells show dose-dependent increase in focal adhesion area ($p<0.05$ ANOVA) with presence of high variability in the results obtained. The results were not significant using Holm-Sidak test to compare treated groups to control. n = 100 cells per group, error bars indicate +1 SD.

CHAPTER 3

EFFECTS OF SPARSTOLONIN B ON THE BIOCHEMICAL COMPONENTS OF ENDOTHELIAL CELLS

3.1 *Introduction*

In an effort to determine how SsnB inhibits angiogenesis at the molecular level, we have investigated the expression of immediate early response genes such as c-Fos, c-Jun, and c-Myc. Early response genes are a set of genes that are induced in response to both cell-extrinsic and cell-intrinsic signals and do not require *de novo* protein synthesis for their expression (Fowler et al. 2011). The AP-1 protein family, including c-Fos and c-Jun, is associated with cell proliferation through their ability to regulate the expression and function of cell cycle regulators. The expression of c-Myc is significant for endothelial cell proliferation, migration, and therefore angiogenesis. Previously published cancer studies demonstrate an important rule of c-Myc and c-Jun in tumor angiogenesis; these oncogenes possess the ability to suppress the expression of anti-angiogenic factor thrombospondin-1 (TSP-1) to provoke angiogenesis in progressed tumors. These genes contribute effectively to angiogenesis in various tumor types, and the same concept can be extended to angiogenesis in plaques (Vleugel et al. 2006; Baudino et al. 2002; Shaulian and Karin 2001; Deed et al. 1997).

3.2 *Materials and Methods*

Compounds—Sparstolonin B (SsnB) was purified from the plant *Sparganium stoloniferum* as described in a previously published paper (Liang et al. 2011). Recently, the compound has been synthesized and tested for efficacy (Hu et al. 2012). For the *in vitro* experiments, SsnB was dissolved in DMSO (74.5 mM and 100 mM stock solutions) and freshly diluted in HUVEC medium before use.

Cells—Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Hopkinton, MA) and cultured on petri plates (100 X 20 mm) coated with 0.1% gelatin. HUVECs were cultured in endothelial cell medium supplemented with 10% fetal bovine serum (FBS) and endothelial cell mitogen / growth supplement (Biomedical Technologies, Stoughton, MA). The endothelial cell medium was replaced every 2-3 days, and the cells were passaged after complete confluence was reached. Cells were used between third and fifth passage.

Immunoblot analysis— HUVECs were cultured in 100 X 20 mm polystyrene culture plates coated with 0.1% gelatin to approximately 80% confluence. Cells were treated with 100μM SsnB for different incubation times (1 hr, 2 hrs, and 3 hrs), and control cells were incubated with0.1% DMSO vehicle for the same time intervals. After lysing cells of each group with 200 μl Berk's lysis buffer (BLB), total protein amount was measured using a DC (detergent compatible) protein assay (Bio-Rad Laboratories) in which bovine serum albumin (BSA) was used as the protein standard. Thirty micrograms of total protein from each sample were mixed with loading dye buffer, boiled, and electrophoretically separated on 10% SDS polyacrylamide gels. Proteins were electrotransferred to PVDF membranes (Bio-Rad). Membranes were blocked in 5%

nonfat milk in Tris-buffered saline containing 0.05% Tween (TTBS) and incubated overnight with primary rabbit antibodies against c-Myc, c-Fos (Cell Signaling Technologies) or c-Jun (Santa Cruz Biotech), at 1:1000 dilutions in 1% milk-TTBS. After washings, membranes were incubated in 1:3000 dilution of horse-radish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Bio-Rad) in 1% milk-TTBS. Immunoreactive band specificity was determined by enhanced chemiluminescence detection (Pierce). After initial probing, membranes were stripped with a buffer containing 0.6M Tris-Cl, 2% SDS, 0.7% β-mercaptoethanol at room temperature for 30 min, and then reprobed with (1:1000) β-actin antibody (MediMabs, Canada) as a loading control. Results are representative of three separate experiments.

Densitometric measurements of Western blots—Image Pro Plus 7 software was used to measure the density of the imaged bands. The brightness and contrast of the blot image were adjusted to separate the bands, appearing as bright objects, from the background. The average intensity of each band was measured. The experimental bands were normalized to β-actin bands, and then the ratio between the normalized value of each band and the vehicle control (at 0 hour) was obtained. The ratio represents a quantitative measurement of c-Myc protein expression.

Real Time- PCR— HUVECs were chosen for real time PCR from 90% confluent plates (100 X 20 mm polystyrene, tissue culture-treated petri plates coated with 0.1% gelatin). Cells were trypsinized and seeded (400,000 cells per well) overnight in 6 well polystyrene culture plates coated with 0.1% gelatin. Half of the wells received HUVEC medium containing 100 μM SsnB, and the remaining plates received vehicle control (0.1% DMSO in complete growth medium). The plates were incubated for several time

intervals (1 hr, 2 hrs, and 3 hrs) to allow SsnB to have an effect on the expression of early response genes. Following incubation, the cells were lysed using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Total RNA was extracted using chloroform, and then samples were centrifuged (13,000 rpm) for 15 min at 4C. Approximately 350 µl of aqueous layer was collected, and isopropanol was added to precipitate the RNA followed by centrifugation for 10 min under the same conditions. The RNA pellet was washed by addition of 1 ml of 75% ethanol and centrifuged at 13,000 rpm for 5 min. The pellet was re-suspended in 30 µl nuclease free water. The concentration of RNA was determined spectrophotometrically at 260 nm and the purity of RNA was assessed by measuring the 260 nm/ 280 nm ratio. First strand cDNA was prepared from the RNA with reverse transcriptase (iScript cDNA synthesis kit, BioRad). The RNA was amplified using c-Myc, c-Fos, or c-Jun primers (Qiagen) and iQ SYBR Green super mix (BioRad). One-step RT-PCR reactions were completed on the BioRad CFX Connect thermal cycler system in the Instrumentation Resource Facility at the USC School of Medicine. The expression levels were normalized to the housekeeping gene GAPDH, and RNA levels were quantified and compared between groups with the relative Pfaffl method for c-Myc (Pfaffl 2001). In addition, we used the ΔΔCT method for c-Fos and c-Jun to compare RNA levels between groups. Representative results from three separate experiments are shown.

Statistical Analysis—Data were represented as mean +SD for each group. Comparisons among mean values of the treatment groups and control group were done using one-way and two-way ANOVA. Holm-Sidak test was used for pairwise comparisons between groups for the data that showed significance by ANOVA ($p<0.05$).

3.3 *Results*

SsnB effects on early response gene expression— Western blot analysis was used to investigate the effect of SsnB on c-Fos, c-Jun, and c-Myc protein levels. We did not detect any signal for c-Fos protein, and it was hard to detect c-Jun signal because of the appearance of several nonspecific bands near the expected position of c-Jun. However, Western blot analysis showed that treatment with vehicle control (0.1% DMSO) resulted in increased expression of c-Myc protein which peaked at 2 hours and then diminished. This effect appeared to be abrogated by treatment with 100 μM SsnB; however, the data did not reach statistical significance. For verification purposes we investigated the effect of SsnB on c-Myc mRNA levels. qRT- PCR analysis demonstrates a decrease in c-Myc gene expression after SsnB treatment compared to the control group, which supports the results obtained from immunoblot analysis ($p<0.05$ ANOVA, Sidak's test) [Figure 3.1]. In addition, we investigated the effect of SsnB on c-Fos and c-Jun mRNA level. Our preliminary data shows that SsnB treated cells reveal a decrease in expression of both genes compared to the control group [Figure 3.2]. This reduction effect was significant for c-Fos ($p<0.05$ ANOVA, Sidak's test), but it was insignificant for c-Jun. The data suggests that SsnB treatment may interfere with or inhibit up-regulation of c-Myc, c-Fos and c-Jun. This effect may relate to the inhibition of cell cycle progression by SsnB treatment, which has been demonstrated previously (Bateman et al. 2013).

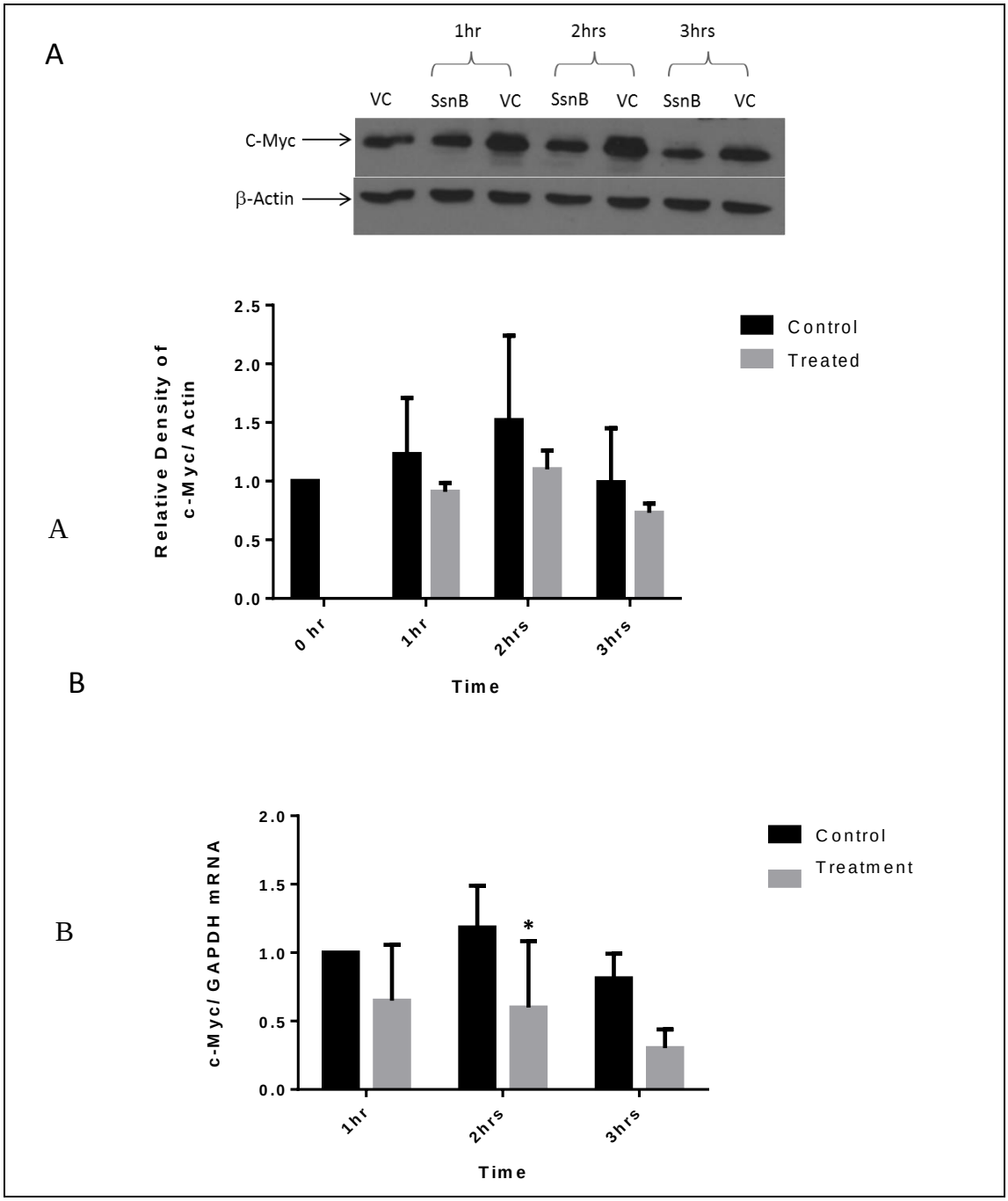

Figure 3.1 SsnB effect on c-Myc expression. (A) Western blot analysis and quantitative densitometry of c-Myc protein expression. When data are normalized for protein loading (actin) and compared to control, c-Myc shows an apparent decrease in expression in SsnB-treated cells relative to control, which did not reach statistical significance. (B) Real Time-PCR shows a decrease of c-Myc mRNA expression (normalized to housekeeping gene GAPDH) in response to SsnB treatment (*$p<0.05$ ANOVA, Sidak's test). All data represent three observations for each group and are expressed as mean values +SD; all groups were compared to 1hr vehicle control.

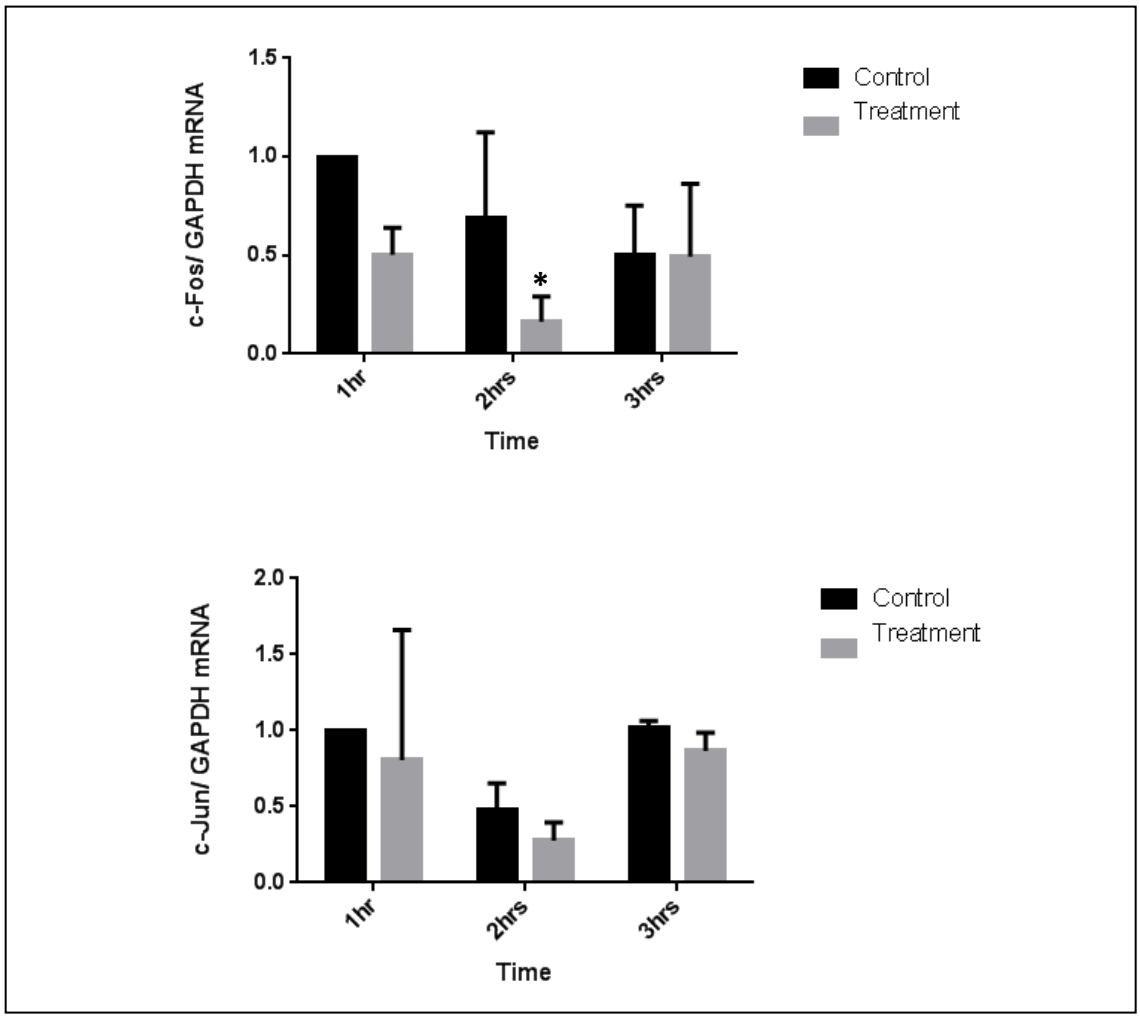

Figure 3.2 Effects of SsnB on relative c-Fos and c-Jun mRNA expression. Real Time-PCR shows a decrease in expression of both c-Fos and c-Jun mRNA (normalized to housekeeping gene GAPDH mRNA) in response to SsnB treatment. (A) c-Fos data were statistically significant at the 2-hr time point (*$p<0.05$ ANOVA, Sidak's test); all groups were compared to 1 hr vehicle control. However, c-Jun data (B) were statistically insignificant. Data represent three observations for each group and are expressed as mean values +SD.

CHAPTER 4

DISCUSSION

In the present study, we sought to assess the anti-angiogenic effect of SsnB at the cellular and molecular level of endothelial cells. Our results demonstrate that HUVECs treated with high concentrations of SsnB (10 and 100 μM) show an increase in formation of actin stress fibers compared to controls. Furthermore, our data shows that focal adhesion area, which is related to stress fiber formation, increased in cells treated with high SsnB concentrations. Qualitative analysis of focal adhesion formation was unreliable because of cell population variability; some cells were bigger in size than others, so it was hard to conclude a difference in focal adhesion formation between control and treated groups. Quantitative analysis was used where focal adhesion area per cell was determined and statistically represented as shown previously in [Figure 2.3.6]. These changes affect the morphology of endothelial cells. SsnB-treated cells demonstrate larger perimeter/cell area ratio than control cells, and become irregular in shape. Taken together, the results suggest that cells treated with SsnB increase their attachment to the substrate, which may play a role in inhibiting their migration, an essential process in angiogenesis. Actin stress fibers play an important role in cell migration; however, stress fibers contribute to cell adhesion as well through formation of stable actin bundles and focal adhesions (Tojkander et al. 2012). Previous work has shown that the tension and contractility of actin stress fibers can send signals that are important for focal adhesion

maturation and dynamics (Johnson et al. 2007). These results support the data shown by Bateman et al. in a previous study that demonstrated inhibition of endothelial cell migration in a Transwell migration assay in response to SsnB treatment (Bateman et al. 2013).

Microtubules are structural components of the mitotic spindle, and microtubule dynamics play an important role in several cellular processes including intracellular trafficking, cell migration and cell division (Müsch 2004). The inhibition of microtubule assembly dynamics has been shown to be the mode of action for several clinically successful anticancer drugs including vinblastine and vincristine (Dumontet and Jordan 2010; Singh et al. 2008). SsnB showed the ability to interfere with cell cycle progression in mitosis through down regulating some cyclins and cyclin-dependent kinases, regulatory proteins that control cell cycle progression, including CCNE2, CCNB1, CDC6, and CDC2. In our study, we examined whether SsnB could perturb microtubule assembly *in vitro*, but we did not obtain any meaningful results. The microtubules in stained cells, both control and SsnB treated, were fragmented and disconnected. We tried several approaches to determine why the anti-tubulin staining was unsuccessful. We tried to modify the staining procedure by using cold methanol as a fixative instead of formaldehyde. We also tried to use different concentrations of permeabilization solution, PBS- 0.5%- 0.1 %Triton X-100. Moreover, we were careful to ensure that the cells were healthy by using a new passage for each experiment, but all ended with the same conclusion. However, we will try to determine whether the antibody itself is the source of the problem. Since there is an identified connection between microtubules and cell

division, which SsnB interferes with, we are planning to continue to investigate the effect of SsnB on microtubule assembly in the future.

We observed either reduced upregulation or downregulation of the early response genes, specifically c-Myc, c-Fos, and c-Jun, in HUVECs after SsnB treatment. The effect of SsnB on c-Myc was more powerful in this study because it affected both c-Myc protein and mRNA. Immunoblotting of c-Myc protein revealed a decrease of expressed protein in response to SsnB treatment relative to vehicle control, and data were confirmed by qRT-PCR. The c-Myc mRNA level was reduced. This could be due either to a decrease in transcription or to an increase in its mRNA degradation, which remains to be determined. c-Myc functions are necessary and sufficient for the entry of most cells into the DNA synthetic (S) phase of the cell cycle (De Alboran et al. 2001). Studies of c-Myc in cancer showed that c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Deletion of c-Myc has a lethal effect on c-Myc-deficient embryos. c-Myc is also required for the proper expression of angiogenic factors such as VEGF; however, the precise mechanism by which c-Myc controls their expression is not resolved. Also, it is assumed that VEGF regulation by c-Myc to be indirect (Baudino et al. 2002).

qRT-PCR analysis demonstrates a reduction of c-Fos and c-Jun mRNA levels between 1 and 2 hours after SsnB treatment, even though no corresponding protein expression data were obtained by immunoblotting. c-Fos and c-Jun, members of the AP-1 protein family, have biological functions in controlling cell proliferation, survival and death. One of the major mechanisms that modulates AP-1 activity is the differential expression of AP-1 proteins in response to extracellular stimuli (Shaulian and Karin

2001). A study revealed new ways to block tumor angiogenesis by targeting Jun/AP-1, and established a functional, *in vitro* link between activated c-Jun and angiogenesis in breast cancer (Vleugel et al. 2006). Another study showed that the nuclear oncogene c-Fos regulates c-Fos-induced growth factor/vascular endothelial growth factor D (Figf/VEGF-D). This regulation is involved in transformation and in regulation of cell growth and differentiation of various tissues. Also, it has been demonstrated that developed tumors in c-Fos deficient mice appear lacking in vascularization (Marconcini et al. 1999).

In conclusion, our data demonstrates that early effects of SsnB on endothelial cells include promoting formation of stress fibers and focal adhesions, and reduction of early response gene expression. These events may contribute to the anti-angiogenic effect of SsnB. Future additional studies are required to fully understand the effect of SsnB on microtubule assembly, and to examine whether SsnB disrupts mitotic spindle and microtubule dynamics. Also, we would like to examine whether the effect on cytoskeleton is reversible after removing SsnB treatment *in vitro*. Moreover, we will investigate the effect of SsnB on c-Fos and c-Jun gene expression by using the Pfaffl method (Pfaffl 2001) to compare experimental genes and to the housekeeping gene.

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APPENDIX A- SUPPLEMENTARY DATA

HUVEC medium:

446 ml	K12 Medium
50 ml	FBS
50 mg	Heparin
50 mg	Endothelial Mitogen
4 ml	Non-Essential MEM Medium

Berk's Lysis Buffer (BLB) pH 7.5:

1.211 g	Tris base
8.766 g	NaCl
0.186 g	KCl
2.099 g	NaF
6.48 g	β-glycerophosphate
0.184 g	Na_3VO_4
5 ml	Triton X-100
5 ml	Nonidet P-40
Up to 1 L	H_2O

Cytoskeleton Buffer with sucrose (CBS)

1.07 g	MES pH 6.1
5.14 g	KCl
0.30 g	MgCl
0.37g	EGTA

Sucrose added fresh on the day of use of the buffer to final 0.32 M.