ABSTRACT

Title of Document: Salvianolic Acid B Inhibits Growth of Cervical Cancer Cells *in vitro* via Induction of Apoptosis through the Extrinsic Pathway

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In 2014 alone, over 12,000 women are expected to be diagnosed with cervical cancer. Of these women who are diagnosed, about 3,909 will result in death. Despite developments in prevention methods, cervical cancer remains a major health concern for women. Growing evidence suggests that Salvianolic acid B (Sal B), a major component of the Chinese herb Danshen, may inhibit cancer cell growth and help fight against cervical cancer. This study characterizes the potential of Sal B as a cervical cancer drug through *in vitro* testing on HeLa cells. We hypothesized that application of Sal B to HeLa cells will result in decreased cell viability and increased apoptosis in a dose dependent manner. HeLa cells were treated with varying concentrations of Sal B: 25µM, 50µM, 100µM, and 200µM. Cell viability was determined through colony formation assay, cell death ELISA, and nuclear morphology. An inhibitor study was also conducted for further apoptosis pathway analysis. Colony formation assay demonstrated a significant decrease in cell viability with increasing concentrations of Sal B with 75% viability at 50µM down to 0% viability at 200µM. Cell death ELISA and the analysis of nuclear morphology via Hoechst staining reported significant levels of apoptosis at concentrations equal to 50µM and greater. Furthermore, experiments using caspase inhibitors indicated that Sal B’s apoptotic effects are caspase-8 dependent. In conclusion, our results demonstrate that Sal B inhibits cancer cell growth by a mechanism that involves apoptosis induction through the extrinsic pathway.

Salvianolic Acid B Inhibits Growth of Cervical Cancer Cells *in vitro* via Induction of Apoptosis through the Extrinsic Pathway

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Table of Contents

Acknowledgements i

Table of Contents ii

List of Figures vi

1 – Introduction 1

1.1- Common uses of Danshen and Sal B 2

1.2- Importance of Wound Management 4

1.3- Complexity of Tissue Regeneration 4

 1.3.1- The Three Stages of Wound Repair 5

 1.3.2- Inflammation 5

1.3.3- New Tissue Formation 6

1.3.4- Remodeling 7

1.3.5- Angiogenesis 7

 1.3.6- Effect of Sal B on Wound Healing 8

1.4- Prevalence of Cervical Cancer 9

1.5- Cancer Background 9

1.6- Western Medicine and Traditional Chinese Medicine 13

 1.6.1- Current Treatments of Cancer 13

1.7- Apoptosis and Cancer Treatment 14

1.8- Salvianolic Acid B and Cancer 17

1.9- Sal B Paradox 21

2- Materials and Methods 22

2.1- Endothelial Cells 22

 2.1.1- Culturing 22

2.2- *In vitro* Scratch Assay 22

 2.2.1- Materials 22

 2.2.2- How the Assay Works 23

 2.2.3- Application to Our Research 23

 2.2.4- Our Experimental Setup 23

2.3- Cervical Cancer Cells 24

 2.3.1- Culturing 25

2.4-Colony Formation Assay 25

 2.4.1- Materials 25

 2.4.2- How the Assay Works 25

 2.4.3- Application to Our Research 25

 2.4.4- Our Experimental Setup 26

2.5- Cell Death Enzyme-Linked Immunosorbant Assay (ELISA) 26

 2.5.1- Materials 26

 2.5.2- How the Assay Works 27

 2.5.3- Application to Our Research 27

 2.5.4- Our Experimental Setup 27

2.6- Hoechst 33342 Staining 28

 2.6.1- Materials 28

 2.6.2- How the Assay Works 28

 2.6.3- Application to Our Research 29

 2.6.4- Our Experimental Setup 29

2.7- Hoechst Stain: Inhibitor Study 29

 2.7.1- Materials 29

 2.7.2- How the Assay Works 29

 2.7.3- Application to Our Research 30

 2.7.4- Our Experimental Setup 30

2.8- Human TNFα ELISA 30

2.8.1- Materials 30

 2.8.2- How the Assay Works 31

 2.8.3- Application to Our Research 31

 2.8.4- Our Experimental Setup 31

3- Results 32

3.1- Sal B did not affect RAOEC migration 32

3.2- Dose-dependent increase of HeLa cell death by Sal B 34

3.3- Dose-dependent increase of apoptosis in HeLa cells by Sal B 35

3.4- Caspase-8 inhibitor and pan-caspase inhibitor inhibit Sal B-induced apoptosis 38

3.5- Sal B Decreases TNF expression 40

4- Discussion 41

 4.1- Wound Healing 41

 4.2- Cancer 42

5- Future directions 45

6- Conclusion 47

Appendix A- RAOEC maintenance 48

Appendix B- RAOEC passaging 48

Appendix C- RAOEC staining 49

Appendix D- HeLa Media Change 49

Appendix E- HeLa Passaging 49

References 51

List of Figures

Figure 1. Effect of Sal B on RAOEC migration in an *in vitro* wound healing model 33

Figure 2. Colony formation assay demonstrates cell death induction by Sal B 35

Figure 3. Sal B induces apoptosis in HeLa cells 38

Figure 4. Pan-caspase and caspase 8 inhibitors prevent Sal B-induced apoptosis in HeLa cells 39

Figure 5. 200μM Sal B significantly reduces TNF expression in HeLa cells 40

1-Introduction

Traditional Chinese Medicine (TCM) has been used more than 2,500 years and is based on herbal formulas [1]. It was originally developed through clinical and theoretical studies for an array of treatments and prevention ailments. Over the years, rising interest in natural medicine has led to an increase of scientific, laboratory research on the effects and mechanisms of TCM [1]. TCM has proven to have fewer side effects than Western medicine, making TCM a very appealing alternative [1]. Today, Eastern medicine is more commonly accepted and applied to various diseases. It has been used to complement medical therapies for diseases such as cancer, rheumatoid arthritis, leukemia, and migraine [2].

One approach of using TCM is combining Western medicine and TCM. This approach has led to the discovery of Taxol, an extraction from the Pacific yew tree, which is now being used as a cancer treatment drug [3]. Taxol is just one example of the power and ability that TCM has to offer; however the difficulty stems from the isolation of the compounds from its natural form. Taxol was discovered in the 1960s and was not approved by the FDA until thirty years later in 1992 for treating ovarian cancer [4]. As seen in the development of Taxol, the precise pharmacological actions of the active ingredients in crude extracts must be analyzed in order for TCM to be approved for prescription. In our study we plan to do exactly this for Danshen, the dried root of *Salvia miltiorrhiza,* by studying the effects of one of its active compounds, Salvianolic acid B (Sal B), on wound healing and cervical cancer. We hope to reach a better understanding of Danshen’s and Sal B’s potential uses.

Danshen, which is the root of *Salvia miltiorrhiza*, is a traditional Chinese herb used to improve blood circulation for thousands of years. Recently, scientific findings show Danshen is also effective in the treatment of cardiovascular diseases and cerebrovascular diseases [5]. In addition, numerous Danshen products are commercially available as tablets, capsules, through intravenous injections, oral liquids, or dripping pills [6]. In 2002, the medicinal herb created a market worth more than $120 million [6]. Many clinical trials demonstrate that Danshen products are effective and safe for the treatment of cardiovascular diseases [6]. However, large randomized clinical trials and further scientific research to determine Danshen’s mechanisms of actions are necessary to ensure safe and effective usage. More than 50 compounds have been isolated from Danshen. The compound our study will focus on is Salvianolic acid B (Sal B) which is a major component of most Danshen products. Sal B is a water-soluble compound extracted from Danshen. Water-soluble compounds are easier and more versatile to work with in drug development [6].

1.1- Common uses of Danshen and Sal B

Danshen has been shown to be effective in activating and improving blood circulation [7,8]. The study of Danshen and its effects on cardiovascular disease began when eleven patients with angina pectoris were treated with Danshen injections in Shanghai’s Third People’s Hospital and saw symptomatic improvements within 24 hours [7]. This led to the creation of The Shanghai Cooperative Group for Study of Danshen in 1971, which continued testing Danshen’s effects on cardiovascular diseases and yielded favorable results [7].

The properties of Danshen help dilate coronary arteries, suppress the development of thromboxane, prevents blood clots, and impede myocardial ischemia [8]. Danshen has also been shown to have the ability to regulate key early events in atherosclerosis, a disease that results in the thickening of arterial walls [9]. Danshen suppresses acetylated low-density lipoprotein (LDL) uptake by human macrophages and increases the level of intercellular adhesion molecule expression and the adhesion of monocytes to endothelial cells [9]. Danshen has been used individually and in combination with other herbs for patients suffering from coronary artery disease in numerous countries including China and the United States [8].

Salvianolic Acid B is one of the major and most bioactive components of Danshen [10]. The specific mechanism through which Danshen and Salvianolic Acid B, activates and improves blood circulation was investigated through various studies on cardiovascular diseases [7,11-13].  One study focused on infarct-induced left ventricular remodeling, a possible side effect of myocardial infractions, which can lead to cardiovascular disease [14,15].  This remodeling can ultimately lead to congestive heart failure, which makes it a serious health concern [14,16].

Some research shows that Sal B may also have anti-cancer properties. In particular, a study done on head and neck squamous cell carcinoma both found Sal B to inhibit cyclooxygenase-2 (COX-2), a major protein in cancer development [17]. Other studies have shown that Sal B can promote the closure of a wound by up-regulating related growth factors and their receptors [18].

1.2- Importance of Wound Management

Infections caused by open wounds after surgery are a major concern in modern medicine. In the United States alone, 35 million cutaneous wounds serious enough to require intervention occur every year. In addition, an estimate of 2-5 million chronic wounds also occur annually in the United States [19]. If a wound is left unrepaired, the wound is likely to become infected, which causes a large portion of surgical patient deaths [19] . Although certain drugs can minimize the chance for infections, they have side effects and are not guaranteed to prevent infections. Therefore, rapid wound closure is the main priority of wound management. Understanding the intricate processes of wound repair and tissue regeneration is crucial to treating a wound [19].

1.3- Complexity of Tissue Regeneration

Wound repair is one of the most biologically complex processes [20]. Immediately following an injury or a wound, the body responds by activating several different cellular pathways. Components of the immune system rush towards the site of injury to fight bacteria and other infectious agents. The blood coagulation cascade is also activated to stop blood loss.

Many different types of cells are involved in the process and the injury triggers the cells’ proliferation, differentiation, and migration. Some of the cells involved are neutrophils, monocytes, lymphocytes, dendritic cells, endothelial cells, keratinocytes, fibroblasts, and epidermal cells. Once the first responses from these pathways are successful; these processes must then be terminated in a specific order while other processes become activated. Wound repair is such a complex process, scientists have classified wound repair into three major stages [21].

1.3.1- The Three Stages of Wound Repair

Scientists have identified the three classic stages of wound healing to be inflammation, new tissue formation, and remodeling [69]. Of particular interest to us is the second stage, new tissue formation, since our research method involves measuring the rate of cell migration as a way of measuring wound repair speed.

1.3.2- Inflammation

The first stage of wound repair is inflammation, which happens immediately after tissue damage in an attempt to restore homeostasis [69]. The main functions of the inflammation stage are to prevent blood and fluid loss, remove dead and dying tissues, and prevent infection. During this stage, components of the immune system, coagulation cascade, and inflammatory pathways are recruited. In the first step of this stage, the formation of platelet plug stops further blood loss, which is then followed by the formation of the fibrin matrix. Next, degranulation of platelets and products of bacterial degradation will recruit neutrophils to the wound site [22]. Following after, the neutrophils remove foreign material, bacteria, non-functional host cells, and damaged matrix components at the wound site. The removal occurs through an engulfing process known as phagocytosis, which clear away dead and dying tissues, thus preventing infection [23]. Monocytes at the wound site will differentiate into macrophages after 2-3 days and are considered critical in the coordination of future events. Without both neutrophils and macrophages, small wounds can still repair but the scarring response is far less than normal [21].

1.3.3- New Tissue Formation

The second stage of wound repair is new tissue formation, which occurs 2-10 days following the injury [69]. The main events of the new tissue formation stage are cell proliferation and migration. First, keratinocytes migrate over the injured inner layer of skin. Angiogenesis follows and capillaries connecting fibroblasts and macrophages replace the previous fibrin matrix [69]. The keratinocytes behind the leading edge undergo proliferation and eventually restore the barrier function of the epithelium. Later in this stage, fibroblasts stimulated by macrophages differentiate into myofibroblasts, which are contractile cells that connect the edges of a wound over time in a process known as re-epithelialization [24]. Together, fibroblasts and myofibroblasts produce an extracellular matrix in the form of collagen which ends up forming a large portion of the scar [21].

1.3.4- Remodeling

The third stage of wound repair is remodeling, which occurs 2-3 weeks following the injury, lasting for a year or more [69]. All previously activated processes terminate during this stage and most of the endothelial cells, macrophages, and myofibroblasts will either undergo programmed cell death called apoptosis or exit the wound site. What remains at the wound is mostly collagen, extracellular-matrix proteins, and the few cells that did not go through apoptosis or leave the wound. Next, interactions between the epithelium and mesenchyme regulate skin integrity and maintain homeostasis [25]. Matrix metalloproteinases (MMP) secreted by fibroblasts, macrophages, and endothelial cells are responsible for the remodeling process. Remodeling strengthens the repaired tissue, but the tissue will never regains the properties it had before the injury [21].

1.3.5- Angiogenesis

Another crucial component of the wound healing process is angiogenesis, which is the formation of new branches from already present blood vessels. This process occurs during the second stage of wound repair: new tissue formation. Angiogenesis is considered a critical component of wound healing since blood vessels are responsible for transporting oxygen and nutrients to the proliferating cells at the wound site [19]. While this process is integral to the process of wound healing, it also has recently been indicated as a contributing factor in cancer development and progression[19,26]. By releasing growth factors that cause the branching of existing blood vessels, cancerous tumors can bring in nutrients and flush out waste products [27]. This increased vascularization of the tumor also aids in metastasis[27,28].

1.3.6- Effect of Sal B on Wound Healing

Because wound healing is still a major issue, it is important to find an agent to aid the wound healing process. Both *in vitro* and *in vivo* studies have shown that Sal B facilitates the wound healing process [18,29]. In a 2003 study conducted by Lay and his colleagues, they observed angiogenesis in an *in vitro* model utilizing a murine endothelial cell line, SVR [18]. The study concluded that Sal B enhances the wound healing process, as evident by the up-regulation of VEGF (Vascular endothelial growth factor), an important protein involved in the wound repair process, and its receptors VEGF-R1 and VEGF-R2. Up-regulation of VEGF and its receptors were observed in the SVR cells after treatment with Sal B [14]. The possibility of Sal B enhancing the process of wound healing is the basis for the wound healing part of our research, and to do that we must understand the mechanism by which would repair occurs.

1.4- Prevalence of Cervical Cancer

Less than a decade ago cervical cancer was the leading cause of cancer death for women in the United States.  Although cervical cancer awareness has greatly improved, cervical cancer is still a major health issue for women. In 2009, 12,357 women in the United States were diagnosed with cervical cancer, and of these 12,357 women, 3,909 women died [30].  Most of the women affected were Black, Hispanic, or American Indian; indicating that cervical cancer is a bigger issue for women of minority groups.  In the past decade the incidence of cervical cancer has decreased in all groups while the incidence of cervical cancer in American Indian women has remained the same from 2000 to 2009 [30]. It is apparent then, that cervical cancer is a major health issue once a woman has been diagnosed. Therefore our team sought to focus on Sal B’s action on cervical cancer cells.

1.5- Cancer Background

A primary part of our research is to investigate if Sal B has an effect on cancer cell growth. In order for us to study cancer cell behavior, we must first understand the mechanism by which cancer develops. Cancer is a disease of unregulated cell growth [31]. Each time the cancer cells duplicate, the newly replicated DNA contains errors. Error-correcting proteins and cell cycle checkpoints usually detect and correct these mistakes in normal cells [32]. However, in cancer cells, these proteins become either overactive or inactive [32]. Such mutations lead to the six qualities that allow for cancer cells to grow, which are sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [32].

These six qualities are a way of describing the attributes of cancer, and are thought to be caused by two major enabling characteristics, genomic instability and tumor-promoting inflammation [32]. Cancer cells are able to continue dividing despite the lack of cellular growth factors, which leads to the uncontrolled growth that is normally attributed to cancer cells. They are able to achieve this in two ways; they can either overexpress growth factor ligands or stimulate the receptors on the cancer cells similar to autocrine signaling, or they can signal other cells, which will then send the cancer cells growth factors. Cancer cells can also cause the breakdown of negative feedback loops, resulting in a loss of homeostasis in the tumor-environment and no more regulation of cell division leading to unregulated growth [32].

To stop this growth, most normal cells contain growth suppressors, whose purpose is to kill any cell that has lost crucial growth regulatory mechanisms. However, in cancer cells these growth suppressors are compromised. The two key growth suppressors include TP53 and RB, both of which when individually knocked out showed no difference in animal physiology, which hints at them being part of a redundancy system. Tumor genesis is shown to be able to make both key growth suppressors lose function as well as compromise the contact-mediated suppressors, leading to the second hallmark of evading growth suppressors [32].

Genomic instability also contributes to cancer virulence. These mutations are allowed to run because the cell machinery, which is in charge of error-checking, suffers from loss-of-function in most cancer lines. These error-checking molecules signal to a normal cell to induce apoptosis if it detects notable levels of DNA damage, or sudden extreme changes in the cell environment. If mutations accumulate in normal cells, where they can no longer be fixed the cells will undergo apoptosis, a form of programmed cell death. However, cancer cells are resistance to this pathway, as well for similar reasons, the proteins that control the apoptosis pathway can be inactive or overactive, which prevents the cell from undergoing apoptosis [31-33].

Research has shown that unlike normal cells, cancer cells do not undergo programmed cell death once they have reached the end of their predetermined lifespan [32,34]. In normal cells, after a certain amount of divisions, telomere shortening limits the lifespan of the cell. In cancer cells, telomere shortening is greatly reduced because of significantly high levels of telomerase, leading to a lack of limited cell division creating the trait called “immortalization”. Some cancer cells have a loss of function in telomerase which contradictory to initial thought, leads to cancer growth because of extreme telomere shortening leading to DNA damage [32].

With all of this growth, cancer cells still have to be able to get the required nutrients and gas exchange that normal cells do. Inducing angiogenesis, the fifth hallmark, describes the neovasculature structure that arises during angiogenesis. The neovasculature structure keeps making vessels, which deliver nutrients to the cell; this allows the cancer cell to continue its prolific division. Angiogenesis was also found to be induced by the same oncogenes that cause the onset of other hallmarks, which showed that different hallmarks can be attributed to the same inducing molecule. Endogenous angiogenesis inhibitors are what regulate angiogenesis in normal cells, however in cancer cells they suffer from loss-of-function, which causes the development of the neovasculature and the nutrient delivery system [27,32].

The last hallmark of cancer is metastasis, the process by which cancer cells will leave their origin and begin growing in a new location. Metastasis starts by changing the cell adhesion of the cell. Normal cells display E-Cadherin, which acts as a tumor-suppressor by keeping nearby cells together in an epithelial sheet. In cancer, E-Cadherin loses function resulting in the segregation of cancer cells from any nearby cells. This goes into the invasion-metastasis cascade, which is the pathway for many cancer cell lines. Local invasion of the cancer cells then goes into the bloodstream, allowing for faster travel throughout the body, followed by cancer cells going into the parenchyma of distant tissues. Small nodules of cancer cells appear after this, and finally colonization occurs which is when micrometastatic lesions become macroscopic tumors [32]. It is clear then, that there are many ways by which potential cancer treatments can affect cancer cell growth, division, and invasion. Currently, many western cancer treatments are synthetically derived; however, as seen with the Taxol example, it is possible that new effective cancer treatments can be derived from natural sources.

1.6- Western Medicine and Traditional Chinese Medicine

1.6.1- Current Treatments of Cancer

The decline of cervical cancer incidences in the United States, as mentioned before, can be attributed to an increase in cervical cancer screening known as a Pap Test.  In 2008 73% of women reported having had a Pap Test in the past 3 years. 16% of these women were Black, 15% were Hispanic, 15% were White, and 14% were American Indian.  The fact that black women have the highest percentage for getting tested, but also have the highest rate of incidence proves that although Pap Tests are detecting cervical cancer, it is far from the solution to this problem.

Cervical cancer is almost exclusively caused by certain strains of human papillomavirus or HPV [35].  HPV is a sexually transmitted infection (STI) that can cause cervical cancer or genital warts.  HPV is one of the most common STIs and most women will have contracted the disease at some point in their lives.  Most times HPV will cause no symptoms and will go away within two years, however the longer the body takes to get rid of the HPV the higher the risk for cervical cancer to develop [35].

Similar to other cancer treatments, cervical cancer is treated through radiation, chemotherapy, and/or removal of the uterus in this case it is called a hysterectomy [35].  In addition to these treatments there are also several FDA approved drugs that are used to treat and prevent recurrence of cervical cancer.  Bleomycin, Cisplatin, and Topotecan are a few of these drugs [35].  Topotecan is the only drug that actually kills cancer cells, Cisplatin and Topotecan only slow or stop the growth of cancer cells [35].  All of these drugs have adverse side effects from less serious ones like hair loss to more serious ones such as loss of vision and difficulty walking [35].

In order to develop an effectively treat cancer; a potential cancer drug should induce death in cancer cells. There are two major pathways through which cell death can be induced, apoptosis and necrosis. Necrosis is often non-specific, meaning that it could induce cell death in normal cells as well as cancer cells. Apoptosis is a more favorable form of cell death; therefore effective cancer treatments should induce apoptosis in cancer cells.

1.7- Apoptosis and Cancer Treatment

A hallmark of a potentially effective anti-cancer treatment is the ability to induce cell death in cancer cells [33,36]. In order to be effective, a potential anti-cancer agent should cause cancer cells to die off, while sparing as many normal cells as possible [33]. It is possible to kill cells through necrosis - the process by which a cell’s membrane will become structurally unstable, eventually leading the cell to burst open, or by apoptosis - a process by which the cell’s DNA breaks down and the cells fragment into apoptotic bodies [34,37]. Typically, necrosis is a non-specific process; cells that are affected by an agent that causes necrosis will die, independent of cell type. Apoptosis, often called programmed cell death, proceeds through a series of steps that lead to the formation of cell fragments, and their eventual digestion by immune cells [31,38,39].

Apoptosis, because it relies on several checks and balances within a cell, can be very cell-type specific [40]. This kind of programmed cell death can be triggered by exogenous agents largely because it is a process that multi-cellular organisms rely on for normal growth, maturation, and physiological function [37,41,42]. Cells that have suffered damage to their genetic material will proceed through apoptosis in order to prevent harm to the organism as a whole. This “cell suicide” is intrinsic to the life cycle of cells, and the disruption of this process is a hallmark of disease - specifically, cancer [33,43].

Through the selective culling of damaged cells, an organism prevents the proliferation of damaged cells; however, in cancer, this process is de-regulated [44]. By becoming resistant to mechanisms that would normally cause apoptosis, these damaged cells continue to grow and divide, despite genetic damage that may prevent them from carrying out their normal physiological responsibilities [45]. As these cells continue to grow, mutations in the genome of these cells will continue to accumulate, eventually leading to a mass of cells that can gain the ability to move to other sites in the organism - in other words, a progression into cancer [31,46,47].

In order to cause apoptosis in a cancer cell, an agent must cause the cell to proceed through the steps necessary to complete the process. The pathway to apoptosis can be initiated by factors either inside the cell, through the intrinsic pathway, or those from outside of the cell, through the extrinsic pathway [45]. The intrinsic pathway is triggered by damage to the DNA, the genetic material of the cell, hypoxia, or lack of growth factors [31,37,48,49], while the extrinsic pathway is triggered by the binding of a factor to a cell death receptor on the surface of the cell which will lead to apoptosis [31,37,50,51]. Each of these pathways proceeds through separate mechanisms through the activation of different proteins. In the extrinsic pathway a ligand binds to the extracellular receptor, then the next protein in the pathway, DISC, recruits and activates Caspase 8 [50]. After Caspase 8 attaches to another Caspase 8 protein (dimerization), it will then proceed to activate Caspases 3, 6, and 7, thus carrying out its pro-caspase activity, and leading to cell death [52]. The intrinsic pathway is activated through damage to the DNA, lack of oxygen (hypoxia), removal of growth factors, and the activation of oncogenes [50]. Each of these signals will eventually cause the mitochondrial membrane to become porous, and allow factors that would normally be contained within the two membranes of the mitochondria to be released [37]. These factors, including cytochrome c, will aid in the formation of the apoptosome, a large protein complex that is crucial to inducing apoptosis [37,53,54]. The activation of these pathways, either extrinsic or intrinsic, will result in the fragmentation of the nucleus of the cell, as well as chromatin condensation and DNA laddering [55,56]. These fragmenting and dying cells will eventually be digested by cells of the immune system [37-39]. In order to study Sal B’s efficacy, our team sought to look at its effect on cancer cell apoptosis, as well as other cancer cell growth mechanisms.

1.8- Salvianolic Acid B and Cancer

Salvianolic Acid B, or Sal B, is an interesting target for cancer research [57,58]. The compound, isolated from the root of *Salvia Miltiorrhiza*, and its derivatives have been studied extensively for its effects on the cardiovascular system [6-9,59,60], the nervous system[61,62], the renal system [63], hepatocytes and hepatocellular carcinomas [5,64-66], and for its ability to prevent ischemia [11,62,67]; however, the study of Sal B in cancer is a relatively new field of study [58]. What makes Sal B an interesting target is how it seems to have contradictory effects depending on what cells are treated with it [18,58,64,68]. While Sal B has been shown to decrease apoptosis in endothelial cells exposed to oxidative stress [69], it has also been shown to up-regulate apoptosis through a caspase dependent pathway in Head and Neck Squamous Cell Cancer [17,58]; moreover, it has been shown to attenuate hepatocyte apoptosis [64], while also decreasing hepatocellular carcinoma cell viability and motility [65,66]. This paradoxical action of the compound makes it a particularly interesting candidate for research in cancer. Furthermore, the modulation of inflammation and cell migration by Sal B has been targeted as an area of research for its implications in cancer pathogenesis [58].

Salvianolic Acid B has been shown to cause apoptosis and decreased cancer cell viability in several cancer cell lines [13,17,70]. In head and neck squamous cell cancer lines [17,57,71], liver cancer cell lines [65,66], and leukemia [70]. Sal B promotes cell death through an apoptotic pathway in these cell lines. This is accomplished through the activation of the caspase family of proteins [52]. Other inducers of apoptosis in cancer cells include tumor necrosis factor and the presence of reactive oxidant species (ROS); however, it seems that Sal B attenuates oxidative damage by acting as an anti-oxidant [68]. Despite this attenuation, Salvianolic Acid B still up regulates apoptosis in these cancer cell lines. Salvianolic Acid B has also been targeted for its anti-inflammatory properties [57].

Inflammation plays a crucial role in cancer development and progression [72,73]. Inflammation is an important part of the immune response to microbes and other objects foreign to the body. It is also integral to the process of wound healing, stimulating the division of cells to close openings in endothelial tissue. This stimulation of growth, and encouragement of cells to proceed through the cell cycle, is a normal physiological process; however, when cells are frequently stimulated to grow and divide, it can lead to the development of cancer [72,73]. This chronic inflammation is the cause of hepatocellular carcinoma in patients infected with Hepatitis. The virus, a foreign object, stimulates inflammation in immunocompetent patients, and will stimulate the cells of the liver to divide [64,74]. Eventually, this continual cell division will result in the accumulation of mutations in the cell, and lead to cancer. Cancer progression and prognosis are also tied to inflammation [72]. Sal B has been shown to be a potent inhibitor of Cyclooxygenase 2, and important regulator of inflammation [71]. This anti-inflammatory property makes Sal B a particularly interesting target for research in cancer, especially types of cancer that have inflammation as an integral part of its pathogenesis, such as head and neck squamous cell cancer. The ability to simultaneously induce apoptosis in cancer cells, protect non-cancerous cells from oxidative damage, and prevent inflammation makes Salvianolic Acid B a strong candidate for a chemopreventive agent [57].

Sal B is an antioxidant, which can eliminate reactive oxygen species (ROS) and reduce oxidative stress [75]. Although this contributes to the vaso-protective properties of Sal B mentioned previously, previous research has postulated that this may also be related to its ability to kill cancer cells [60,68]. As mentioned previously, the two possible mechanisms for triggering apoptosis are intrinsic and extrinsic. Extrinsic apoptosis would mean that Sal B binds to an extracellular death receptor, and intrinsic apoptosis could be achieved through various intracellular stressors, one of such is through regulation of ROS activity.

Reactive oxygen species contribute to mutagenesis and carcinogenesis by damaging cellular DNA. They are also produced by HeLa cells, due to elevated metabolic activity or the presence of tumor inducing agents, which leads to further mutations [76]. Thus in many types of cancer cells, there is an elevated level of reactive oxygen species. Although cells normally activate apoptosis in response to elevated ROS to prevent carcinogenesis, the regulatory mechanism that activates this is dysfunctional in cancer cells. One such mechanism is the PI3-kinase/Akt pathway. Upregulation of this pathway reduces a cell’s response to stress signals, which normally triggers apoptosis [77]. It does so by inactivating pro-apoptotic factors such as Bad and caspase 9, which are produced during intrinsic apoptosis. The PI3-kinase/Akt pathway is regulated by the balance of levels of phosphotases, which dephosphorylate molecules, and kinases which phosphorylate molecules and upregulate the pathway to trigger apoptosis. ROS molecules produced by cancer cells can inhibit phosphatases by modifying their active site, leading to an increase in phosphorylation and a decreased apoptotic response to cellular stress, increasing tumor viability [77,78]. In addition to Sal B’s effects on cancer cells, as well as the aforementioned effects on wound healing, our team sought to study the seemingly contradictory effects of Sal B on angiogenesis in cancer and wound healing.

1.9- Sal B Paradox

Currently, Sal B is believed to have paradoxical effects on angiogenesis in the wound healing and cancer treatment. To improve the wound healing process, one would expect angiogenesis to be enhanced so as to improve transport of nutrients to proliferating cells. However, in the treatment of cancer, one would expect the process of angiogenesis to be down-regulated so as to inhibit the growth of cancer cells. As established by Lay and his colleagues, Sal B promotes angiogenesis in terms of up-regulation of VEGF and its receptors [18]. However, as concluded in a study by Yang, Sal B is associated with anti-angiogenic effects on oral cancer cells. Yang proposes a possible explanation for this contradiction. Their study suspects that Sal B prevents the formation of new vessels by promoting vascular maturation. Since angiogenesis in tumors tends to form unorganized and leaky vessels unable to support efficient blood flow. They predict that the effects of Sal B on blood circulation inhibits the formation of the leaky vessels in tumors while promoting the maturation of normal, healthy vessels, hence explaining the contradictory results from studies on wound healing and cancer [71].

Due to the results of these studies on Sal B our research goal is to show if Sal B has anti-cancer effect on other forms of cancer such as cervical cancer. Our research is also interested in investigating if Sal B can increase the rate of cell migration and proliferation in wound repair related cells such as endothelial cells.

2- Materials and Methods

In order to investigate Sal B’s effect on wound healing and cancer inhibition, we conducted research on two different cell types that are relevant to each case.

2.1- Endothelial Cells

To determine Sal B’s effect on wound repair, we used endothelial cells from the healthy aorta from adult female Sprague Dawley rat (RAOEC) purchased from (Cell Applications, Inc.) and established an *in vitro* wound healing model.

2.1.1- Culturing

The RAOEC cells were cultured in T-75 flasks purchased from (Fisher Scientific) and kept in Dulbecco's Modified Eagle Medium, DMEM (Life Technologies) media supplemented with 10% Fetal Bovine Serum, FBS (Gibco, Invitrogen). The cell cultures were incubated in NUAIRE cell culture incubator and kept at 37oC with 5% carbon dioxide. The RAOEC cells were fed new media (Appendix A) every two to three days and passaged (Appendix B) every four days.

2.2- *In vitro* Scratch Assay

2.2.1- Materials

The materials we used for the *in vitro* scratch assay include Sal B (Easy Buy Chemicals, eBioChem), BD Falcon 24-well tissue culture plate (Fisher Scientific), P-200 pipet tips (Fisher Scientific), methylene blue dye solution (Sigma-Aldrich), ImageJ software (downloaded for free from <http://www.nih.gov/>) , and other standard laboratory equipment.

2.2.2- How the Assay Works

The assay involves growing a monolayer of cells and then disrupting that monolayer by artificially inducing a scratch to simulate a wound. Cells were then stained at desired time points to allow better visualization of the cells. Cell migration rate is then observed visually and quantified by measuring the distance of the induced gap using computer software.

2.2.3- Application to Our Research

By quantifying the rate of migration of endothelial cells when exposed to media containing differing levels of Sal B, we can find out if Sal B has an effect on the cell migration rate and determine if Sal B enhances migration, which is what would be expected if Sal B enhances wound healing. Cell migration is a crucial part of new tissue formation, the second stage of wound healing [21]. Hence, increase in migration and proliferation of the endothelial cells when treated with Sal B will indicate that Sal B enhances the new tissue formation stage, and therefore enhances wound healing [21].

2.2.4- Our Experimental Setup

ROAEC cells were grown in DMEM supplemented with 10% FBS. 1 x 105 cells were seeded onto 24-well tissue culture plates and allowed to reach a confluent monolayer after 24 hours of growth. A scratch in a straight line in the vertical direction was induced across the monolayer by slowly and gently moving a sterile P-200 pipette tip across the well. While scratching across the surface of the well, the long-axial of the tip remained perpendicular to the bottom of the well to ensure equal gap distance across the scratch. Medium was then removed and replaced with appropriate medium. The control wells were replenished with normal medium while the experimental wells were replenished with media containing 1, 5, 10, and 20 μM of Sal B respectively. At the 0, 12, 24, and 36 hour time points, cells were stained with methylene blue dye solution and fixed to the well (Appendix C). The software ImageJ was then used to quantify the cell migration rate. Since the experiment was done in triplicates, there were 3 wells for each concentration of Sal B for each time point. By taking 3 pictures for each well and taking 5 measurements for each picture, we obtained 45 measurements to represent the gap distance for each concentration of Sal B at each time point. The data was then plotted to observe the decrease in the gap distance over time (Figure 1E).

2.3- Cervical Cancer Cells

To determine Sal B’s effect as a cancer treatment drug, we cultured cervical cancer cells (HeLa cells) and used various assays to examine if Sal B reduces cell viability and apoptosis.

2.3.1- Culturing

HeLa cells were cultured in T-25 flasks purchased from Fisher Scientific and kept in DMEM (Life Technologies) media supplemented with 10% FBS (Gibco, Invitrogen). The cell cultures were incubated in NUAIRE cell culture incubator and kept at 37oC with 5% carbon dioxide. The HeLa cells were fed new media (Appendix D) and passaged (Appendix E) every three to four days.

2.4-Colony Formation Assay

2.4.1-Materials

The materials we used for the colony formation assay were DMEM media, FBS, 24 well plates, Sal B, staurosporine (LcLabs), and PBS (Life Technologies).

2.4.2- How the Assay Works

The colony formation assay evaluates the effect of a substance on cell viability. As part of the assay, a small number of cells are seeded on a plate and allowed to grow in the presence of the substance of interest. After a chosen amount of time, colonies of at least fifty cells are counted to measure the cell’s rate of growth. A larger number of colonies imply higher cell viability. The assay also evaluates the number of cells killed, and whether cells stopped replicating [79].

2.4.3- Application to our research

The colony formation assay provides information about cell viability. An effective cancer treatment drug would ideally be able to kill cancer cells and inhibit their growth. Hence, one of the goals of our research is to investigate whether Sal B can inhibit the growth of cancer cells. The cell viability knowledge obtained from the colony formation assay would let us know if Sal B has the ability to inhibit growth of cancer cells [79]. If number of colonies formed from cells treated with Sal B is less than those of the control, then we know that Sal B lowers the cell viability of cancer cells, which would support our research hypothesis.

2.4.4- Our Experimental Setup

HeLa cells were grown in DMEM media containing 10% FBS. Next, the cells were seeded in 24 well plates at a density of 50 cells per well. After the cells were allowed to settle over two hours, the experimental groups were treated with 1, 5, 10, 20, 50, 100, and 200 µM of Sal B while the control was not treated and the positive control was treated with 50 nM of staurosporine. The cells were incubated at 37oC for 24 hours with treatment. Next, the media for all cells were removed, washed with PBS, and cells were then supplemented with DMEM (10% FBS) without Sal B or staurosporine. Cells grew for seven days before viable colonies were counted (Figure 2).

2.5- Cell Death Enzyme-Linked Immunosorbant Assay (ELISA)

2.5.1- Materials

The materials we used for Cell Death ELISA are HeLa cells, 2 mL centrifuge tubes, staurosporine, Sal B, and the materials found in the ELISA kit (Roche Applied Science).

2.5.2- How the Assay Works

The cell death ELISA measures the quality and quantity of DNA fragments after cell death. Cells are exposed to a growth agent conjugate, and then supplemented with an illuminator. After a chosen amount of time, the assay is measured by relative light units (RLU), which indicates the brightness of the cells. A higher RLU indicates a higher amount of cell death.

2.5.3- Application to Our Research

When cells go through apoptosis, the nucleus dissolves and the DNA fragments. The presence of these DNA fragments can be detected and quantified through the cell death ELISA. After cells are lysed, the supernatant, containing the fragmented DNA, is exposed to a surface antibody that binds exposed DNA histones. A secondary antibody can then be added which binds to the primary antibody, producing a blue-colored complex. The quantity of the complex can be measured by its light absorbance, in units of RLU (relative light units). A higher RLU value indicates the presence of more fragmented DNA, and more apoptosis.

2.5.4- Our Experimental Setup

5 x 104 HeLa cells were seeded in 2 mL microcentrifuge tubes with 1 mL DMEM media. Then, the experimental cells were treated with 50, 100, and 200 µM of Sal B and the positive control were treated with 50 nM staurosporine while the control was untreated. The cells were then incubated for 24 hours and the ELISA was carried out following Roche protocol at 405 nm (Figure 3F).

2.6- Hoechst 33342 Staining

2.6.1- Materials

The materials required are Hoechst 33342 stain (Sigma-Aldrich), HeLa cells, microscopy slides, Sal B, and staurosporine.

2.6.2- How the Assay Works

Hoechst 33342 stain binds to cellular DNA and fluoresces blue in response to ultraviolet light in the 460-490 nm region. It can therefore be used to visualize cell nuclei, which varies in morphology depending on whether cells are viable or apoptotic. Live cell nuclei appear large and circular within the cell. Apoptotic cell nuclei stain brightly due to chromatin condensation which puts DNA into a more compact state, and occurs as a precursor to DNA degradation [80]. Due to this condensation, nuclei also can appear to have more frayed edges, and in later stages of apoptosis the nuclei is fragmented, appearing as a mass of small, bright particles. The percent apoptosis is calculated by counting the total number of apoptotic cells in a field of view and dividing by the number of normal viable cells.

2.6.3- Application to Our Research

Our project determines the potential of Sal B as an anticancer drug. Apoptosis is a measure of cell death, and thus if Sal B causes a significant amount of apoptosis, it would be a potential anticancer agent. Measuring apoptosis is different than measuring just cell viability, which measures whether an agent causes cell death as well as arrests growth. Cell death can result from apoptosis, or necrosis. Necrosis is undesirable, because as previously stated, necrotic cancer cells burst and release proteins and growth factors which can have negative effects. Therefore, the ability of Sal B to cause apoptosis is important to assess

2.6.4- Our Experimental Setup

HeLa cells were seeded at 1 x 105/mL on each well of a 3-well microscope slide, and subjected to different treatments. Treatments included 50, 100, 200 µM Sal B and 50 nM staurosporine. Cells were incubated for 24 hours, and then stained with Hoechst 33342 for quantification (Figure 3).

2.7- Hoechst Stain: Inhibitor Study

2.7.1-Materials

The materials required are Hoechst 33342 stain, HeLa cells, microscopy slides, Sal B, caspase 8 inhibitor, caspase 9 inhibitor, and pan caspase inhibitor.

2.7.2- How the Assay Works

See Hoechst 33342 staining above for how a Hoechst stain works.

2.7.3- Application to Our Research

The assay is necessary in order to further study Sal B’s mechanism of action, specifically whether Sal B causes intrinsic or extrinsic apoptosis. Intrinsic apoptosis involves recruitment of caspase 9, and extrinsic apoptosis involves caspase 8. The active caspase protein can be identified by staining HeLa cells treated with Sal B, as well as with different caspase inhibitors. That is, if cells treated with Sal B and a specific caspase inhibitor does not go through apoptosis, it can be inferred that the specific caspase is essential to the apoptotic pathway of Sal B.

2.7.4- Our Experimental Setup

HeLa cells were seeded at 1 x 105/mL in each well of a 3-well microscope slide and treated with 200 uM Sal B. Cells were also treated with 50 nM of either Caspase 8, 9, or pan-caspase inhibitors. As above, Cells were incubated for 24 hours, and then stained with Hoechst 33342 for quantification (Figure 4).

2.8- Human TNFα ELISA

2.8.1- Materials

The materials required are anti-TNFα capture antibody (Pharmingen), binding solution (0.1M Na2HPO4), HeLa cells, 96-well plate, staurosporine, Sal B, PBST, TNFα standard tube, biotin anti-human TNFα (Pharmingen), streptavidin-AP, PNPP tablet, aluminum foil, parafilm, plate reader.

2.8.2- How the Assay Works

The TNFα ELISA quantifies the amount of TNFα present in the samples. See Cell death ELISA above for how an ELISA works.

2.8.3- Application to Our Research

Tumor Necrosis Factor-alpha (TNFα) is a protein, which has cytotoxic effects on a wide range of cells including tumor cells. By quantifying the amount of TNFα present, we can determine if Sal B is using the TNF pathway to signal cell death. If Sal B is using the TNF pathway or is mimicking it, we should observe higher amounts of TNF present. If Sal B does not use the TNF pathway we should observe low amounts of TNF. This assay will help us understanding the mechanism by which Sal B induces cell death.

2.8.4- Our Experimental Setup

1 x 104 HeLa cells were seeded per well in 96 well plates. The experimental cells were treated with 50, 100, and 200 µM of Sal B in triplicates and the positive control were treated with 50 nM saurosporine while the control was untreated. The cells were then incubated for 24 hours and the ELISA was carried out following the Pharmingen protocol at 405 nm and 620 nm.

3- Results

3.1- Sal B did not affect RAOEC migration

To analyze the effect of Sal B on new tissue formation during wound healing, RAOEC were exposed to Sal B ranging from 1 to 20μM and RAOEC migration rates were measured with the *in vitro* scratch assay over 36 hours. Gap distance decreased uniformly across all Sal B concentrations (Figure 1E). However, Sal B had no significant effect on RAOEC migration relative to the control of no Sal B (Figure 1E).



**Figure 1. Effect of Sal B on RAOEC migration in an *in vitro* wound healing model.** Scratch assays were performed on confluent monolayers of RAOEC as described in the methods section. RAOEC were visualized with light microscopy at 4x 0hr (A), 12hr (B), 24hr (C), and 36hr (D) post-scratch and incubation in 37°C, 5% CO2. RAOEC were exposed to concentrations of 0.01, 0.1, 1, and 10μM Sal B immediately post-scratch, and gap distance was measured over 36 hours post-scratch in triplicate in three independent trials.

3.2- Dose-dependent increase of HeLa cell death by Sal B

The effect of Sal B on HeLa cell viability was determined with the colony formation assay. In this study, a viable colony consisted of at least 50 cells (Figure 2A). As shown in Figure 2C, Sal B decreased HeLa cell viability in a dose-dependent manner. Sal B at concentrations 25 μM (78.9 ± 30.0%), 50 μM (72.2 ± 22.6%), 100 μM (47.1 ± 21.4%), and 200 μM (0.0%), significantly decreased HeLa cell viability normalized to the untreated control (100%). HeLa cells treated with 200 μM Sal B showed 0% cell viability, which was lower than the cell viability of the positive control, staurosporine (10.2 ± 6.9%).



**Figure 2. Colony formation assay demonstrates cell death induction by Sal B.** (**A)** viable HeLa cell colony (over 50 cells) visualized at 100x. (**B)** non-viable HeLa colony. (**C)** dose dependent effect of Sal B on the number of viable HeLa cell colonies. Cell viability was determined by counting the number of viable colonies as described in the methods section. All experimental groups were normalized to the average control value, which was set to 100% viability. \* p < 0.05 when compared to the untreated control cells

3.3- Dose-dependent increase of apoptosis in HeLa cells by Sal B

Hoechst nuclear staining was performed in addition to the colony formation assay to distinguish whether the decrease in HeLa cell viability was due to necrosis or apoptosis--the latter being more favorable. Apoptotic HeLa cells were quantified with Hoechst nuclear staining. Apoptotic cells were visualized by fragmented nuclei (Figure 3A-D). Sal B increased the percent of apoptotic cells in a dose-dependent manner (Figure 3E). Sal B had a significant effect on the percent of apoptotic cells at concentrations 50 μM (5.5 ± 2.1%), 100 μM (10.8 ± 2.7%), and 200 μM (17.6 ± 4.0%) compared to the untreated control (1.5 ± 0.9%). HeLa cells treated with 200 μM Sal B had a greater percentage of apoptotic cells than that of the positive control staurosporine (15.5 ± 3.9%). Cell death ELISA was performed in addition to the Hoechst nuclear staining to further determine Sal B’s effect on apoptosis in HeLa cells. Cell death ELISA data show 100 μM and 200 μM Sal B significantly increased DNA fragmentation in HeLa cells by 1.4-fold and 1.8-fold respectively, normalized to the control cells without Sal B (Figure 3F).



**Figure 3. Sal B induces apoptosis in HeLa cells.** HeLa treated with different concentrations of Sal B were stained with Hoechst and visualized via fluorescence microscopy at 100x as described in methods. (**A)** control; (**B**) 50 nm staurosporine; (**C)** 100 uM Sal B; (**D**) 200 uM Sal B. Arrows indicate apoptotic cells. (**E)** Dose-dependent effect of Sal B on the percent of apoptotic HeLa cells. Percent Apoptotic cells was determined by dividing the number of apoptotic cells by the total number of cells in each experimental group. Asterisks indicate p<.05. (**F)** Apoptosis was determined using the Roche Cell Death ELISA kit. All absorbance values were normalized to the average value of the control group. Experiments were performed in triplicate with 3 technical replicates. \* p < 0.05 as compared to the untreated control cells.

3.4- Caspase-8 inhibitor and pan-caspase inhibitor inhibit Sal B-induced apoptosis

Using Hoechst nuclear staining, apoptosis was detected in both HeLa cells treated with 200μM Sal B alone and HeLa cells treated with Sal B + caspase 9 inhibitor (Figures 4B, 4D). Apoptosis was not as frequently visible in both HeLa cells treated with Sal B + caspase 8 inhibitor and HeLa cells treated with Sal B + pan-caspase inhibitor (Figures 4C, 4F, 4E). Both groups treated with pan-caspase inhibitor and caspase-8 inhibitor had significantly low percentages of apoptotic cells--3.5 ± 1.3% and 5.2 ± 0.9% respectively (Figure 4F).



**Figure 4. Pan-caspase and caspase 8 inhibitors prevent Sal B-induced apoptosis in HeLa cells.** HeLa cells were treated with 200 μM Sal B and different caspase inhibitors, stained with Hoechst, and visualized with fluorescence microscopy at 100x. (**A)** control; (**B)** Sal B; (**C)** Sal B + pan-caspase inhibitor; (**D)** Sal B + caspase 9 inhibitor; (**E)** Sal B + caspase 8 inhibitor. Arrows indicate apoptotic cells. **F.** Quantitative inhibition of Sal B via caspase inhibitors. Apoptotic cells were identified using the Hoechst staining method. Percent apoptotic cells were determined by counting the number of apoptotic cells as compared to the total number of cells as described in methods. \* p < 0.05 as compared to the untreated control cells.

3.5- Sal B Decreases TNF expression

Human TNF ELISA was performed to further determine Sal B’s effect on apoptosis in HeLa cells. The data shows that 200 μM Sal B significantly decreased TNF levels in HeLa cells almost twofold, normalized to the control cells without Sal B (Figure 5).

**Figure 5. 200μM Sal B significantly reduces TNF expression in HeLa cells.** TNF levels were quantified using Human TNF ELISA (Pharmingen) in HeLa cells with Sal B or 50 nM staurosporine, All absorbance values were normalized to the average value of the control group. Experiments were performed in triplicate with two technical replicates. \* p < 0.05.

4- Discussion

4.1- Wound Healing

Sal B has been shown to increase angiogenesis both *in vitro* and *in vivo*; however, our results demonstrated no significant difference in the rate of wound healing between the Sal B treated cells and the control cells [18,29]. The discrepancy between the literature and our results can be explained by the simple nature of our model and experimental design. Wound healing is a complex and intricate process, which involves various growth factors and tissue types. Our model, however, consisted only of a single cell type, endothelial cells [19,21,23,81,82].

The simplification of the wound healing model to a single tissue creates the possibility of missing interactions that would normally occur *in vivo.*

Since our model was comprised of one cell type, the target of Sal B or interactions between different tissue types may have been missed since these elements are not present in our model. Furthermore, our study used RAOECs, a primary cell line, whereas other researchers who observed wound healing after the application of Sal B used transformed endothelial cell lines, which differ in form and function due to the properties of an immortalized cell line [29]. Even though our study did not find a significance increase in wound healing, the results do not mean that Sal B has no effect on wound healing, but simply that our particular study did not observe this phenomenon.

4.2- Cancer

Salvianolic Acid B is a compound that has been studied heavily for its cardiovascular effects, and has recently been the target of research into its anti-cancer properties. Sal B has also been shown to decrease COX-2 associated inflammation in Head and Neck Squamous Cancer Cell lines, making Sal B a potential chemopreventative agent for cancers associated with inflammation as one of their etiologies. Furthermore, Sal B has been shown to have interesting effects in hepatocellular carcinoma cell lines – both inducing apoptosis in cancerous liver cells, while simultaneously decreasing the rate of healthy hepatocyte apoptosis [64,66]. Sal B has also been shown to increase the rate of wound healing in endothelial cell lines, as well as act as an antioxidant [18,68]. Sal B’s seemingly contradictory effects make it an interesting target for research, and warrants further research into its mechanism of action.

Here, we report that our data indicates that Sal B has anticancer effects on HeLa cells similar to its effects on HNSCC lines, and acts through extrinsic, and not intrinsic apoptosis. Our experimentally determined IC50 was approximately 100 µM, which is consistent with the value previously found by Hao et al. (2009) for head and neck cancer JHU-013 cells, at 50 µM. In order to understand the mechanism of Salvianolic Acid B, our team first established its effects on our model system through the clonal assay. Our data showed a dose dependent decrease in cancer cell viability at concentrations above 25 µM, reaching a 100% decline in viability (n>50) at the 200 µM concentration. This assay shows that Sal B has a significant effect on cancer cell viability. The standard deviation values for the experimental groups was higher than expected, which may have been due to difficulties in consistently plating 50 cells per well. This was because the distribution of cells in suspensions is often not even, as cells tend to clump together and settle on the bottom of flasks. It was also difficult to obtain a specific number of cells through serial dilutions due to the random distribution of the cells. Despite this, we were able to identify a number of concentrations of Sal B that caused significant decreases in viability.

To further characterize Sal B’s effects on HeLa cells, experiments were performed to determine the mechanism of this action. Nuclear staining assays and cell death ELISA were performed to determine Sal B’s effects on viability were due to apoptosis. The results of the nuclear staining assay confirmed that Sal B caused apoptosis, with 200 µM Sal B achieving a level of apoptosis comparable to 50 nm staurosporine. The results of the cell death ELISA corroborated this. In both apoptosis studies, although the overall apoptotic effect of Sal B did, not appear to be as large as its effect on viability in the colony formation study, reaching a maximum of 17.6% apoptotic cells at 200 µM. However, this was to be expected due to the methodology. The colony formation assay works by counting the total number of viable colonies that can be produced from single cells. Cells that have died by apoptosis and necrosis, as well as cells which are unable to replicate, are considered not viable. In contrast, the nuclear stain assay only counts cells that have gone through apoptosis. In the nuclear staining assay, 25 µM was not significant, although the p value in both cases was close to .05, with p=.03 in the viability assay and p=.07 in the nuclear staining assay. Overall, the data shows that Sal B causes a significant decline in HeLa cell viability, part of which is achieved through apoptosis. With these anticancer properties, as well as an already proven ability to increase wound healing and prevent cardiovascular damage, Salvianolic Acid B is a promising drug for chemo-preventative treatment [18,29].

Hao et al. (2009) showed that Sal B acted by upregulating COX-2 and caspase-3 in head and neck squamous cell cancer cells, so we chose to investigate the mediators upstream that are responsible for these changes [17]. Prior to our experiment, the major theories about how Sal B interacted with cancer cells, were that Sal B acted in cancer cells by binding to an extracellular receptor, or by mediating oxidative stress [68]. Although Sal B is an antioxidant, and the presence of reactive oxygen species is reported to trigger apoptosis in some cancer cells, there are other known antioxidative agents which have also been shown to trigger apoptosis in cancer cells through modulation of regulatory pathways that lead to intrinsic apoptosis [77]. However, our data did not support this theory in our HeLa cell model, as the mechanism of Sal B was found to be caspase 9-independent, and therefore not intrinsic. Instead, the mechanism involved caspase 8, which is associated with extrinsic apoptosis.

5- Future directions

In regards to the effects of Sal B on wound healing, future studies could utilize different wound healing models. The *in vitro* model is too simple to adequately capture the complex process of wound healing; therefore an *in vivo* model would be required to test the effectiveness of Sal B on wound healing. A mouse or rat model can accurately mimic the wound healing process, which would prevent the lost interactions that appeared in the *in vitro* model. A study in an *in vivo* model could prove the effectiveness of Sal B as a potential wound healing agent or reaffirm our initial findings.

Our data indicates that Sal B causes apoptosis in HeLa cells through extrinsic apoptosis, through binding of a ligand to an extracellular death receptor. Future studies are necessary in order to determine the identity of the receptor. A prior study on the effect of Sal B in cardiovascular cells found that Sal B is a competitive inhibitor of the MMP9 receptor [12]. MMP9 dissolves the extracellular matrix, and allows cells to invade tissues. It also induces angiogenesis in endothelial cells. In cardiovascular cells, inhibition of MMP9 prevents structural damage to the heart after myocardial infarction, and does not affect cell viability [12]. However, in cancer cells, MMP9 is known to promote metastasis and invasion of other tissues [83,84]. Also, MMP9 stimulates cancer cell proliferation, and its inhibition has been shown to trigger apoptosis in prostate cancer cells through the extrinsic pathway [85,86].

Because our data indicates that Sal B acts through the extrinsic apoptosis pathway and not the intrinsic pathway, and MMP9 is necessary for cancer cell viability, it is plausible that the effects of Sal B on cancer cells are due to its MMP9 receptor-binding properties. Future research should confirm this by investigating the effects of Sal B on MMP9 receptor expression, and other mediators of the extrinsic apoptosis pathway in HeLa cells. This theory is also consistent with prior research indicating that Sal B down-regulates COX-2 production, which is also involved in the cancer inflammation and angiogenesis pathway [17].

This may also account for the “Sal B paradox” found in previous research: that Sal B seems to have different effects on apoptosis and angiogenesis in different cell lines. Although MMP9 is necessary for cancer cells to remain viable, it is has less importance for other cell types. MMP9 also upregulates other pro-angiogenic factors, so inhibiting it would reduce angiogenesis. Also, triggering apoptosis in cancer cells would eliminate the production of other proteins that could induce angiogenesis. In an *in vivo* system, MMP9 downregulation would also inhibit angiogenesis by decreasing liberation of VEGF from the extracellular matrix [86]. This contradiction in the effects of Sal B may be the result of the different oxidative profiles of the different cells, and their differing responses to oxidative stress. Although in cancer cells, Sal B’s antioxidant properties do not trigger changes in viability, it can reduce extracellular ROS. Cancer cells are not as sensitive to increased ROS due to signaling changes and overall elevated basal levels, however normal endothelial cells are. Thus reducing ROS in endothelial cells protects against apoptosis and contributes to cell viability, a protective effect reported in previous studies [75]. Future study will help uncover the pathway, which Sal B uses to induce apoptosis in cancer cells. Understanding the pathway is important for its future use as an anticancer therapy.

6- Conclusions

Our data indicates that Sal B causes apoptosis in HeLa cells through extrinsic apoptosis, with an IC50 of 100 µM. However, contrary to previous research we did not find any effects of Sal B on angiogenesis, possibly due to the simplicity of our wound healing model. Overall, the cellular mechanisms by which Salvianolic Acid B causes these diverse effects is still an excellent target for research in cancer, and in other fields, such as wound healing, cardiac remodeling, inflammation, and cellular migration. The diversity of the observed properties of Salvianolic Acid B makes isolating its mechanism of action, and changes depending on cellular profile, an important candidate for further research.

**APPENDICES**

**Appendix A: RAOEC maintenance**

1. RAOEC cultures should be fed 24 hours after plating and then every 48 hours afterwards.
2. Remove flask of cells from incubator and check them under the inverted microscope at 10x magnification.
3. Place media in the 37oC water bath. Remove media after it is warm, wipe off water and spray with 70% ethanol. Place solutions and flasks in the biohood.
4. Unscrew the cap of the flask and place aside.
5. Move the media to the corner top of flask – no cell area. Aspirate off the media using a Pasteur pipet attached to a vacuum line. Be careful not to disturb the cells in the flask as well as avoiding the neck of the flask.
6. Pipet necessary volume of media into the flask.
7. Make sure that the cell plated side is covered with media by rocking the flask.
8. Place cap back onto the flask and put it into the incubator.

**Appendix B: RAOEC passaging**

Preparation:

After being sure the cultures are confluent.

Get media for new flasks. Let sit out until room temp.

Ready new flasks for cultures using attachment factor.

Warm Trypsin, but not quite to 37 °C.

1. Turn on biohood blower and let air flow through for 15 minutes.
2. Sterilize biohood with EtOH.
3. Ensure that all pipettes, pipette tips, and reagent solutions are sterile.
4. Remove media using vacuum hose.
5. Pipet on 5 mL PBS (acts as a buffer, cleans surface of residual media/proteins.
6. Rock back and forth across cells then remove using vacuum hose.
7. Add Trypsin 3-4 mL for the T-75 flasks (about 5-7 for the T-150 flasks)
8. Let sit for 5 minutes initially. Check under microscope to see if cells have been freed and are floating. If not, check every 3 minutes. DO NOT wait more than 15 minutes to go onto the next step.
9. Add an equal amount (as in step 7) of media to the flask. Spray directly on the cell side in a fan-like pattern and rock back and forth over the surface.
10. Put in smaller 15 mL tube
11. Centrifuge into a pellet. Set Centrifuge to 5xg (setting 2) for 5 min.
12. Balance the opposite side of centrifuge with “weights” of equal volume. They are to the right of the centrifuge.
13. Clean the Green pipet (located on hooks attached to left side of incubator) by spraying a chem wipe with ethanol. Not by spraying directly. The tips for pipet are in the Green sterile box. Set pipet to 100 µL.
14. Remove all but the pellet within the 15 mL tube with the vacuum hose.
15. Resuspend cell pellet in 10 mL of solution. Mix well until homogenous.
16. Micro pipet 100 µL into a centrifuge container (to count).
17. Stain Trypan blue to count on hemacytometer. Use Red pipet with the containers with filter (look like white dots in bottom).
18. Pipet only 10 µL into 2nd container along with 10 µL of the stain.
19. Out into hemacytomer under cover slip via the triangle opening in the side.
20. Count only the cells in the four 4x4 corner areas of hemacytometer.
21. To calculate the number of cells currently present in entire sample: (# counted)/4 \* 2 \* 10^4 = cells/mL
Then multiply by total volume for total number of cells
22. To clean hemacytometer/cover slip:
Spray with ethanol until blue is no longer visible
Air dry before replacing them in cabinet
23. Remove attachment factor from flask.
24. Divide up cell solution evenly among the new flasks.
25. Add additional media until the total volume present is 15 mL (for T-75, for T-150, 22 mL).
26. Place flasks in incubator.

**Appendix C: RAOEC staining**

1. Remove cell culture plates from incubator.
2. Remove media from cell culture plates.
3. Wash cells with PBS.
4. Remove PBS.
5. Add 500 µL of methylene blue dye to cells and wait 30 minutes.
6. Remove methylene blue dye.
7. Wash with water.
8. Observe under microscope.

**Appendix D: HeLa Media Change**

1. Take out cells from incubator. Remove medium from cells.
2. Add medium.
3. Return to incubator.

**Appendix E: HeLa Passaging**

1. Make 1:10 dilution of (0.25%) trypsin.
2. Take out cells from incubator. Remove medium from cells.
3. Wash cells with PBS.
4. Add diluted trypsin to cells. Incubate cells at 37ºC for 3 min. Check for cell detachment under microscope. If not detached, incubate longer.
5. Add medium to trypsinized cells to inactivate trypsin.
6. Transfer cells to 15 mL tube.
7. Centrifuge at 300g for 3 min.
8. Remove supernatant.
9. Resuspend cell pellet in medium.
10. Add cells to new container.

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