Drug discovery: identification of anticancer properties of podophyllotoxin analogues

By

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

May 2020

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ABSTRACT

The purpose of this research involves the study of plant compound podophyllotoxin and the potential benefits of podophyllotoxin analogues on cancer therapy. Podophyllotoxin has been found to have anticancer properties due to its role in tubulin depolymerization within the cell cycle. Cancer is a growing concern throughout the United States and across the world. The most well-known cancer therapies are synthetic in nature and can be very harmful to the body, leaving the growing need for natural product plant-derived anticancer therapies. In this study, the potential anticancer properties of structural analogues of podophyllotoxin will be evaluated for their effects on cell viability, apoptosis induction, cell-cycle control, and migration. Podophyllotoxin analogues will be screened using cell lines A549, MCF7, MDA-MB-231, SK-MEL-28, and Jurkat, with the aims of discovering more potent compounds.

Acknowledgements

I wish to thank and acknowledge everyone who truly made this thesis possible. Dr. Sev, thank you for entrusting me with this project from the beginning. You genuinely are one of my biggest inspirations and I aspire to continue growing into the researcher you taught me to be; I hope to make you proud beyond my years at YSU. Thank you for pushing me to the greatest of my abilities and for never giving up on me. Dr. Walker, thank you for immediately thinking of me when this project was first introduced to YSU. Your guidance in and outside of the lab will always be extremely appreciated. You were the first person who began teaching me how to be a scientist, and for that I am eternally grateful. Dr. Steelant, thank you for taking the time to be on my committee, and pushing me to think professionally and scientifically. Dr. Benyo, you have made more of an impact on me than you probably realize. You are so encouraging and supportive, and one of the biggest reasons I chose to continue my education and earn a PhD. I will never stop trying to make you proud. To all of my other professors, thank you for preparing me for graduate school in my undergraduate years, and shaping me into a scientist during graduate school.

Youngstown State University, you will be missed.

To my family, you sincerely do not know the impact you have had on making my education a success. I'm in tears writing this because I am so beyond blessed to have a family as supportive as you. Everything I am and hope to be, is because of you and I'll never be able to thank you enough. To my friends and lab teammates, I wish I could write something for each of you, but I'd take up too much room, so I'll leave it at this: y'all are the very best. Every time I thought I couldn't do it and every time I doubted myself, you all believed in me and lifted me up. The amount of support you gave me in the lab is immeasurable and even though it didn't always seem like it, you didn't go unnoticed or unappreciated (you know who you are). My best friends outside of the lab, I couldn't have possibly done any of this without you, thank you for always wiping the tears away and being the absolute best cheerleaders.

To everyone involved, and everyone in my life: much love and thanks.

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Chapter 1. Introduction

Cancer is currently the second leading cause of fatalities worldwide [1]. Approximately 38.4% of men and women in the United States, alone, will be diagnosed with cancer during their lifetime, with around 35% of diagnosed cases resulting in death [2]. Due to the advancement of medicine and technology, early detection and treatment of cancer have been shown to extend the lives of many cancer patients [1]. Cancer can occur at any location and can rapidly spread to other parts of the body via the cells traveling through the circulatory system. The human body consists of trillions of cells, as cells form the foundation for all living things. All cells in the body have specialized functions and duplicate their DNA when needed for growth, repair, or reproduction. Unfortunately, cells in distinct body regions may undergo abnormal proliferation. This uncontrolled rapid growth of damaged cells is a definitive cancer characteristic [19]. Cancer is a complex disease with several factors influencing its development, with many unknown mechanisms speculated to remain.

Currently, chemotherapy, radiation therapy, and surgery are among the most common cancer treatment methods. Chemotherapy treatment has proven effective for various types of cancer; however, the adverse effects of chemotherapy are very damaging to the body. Chemotherapy not only kills the cancer cells, but cannot be administered to solely target the cancer, leading to the death of healthy cells in the body as well [4]. Radiation treatment and surgery also pose their own threats to the human body, which led researchers to have the desire to find less harmful cancer treatments. Chemotherapy is the most widely used

cancer treatment; however, chemotherapy drugs are synthetic in nature, meaning they are formulated from chemicals in a laboratory. Natural or semi-synthetic drugs are derived from natural products and are becoming more prevalent in cancer research because of their potential to have fewer harmful effects on the body.

1.1 Cancer

1.1.1 Introduction

Cancer is generally characterized as a stem cell disease in which case stem cells lose their normal control of cell division. One distinctive feature of cancer is uncontrolled rapid proliferation of damaged cells. Proliferation is evident by altered expression and/or altered activity of proteins involved in the cell cycle [24]. As previously stated, cells duplicate when needed, typically to replenish lost cells generally lost to aging. Most cells within the human body reproduce at a constant rate and because of this several cells may become damaged. Damage to cells occurs most frequently as a result from the harsh molecules humans consume via food products, pharmaceuticals, or environmental contaminants. Products produced from natural energy-production including glucose and free-radicals are of the most damaging to fats, proteins, and the DNA within cells [18]. Ordinarily, the body is able to repair these cell damages or undergo apoptosis; however, occasions arise when the damages aren't repaired prior to division and apoptosis fails thus the damages are passed to the daughter cells during division. The damages may then continue through generations of cells, accumulating an abundance of impaired cells.

1.1.2 Historical Review

Cancer is a highly complex disease and the exact cause of every type of cancer may never be fully understood. However, historically speaking, several causes of cancer have been identified. The International Agency for Research on Cancer (IARC) has classified pharmaceuticals, hormones, alcohol, bacteria, fungi, sunlight, tobacco, among some of the primary carcinogenic agents [25]. The exact cause of cancer has fascinated people for thousands of years [25].

Early studies, dating back to the 1700s, sought to identify carcinogens involved the analysis of specific occupations with the implication that cancers were caused from environmental exposures. Early research was inherently observation based prior to the discovery of advanced diagnostic tools. Individuals sharing an occupation would often present with similar cancer development, including nuns suffering from breast cancer, chimney sweepers and coal tar distillers developing scrotal cancer, and early radiologists expiring from skin cancers [25].

The theories of what causes cancer began to expand into experimental studies. It was hypothesized chronic irritation, displaced embryonal tissue, and/or infectious agents also caused cancer development. Utilizing the theories hypothesized, researchers attempted to generate cancer in experimental animals; however, malignant growths failed to form, and extracting microbes to produce cancer was unsuccessful [25].

Several years later the observations made from early occupational studies intrigued researchers into looking at specific compounds in coal tar that may

produce cancer. Dibenz(a,h)anthracene was identified as the first pure chemical compound, along with several other chemicals, to produce cancer when introduced into experimental animals. This discovery was monumental, as the realization of chemicals being carcinogenic led to pharmaceutical studies wherein synthetic agents causing cancer was being reviewed. Numerous studies involving synthetic carcinogens revealed a substantial amount of chemicals at low levels may have biological effects; thus, indicating synthetic agents, although approved for pharmaceutical use, may have carcinogenic effects [25].

After establishing carcinogens in pharmaceuticals and resulting from certain occupations, research methods were more established and led scientists to question if cancer could result from a virus. Through studying blood cells, it was discovered that viral RNA replicate via the reverse transcriptase enzyme, which led to the identification of cancer-causing viruses by detecting viral reverse transcriptase via enzyme assays [25]. The ultimate outcome was the uncovering of a T-cell retrovirus, termed HTLV-1. Nearly all patient's diagnosed with T-cell leukemia tested positive for HTLV-1. Cells containing HTLV-1 revealed the monoclonal form of HTLV-1 integrates into the host genome, illustrating the virus joins the host genome during its typical replication cycle; thus, demonstrating that HTLV-1 infection and the genome integration occur prior to viral expression within the tumor cells. HTLV-1 discovery proved the principle that viruses may cause cancer in humans [25].

With the introduction of HTLV-1 retrovirus, studies focusing on the mechanisms by which various viruses caused cancer became more prevalent. The

hepatitis B virus, human papillomavirus, Epstein-barr virus, human immunodeficiency virus, to name a few, were all ascertained as having mechanisms directly relating to cancer progression [25]. The mechanism by which the human immunodeficiency virus induces cancer is complex. T cells and macrophages are infected with HIV which stimulates a release of a HIV encoded protein. Surrounding cells in the microenvironment take up the encoded protein which has been concluded to promote carcinogenesis [25]. HIV causing immunosuppression has been established to be a cofactor in specific sarcoma development as well as lymphomas [25].

Researchers have hypothesized that an initiating event occurs during fetal development of females which makes their breast tissue prone to promoting estrogen, correlating to increased rates of breast cancer [25].

Elements of the human genome should also be considered when looking at the causes of cancer, as infectious or genetic carcinogens. Knowing viruses have the capability of being carcinogenic, it is important to note that in the human genome, a remarkable amount of repeat protein coding sequences are transposable elements which are homologous with viruses. Long terminal retroviruses, generally referred to as human endogenous retrovirus, resemble ancient retroviruses. Most women diagnosed with melanoma or breast cancer show increased expression of human endogenous retrovirus from their cancer tissue biopsy and in their serum [25].

Obesity, natural chemicals, synthetic agents, all have been associated with increased risk of cancer development as shown from several scientific studies [25].

Several mechanisms and carcinogens have been identified as inducing cancer development. Regardless of etiology, ten hallmarks of cancer have been discovered and remain fairly consistent (Fig 1). In reality, there are so many factors leading to the development of cancer, the exact cause of every cancer type in all patients is not fully understood and may never be absolutely concluded. However, it has been discovered that one main cause of cancer results from damages in the cell cycle and imperfections performing apoptosis.

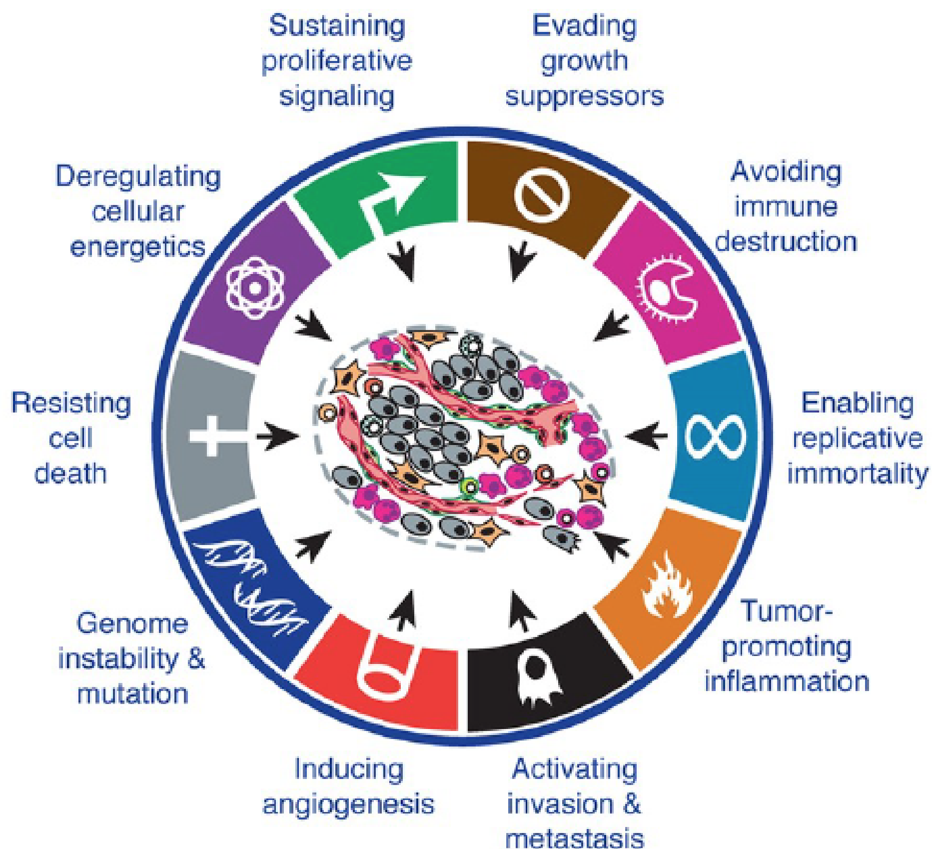

Figure 1. *Hallmarks of Cancer.*
Retrieved from: Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. Cell, 144(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

1.1.3 Cell Cycle

A component of cancer development has been found to be when damages occur within the cell cycle. Somatic cells undergo division utilizing the cell cycle process (Fig. 2). The cell cycle consists of four phases, G1, S, G2, and M, all constituting interphase. Cells begin their preparation to divide during the G1 phase characterized by the extension of the chromosome state. Chromatin, an essential component in packaging DNA to fit into the cellular nucleus, slenderizes and fully extends to allow for transcription [30] in order to produce a copy of the RNA genomic sequence. Transcription synthesizes RNAs to generate tRNA, rRNA, and mRNA, as well as proteins necessary to initiate the DNA replication process [30]. The G1 phase contains a significant checkpoint to initiate DNA synthesis [30]. During the G1 phase, the cell may choose which path to take, enter the S phase to proceed with the cell cycle, undergo a resting phase G0, or stop the cell cycle to inhibit replication in order to differentiate for a specific role [31]. After choosing to proceed with the cell cycle, the cell will move into the S phase in which the cell replicates its DNA utilizing DNA synthesis [29]. The number of chromosomes is doubled via DNA replication with the help of associated proteins. When chromosomes are doubled, sister chromatids become attached to the centromeric region; the centrosome is duplicated during S phase as well [29]. A copied set of DNA is produced, the entire genome is replicated, and in order to organize the additional genetic material, the cell moves into the G2 phase. During the G2 phase the cell condenses the second set of genetic material and prepares to undergo mitosis and divide during the M phase. The cell enters the M phase in order to split genetic material into two daughter cells [29]. M phase consists of

five subphases: prophase, metaphase, anaphase, telephase, and cytokinesis (Fig 2). Prophase occurs when the DNA compresses into the sister chromatids, entering into metaphase in which the sister chromatids align and attach to the centromeres before separating at the centromere during anaphase. During anaphase, the separated chromatids are pulled by the mitotic spindle and reach opposing poles of the cell during telephase. DNA strands are unwound, spindles begin disappearing, and the nuclear membrane becomes nonexistent. The phase in which the cell membrane officially splits is known as cytokinesis. When cytokinesis finishes, two new daughter cells exist, each with their own set of DNA. The cell cycle may then begin again at G1, or the cell may enter the resting G0 phase until the cell cycle is needed to proceed with G1 [30].

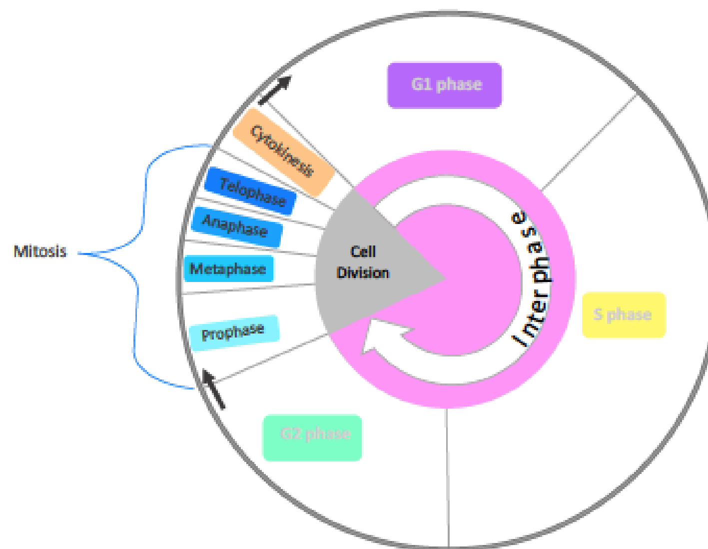

Figure 2. *Cell Cycle Mechanism.*

In order to prevent uncontrolled division of damaged cells, vital checkpoints occur at specific locations within the cell cycle. Three important

checkpoints occur at the G1/S phase transition, at the G2/M phase transition, and a spindle checkpoint between the metaphase and anaphase transition [32]. Checkpoints play the decisive role of examining internal and external signals in order to decide whether to proceed to the next steps in the cell cycle. The mechanism which controls the cell cycle checkpoints is driven by cyclin-dependent kinases (CDKs) [33]. CDKs are responsible for phosphorylation of substrates which promote DNA synthesis and M phase progression [33]. Checkpoints help regulate damages in the DNA, errors in replication, cellular size, molecular signals to ensure appropriate growth factors, and assurance that the cell has adequate energy and nutrients to complete full division [32]. At the various checkpoints, if an error or damage is detected, a normal healthy cell will undergo apoptosis to prevent production of numerous abnormal cells.

1.1.4 Apoptosis

Apoptosis is programmed cell death which occurs as a routine component of cell development when damages occur to the cell. Rather than undergoing apoptosis, as a normal healthy cell would endure a mutation if these damages occurred, cancerous cells continue to grow, divide, and replicate, forming additional abnormal cells, referred to as a malignant mass or tumor [1]. Apoptosis does not occur naturally within cancerous cells because of the inhibitions or mutations in the cell cycle mechanism. The regulating checkpoints within the cell cycle become impaired and are unable to detect the damaged DNA within the cell, thus accounting for the accelerated cell growth of the abnormal cells [3]. When apoptosis does not continue, and mutated cells accumulate, cancer will develop

(Fig 3). Apoptosis is a highly complex mechanism involving many pathways, making the exact understanding difficult [20]. Not only may the damages in apoptosis signaling result in malignant tumor formation but may also contribute to anticancer drug resistance. One specific pathway of reduced apoptosis involves the downregulation of the p53 tumor suppressor gene. The response to the p53 activation stimulus is reduced or suppressed, causing reduced apoptosis which leads to inactivation of p53 strengthening the tumor development process. Unfortunately, the inactivation of the p53 gene has been associated with many cancer diagnoses in humans [20].

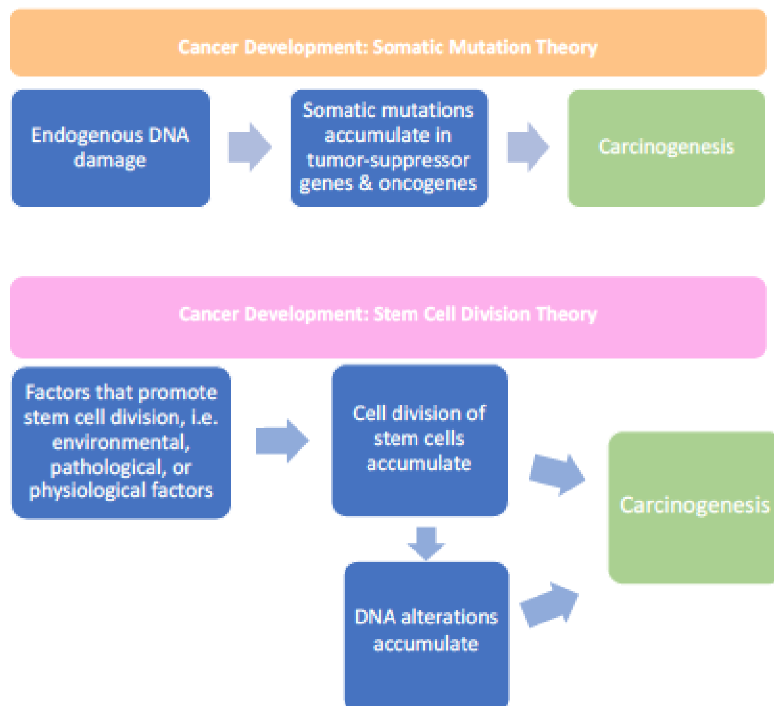

Figure 3. *Theories of Cancer Development.*

1.1.5 Metastasis

Cancer is a very damaging and potentially deadly disease. Fatalities typically occur when the primary cancer cells travel through circulation away from their initial location, spreading to other parts of the body in a process known as metastasis. Cancer cells have the ability to grow outside their original parameters, making metastases dangerous. Metastases may develop into the tissue directly surrounding the primary tumor, cancerous cells may travel in circulation to distant locations from the primary site, and/or cancerous cells may travel through the lymphatic system affecting nearby or distant lymph nodes. The metastatic cascade can be summarized in Figure 4.

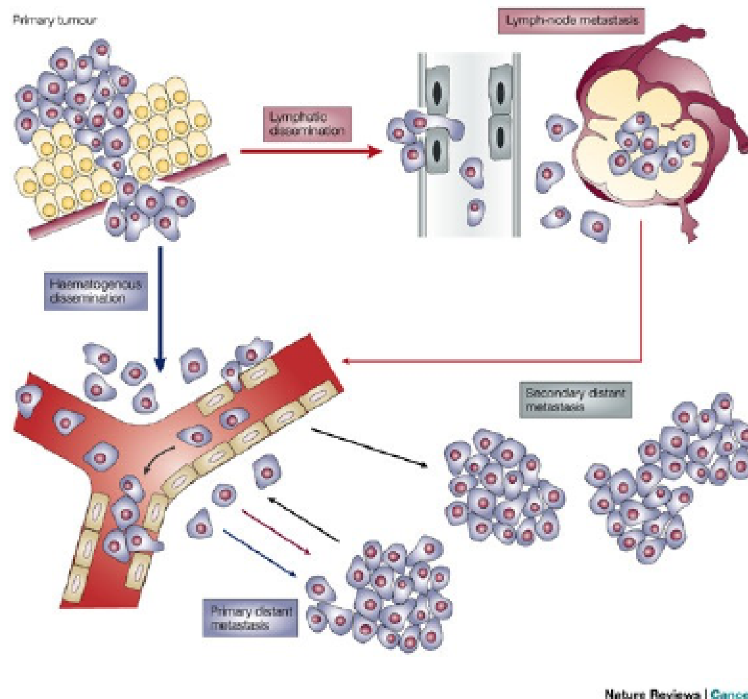

Figure 4. *Metastatic Cascade.*
Obtained from: Pantel, K., Brakenhoff, R. Dissecting the metastatic cascade. *Nat Rev Cancer* 4, 448–456 (2004). https://doi.org/10.1038/nrc1370

The primary stage of metastasis begins with an abnormal cell becoming malignant. The malignancy undergoes uncontrolled proliferation, avoids destruction from the immune system, and forms a primary tumor with its own blood supply [34]. After the primary tumor is established, an invasion or migration stage begins in which some malignant cells detach from the primary tumor site before traveling to other sites in the body via the circulatory system. Once detachment occurs, the intravasation stage will commence, involving invasion and disregard of the extracellular membrane. Intravasation involves the permeation of the blood or lymph vessels to obtain access to the circulatory or lymphatic system for travel to unaffected areas of the body [34]. Extravasation is then the opposite of intravasation. Extravasation occurs when the migratory cell leaves the transport system and infects an unfamiliar organ or tissue [34]. Cancer commonly metastasizes into the brain, bones, and lungs, but has the ability to metastasize to any organ or gland [28]. Cell proliferation occurs in the new location and another colony of malignant cells is built forming a small secondary tumor without angiogenesis [34]. A secondary tumor enters macrometastasis when it develops its own blood supply for sustained growth. Metastatic cancer makes treatment of cancer increasingly difficult, as then the primary and secondary sites must be addressed.

1.1.6 Treatment Methods

In an attempt to control the growth of the abnormal cancerous cells, various treatment modalities have been innovated and put into practice. Chemotherapy, radiation therapy, and surgery are among the most common

treatment methods. Chemotherapy is the most commonly used method and uses vigorous chemicals to kill the rapidly growing cancer cells; however, chemotherapy drugs do not solely target cancer cells [4]. Chemotherapy kills or slows the growth of cancerous cells as well as the surrounding healthy cells, which in turn can provoke several adverse effects. Radiation treatment may be more controlled in that the high dose of radiation waves administered to patients can be used to target specific tumors. By targeting tumors, the radiation acts to shrink the tumor and kill the cancer cells [4]. Once a tumor is reduced in size, surgery may be warranted to remove the remaining malignancy. Unfortunately, adverse effects are highly common when receiving chemotherapy or other cancer treatments involving harsh chemicals because of the synthetic nature of the treatments. The nature of chemotherapy does not allow for the control over which cells are being killed, as chemotherapy is typically administered intravenously into the circulatory system, or administered orally via a pill, indicating the medication is traveling to all parts of the body, despite the cancer being localized. The harshest effect of chemotherapy treatment is concluded as the compromising of the patient's immune system. The damage and death of the normal healthy cells throughout the body results from chemotherapy treatment. While the immune system would typically fight pathogens in a healthy patient, cancerous patients have a weakened immune system due to the lack of healthy cells. Immunocompromised patients are thus at a higher risk for infection and contraction of other pathogenic diseases. Considering the mechanism of action of chemotherapy treatment, the most common adverse effect is total body hair loss,

along with nausea, emesis, constipation or diarrhea. More severe effects may include liver, lung, bladder, heart, and/or nervous system damage, which could require additional medications to reverse the damages [21]. Chemotherapy medications as of now are developed synthetically, indicating they are made in a laboratory with the use of harsh chemicals. Fortunately, cancer treatments involving the use of natural products are becoming more prevalent and are hopeful to be less harmful.

1.1.7 Natural Products

According to the FDA, a therapeutic natural product may be defined as: (i) unregulated organisms or naturally occurring materials; (ii) unmodified naturally occurring materials or organisms regulated by the FDA; (iii) a semisynthetic compound, which is a chemically modified naturally occurring compound; and (iv) a purely synthetic compound modified after a natural compound [26]. Near the end of 2013, 547 new natural products and derivates, 25% of which were derived from plants, were approved by the FDA [26]. Plants contain considerable pharmacologic functions including anticancer, antimicrobial, antioxidant, and antiparasitic effects [27].

Within the past thirty years, nearly all of the anticancer drugs approved by the FDA are derived from natural products [5]. Drug discovery and development of cancer therapies comes from natural extracts, pure compounds, dietary sources, organisms, and more [5]. Previous studies done using various cancer cell lines within animal models show the antiproliferative and cancer therapeutic activities against cancerous cells [5]. Many natural products used in clinical trials have

exhibited various anticancer advantages including apoptotic activity, anti-invasive activity, alteration of cell cycles, and antimetastatic activity; which, will be the four main focuses when looking at the anticancer properties of podophyllotoxin analogues. Alternative anticancer therapies focus on utilizing the phytochemicals (secondary metabolites [27]) in plants to promote healing and avoid the toxicity that comes with chemotherapy and other synthetic cancer therapies [6]. Phytomedicine utilizes plants for medicinal therapeutic uses in treating diseases and overall improving health in humans [27]. These phytochemicals contain active ingredients which have been considered for medical purposes as a drug and may be classified based on their chemical compositions [27].

Research has shown that drugs derived from natural products may be used as a secondary anticancer therapy, complimenting the established conventional treatments [6]. The fact that a multitude of studies have shown an individual's diet may affect cancer risk, leads researchers to continue to involve natural products, specifically plants, into their drug discovery search, as it is highly documented that specifically herbal plants and derivitaves play a major role in recent drug development [27]. The mechanism of developing natural products notably involves the use of naturally occurring substances; thus, the lack of availability of said products is a major risk when developing new natural treatments.

1.1.8 Preventions

Dietary factors including diets high in red meat, diets heavy in processed meats, low fiber diets, and obesity have also been identified as contributing

factors for increased cancer risk by the American Institute of Cancer Research and the World Cancer Research Fund.

It has been predicted that a fairly large percentage of cancers may be prevented by lifestyle and nutrition choices [7]. Researchers have studied specific dietary factors related to increased cancer risk, as well as those that have been shown to reduce cancer risk. Obesity particularly has been linked to increased cancer risk based on the fact that obesity leads to a higher risk of malignancy. Knowing that obesity leads to increased malignancy risk, it has been concluded that chronic caloric surplus is a cancer risk factor. Looking back into history, cancer cases were not as prevalent as in present day. A slew of factors may attribute to this fact; however, proving that poor diet and physical inactivity are cancer risk factors, an increase in cancer cases worldwide is logical, as overall individuals have adapted to an unhealthier lifestyle compared to decades ago. In a study focusing on correlations between obesity and mortality from cancer in adults, it was found that 14 percent of cancer deaths in men and 20 percent in women were positively associated with those adults being overweight or obese [7]. Of those fatalities, researchers from this prospective cancer prevention cohort found a correlation with higher death rates among 13 specific types of cancer [7]. Overweight and obese adult cancer patients had a significantly higher mortality rate, concluding that obesity alone stands as a cancer risk factor. Avoiding a caloric surplus is advantageous to lowering cancer risk; however, other nutritional choices have also been proven to decrease risk of cancer. Changes in daily dietary intake by adding copious amounts of fruits, vegetables, vitamins, and minerals

can abate risk of developing various types of cancer. Specifically, cruciferous vegetables containing sulforaphane, vitamins C and D found in many fruits and vegetables, selenium, and chlorophyll, were studied and tested positive for containing anticancer properties [7]. Not only have many natural compounds been found to contain properties that decrease risk of developing cancer, but a majority of these compounds also have anticancer properties, meaning the compounds could potentially treat cancer after the cancer has developed.

Knowing that many naturally occurring compounds contain anticancer properties has led researchers to continue to search for additional natural treatments for cancer. Compounds that have been successful in treating cancer have mostly been derived from plant microbes, with the microbes having anti-tumor activity [8]. Additionally, anticancer properties have been found in plant toxoids, alkaloids, and podophyllotoxins [8].

1.2 Podophyllotoxin

1.2.1 Introduction

Podophyllotoxin is a naturally occurring plant compound found in resin of plants in the genus Podophyllum. First isolated in 1880 from podophyllum species, podophyllotoxin wasn't reported useful in clinical trials until the 1950s [9]. Podophyllotoxin is a 'non-alkaloid toxin lignin extracted from the roots and rhizomes of *Podophyllum* species' [10]. Podophyllotoxin contains a five-system ring (A, B, C, D, and E) with four chiral centers, C-1 to C-4 [10] (Fig 5). Synthesizing the podophyllotoxin analogues involves manipulation of the rings, as modifications in each ring have been shown to have different effects;

specifically, research has shown that free-rotation of the E-ring is a critical component for compounds to have antitumor activity [10].

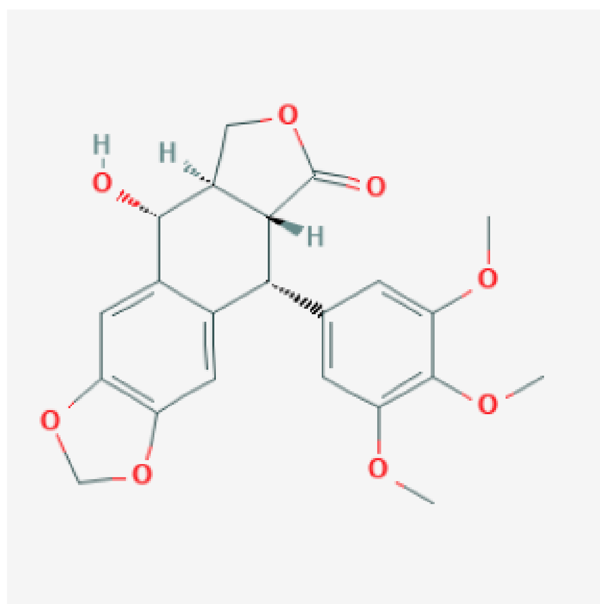

Figure 5. *Parent compound Podophyllotoxin structure.*
Obtained from: National Center for Biotechnology Information. PubChem Database. Podophyllotoxin, CID=10607, https://pubchem.ncbi.nlm.nih.gov/compound/Podophyllotoxin.

1.2.2 Phytochemistry & Cytotoxicity

The cytotoxicity of podophyllotoxin is becoming more prevalent in research, and therefore the Podophyllum species is beginning to dwindle. Because of limited supplies of podophyllotoxin, the importance for developing analogues is increasing due to the need for continued development of anticancer therapies derived from natural products. Anticancer agents such as etoposide, teniposide, and etoposide phosphate have been developed via utilization of podophyllotoxin and been used as successful treatments for skin cancer and HPV [9,11]. The anticancer properties of podophyllotoxin can be attributed to its ability to inhibit tubulin polymerization within a cancerous cell [12]. Disruption of the cytoskeleton can be easily observed when a cell is treated with the

podophyllotoxin compound [12]. The inhibition of tubulin polymerization leads to the disruption of cell division or induces apoptosis [13]. Tubulin is a principal protein constituent of microtubules in living cells. Tubulin is a vital protein because it polymerizes into the filaments that form the skeletal system of cells [22]. Inhibiting the polymerization of tubulin would result in the disruption of the cell cycle, as the ability for the cell to divide would be constrained (Fig 6).

Podophyllotoxin itself has been reported to cause adverse effects including nausea, emesis, and diarrhea, as well as the inability to target select cells [13]. However, few analogues of podophyllotoxin have been reported to have less potency, causing fewer side effects, while maintaining the cytotoxicity level needed in anticancer therapy [13]. Unfortunately, many unknowns exist within the development of natural product cancer therapies. Due to many unknowns about the specific molecular mechanisms of action and potential toxic side effects, a vast majority of natural product cancer therapies have not yet reached actual human subject clinical trials [14].

Extremely extensive research must be completed before any research can reach human clinical trials, and for natural product derived anticancer treatments the first step is to determine if the product has any effect on cell viability, which may be assessed using various assays. For the purpose of this study, ten podophyllotoxin analogues will be used to determine if analogues contain the same anticancer properties as the parent podophyllotoxin compound (Fig 8 & 9). Experimental outline reviewed in Figure 7.

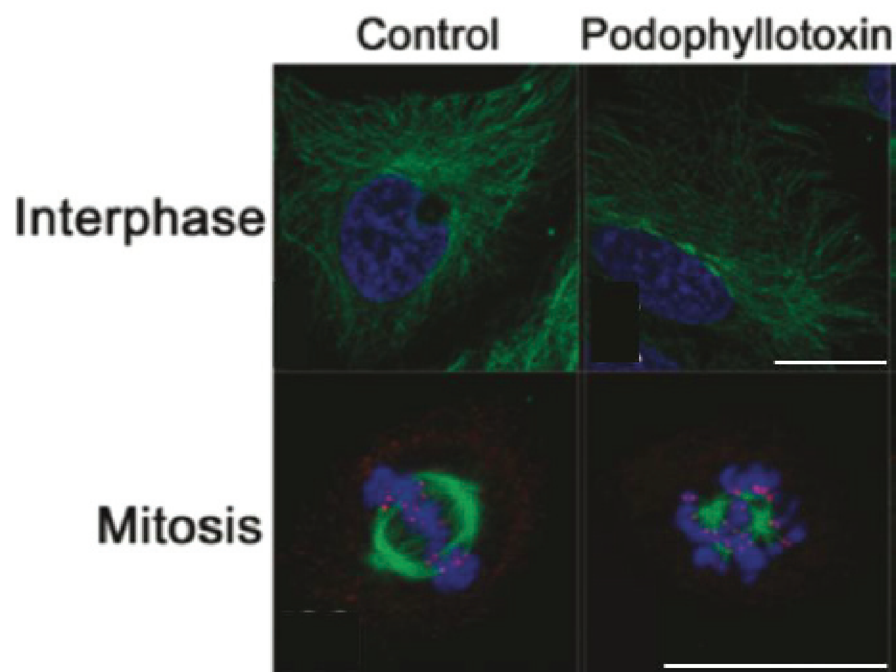

Image adapted from: Igor V. Magedov; Liliya Frolova; Madhuri Manpadi; Uma devi Bhoga; Hong Tang; Nikolai M. Evdokimov; Olivia George; Kathy Hadje Georgiou; Steffen Renner; Matthias Getlik; Tiffany L. Kinnibrugh; Manuel A. Fernandes; Severine Van slambrouck; Wim F. A. Steelant; Charles B. Shuster; Snezna Rogelj; Willem A. L. van Otterlo; Alexander Kornienko; *J. Med. Chem.* 2011, *54*, 4234-4246

Figure 6. *Microtubule organization in interphase and mitotic HeLa cells treated with parent compound Podophyllotoxin.*

Microtubule organization in interphase and mitotic HeLa cells treated with Podophyllotoxin compared to an untreated control. Microtubules (green), the kinetochore marker Hec1 (red) and Hoechst 33342 (blue). Scalebars are 10 µm.

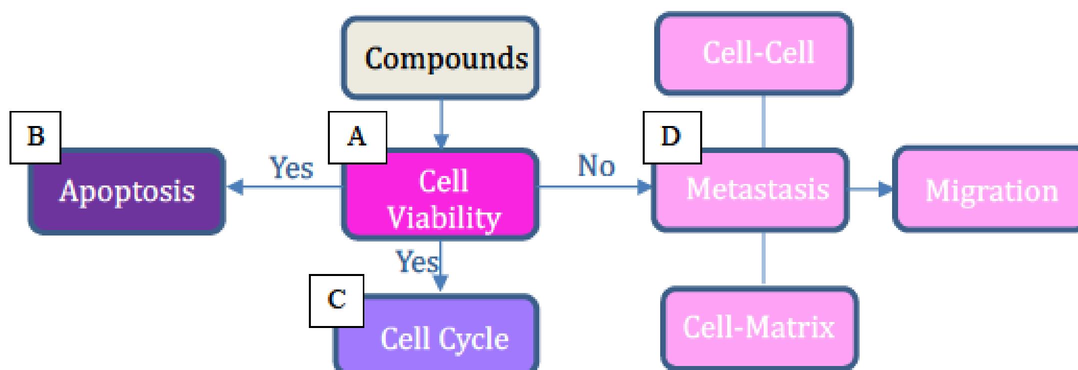

Figure 7. *Experimental Design.*
Podophyllotoxin analogue compounds were tested for their effect on cell viability (A). Analogues which revealed cytotoxic properties were analyzed to determine their effect on apoptosis induction (B) or their effect on cell cycle control (C) using flow cytometry. Analogues that did not have a significant effect on cell viability were further analyzed using scratch plate analysis for their effect on cell migration (D).

Chapter 2. Materials & Methods

2.1 Podophyllotoxin Analogues

Podophyllotoxin analogues were provided in collaboration by Willem van Otterlo (Stellenbosch University Stellenbosch, Western Cape, South Africa).

MGB10-01
MGB10-02
MGB10-04
MGB10-06
MGB10-07
KG201
KG203
KG210
KG216
KG217

Compound Code	Molar Mass ($g.mol^{-1}$)	Mass (mg)	Structure
MGB10-01	592,6196	4,0	
MGB10-02	668,3757	5,2	
MGB10-04	910,1482	4,6	
MGB10-06	671,6964	4,2	
MGB10-07	470,4767	4,4	

Figure 8. *Podophyllotoxin analogues 1-5.*
Obtained from collaborators via Willem van Otterlo.

Compound code	Structure	Amount in mg	previously obtained IC50 in microMolar	Mol formula
KG201		9.77	347	C24H23NO9
KG203		8.85	118	C25H25NO9
KG210		10.87	94	C23H19NO7
KG216		3.66	N/a	C28H24N2O9
KG217		8.86	N/a	C27H22N2O9

Figure 9. *Podophyllotoxin analogues 6-10.*
Obtained from collaborators via Willem van Otterlo.

2.2 Cancer Cell Lines

Obtained from American Type Culture collection (ATCC)

A549 (ATCC® CCL-185™) – epithelial lung carcinoma cells

MCF7 (ATCC® HTB-22™) – mammary gland epithelial breast adenocarcinoma cells

SK-MEL-28 (ATCC® HTB-72™) – polygonal malignant skin melanoma cells

MDA-MB-231 (ATCC® HTB-26™) – triple negative mammary gland breast adenocarcinoma cells

Jurkat (ATCC® TIB-152™, E6-1 clone) – lymphoblast acute T-cell leukemia T-lymphocyte cells

2.3 Cell Culture

Cancer cell lines MCF7, A549, SK-MEL-28, and MDA-MB-231, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1.0 mM sodium pyruvate. Jurkat cells were cultured in full RPMI-1640 supplemented with 10% (v/v) FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.01 mg/ml bovine insulin (Thermo Fisher Scientific, Waltham, MA). All cell lines were maintained on tissue culture plastic substrate and kept at 37°C in a humidified atmosphere containing approximately 5% CO_2.

2.4 Assay for Cell Viability

Cell viability assays will screen for the effect on cell proliferation versus cytotoxicity effects leading to apoptosis [15]. An important and successful assay in determining cell viability is a colorimetric assay involving mitochondrial dehydrogenase. The MTT assay utilizes mitochondrial dehydrogenase enzymes to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

into purple formazan crystals [16]. MTT was used for analysis of MCF7, A549, SK-MEL-28, and MDA-MB-231. Jurkat cells were analyzed using a sulforhodamine B (SRB) assay; the SRB assay is a more convenient assay for non-adherent cells such as Jurkat. Cell viability was tested in accordance with Romijn *et al.* [23]. Succinctly, mitochondrial dehydrogenase activities were measured by an MTT reagent. Cells were seeded in 96-well plates at an initial density of $1.5x10^4$ cells in 100µl of DMEM culture medium. After overnight incubation, cells were treated with various podophyllotoxin analogues, podophyllotoxin, and DMSO as a control, with a range of concentrations (concentrations of 100µM, 50µM, 25µM, and 10µM) to determine IC_{50} (half-maximal inhibitory concentration) values. After a 48-hour post-treatment incubation, 100µl of medium was removed from each well prior to the addition of the MTT reagent. The formed formazan crystals were then dissolved in 200µl DMSO (ThermoFisher Scientific, Waltham, MA, USA). After adequate mixing of each well via micropipetting, absorbances were read using a spectrophotometer at a wavelength of 570 nm. At least three independent experiments were completed to determine the mean absorbance referring to cell viability, using a Cytation™ 3 Cell Imaging Multi-mode reader with Gen5 software (BioTek Instruments, Inc., Winooski, VT, USA). Absorbances were expressed in percentage format as compared to DMSO-treated control cells. In each experiment, eight wells were used per condition. A condition in this case is defined as one cell line per 96-well plate, the first and last columns containing only DMEM growth medium, the second column on the plate containing DMSO as a control, with each of the

remaining nine columns on the plate containing a different concentration of an indicated treatment.

The SRB assay was used for cell cytotoxicity determination. Cells were seeded in 96-well plates a density of $1.5x10^4$ cells per 100µl of RPMI culture medium. After overnight incubation, cells were treated with various podophyllotoxin analogues, podophyllotoxin, and dimethyl sulfoxide as a control, with a range of concentrations (concentrations of 100µM, 50µM, 25µM, and 10µM) to determine IC_{50} (half-maximal inhibitory concentration) values. After a 48-hour post-treatment incubation, cells were fixed using 50µl of 50% trichloroacetic acid (TCA) (ThermoFisher Scientific, Waltham, MA, USA) added to each well, and then incubated for one hour at 4° C. Each plate was then gently rinsed with tap water approximately five times prior to completely drying the plates either in a scientific oven, incubator, or sterile hood. After each plate has dried entirely, 100µl SRB was added to each well before a 30-minute room-temperature incubation period. Each well on each plate was rinsed approximately four times with 1% glacial acetic acid and each plate was once again dried thoroughly. After complete dryness, 200µl of 10mM Tris buffer was added to each well, and each well was mixed adequately via micropipetting prior to reading absorbances using a spectrophotometer at a wavelength of 570nm. At least three independent experiments were completed to determine the mean absorbance referring to cell viability, using a Cytation™ 3 Cell Imaging Multi-mode reader with Gen5 software (BioTek Instruments, Inc., Winooski, VT, USA). Absorbances were expressed in percentage format as compared to DMSO-treated

control cells. In each experiment, eight wells were used per condition. A condition in this case is defined as one cell line per 96-well plate, the first and last columns containing only RPMI growth medium, the second column on the plate containing DMSO as a control, with each of the remaining nine columns on the plate containing a different concentration of an indicated treatment.

2.5 Spectrophotometry

After the finalization of the MTT and SRB assays for cell viability, the Cytation™ 3 Cell Imaging Multi-mode reader with Gen5 software (BioTek Instruments, Inc., Winooski, VT, USA) read absorbance values at a 570nm wavelength in each well of the 96-well plates. Absorbance values are automatically generated, and data is saved in an excel spreadsheet. Absorbance values are then used to find the average absorbance, standard deviation, and the percent relativity comparing each of the well averages to the average of the DMSO control in column B of the 96-well plate. An IC_{50} value is determined from the percent relativities. If the average percent relativity is at or below 50%, that constitutes an IC_{50} and the concentration of compound required to decrease cell growth by at least 50% is distinguished. The compounds that revealed an IC_{50} value are assumed to affect cell viability.

2.6 Scratch Assay

Following appropriate cell culture methods, cell lines A549 and MCF7 were grown to confluency. After ~90% cell confluency was reached, cells were seeded in Corning® 96-well Clear TC-Treated Plates (BioTek) at an initial density of 1.5×10^4 cells in 100 µl of DMEM culture medium. Cells were

incubated overnight at 37°C in a humidified atmosphere containing approximately 5% CO_2. BioTek's AutoScratch™ Wound Making Tool was auto-cleaned appropriately following the protocol supplied by BioTek. The eight scratch pins underwent a wash step using MultiFlo FX Multi-Mode Dispenser with 12mL sterile deionized water, 12mL sterile ethanol, 12mL Alconox solution, and a second well of 12mL Alconox solution. Following the scratch pin wash step, the confluent cells in the 96-well plate was placed in the AutoScratch machine which automatically created high consistency wounds in each well of each 96-well plate. After the automatic formation of the wounds, 80µl solution was removed from each well and replaced with 80µl warmed DMEM, using a multiload pipette. Debris may remain in each well as a result of the automated scratch, requiring the DMEM wash step to be repeated twice to remove any excess debris from each well. Following the media wash steps, cells were treated with podophyllotoxin analogues KG201, KG203, KG210, KG217. These particular compounds were chosen for treatments because they revealed no significant effect on cell viability; therefore, were assumed to not be a confounding factor during the migration assay. DMSO was used as a negative control and each treatment/control were at 100µM concentration. Two columns of untreated cells were included in analysis. Plates were imaged using the Cytation™ 3 Cell Imaging Multi-mode reader with the Scratch Assay App software (BioTek Instruments, Inc., Winooski, VT, USA). Images were taken at 0 hours, 24 hours, and 48 hours after initial scratch and recorded as t=0, t=24, and t=48. Wound margins were calculated and recorded in Table 2 and Table 3.

2.7 Flow Cytometric Analysis

Nonadherent cell type Jurkat was used as the model for induction of apoptosis and cell cycle control. Following appropriate cell culture methods, Jurkat cells were grown in 6-well plates and treated with 25µM Podophyllotoxin, 50µM Podophyllotoxin, and 100µM Podophyllotoxin. 5µM Camptothecin (Cell Signaling Technology, Inc., Danvers, MA, USA) served as a positive control for the induction of apoptosis and cell cycle arrest, compared to DMSO and untreated conditions as negative controls. Initial treatment used to set the apoptosis and cell cycle flow gates stained with an Alexa Fluor® 488 Annexin V and propidium iodide (PI) kit (ThermoFisher Scientific, Waltham, MA, USA) utilizing the manufacturer's protocol.

Jurkat cells were grown to confluency and treated for 48 hours with podophyllotoxin analogues at respective IC_{50} values. After treatment, cells were washed with cold PBS for removal of potential debris. For apoptosis detection, cells were incubated in the dark with Alexa Fluor® 488 Annexin V and PI in cold 1X annexin-binding buffer for fifteen minutes. After the fifteen-minute dark incubation period, samples were resuspended in the 1X annexin-binding buffer and analyzed within thirty minutes, measuring the fluorescence emission at 530nm using 488nm excitation. Samples analyzed using the Invitrogen Attune NxT Flow Cytometer (ThermoFisher Scientific, Waltham, MA, USA).

Cell cycle control analysis was also analyzed on Jurkat cells. Jurkat cells were grown to confluency and treated for 48 hours with podophyllotoxin analogues at respective IC_{50} values. Vybrant® DyeCycle™ Green Stain

(ThermoFisher Scientific, Waltham, MA, USA) was added to a 1mL cell suspension to a final concentration of 250nM. Following a thirty-minute incubation period at 37°C, samples were analyzed using the Invitrogen Attune NxT Flow Cytometer (ThermoFisher Scientific, Waltham, MA, USA). Untreated cells in RPMI media and cells treated with DMSO were used as negative controls and Camptothecin (Cell Signaling Technology, Inc., Danvers, MA, USA) treated cells were used as the positive control.

Chapter 3. Results

3.1 The Effect of Podophyllotoxin Analogues on Cell Viability

Each cancer cell line was treated for 48 hours with each podophyllotoxin analogue at concentrations of 100μM, 50μM, and 10μM. Analogue effect on cytotoxicity was determined utilizing MTT and SRB assays. The effect each analogue has on cytotoxicity is expressed as the mean value with standard deviation. The values are derived from comparison of treated cells versus untreated cells and expressed as a percent to determine an IC_{50} value (Table 1). The analogues had the most potent effect on the MDA-MB-231 human breast cancer cells. Ten analogues were tested, and seven of the ten compounds were found to decrease cell viability by 50% in the MDA-MB-231 cells. Although not as prevalent, analogues also revealed an effect on SK-MEL-28 melanoma cells, MCF7 breast cancer cells, and T cell lymphoma Jurkat cells. Analogues did not have any cell viability effect on A549 human lung adenocarcinoma cells, as all mean IC_{50} values were greater than 100, indicating the concentration of analogues

used would need to be greater than 100μM to produce any cytotoxic effect. Compound MGB10-04 could not be tested on cell lines SK-MEL-28 or Jurkat and testing with compound MGB10-06 could not be completed on MCF7 and MDA-MB-231, as there was not a sufficient amount of compound remaining to complete experimentation.

	A549	MCF7	MDA-MB-231	SK-MEL-28	Jurkat
MGB10-01	*>100 **	*40±10*	*38.5±4.9*	*40-100*	*41 ± 5.657*
MGB10-02	*>100 **	*between 50-100*	*21.5±17.6*	*40-80*	*59.167 ± 30.807*
MGB10-04	*>100*	*~ 100*	14.5±7.8	*	*
MGB10-06	*>100*	*ranges from ~ 40 to 100*	*no data*	*51±12.7*	*>100*
MGB10-07	*>100*	*>100*	*92.5±17.7*	*>100*	*93.75 ± 2.475*
KG 201	*>100 **	*>100**	*27±24.0*	*29.7±24.2*	*>100*
KG 203	*>100 **	*>100 **	*6.6±3.2*	*12.7±6.4*	*>100*
KG 210	*>100*	*>100 **	*<10*	*7.3±0.6*	*>100*
KG 216	*>100*	*>100*	*18.5 ± 12.021*	*>100*	*
KG 217	*>100 **	*>100*	*ranges from 10-100*	*10-50*	*>100*
Podophyllotoxin	*~ 10*	*~ 10*	*<10*	*<10*	*TBD*
Camptothecin	*~10*	*~10*	*<10*	*<10*	*TBD*

Table 1. *The Effect of Podophyllotoxin Analogues on Cell Viability.*
Values are the μM concentrations ± standard deviation by which each podophyllotoxin analogue compound decreased cancer cell viability by 50% relative to the DMSO controls. **cell viability at 55-60% at 100μM,* needs confirmation for reliability, out of compound.

3.2 Flow Cytometry: Apoptosis of Jurkat Cells

During flow cytometric analysis, Alexa Fluor® 488 Annexin V/PI double staining was used to determine the effects of podophyllotoxin analogues on apoptosis induction in T cell lymphoma Jurkat cells. The double staining provides the benefit of evaluating live cells, early stage apoptotic cells, late stage apoptotic cells, and necrotic cells. Phosphatidylserine is a phospholipid protective layer on the inner plasma membrane of a normal cell. When a cell begins to undergo apoptosis, phosphatidylserine residue is flipped to the outer leaflet of the plasma

membrane. When an apoptotic cell is stained with Alexa Fluor® 488 Annexin V, the Annexin-V will bind to the phosphatidylserine, making Annexin-V an apoptosis marker. Annexin-V will not indicate necrotic cells, because necrotic cells do not contain phosphatidylserine. Instead, necrotic cells will be stained with propidium iodide due to the loss of membrane integrity in necrotic cells. Live cells are indicated by neither a staining by Annexin-V nor PI. Figure 10 shows parent compound podophyllotoxin at varying concentrations in order to show proper gate setup.

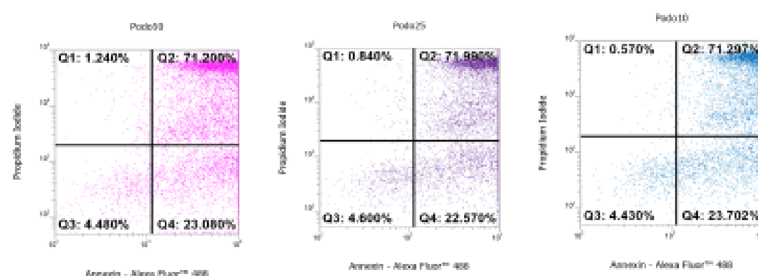

Figure 10. *Podophyllotoxin gate set-up*
Podophyllotoxin parent compound used at concentrations 50µM, 25µM, and 10µM to ensure proper gates. Podophyllotoxin was used in gate set-up due to it being the parent compound.

Following a 48-hour treatment incubation with analogues KG201, KG203, KG210, and KG217 each at 100µM concentration, flow cytometry was utilized to analyze any effect analogues had on the induction of apoptosis in Jurkat cells when compared to the Camptothecin control. DMSO was used as the negative control because analogue compounds were initially dissolved in DMSO, and 5µM Camptothecin used as positive control (Fig 11). KG201, KG203, KG210, and KG217 analogues were chosen because these compounds were the only compounds remaining in this study. The six other compounds tested against cell viability were used up in the cell viability assays. To learn the flow cytometry process, the four remaining compounds were used, despite showing no effect on

cell viability. Typically, only compounds indicating an effect on cell viability would be used to analyze apoptosis induction; however, compound analyzation continued despite the initial IC_{50} values for KG201, KG203, KG210, and KG217 being greater than 100 μM. IC_{50} values are arbitrary and are only used as definitions for protocols.

When compared to Camptothecin, the four podophyllotoxin analogues tested were not shown to increase the induction of apoptosis (Fig 12). Flow cytometric analysis of Jurkat treated with Camptothecin revealed greater than 80% of cells were in late stage apoptosis, 17.6% in early apoptosis, with only 1.6% of viable cells remaining, which was to be expected considering Camptothecin induces apoptosis. DMSO, as the negative control, revealed about 77% of cells were in late stage apoptosis, 13.9% of cells in early stage apoptosis, with 7.6% of viable cells remaining. Untreated culture conditions served as a negative control and reveal conflicting results. Untreated conditions are expected to have the majority of cells remain viable with fewer apoptotic and necrotic cells. However, in this case, 41.9% of untreated cells were apoptotic, thus there is an evident error in the RMPI control. The large number of apoptotic cells in the RPMI control are likely due to poor culture conditions of the Jurkat cells, or the method of mixing was too vigorous during different stages of protocol, ultimately damaging the cells during any mixing process prior to analyzation.

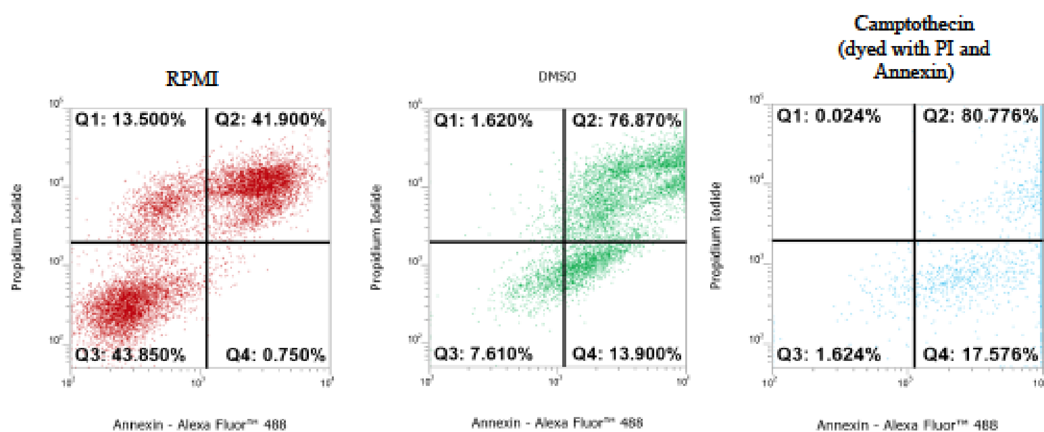

Figure 11. *Positive and Negative Controls.*
Untreated Jurkat cells in RPMI media served as a negative control. DMSO was a solvent control and also served as a negative control. Cells treated with Camptothecin (dyed with PI and Alexa Fluor® 488 Annexin V) used as positive control. Q1 represent necrotic cells. Q2 represent late stage apoptotic cells. Q3 represent live viable cells. Q4 represent early stage apoptotic cells.

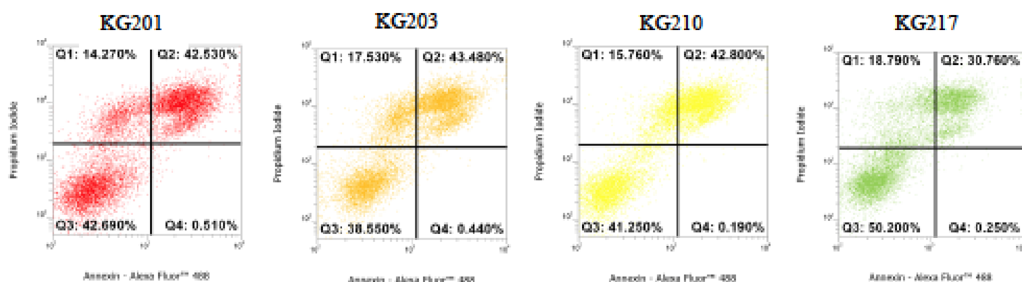

Figure 12. *The Effect of Podophyllotoxin Analogues on apoptosis of Jurkat cells*
Compared to the DMSO control in Figure 11, each compound did not increase the induction of apoptosis. The percentage of live cells, apoptotic cells, and necrotic cells after treatment with analogues remained consistent with the RPMI media control, indicating the analogues did not increase apoptosis induction. Q1 represent necrotic cells. Q2 represent late stage apoptotic cells. Q3 represent live viable cells. Q4 represent early stage apoptotic cells.

Podophyllotoxin analogues revealed 30-43% cells in late stage apoptosis, with <1% cells in early apoptosis. The majority of cells after treatment with a podophyllotoxin analogue were shown to be live cells, at 38-50% of treated cells remaining viable. The results of the analogue testing are nearly identical to the RPMI negative control (Fig 11). An error occurred with the RPMI control, indicating that the analogue results (Fig 12) are insignificant. Without a reliable control for comparison, analogue results cannot be properly analyzed. The error in

the untreated control indicates difficulty in adequate analyzation of the analogue results; because, like the RMPI control, the analogue results showing a large portion of cells in late stage apoptosis may be due to cell damage that occurred during mixing or poor cell culture conditions.

3.3 Flow Cytometry: Cell Cycle of Jurkat Cells

The effects of four podophyllotoxin analogues on cell cycle control were analyzed using flow cytometric analysis on Jurkat cells. Following a 48-hour treatment incubation period, Jurkat cells were stained with Vybrant® DyeCycle™ Green Stain which is a permeable DNA dye used for cell cycle analysis. Parent compound podophyllotoxin was used at concentrations 10µM, 25µM, and 50µM to set up appropriate gate control. Podophyllotoxin inhibits tubulin polymerization, inhibiting cells from completing the normal stages of mitosis, leading to an increase in cell count in the G2/M phase. Podophyllotoxin was used as a positive control for comparison of cell cycle activity of the analogues to the parent compound. Camptothecin is also used as a positive control, as Camptothecin induces apoptosis which leads to an increase in cell count in the G0 preapoptotic phase. RPMI represents the typical cell cycle without any alteration from treatments. Figure 13 represents all controls.

All events:

G0 phase
G0 G1 phase
S phase
G2 M phase

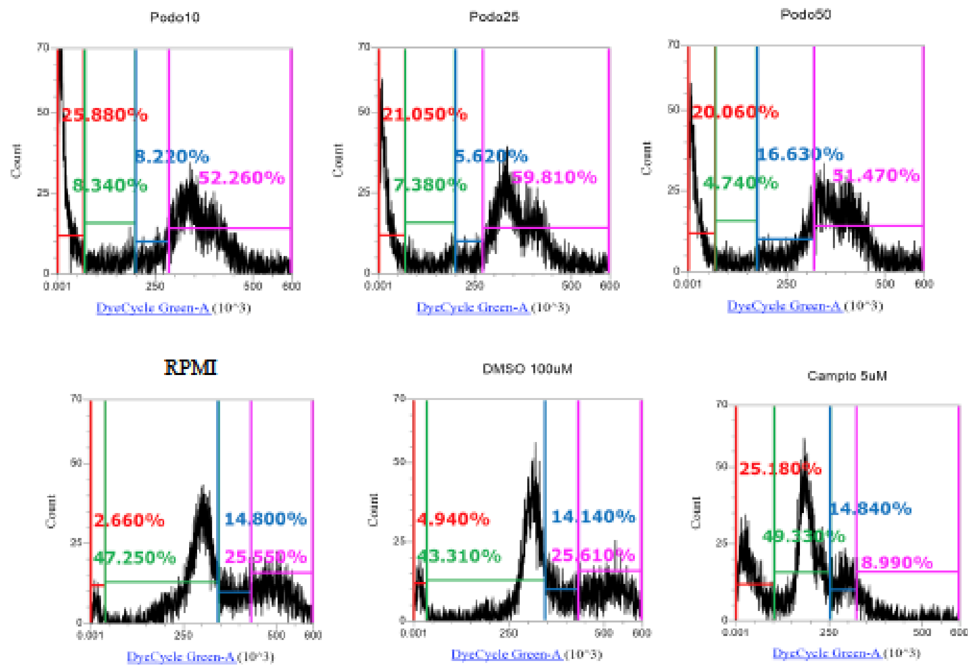

Figure 13. *Positive and Negative Controls.*
DMSO was used at the solvent control, with Camptothecin and podophyllotoxin used as positive controls.
RPMI negative control represents the typical cell cycle without any alteration from a treatment.

The G0 phase represented in red, is the preapoptotic phase. The typical cell cycle for Jurkat cells should show the largest spike in cell count during the G0/G1 phase (green), with a dip in cell count during the S phase (blue), followed by a slight elevation during the G2/M phase (pink). The RMPI media control follows this pattern as expected. Since Camptothecin induces apoptosis, it is expected that cell counts be higher in the G0 phase when treated with Camptothecin (Fig 13). Camptothecin has 25% of cells in the G0 phase, which is more than a 20% increase in cells compared to DMSO and RPMI in the G0 phase.

Jurkat cells were treated with 100µM podophyllotoxin analogues KG201, KG203, KG210, and KG217. These particular analogues were chosen for treatments because, like in the apoptosis analysis, these compounds were the only compounds remaining in this study. Analogue results were compared to the DMSO negative control, and Camptothecin and Podophyllotoxin positive controls (Fig 14).

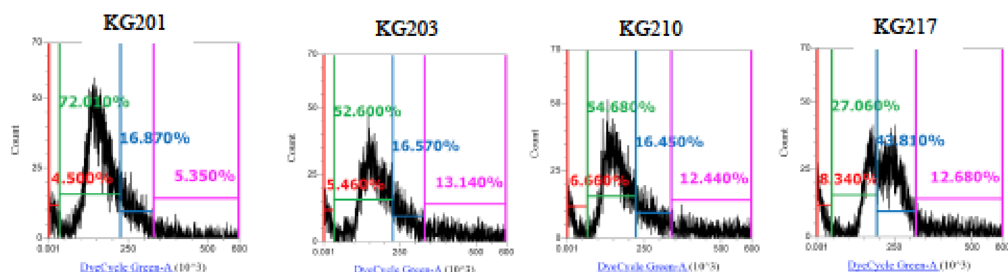

Figure 14. *The Effect of Podophyllotoxin Analogues on Jurkat cell cycle control* Podophyllotoxin analogues showed no elevation in cell counts in the M phase of the cell cycle. This indicates after treatment with analogues, these Jurkat cells continued with their regular cell cycle division, with no inhibition of the M phase.

DMSO is used as a negative control to ensure potential alterations in the cell cycle are not resultant of the DMSO solvent. Analogues KG201, KG203, KG210, and KG217 show elevated cell levels in the G0/G1 and S phases, with a decreased percentage of cells in the G2/M phase as compared to the DMSO control. Compared to Podophyllotoxin, it appears that the analogues are not acting similarly to the parent compound. Podophyllotoxin has significantly elevated cell counts specifically in the G2/M phase, whereas each of the four analogues show a majority of cells remaining in the G0/G1 phase. The podophyllotoxin analogues do not follow the same pattern as the podophyllotoxin parent compound. Compared to the Camptothecin control, analogues do not follow the same pattern of cell cycle alteration as shown in the control. Camptothecin shows a large

elevation in cell counts in the G0 phase with a significant decrease in cell counts in the G2/M phase; the analogues do not show similar results.

Although percentages in analogue cell count numbers are elevated compared to the DMSO control, the analogues follow a similar pattern as DMSO. Compound KG217 is the exception. KG217 shows a significant increase in cell counts in the S phase and a decrease in the G0/G1 phase. A majority of cells (43.8%) in the S phase is unlike any of the controls and would need to be tested again for confirmation.

These four analogues were tested for their effect on cell cycle control each at 100μM concentration. These analogues did not initially show any significant effect on decreasing cell viability, which explains the analogue cell cycle results being most similar to the DMSO solvent control and untreated RMPI control patterns.

3.4 Scratch Plate Analysis: Wound Healing

Scratch plate analysis was used to analyze the effect of podophyllotoxin analogues on wound healing. After the initial scratch, each plate was treated with analogues KG201, KG203, KG210, and KG217 each at 100μM concentration. These particular compounds were chosen for treatments because they revealed no significant effect on cell viability of A549 or MCF7 cell lines; therefore, were assumed not to have a confounding factor during the migration assay. Having no effect on cell viability indicates these chosen compounds may be analyzed for their effect on migration without interference of simultaneous decrease in cell viability.

Images of the wounds were taken at 0 hours, 24 hours, and 48 hours and recorded as time points t=0, t=24, and t=48 (Fig 15-20). Using the wound margins provided, migratory velocities were calculated, that is, how quickly the wound is closing in micrometers per hour. Scratch plate analysis uses DMSO as the solvent control.

As seen in the images (Fig 16), the wound created in the DMSO control had nearly completely closed after 24 hours post-initial scratch and had fully closed by 48 hours, which was to be expected as DMSO should have no effect on slowing migration. When comparing each of the analogue results to the DMSO images, it is clear that after 48 hours the wounds had not fully closed, implying the podophyllotoxin analogues had slowed the rate of migration for the A549 cell line.

In order to determine the rate at which each analogue effected migration, migratory velocity was calculated. When each scratch image was produced, a respective graph of the wound margin (in μm) at that time point was also produced. The wound margin graphs do not combine with each other at varying time points, meaning separate graphs for each image at each time point were produced. Wound margin graphs did not indicate the exact wound margin measurement, but rather gave a point on a graph with no coordinates; therefore, the plotted point for each wound margin had to be closely guesstimated. Using the wound margins, a migratory velocity was calculated between the time points (Table 2).

3.4.1 Effect of analogues on A549 wound healing

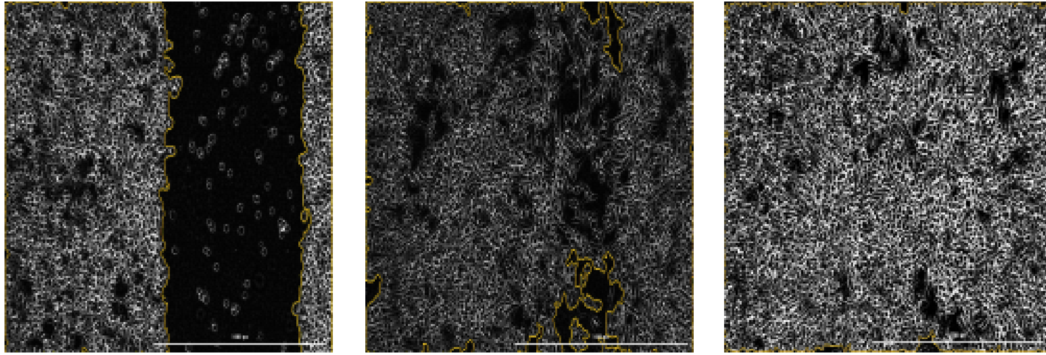

Figure 15. *A549 cells DMEM media control at t=0, t=24, and t=48, respectively.*

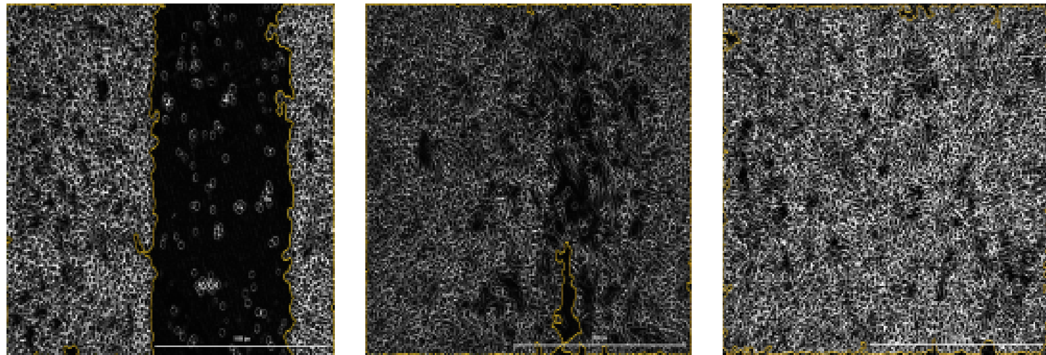

Figure 16. *A549 cells treated with DMSO control at t=0, t=24, and t=48, respectively.*

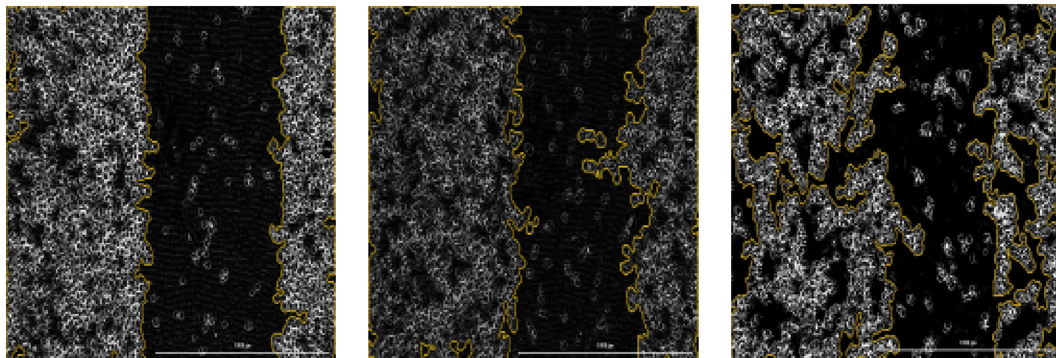

Figure 17. *A549 cells treated with analogue KG201 at t=0, t=24, and t=48, respectively*

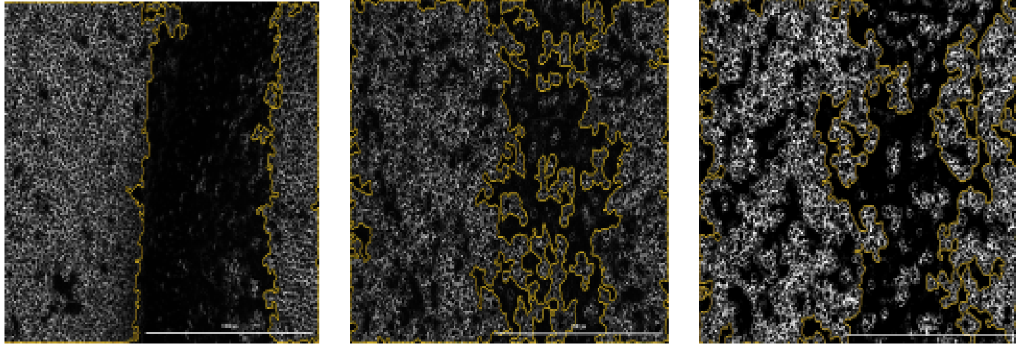

Figure 18. *A549 cells treated with analogue KG203 at t=0, t=24, and t=48, respectively*

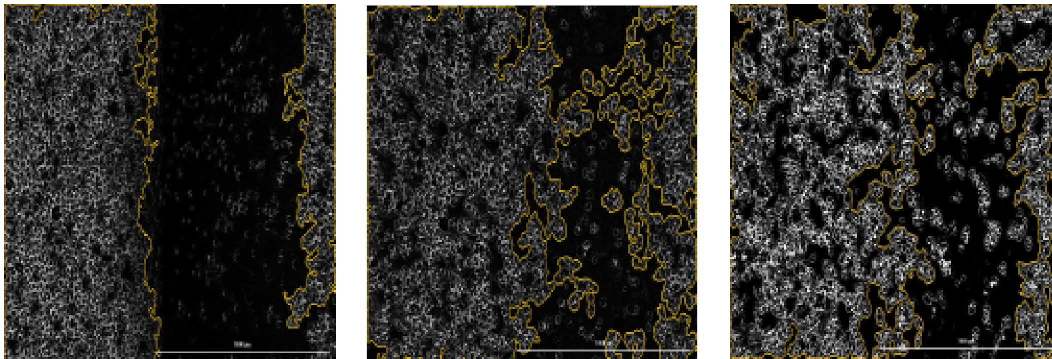

Figure 19. *A549 cells treated with analogue KG210 at t=0, t=24, and t=48, respectively*

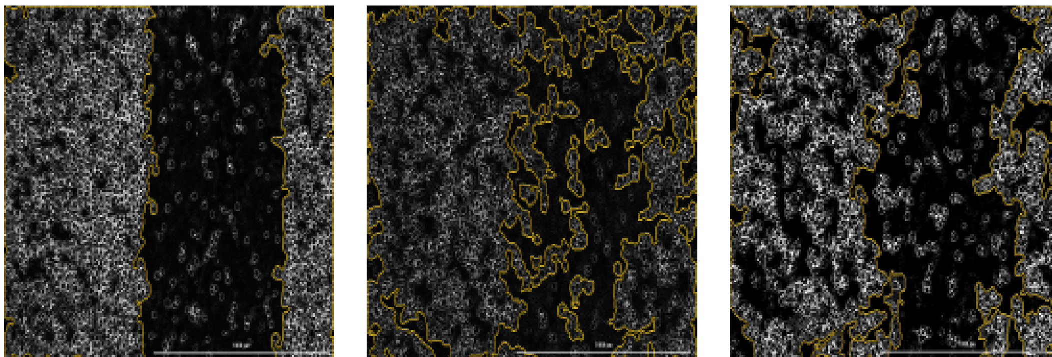

Figure 20. *A549 cells treated with analogue KG217 at t=0, t=24, and t=48, respectively*

Treatment	Wound Margin t=0	Wound Margin t=24	Migratory Velocity at 24 hrs	Wound Margin t=48	Migratory Velocity at 48 hrs
DMEM	776 µm	94.8 µm	28.38 µm/hour	33.4 µm	15.47 µm/hour
DMSO	775 µm	35.48 µm	30.81 µm/hour	33.68 µm	15.44 µm/hour
KG201	798.6 µm	660 µm	5.775 µm/hour	842.1 µm	-0.906 µm/hour
KG203	774 µm	473.5 µm	12.52 µm/hour	672 µm	2.125 µm/hour
KG210	905 µm	602.98 µm	12.58 µm/hour	740 µm	3.44 µm/hour
KG217	776 µm	626.6 µm	6.225 µm/hour	808.5 µm	-0.677 µm/hour

Table 2. *Migratory Velocities A549 cell line.*
Migratory velocities were calculated using formula $\left(\frac{\Delta d}{\Delta t}\right)$ where Δd = change in distance of wound margins between time points of interest in unit micrometers (µm) and Δt = change in time between time points in unit hours. Migratory velocities were calculated for time point t=24 and t=48, so that the difference in migratory velocities over time may also be analyzed.

When comparing the analogue treated cells to the DMSO control, it is evident that the analogues did have an effect on migration by slowing cell migratory velocity as evidenced by the results in Table 2. At 24 hours after initial scratch, the DMSO control had a migratory velocity of 30.81µm/hour, which significantly decreased in the cells treated with the analogues. Compound KG201 showed the greatest decrease in migration rate by indicating a migratory velocity of 5.775µm/hour after 24 hours, slowing cell migration rate by greater than 25µm/hour. Each of the four analogues tested decreased the rate of migration by more than 50%, demonstrating that podophyllotoxin analogues KG201, KG203, KG210, and KG217 each at 100µM concentration, did effect migration by significantly slowing the rate of migratory velocity.

Although it is assumed that all four compounds tested did affect the rate of migration, the increase in wound margins at the 48-hour time point should be noted. Although these particular compounds did not reveal any significant effect on cell viability, this definition of affecting cell viability in this study is by an

IC_{50}. In order to have a notable effect on viability, cell numbers had to decrease by at least 50% during the cell viability assays. Due to wound margin numbers increasing at the 48-hour time point, it may be due to these compounds having an insignificant effect on cell viability. This small effect would not have been significant enough to define these compounds as effecting cell viability, but nonetheless after a 48-hour treatment incubation period, the analogue compounds may have started to effect viability, causing the wound margins to be measured as larger than the earlier time point.

3.4.2 Effect of analogues on MCF7 wound healing

Scratch plate analysis was also utilized in analyzing the effect of podophyllotoxin analogues on MCF7 cell migration. Analogues KG201, KG203, KG210, and KG217 at 100 µM concentration were used for treatments. Treatments occurred at time point t=0 after initial scratch. Images were taken at 0 hours and 24 hours after the initial wound was made and recorded as time points t=0 and t=24 (Fig 21-26). Using the wound margins provided, migratory velocities were calculated and recorded in micrometers in Table 3.

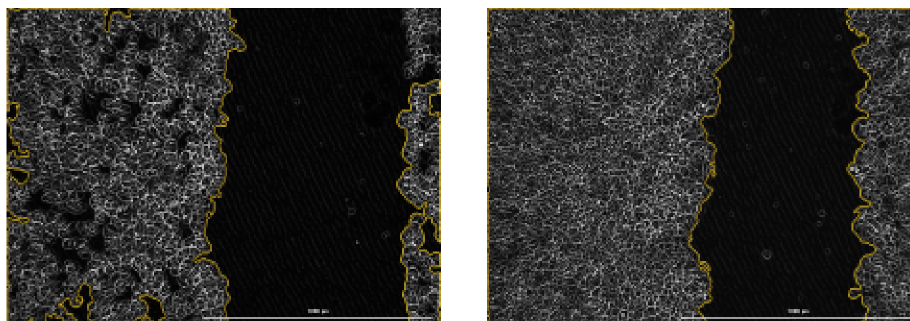

Figure 21. *MCF7 cell line with DMEM media control at t=0 and t=24, respectively*

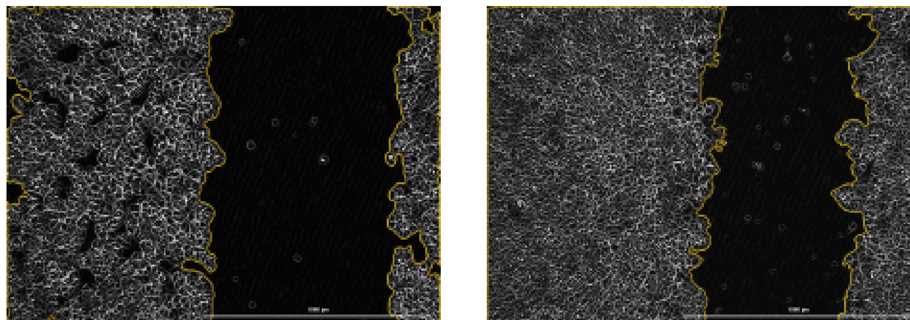

Figure 22. *MCF7 cell line with DMSO control at t=0 and t=24, respectively*

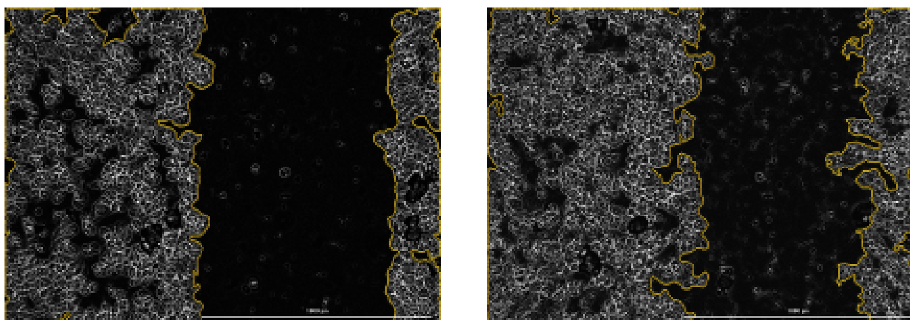

Figure 23. *MCF7 cell line treated with analogue KG201 at t=0 and t=24, respectively*

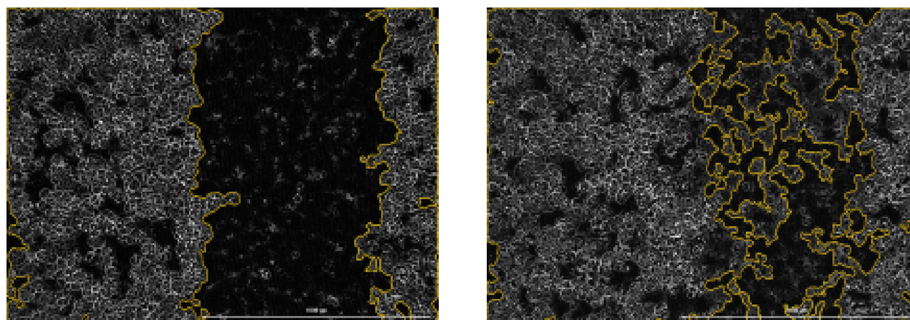

Figure 24. *MCF7 cell line treated with analogue KG203 at t=0 and t=24, respectively*

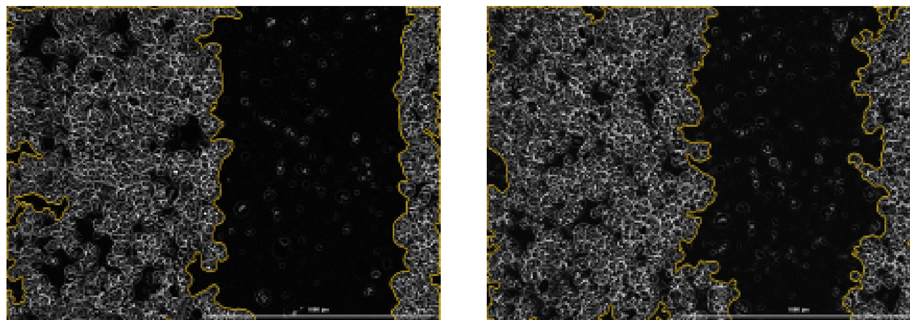

Figure 25. *MCF7 cell line treated with analogue KG210 at t=0 and t=24, respectively*

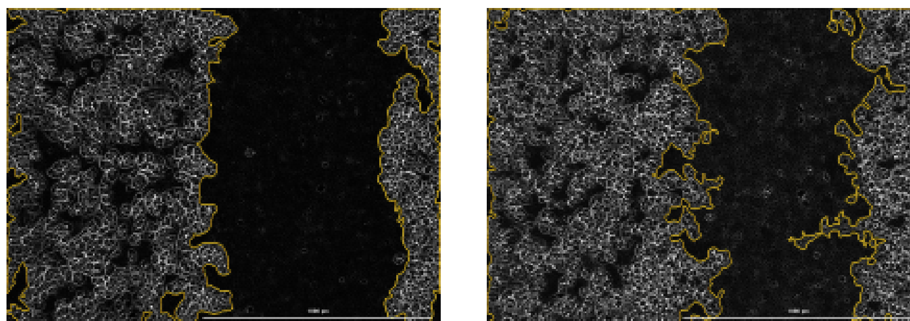

Figure 26. *MCF7 cell line treated with analogue KG217 at t=0 and t=24, respectively*

Treatment	Wound Margin t=0	Wound Margin t=24	Migratory Velocity
DMEM	879 µm	657.38 µm	9.23 µm/hour
DMSO	846.1 µm	637.95 µm	8.67 µm/hour
KG201	878.9 µm	761.83 µm	4.78 µm/hour
KG203	800.4 µm	417.8 µm	15.94 µm/hour
KG210	831.8 µm	479.5 µm	3.43 µm/hour
KG217	832.8 µm	690 µm	5.95 µm/hour

Table 3. *Migratory Velocities MCF7 cell line.*
Migratory velocities were calculated using formula $\left(\frac{\Delta d}{\Delta t}\right)$ where Δd = change in distance of wound margins between time points of interest in unit micrometers (µm) and Δt = change in time between time points in unit hours.

Scratch plate analysis of the analogues were compared to the DMSO control. It is evident that at 24-hours post-initial scratch, all four of the analogues tested did effect migration. Compounds KG201, KG210, and KG217 appear to

considerably slow the rate of migration of the MCF7 cells. The DMSO control had a migratory velocity of 8.67µm/hour at the 24-hour time point. Compound KG210 most notably decreased the rate of migration with a migratory velocity of 3.43µm/hour after 24 hours. Interestingly, compound KG203 seems to have increased the rate of migration after 24 hours with a migratory velocity of 15.94µm/hour. Compared to the DMSO control, the KG203 analogue had a great decrease in wound margin after 24-hours, equating to the large increase in velocity because the wound began closing at a quicker rate than the DMSO control.

Chapter 4. Discussion

Natural products are an important aspect of drug discovery. Natural products aren't necessarily any safer than current cancer therapies; however, the availability of natural products in nature makes natural product drug discovery more diverse. Parent compound podophyllotoxin has known anticancer properties. The goal is to use semisynthetically made podophyllotoxin analogues to discover more potent compounds. Overall, several podophyllotoxin analogues have the potential to contain potent anticancer properties. Additional studies must be completed in order to have enough reliable data to definitively state which compounds contain anticancer properties against which cancer cell lines. The experimental design of this study is outlined in Figure 7.

Several IC_{50} values were obtained from MTT and SRB assays, but due to initial human error in data analyzation and lack of compounds, sufficient data was not produced to decisively confirm reliable results. Experiments for reliability

were run in duplicate due to low amounts of compound; therefore, one more experiment testing each podophyllotoxin analogue against each of the five cell lines needs to be completed for final confirmation of the effect of each analogue on cell viability. Cell viability of A549 cells did not show to be sufficiently affected by any of the ten analogues tested. A549 may have interacted negatively with the coating on the 96-well plates used, contradicting initial full growth of the A549 cells. When testing for cell viability in the future, initial cell growth needs to be adequate and reliable prior to proceeding with further confirmation studies.

The effects of analogues on induction of apoptosis and cell cycle control in Jurkat cells were tested using flow cytometric analysis. The apoptosis results, when compared to the RPMI, DMSO, and Camptothecin controls, show none of the four analogues tested had an elevated expression of cells in late or early stage apoptosis, but rather had consistent amounts of live cells remaining. In analyzing the RPMI negative control, it is expected that the majority of cells be viable, with a very small number of apoptotic cells present due to some cells undergoing normal apoptosis when needed. However, in this study, the percent of viable cells remaining was nearly equal to the percentage of late stage apoptotic cells. This is an abnormal result and is likely due to mixing methods being too vigorous when following protocol causing cell damage, or cell culture conditions were poor. Future studies should account for the fragility of the cells being worked with, specifically focusing on gentleness when mixing; and, extra care should be taken when handling culture conditions to ensure proper growth of the cells prior to

flow cytometric analysis begins. The error in the RMPI control indicate that the analogue results are insignificant.

As for cell cycle control analysis, none of the four analogues tested appeared to follow the patterns of parent compound Podophyllotoxin, as the analogues did not cause elevated cell counts in the G2/M phase, nor did the analogues follow the pattern of Camptothecin by elevating cell counts in the preapoptotic G0 phase. The analogue treated cells seemed to most closely follow the pattern of the DMSO solvent control. The DMSO and RPMI results were nearly identical, indicating the DMSO solvent was not causing any alteration in the cell cycle; this was to be expected. The KG201, KG203, KG210, and KG217 analogues did not initially show a significant effect on Jurkat cell viability. In this case, the cell cycle results were not beneficial for analysis, as the results did not provide any insight into whether the analogues behave more similarly to podophyllotoxin or Camptothecin. These analogues causing the Jurkat cell cycle to follow most closely with DMSO does not allow for narrowing down how these analogue compounds are behaving. Future cell cycle analysis needs to ensure testing is done on compounds that *do* effect cell viability, in order to determine how the cell cycle is being altered.

Scratch plate analysis was performed on A549 and MCF7 cell lines. A549 and MCF7 were used because they are adhesive cancer cell types and the analogues chosen to complete scratch plate analysis did not produce any effect on cell viability. Using analogues that don't affect cell viability is important to avoid any confounding factors. From observation of the A549 wound images, it is

assumed that podophyllotoxin analogues KG201, KG203, KG210, and KG217 did have an effect on wound healing, implying these analogues may alter migration and metastatic rates of the A549 lung adenocarcinoma cells. Looking at the migratory velocities at 24 hours after initial scratch, migratory velocities of analogue-treated cells decreased compared to the DMSO control, indicating that the analogues slowed the rate of migration of the A549 cells. MCF7 cells showed similar results with one exception. Analogue KG203 showed an increase in migratory velocity compared to the DMSO control, indicating the KG203 analogue affected migration rates of MCF7 cells by increasing the rate of migration. MCF7 cells treated with compounds KG201, KG210, and KG217 showed a decrease in migratory velocity after 24-hours, indicating the analogues slowed the rate of migration of the MCF7 breast adenocarcinoma cells. The DMSO control in the scratch assay had a closed wound at 24-hours after initial scratch. Future studies should narrow down the time frame at which images are taken and wound margins are measured, rather than waiting 24 hours to take the second images. Taking images and measuring wound margins more frequently would allow for the determination of exactly when the wounds begin to close in the DMSO control. Migratory velocities would be more accurate and specific, especially if wound margins were measured every hour. At 48-hours after initial scratch in the analogue-treated A549 cells, wound margins increased compared to at 24-hours. This is likely due to the analogues having a small effect on cell viability despite these analogues showing no significant effect on cell viability initially. In order to have a significant effect on viability, and IC_{50} value must

have been determined, as the IC_{50} values were used as the definition for the protocols used in this study. However, the KG201, KG203, KG210, and KG217 analogues may have, by definition, an insignificant effect on cell viability, but a small enough effect to be a confounding factor in the migration assay. Future studies should first have shorter time points and measure wound margins more frequently, but because of the potential conflicting effect on the viability, the concentration of compounds used should be altered and slightly decreased.

With acquiring additional podophyllotoxin analogue compounds, the additional testing may be completed for final confirmation of their effect on cell viability, induction of apoptosis, effect on cell cycle control, and effect on migration. Several of the podophyllotoxin analogues in this study may contain sufficient anticancer properties and could be confirmed with additional analysis.

Chapter 5. References

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