The Anti-cancer Properties of Podophyllotoxin Analogues

By

Francisco Oliva

A thesis submitted in partial fulfillment of the requirements for the
Master of Science
Biological Sciences Program
Youngstown State University
December 2019
The Anti-cancer Properties of Podophyllotoxin Analogues

Francisco Oliva

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

_____

Francisco Oliva
Student Date

Approvals:

_____

Dr. Gary R. Walker
Thesis Advisor Date

_____

Dr. Séverine Van slambrouck
Thesis Committee Date

_____

Dr. Wim Steelant
Thesis Committee Date

_____

Dr. Salvatore Sanders
Dean of Graduate Studies Date

Abstract

Cancer is one of the major causes of death in the United States resulting in over 20% of annual deaths. Over the last decade the interest of natural products derived from medicinal plants as potential sources of new anti-cancer drugs has increased greatly. Now, the critical role of medicinal plants through the discovery of their 'mechanisms of action' is being widely recognized in the biomedical and pharmaceutical industry. Podophyllotoxin has been extensively used as a lead agent in the development of new anticancer drugs. This study focused on the screening of several podophyllotoxin analogues to determine their effects on cell viability, the induction of apoptosis and cell cycle control on A549, MCF7, Jurkat, MDA-MB-231, and SK-Mel-21 cells.

Several of the podophyllotoxin analogues, such as compounds 5.2 were shown to have a potent effect on cell viability, especially on Jurkat T cell Leukemia cells. When tested to observe their effects on cell cycle control and on the induction of apoptosis in Jurkat cells, the podophyllotoxin analogues were shown to be able induce necrosis and apoptosis as well as arresting cell growth in G2/M in a manner similar to Podophyllotoxin.

Acknowledgements

I would like to acknowledge the people who have believed in me and guided me throughout my career. Dr. Steelant, thank you for being the one who took a chance with me and heling me as I took my first step along this long road we have taken. Dr. Sev, you have always pushed me to be the greatest I could be and always picked me up when I was down. Finally, Dr. Walker, your patience and support in and out of lab made all the difference between my transitions into the program. I hope to keep making you all proud and to continue forward with all the knowledge and skills that you have each given me.

Table of Contents

List of Tables and Figures

Chapter 1. Introduction

Cancer is a global health issue which affects not only patients but their families and loved ones as well (1,2). The current treatment modalities include surgery, radiation, and chemotherapy, with the latter two often having long term negative impacts on the patients' quality of life (3). The toxic side effects of many chemotherapeutic drugs have led to an increased interest in the practice of traditional and herbal medicine by many cancer patients. However, many of these traditional remedies derived from natural products, often lack scientific evidence and may be even more harmful than the existing therapies. Nevertheless, it should be noted that medicinal plants used in herbal medicine have proven to be excellent sources for the discovery of novel active or important lead components *(4*).

The potential of using natural products is recognized by the National Cancer Institute (NCI). In 1992 the NCI developed The *Natural Products Open Repository Program* to screen natural products as potential anti-cancer agents. Eventually the success of the program as well as the need to develop new and more effective drugs has led to the program to cover all human diseases. As such, medicinal plant research offers not only the identification of new components that allow for further development and structure optimization into useful pharmaceutics needed for improvement of existing therapeutic strategies, but may also contribute to our natural products and natural/traditional medicine knowledge to fulfill the present and future health needs in alternative and complementary medicine (*5)*. This is true even more so for anticancer

drugs, of which, over the last 60 years, about 70% of the new drugs were developed using natural products (Fig. 1) (6).

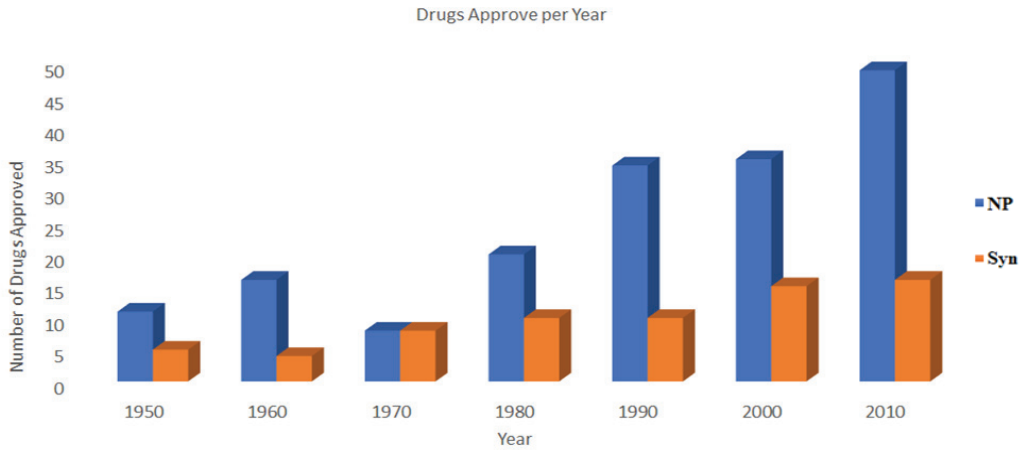

Figure 1. *Natural Products as a New Source of Drugs Over 60 Years.* The number of synthetic (orange bars) and drugs derived from natural products (blue bars) approved per year by the FDA over the last 60 years.

This study was centered on the screening of several podophyllotoxin analogues for their anticancer potential (Figure 2). Initially, these analogues were screened to determine their effect on cell viability, in order to derive an (IC_{50}) value. This value represents the concentrations of each compound, required for 50% inhibition *in vitro* or leaving a minimum of 50% the cells viable (Figure 3A). Analogues which did exhibit an effect on cell viability, those of which we were able to derive an IC_{50}, were further tested to determine their effect on apoptosis and cell cycle control (Figure 3BC). Alternately, analogues which did not have an IC_{50} will later be tested for their effect on metastasis (figure 3D).

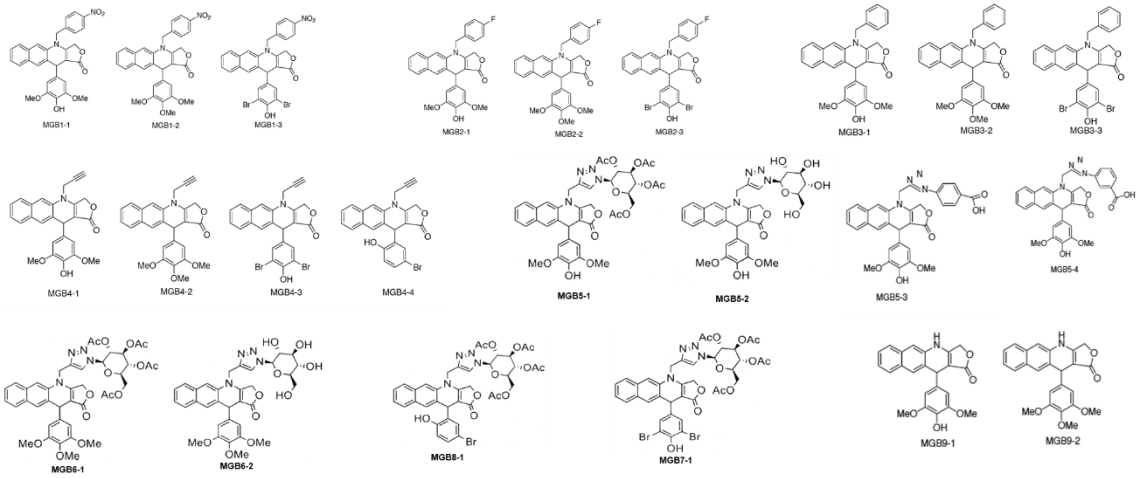

Figure 2. *Podophyllotoxin Analogues.* The structure of the podophyllotoxin analogues.

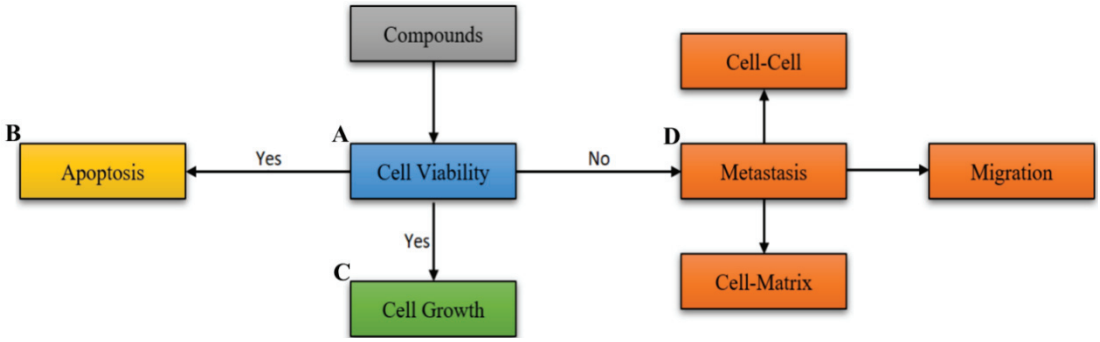

Figure 3. *Research Goals and Objectives*. The Podophyllotoxin analogues (grey) were initially tested to determine their effect on cell viability (A). The compounds which were shown to have cytotoxin properties were then further tested to determine their effect on apoptosis (B) and their effects on Cell Growth (C). Compounds which did not have a marked effect on cell viability will further be tested to determine their effect on metastasis (D).

1.1 Cancer

1.1.1 Introduction

Cancer is generally categorized by the uncontrolled and rapid proliferation of abnormal cells. However, the ultimate underlying cause which leads to the development of cancer is not fully understood. The somatic theory of cancer proposes that mutations which lead to the development of oncogenes and inhibit tumor-suppression genes lead to the development of cancer (10) (Fig. 4). The activated oncogenes result in rapid cell proliferation while the lack of tumor suppression genes inhibits apoptosis and the arrest

of growth during the cell cycle (11 ,12). The stem cell theory of cancer takes a slightly different approach when describing changes in DNA that lead to mutations (Fig 4). This theory considers changes in DNA which do not consist of mutations in DNA (epigenetic modifications, physiological, pathological and environmental factors) but still result in a change in gene expression. As a stem cell undergoes cell division, it accumulates changes in DNA which result in carcinogenesis (13,14).

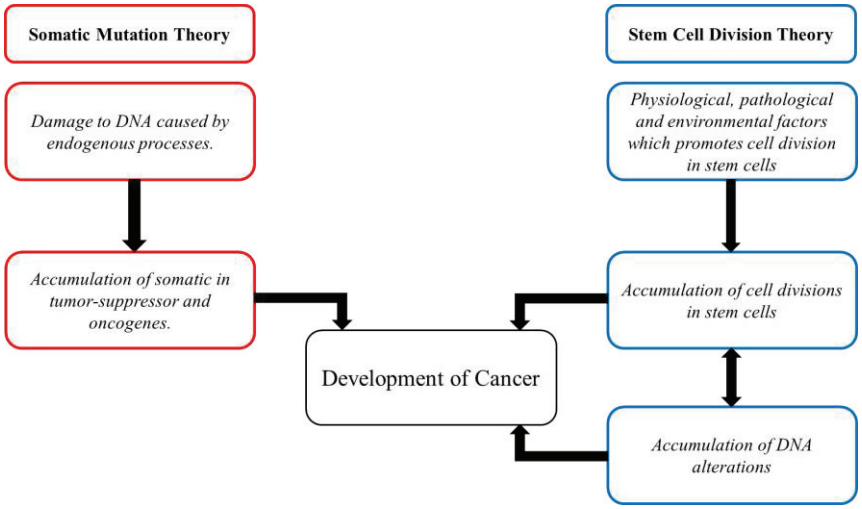

Figure 2. *Stem Cell and Somatic Theories of Cancer Development.*

Regardless of etiology, the hallmarks of cancer remain generally the same. These 10 hallmarks, proposed by Hanahan et al., in 2010 and then updated in 2011, comprise the biological traits which describe the processes of carcinogenesis and metastasis (15). The first six hallmarks describe tumor development and include the (i) stimulation of growth through sustaining proliferative signaling, (ii) insensitivity to inhibitory signals that would inhibit growth and (iii) to signals which would induce apoptosis, (iv) the indefinite proliferation resulting in replicative immortality, (v) the stimulation of growth

of blood vessels to supply nutrients to tumors (angiogenesis), and finally, (vi) the possible invasion of local tissues and metastasis to other locations.

The acquisition of these hallmarks by aberrant cells is due, in part, to the cells genetic instability caused by the multiple mutations which initiate and drive tumorigenesis, as well as by the local chronic inflammation associated with multiple forms of cancer (16, 17,18). Finally, the last four hallmarks, describe how cancer cells to evade detection by the immune system and the altered metabolic pathways used by several forms of cancer (19).

1.1.2 Cell Death

Cell death is a critical and constant process which maintains the homeostatic balance of tissues, through the removal of aberrant and damaged cells. This irreversible loss of vital cellular functions and the disruption of cellular integrity and generally manifests in three distinct forms: Autophagy, Necrosis, and Apoptosis, each of which is characterized by lethal morphologic and physiological changes (21,22,23). Autophagy is the result of cytoplasmic vacuolization which results in phagocytic uptake and subsequent lysosomal degradations. While autophagy is an active catabolic process in response to stress, it seems to accompany rather than initiate cell death, since the initiation of the autophagy pathway represents a cells inability to overcome stress, and may accompany either apoptosis or necrosis, which are mutually exclusive.

Apoptosis is characterized by the condensation of DNA (pyknosis), cellular shrinking, the fragmentation of the nucleus (karyorrhexis), and the formation of apoptotic bodies (24,25). There are two pathways in which apoptosis is triggered, The intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway (26,27). Both

pathways culminate in the activation of caspases and the degradation of the apoptotic cell. Caspases are cysteine proteases, which are responsible for the morphological change's characteristic of apoptosis (28). Both pathways activate initiator caspases which in turn activate executioner caspases which systematically degrade proteins and ultimately induce apoptosis (29).

In response to damaged DNA, intracellular stress, or errors in cell developments, The intrinsic pathway is activated through the permeabilization of the mitochondria and subsequent release of cytochrome c into the cytoplasm (30). Cytochrome c induces the assembly of apoptosome which initiates activation of the caspase cascade (31). The extrinsic pathway however is initiated through the binding of extracellular ligands to cell surface receptors through the binding of the Tumor Necrosis Factors (TNF) or Fas-Fas ligand mediated binding to the Tumor Necrosis Factor Receptors (TNFR) (32)

Necrosis, however, would best be defined the loss of membrane integrity like apoptosis, without pyknosis, cellular shrinking, karyorrhexis, as well as phagocytic uptake, like autophagy, without extensive vacuolization (33). Necrosis is mainly a consequence of factors external of the cell such as exposure to toxins, infection, or physical trauma. Although necrosis can occur as a consequence of irreparable damage to the cell, necrosis can also be actively induced (34, 35). This form of active cell death, necroptosis, is triggered by through signaling cascades which all converge on the activation of Receptor Interacting Protein kinase 3 (RIP3).

In contrast, apoptosis is a naturally occurring programmed and targeted cause of cellular death. While apoptosis often provides beneficial effects to the organism, necrosis is almost always detrimental and can be fatal. Cellular death due to necrosis does not

follow the apoptotic signal transduction pathway, but rather various receptors are activated, and result in the loss of cell membrane integrity and an uncontrolled release of products of cell death into the extracellular space.

However, unlike in apoptosis, necrosis and necroptosis do not involve the activation of caspases. Also, unlike apoptosis in which the apoptotic cell forms membrane blebs and fragments into apoptotic bodies where cellular contents are stored for phagocytosis, necrotic cell death result in the loss of membrane integrity and the release of a cells contents into the extracellular space.

1.1.3 Cell Cycle Control

The cell cycle is a series of events through which a cell is able to replicate its genome, grow and divide (36). The cell cycle is divided into four distinct phases, with each phase occurring sequentially and in a single direction (Fig 5). Briefly cells first undergo an initial period of growth (G1) where several macromolecules needed for growth and division are synthesized (37). This initial growth phase is then followed by a period of DNA synthesis (S) (38). Following another period of growth (G2), cells are able to undergo mitosis (M) (39). The M phase is characterized by two coupled processes: mitosis itself, in which the nucleus is divided, and cytokinesis, where the cytoplasm is divided to form two daughter cells. The first three phases, G1, S, and, G2 are collectively known as interphase, while M phase itself is the process where cell growth stops, and division occurs. However, nondividing cells exit the cell cycle at G1 and enter a “resting phase” or a state of quiescence (G0).

Figure 3. *Hallmarks of Cancer.*
Adopted 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

In order to prevent uncontrolled cell division of aberrant or damaged cells, the cell cycle is tightly controlled through key checkpoints whose requirements must be met in order to proceed through the cell cycle (40). The main mechanism through which these checkpoints are regulated are through the actions of cyclin-dependent kinases (CDKs) which bind to another class of regulatory proteins known as cyclins (41). It is through the binding and activation of specific cyclin-CDK complexes that allow passage through checkpoints and the continuation of the cell cycle.

The three main checkpoints of the cell cycle are G1/S, G2/M and the Metaphase checkpoint. It is during the G1/S checkpoint that the cell becomes committed to entering the cell cycle (42). It is through this rate limiting step that the cell either continues onto S phase or enters a quiescent state and stays in G0. If the cell has damaged DNA or lacks the cellular machinery needed to continue with growth or DNA synthesis, it is arrested at G1. However, if the cell is normal and there is proper cyclin-CDK activation, the cell continues through S phase. Following DNA replication during S phase, the cell undergoes another round of growth. It is here that the G2/M checkpoint ensures that there is not damage to the cell's DNA, as well as ensuring that all mitotic proteins are present. Like the G1/S checkpoint, there needs to be appropriate cyclin-CDK binding and activation in order to proceed to metaphase (43). Then, once the cell has entered M phase, it has become committed to mitosis. While the metaphase checkpoint may be considered a minor checkpoint, the cell needs to ensure that the mitotic spindle has formed properly, the chromosomes are aligned, and that there is bipolar tension in order to continue to anaphase. If at any point during the cell cycle that the requirements are not met in order to enter into the next phase, the cell becomes arrested in its current state. It is the disruption or evasion of these checkpoints, as well as the evasion of apoptosis and necrosis which leads to tumor formation and eventually metastasis.

1.1.4 Metastasis

While the development and uncontrolled growth of cancer is difficult to manage, it is not the most lethal aspect of cancer. There seems to be less consensus in the stages in which cancer metastasizes with most of the disagreement centered around whether a cancerous cell may disseminate before forming a primary tumor (44, 45).

However, for this study, the metastatic cascade will be characterized into three general phases (Fig. 6). Generally, a tumor can be described as malignant or benign. A benign tumor is generally smaller, slow growing, well differentiated, and localized, whereas a malignant tumor is larger poorly differentiated, fast growing and able to invade local tissues and metastasize to other areas of the body.

This metastatic process begins with the detachment of a cancer cell from the primary tumor site, invasion of the surrounding tissues and vasculature, and ending with the formation of a secondary tumor at the metastatic site. Following angiogenesis and tumor formation, a cancer cell develops an invasive phenotype which allows it to detach from the primary tumor. Following its detachment, the cell invades the surrounding tissues. Then as it interacts with the surrounding extracellular matrix the renegade cell migrates towards a blood or lymph vessel to be transported to other unaffected areas. As it circulates, the cell escapes circulation and invades the nearby tissue. Finally, it migrates through the extracellular matrix where it eventually becomes arrested at the metastatic site. Here the cell begins to proliferate, stimulate angiogenesis, and exhibit the same hallmarks as the primary tumor.

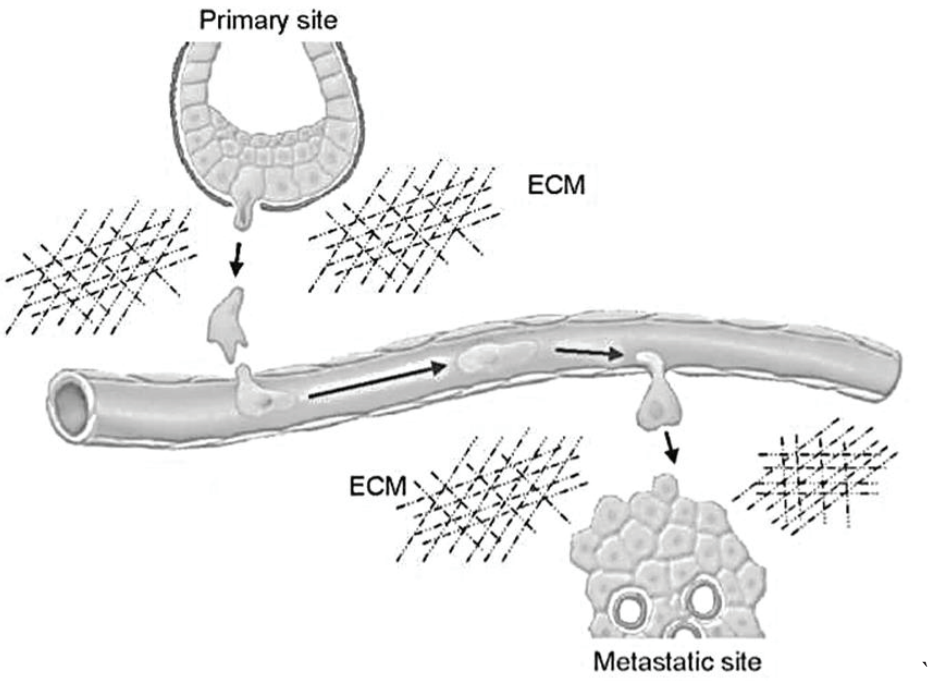

Figure 4*. The Metastatic Cascade*

1.2 Podophyllotoxin

1.2.1 Introduction

Natural products have been used throughout human history to treat a multitude of ailments. According to the World Health Organization (WHO) the use of traditional and folk medicine, which is are largely based around natural products and medicinal plants, is estimated to be used by 65% of the world's population as their primary form of health care (1). This is due to the fact that natural products can be thought of as having been evolutionarily formed into privileged structures (46). That is, compounds which have been selected and developed to exhibit a wide range of biological activities. It is due to this intrinsic value of natural products which has helped them become a major source of new leads for the development of novel drugs, especially for anticancer treatments.

One such natural product is podophyllotoxin, a natural product derived from the rhizomes of the *Podophyllum* genus of plants. Several drugs, especially antitumor drugs, have been derived from podophyllotoxin (47). Podophyllotoxin itself inhibits tubulin polymerization and therefore prevent cytokinesis (48,49). Furthermore, podophyllotoxin and several of its more well-known derivatives, such as etoposide, have also been shown to bind and destabilize DNA, inhibit the activation of topoisomerase II, and induce apoptosis.

1.2.2 Podophyllotoxin: Phytochemistry & Toxicology

Podophyllotoxin is a lignan isolated from the rhizomes of the *Podophyllum* genus of plants. Lignans, a family of natural products formed as a secondary metabolite in plants, have and been shown to have a multitude of biological properties (50). For over a millennium, lignan containing plants have been used as folk remedies in the East to treat a variety of ailments. More recently however, Podophyllotoxin has been shown to be competent antiviral agent, in the treatment of rubeola and type I herpes (51). In fact, as a topical ointment podophyllotoxin has been shown to be effective in the treatment of genital warts, as well as having fewer side effects than other treatment modalities (52).

Several fields of research have been established around studying and developing new drugs from lignans, such as podophyllotoxin, due to their efficacy as cytotoxic and antimitotic agents. However, it is Podophyllotoxins anticancer properties which are of interest to this study. Our collaborators have shown that Podophyllotoxin and several analogues has anti-neoplastic properties as well as being able to induce apoptosis (49). Indeed, they have shown that Podophyllotoxin itself disrupts microtubule polymerization and induces cell cycle arrest during mitosis (Fig. 7).

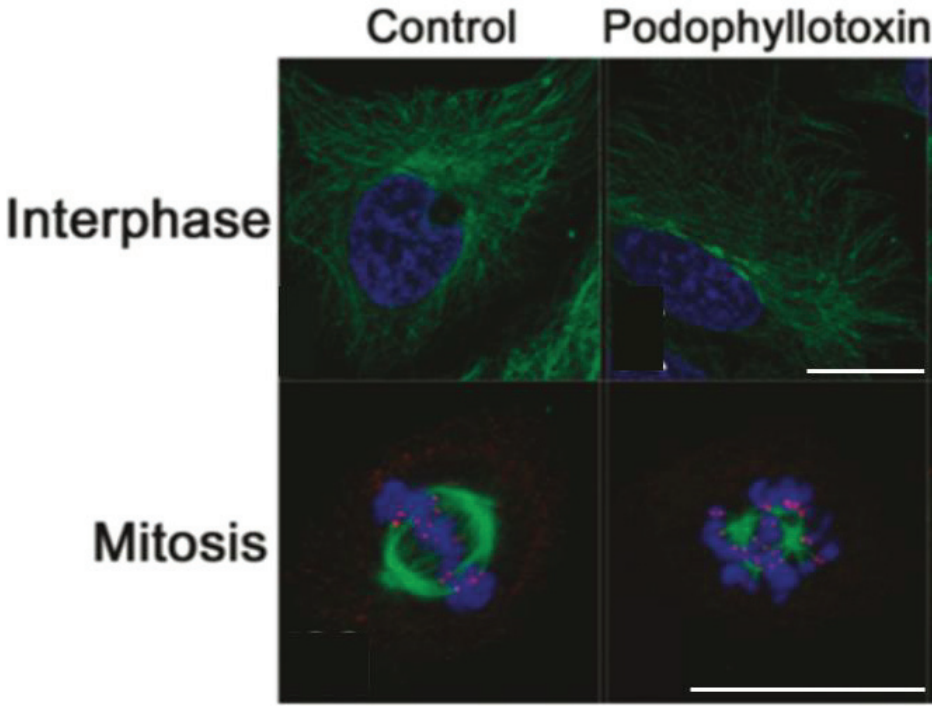

Image adapted from: Igor V. Magedov; Liliya Frolova; Madhuri Manpadi; Uma devi Bhoga; Hong Tang; Nikolai M. Evdokimov; Olivia George; Kathy Hadje Georgiou; Steffen Renner; Matthäus Getlik; Tiffany L. Kinnibrugh; Manuel A. Fernandes; Severine Van slambrouck; Wim F. A. Steelant; Charles B. Shuster; Snezna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

Figure 5. *Microtubule organization in interphase and mitotic HeLa cells treated with Podophylotoxin*
Microtubule organization in interphase and mitotic HeLa cells treated with indicated agents at 5 nM: microtubules (green), the kinetochore marker Hec1 (red) and Hoechst 33342 (blue). Scalebars, 10 µm.

Chapter 2. Materials and Methods

2.1 Podophyllotoxin Analogues

Podophyllotoxin analogues were provided in collaboration by Willem van Otterlo (Stellenbosch University Stellenbosch, Western Cape, South Africa) and Alexander Kornienko (Texas State University, San Marcos, TX).

2.*2 Cell culture*

MCF7 (ATCC® HTB-22TM), A549 (ATCC® CCL-185), SK-MEL-28 (ATCC® HTB-68), and MDA-MB-231 (ATCC® HTB-26) (ATCC, Manassas, VA) cells 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 (ATCC® TIB-152, E6-1 clone) 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 5-10% CO_2.

2.3 Assay for cell viability

Cell viability was tested in accordance with Romijn *et al*. Briefly, 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 100 µl culture medium. After overnight incubation, cells were treated with the analogues with a range of concentrations to determine IC 50 values. After 48 hr, 100 µl of medium was removed prior to the addition of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent (MTT) (Sigma Aldrich, St. Louis, MO). The formed formazan crystals were then dissolved in 200 µl dimethyl sulfoxide (DMSO) (Thermo Fischer Scientific). 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) and were expressed in percentage as compared to DMSO-treated control cells. In each experiment, eight wells were used per condition.

2.4 Cell Counting

Jurkat cells were seeded in 25-cm^2 culture flasks at an initial density of ~1.5×10^5 cells in 5 ml culture medium and were treated with the analogues with concentrations at their respective IC_{50} values. The cells were allowed to grow for 48 hr, then harvested using trypsin/EDTA (Invitrogen, Carlsbad, CA) and counted with a TC20™ automated cell counter (Bio-Rad Laboratories, Hercules, CA) using Trypan Blue

dye solution (Thermo Fischer Scientific). Three independent experiments were performed to determine the mean value, which is presented as a percentage compared to the DMSO-treated controls.

2.5 Flow cytometric analysis.

Due to their sensitivity to the podophyllotoxin analogues as well as their ease of use and analysis due to their nonadherent phenotype, Jurkat cells were used as the model for the induction of apoptosis and cell cycle control. The cells were treated for 48 hr with the analogues at their respective IC_{50} values and were then washed with cold PBS. For the detection of apoptosis, cells were stained with an Annexin V/7-AAD kit (Beckman Coulter, Miami, FL, USA) using the manufacturer's protocol.

In brief, cells were incubated with Annexin V and 7-AAD in ice-cold binding buffer in the dark. After 15 min the samples were resuspended in binding buffer and analyzed within 30 min. As a positive control for the induction of apoptosis, Jurkat cells were treated with 5 μM of camptothecin for 48 hr. Since the analogues were derived from podophyllotoxin, jurkat cells were also treated with 10 μM of podophyllotoxin as a positive control in order to compare the effects of the analogues to their native structure. Cell cycle analysis was also performed on Jurkat cells, following a 48 hr treatment with the analogues at their respective IC_{50} values. Vybrant® DyeCycle™ Green Stain (Thermo Fisher Scientific) was then added to a 1 ml cell suspension at a final concentration of 250 nM. After 30-min incubation at 37˚C, the samples were analyzed by flow cytometry and compared against DMSO-treated and podophyllotoxin controls. All these experiments were performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA) using CytExpert 2.0 software.

2.6 Statistics

Treatments were matched and performed at least in triplicate. Data were analyzed using Excel, for determination of mean, standard deviation (SD) and Student's t-test (95%).

Chapter 3. Results

3.1 The Effect of Podophyllotoxin on cell viability.

Each cell line was treated for 48 hr with each analogue at concentrations of 10, 25 and 50 μM with the effect on cytotoxicity determined by MTT assays. The effects of the podophyllotoxin analogues on cytotoxicity are expressed as means of the derived IC_{50} values in Table 2. Overall, the analogues had a more potent effect on the T cell lymphoma Jurkat cells. Of the 23 analogues tested, 10 compounds were found to decrease cell viability by at least 50% in Jurkat cells. Analogues 1.2, 2.1, 6.2, and, 7.1 had the most notable cytotoxic effect with IC_{50} values lower than 20 μM. Analogues 6.2 and 7.1 had the most pronounced effect on cell viability with IC_{50} values of about 4 μM and 7 μM respectively. While the cytotoxic effect of the podophyllotoxin analogues was not as pronounced when compared to Jurkat cells, there was a marked effect on A549, and, MCF7 cells. 10 of the compounds were able to decrease cell viability in A549 lung cancer cells. Specifically, 1.3, 2.1, 2.2, and 4.4, and 9.2 had the most robust effect on cell viability with IC_{50} values of about of about 18, 15, 17, 16 and 3 μM respectively. While the analogues did not have such a marked effect on MCF7 breast cancer cells when compared to A549 and Jurkat cells, 8 of the podophyllotoxin analogues displayed cytotoxic activity. Analogues 2.1, 2.2 and 4.4 had comparatively low IC_{50} values of about 15, 17, and, 18 3 μM respectively. Interestingly, SK-MEL-28 skin cancer cells only had

3 analogues which were able to decrease cell viability by at least 50%, with the lowest being 6.2 at around 24 μM. Finally, no IC_{50} values were able to be derived for MDA-MB-231Triple Negative Breast Cancer cells (TNBC). It should be noted however, only analogue 5.2 was able to provide a discernable IC_{50} across all cell lines, except MDA-MB-23 (Fig 8). Intriguingly, when A549 cells were treated with compound 9.2, it provided the lowest IC_{50} value, about 3 μM, when compared to all other cell lines.

	Jurkat	A549	SK-MEL-28	MCF-7	MDA-MB-231
Podophyllotoxin	*0.0001±0.00002*	*0.001±0.00031*	*>50*	>50	>50
1.1	*29.1±11.87*	*>50*	*>50*	*>50*	*>50*
1.2	*>50*	*>50*	*>50*	*>50*	*>50*
1.3	*17.8±1.02*	*17.8±3.0*	*>50*	*33.3±13*	*>50*
2.1	*16.1±3.8*	*15.2±2.3*	*>50*	*15.2±2.67*	*>50*
2.2	*23.84±1.04*	*16.9±3.9*	*>50*	*17.7±1.8*	*>50*
2.3	*>50*	*21.5±4.9*	*>50*	*21±4.4*	*>50*
3.1	*>50*	*24.5±0.71*	*>50*	*19.5±2.29*	*>50*
3.2	*>50*	>50	*>50*	>50	*>50*
3.3	*>50*	*26.167±7.75*	*>50*	*20.7±3.4*	*>50*
4.1	*>50*	*>50*	*>50*	>50	*>50*
4.2	*>50*	*>50*	*>50*	*>50*	*>50*
4.3	*>50*	*>50*	*>50*	>50	*>50*
4.4	*>50*	*16.3±2.4*	*34.3±3.62*	*17.75±1.1*	*>50*
5.1	*>50*	*>50*	*>50*	*>50*	*>50*
5.2	*23.3±.7.6*	*42.5±3.54*	*43.5±3.57*	*26.17±7.84*	*>50*
5.3	*>50*	*>50*	*>50*	*>50*	*>50*
5.4	*>50*	*20.5±1.41*	*>50*	*>50*	*>50*
6.1	*>50*	*>50*	*>50*	>50	*>50*
6.2	*4.23±0.67*	>50	>50	>50	*>50*
7.1	*6.75±1.02*	>50	*24.45±5.7*	>50	*>50*
8.1	*34.25±1.06*	>50	>50	>50	*>50*
9.1	*48.24±2.06*	>50	>50	>50	>50
9.2	*36.05±4.63*	*3.1±0.57*	>50	*34.25±1.06*	>50

Table 1. *The Effect of Podophyllotoxin analogues on cell viability*. The concentration at which each compound decreased cell viability by 50% in μM relative to DMSO controls ±% SD from at least three independent experiments as determined by MTT assays.

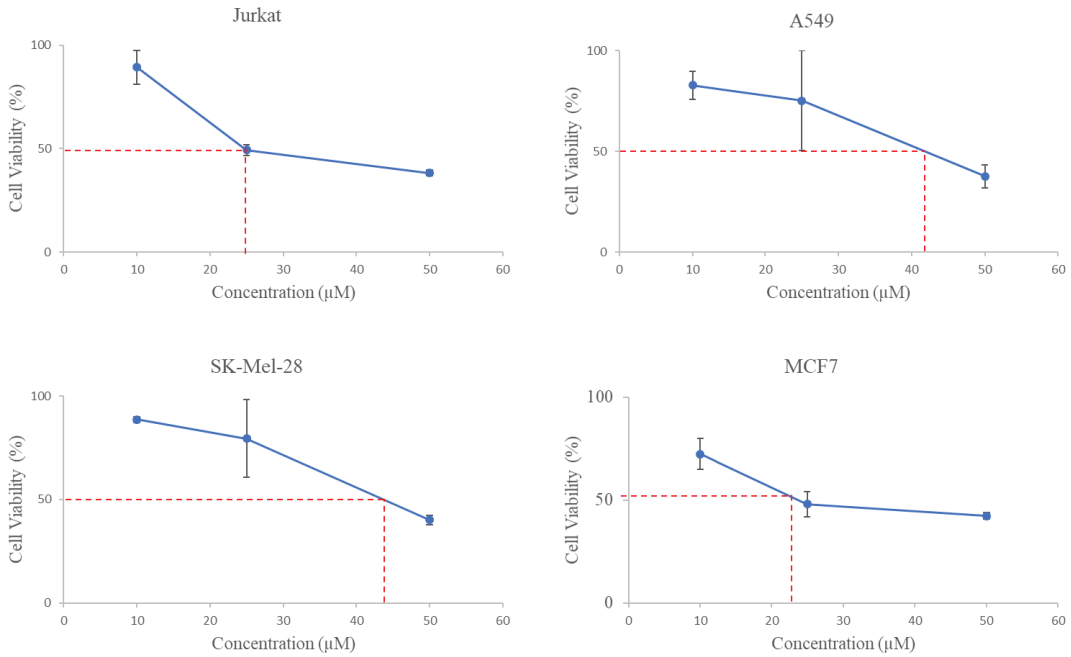

Figure 6. *The Effect of Podophyllotoxin analogue 5.2 on cell viability.* Cells were exposed to various concentrations of compound 5.3 for 48 hr to derive an IC_{50} value. The dash red line indicates the concentration at which the compounds decreased cell viability by about 50%. The percentage of cell viability was determined by MTT assay. Each data point represents the mean and standard deviations of results from at least three independent experiments.

3.2 The Effect of Podophyllotoxin on cell growth on Jurkat cells.

Due to their sensitivity to the podophyllotoxin analogues as well as being the main model used for cytometric analysis, Jurkat cells were used to determine the growth inhibitory effects of Podophyllotoxin analogues via cell counting. Following a 48 hr treatment at their respective IC_{50} values, several of the analogues exhibited a limited effect on the number of cells grown when compared to DMSO controls (Fig 9). Of the podophyllotoxin analogues tested, only analogues 3.1 and 3.2 were somewhat comparable to the data obtained from the MTT-assays, with only about a 40% and 45% reduction in the number of cells respectively. Alternately, analogues 1.1 and 1.3 only had a slight reduction in the number of cells with about 15% and12% respectively.

Interestingly, analogues 2.1 and 5.2 significantly reduced the number of live cells by 60% and 80% when compared to DMSO controls. Jurkat cells were also treated with 5 μM of camptothecin and 10 μM of podophyllotoxin. Camptothecin reduced the number of Jurkat cells by about 40%, whereas podophyllotoxin had about a 55% reduction.

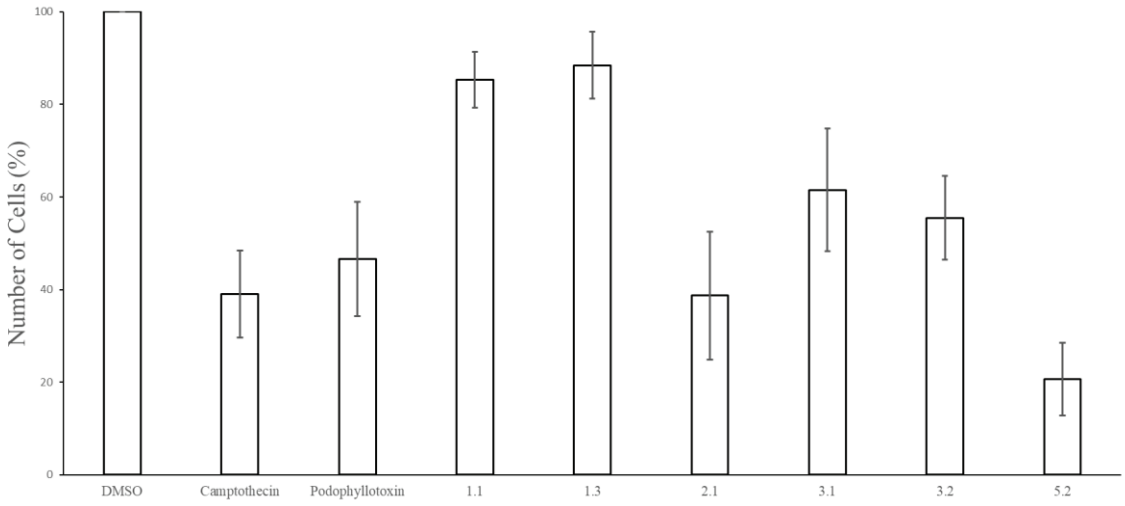

Figure 7. *The effects of Podophyllotoxin analogues on cell growth as determined by cell counts.* Jurkat cells treated with podophyllotoxin analogues at their respective IC_{50} values. The number of cells was determined by counting and is expressed as a percentage of control DMSO treated cells. White bars indicate mean values of at least three independent experiments with error bars expressing standard deviations.

3.3 The Effect of Podophyllotoxin Analogues on apoptosis on Jurkat cells.

The Annexin V-FITC/7-AAD double staining was used to examine the effects of podophyllotoxin analogues on the induction of cellular apoptosis on Jurkat cells. The Annexin V-FITC/7-AAD double staining technique allows for the easy discrimination of apoptotic, necrotic and live cells. Cells entering apoptosis express phosphatidylserine (PS) on the outer leaflet of the plasma membrane which can be stained using annexin V.

However, necrotic cells do not express PS but do lose their membrane integrity which is permeable to the vital dye 7-AAD.

Following a 48 hr treatment, analogues 1.1, 1.3, 2.1 and 3.3 had the most prominent effect on the induction of apoptosis as shown in (Fig 10). Generally, when compared to DMSO controls, the podophyllotoxin analogues were able to increase the induction of apoptosis by an average of 20%. The largest increase in the induction of apoptosis was seen with analogue 1.3 at about 29% and the lowest with analogue 1.1 at around 17%. However, treatment with the analogues only lead to a slight increase of about 3% in the induction of necrosis. Jurkat cells were also treated with 5 μM of camptothecin as a positive control for the induction of apoptosis as well as 10 μM of podophyllotoxin in order to compare the effects of the analogues to their native compound. While the concentration of podophyllotoxin used was significantly higher than the derived IC_{50}, when compared to the DMSO controls, there was about a 27% increase in the induction of necrosis an about a 6% increase in necrosis. Similar to podophyllotoxin, camptothecin had an increase of about 30% of cells in apoptosis, yet,

also increased the number of necrotic cells by about 22%.

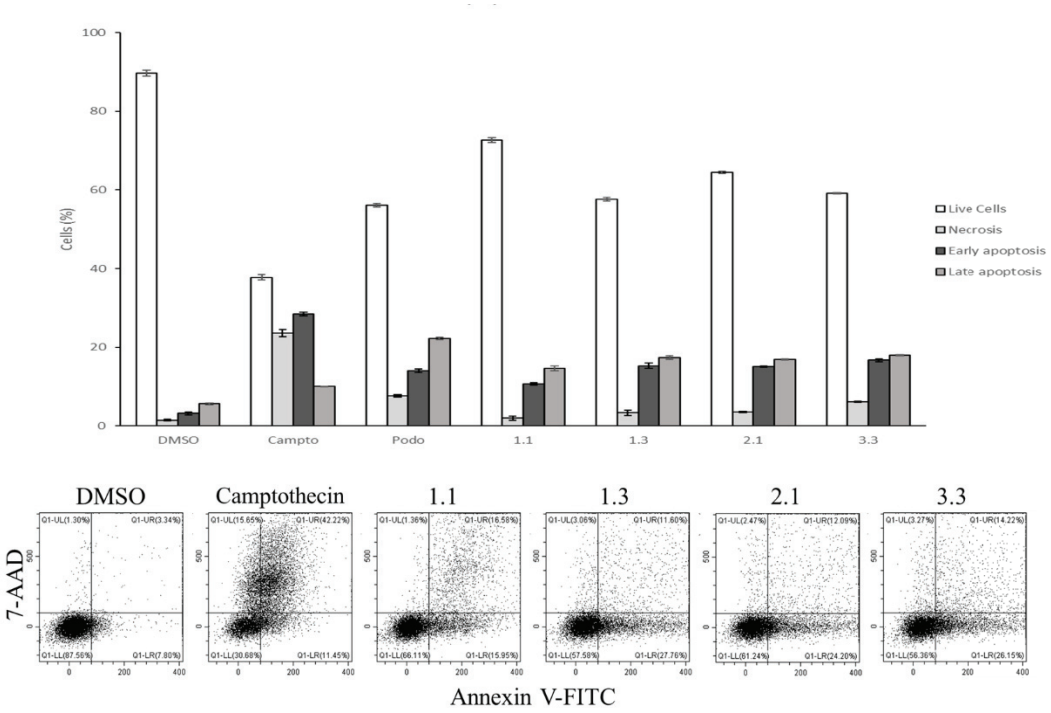

Figure 8*. The Effect of Podophyllotoxin Analogues on apoptosis on Jurkat cells.* Effects of Podophyllotoxin analogues as well as 5 µM camptothecin and 5 µM DMSO as controls (top panel). Percentage of live cells (white bars), early apoptotic (grey bars), late apoptotic (light grey bars) and necrotic (light grey bars) cells, expressed as mean % ± SD of three independent experiments. Representative dot plots describing the effect of podophyllotoxin analogues on jurkat cells following double staining with Annexin V-FITC and 7-AAD (bottom panel).

3.4 The Effect of Podophyllotoxin Analogues on cell cycle control on Jurkat cells.

The effects of podophyllotoxin analogues on the cell cycle were evaluated by performing a Vybrant® DyeCycle™ green staining. Comparison with DMSO-treated cells indicated that that the analogues did not have a marked effect on cell cycle control following a 48hr treatment at their respective IC50 values (Fig 11). Only analogue 1.2 was able to cause a significant difference in any cell cycle population. There was about a 22% increase in sub G0 as well as a 17% decrease in the number of cells in S phase. While the analogues may not have had a statistically significant effect on cell cycle

control, it is worth mentioning that some of the compounds did indeed show remarkable effects on the cell cycle. In particular, analogue 1.3 saw twice the number of cells arrested in G2/M when compared to DMSO-controls, as well as about a 100% decrease in all other phases in the cell cycle. Jurkat cells were also treated with 10 uM Podophyllotoxin. There was a marked increase in the population of cells in sub G0, as well as a dramatic decrease of cells in G0/G1, as well as a slight decrease of cells in S phase. While this concentration is significantly higher than the IC_{50} values, it provided insight as to how the analogous behaved compared to their native compound.

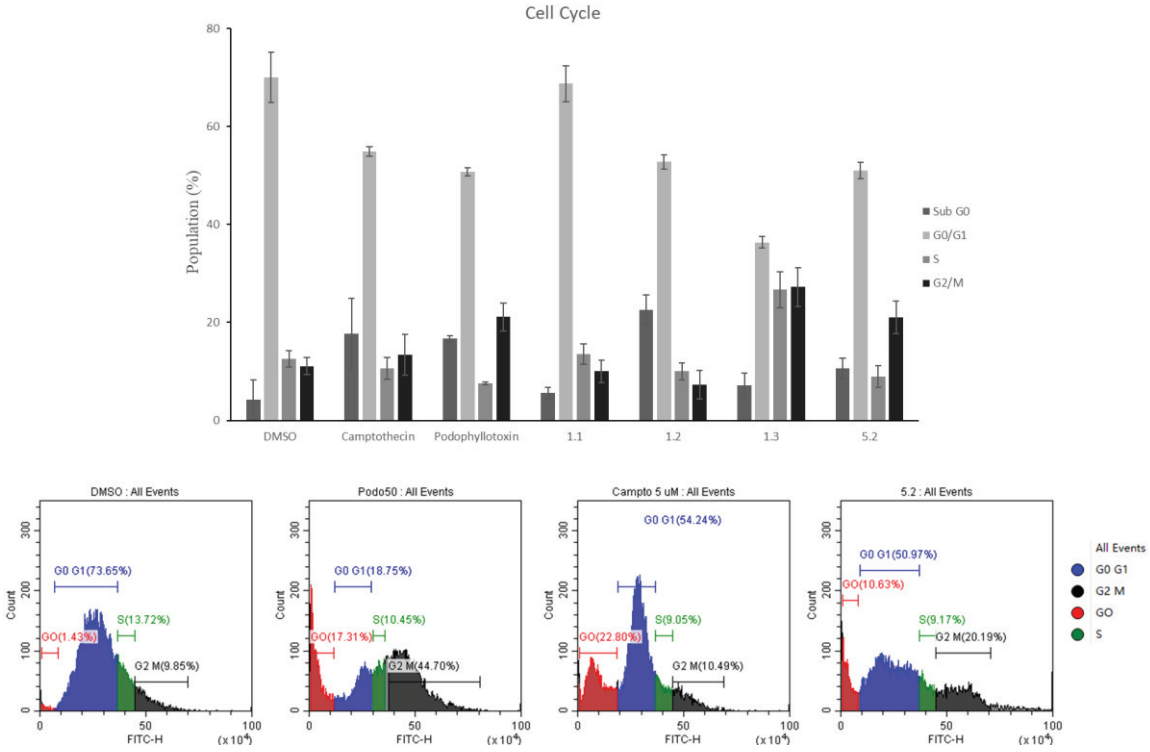

Figure 9. *The Effect of Podophyllotoxin Analogues on Cell Cycle Control on Jurkat cells.* Percentage of population in sub G0 (dark grey bars), G0/G1 (light grey bars), S (grey bars) and G2/M (black bars) cells, expressed as mean % ± SD of three independent experiments (top panel). Representative histograms of the number of jurkat cells stained with Vybrant® DyeCycle™ Green in sub G0 (red), G0/G1 (azul), S (green) and G2/M (black)

Chapter 4: Discussion

Podophyllotoxin has been used as a lead agent in the development of several anticancer drugs. Two of its semisynthetic derivatives are currently used in the treatment of several forms of cancer. The present study reports for the first time on the anticancer properties of novel Podophyllotoxin analogues, which were developed through mimetic scaffolding constructed using one-step multicomponent reactions. Previous studies have shown that the simplified structures of these analogues were able to inhibit in vitro tubulin polymerization and disrupt the formation of mitotic spindles in dividing as well as having cytotoxic and apoptosis-inducing properties similar to podophyllotoxin.

In order to determine the possible anticancer effects of the podophyllotoxin analogues, the study followed an experimental outline as shown in figure 5. The compounds were initially screened across several cell lines to determine their effects on cell viability. Following a 24 hour treatment our results indicated that generally, when compared to DMSO controls, the compounds greatly reduced cell viability on Jurkat cells while there was practically no reduction in cell viability for MDA-MB-231 cells when treated with any of the podophyllotoxin analogues. This seems to be due to Jurkat cells being susceptible to the induction of apoptosis when treated with different chemotherapeutic agents, while MDA-MB-231 cells, a triple negative cell line, is normally more robust and less sensitive to many forms of treatment. Interestingly, there did not seem to be much conformity between how each of the analogues affected the different cell lines. Only compound 5.2 was able to reduce cell viability by 50% across all cell lines, except in MDA-MB-231 which had no discernable IC_{50} values.

Compounds which were shown to decrease viability by 50% were classed as being cytotoxic and further tested to determine their effect on cell cycle control and on the induction of apoptosis. Due to their sensitivity to the podophyllotoxin analogues as well as their ease of use and analysis due to their nonadherent phenotype, Jurkat cells were used as the model for the induction of apoptosis and cell cycle control.

Cytometric analyses revealed that when compared to DMSO and campothecin controls several of the analogues were able to induce apoptosis in Jurkat cells. The compounds also had relatively low populations of necrotic cells, indicating that analogues are more of an apoptosis inducing agent as seen in figure 10. Yet, when the analogues are compared to Jurkat cells treated with 5 uM of Podophyllotoxin, the population of apoptotic cells seem to be lower. However, the concentration of podophyllotoxin is drastically higher than previously derived IC_{50} values. Still, when treated with the podophyllotoxin analogues, proportionally, there were still significantly less necrotic cells when compared to those treated with podophyllotoxin, again suggesting that the analogues are more potent inducers of apoptosis than necrosis.

Further cytometric analyses revealed that several of the podophyllotoxin analogues had a marked affect on cell cycle control. Podophyllotoxin is a known antimitotic agent leading to the arrest of cells in the G2/M phase. Following a 24 hour treatment with podophyllotoxin almost 45% of Jurkat cells were shown to be arrested in G2/M. Again, these cells were treated with concentrations above their previously derived IC50 values in order to show a more distinct difference between the different phases of the cell cycle. Interestingly, compounds 1.3 and 5.2 were shown to have the largest population of cells arrest in G2/M. 5.2 was also the only analogue which was able to

decrease cell viability by 50% across all cell lines, making it an ideal candidate for further testing on other cell lines.

Chapter 5: References

1. World Health Organization, International Agency for Research on Cancer (2015). GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide. Cancer Fact Sheets. Available from: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

2. American Cancer Society. (2011). Global cancer facts and figures(2nd edn). Atlanta, GA: Author. Available from: http://www.cancer.org/acs/groups/content/@epidemiologysurveilance/documents/document/acspc-027766.pdf

3. Lorusso, D., Bria, E., Costantini, A., Di Maio, M., Rosti, G., & Mancuso, A. (2016). Patients' perception of chemotherapy side effects: Expectations, doctor-patient communication and impact on quality of life - An Italian survey. European Journal of Cancer Care, 26(2), e12618. https://doi.org/10.1111/ecc.12618

4. Newman DJ, Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 75: 311-335. doi.10.1021/np200906s. PMID: 22316239

5. Cragg GM, Grothaus PG, Newman DJ (2014) New horizons for old drugs and drug leads. J Nat Prod 77: 703-723. doi: 10.1021/np5000796. PMID: 24499205

6. Newman DJ and Cragg GM: Natural products as sources of new drugs over the last 25 years. J Nat Prod 70: 461-477, 2007.

7. Cimmino, A., Masi, M., Evidente, M., Superchi, S., & Evidente, A. (2017). Amaryllidaceae alkaloids: Absolute configuration and biological activity. Chirality, 29(9), 486–499. https://doi.org/10.1002/chir.22719

8. Kornienko, A., Evidente,A.,2008.Chemistry,biology and medicinal potential of narciclasine anditscongeners.Chem.Rev.108,1982–2014.

9. Pettit, G.R., Pettit III, G.R.,Backhaus,R.A.,Boyd,M.R.,Meerow,A.W.,1993.Cell growthinhibitoryisocarbostyrilsfrom Hymenocallis. J.Nat.Prod.56, 1682–1687.

10. M. Lopez-Lazaro What is the main cause of cancer? Cancer Stud. Ther. J., 1 (1) (2016), pp. 1-2

11. Demetriou, C., Degli Esposti, D., Pullen Fedinick, K., Russo, F., Robinson, O., & Vineis, P. (2018). Chemical carcinogenesis and the hallmarks of cancer: A temporal perspective. European Journal of Clinical Investigation, e12933. https://doi.org/10.1111/eci.12933

12. B. Vogelstein, K.W. Kinzler The path to cancer –three strikes and you're out N. Engl. J. Med., 373 (20) (2015), pp. 1895-1898

13. López-Lázaro, M. (2018). The stem cell division theory of cancer. Critical Reviews in Oncology/Hematology, 123, 95–113. https://doi.org/10.1016/j.critrevonc.2018.01.010

14. Brücher, B. L. D. M., & Jamall, I. S. (2016). Somatic Mutation Theory - Why it's Wrong for Most Cancers. Cellular Physiology and Biochemistry, 38(5), 1663–1680. https://doi.org/10.1159/000443106

15. 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

16. Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). Genomic instability — an evolving hallmark of cancer. Nature Reviews Molecular Cell Biology, 11(3), 220–228. https://doi.org/10.1038/nrm2858

17. Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. Nature, 454(7203), 436–444. https://doi.org/10.1038/nature07205

18. Kawanishi, S., Ohnishi, S., Ma, N., Hiraku, Y., & Murata, M. (2017). Crosstalk between DNA Damage and Inflammation in the Multiple Steps of Carcinogenesis. International Journal of Molecular Sciences, 18(8), 1808. http://doi.org/10.3390/ijms18081808

19. Feron, O. (2009). Pyruvate into lactate and back: From the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiotherapy and Oncology, 92(3), 329–333. https://doi.org/10.1016/j.radonc.2009.06.025

20. Green, Douglas R, and Fabien Llambi. “Cell Death Signaling.” Cold Spring Harbor perspectives in biology vol. 7,12 a006080. doi:10.1101/cshperspect.a006080

21. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, Zitvogel L, Kroemer G. 2007.Cell death modalities: Classification and pathophysiological implications. Cell Death Differ 14: 1237–1243.

22. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, et al. 2012. Molecular definitions of cell death subroutines: Recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 19: 107–120.

23. Green, D. R., & Llambi, F. (2015). Cell Death Signaling. Cold Spring Harbor Perspectives in Biology, 7(12), a006080. https://doi.org/10.1101/cshperspect.a006080

24. Houghton JA, Harwood FG, Tillman DM . (1997). *Proc Natl Acad Sci USA* **94**: 8144–8149

25. Xu, X., Lai, Y., & Hua, Z.-C. (2018). Apoptosis and apoptotic body: disease message and therapeutic target potentials. Bioscience Reports, 39(1), BSR20180992. https://doi.org/10.1042/bsr20180992

26. D. González, I. Bejarano, C. Barriga, A.B. Rodríguez, J.A. Pariente (2010). "Oxidative Stress-Induced Caspases are Regulated in Human Myeloid HL-60 Cells by Calcium Signal". Current Signal Transduction Therapy 5: 181–186. doi:[10.2174/157436210791112172]

27. Wajant, H. (2002). The Fas Signaling Pathway: More Than a Paradigm. Science, 296(5573), 1635–1636. https://doi.org/10.1126/science.1071553

28. Li J, Yuan J. Caspases in apoptosis and beyond. Oncogene. 2008; 27:6194-6206.

29. Thomberry NA, Laxebnik Y. Caspases: enemies within. Science 1998; 281:1312-1316.

30. Garrido, C., Galluzzi, L., Brunet, M., Puig, P.E., Didelot, C. and Kroemer, G. (2006) Mechanisms of cytochrome c release from mitochondria. Cell Death Differ. 13, 1423–1433, https://doi.org/10.1038/sj.cdd.4401950

31. Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 102, 33–42, https://doi.org/10.1016/S0092-8674(00)00008-8

32. Micheau, O. and Tschopp, J. (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114, 181–190, https://doi.org/10.1016/S0092-8674(03)00521-X

33. Yuan J, Kroemer G. 2010. Alternative cell death mechanisms in development and beyond. Genes Dev 24: 2592–2602.

34. Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK. 2009. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137: 1112–1123.

35. Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J. 2009. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325: 332–336.

36. Matson, J. P., & Cook, J. G. (2016). Cell cycle proliferation decisions: the impact of single cell analyses. The FEBS Journal, 284(3), 362–375. https://doi.org/10.1111/febs.13898

37. Nurse, P. (2000). A Long Twentieth Century of the Cell Cycle and Beyond. Cell, 100(1), 71–78. https://doi.org/10.1016/s0092-8674(00)81684-0

38. Howard A Pelc S Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity. 1953; 6: 261-273

39. Mitchison J.M The Biology of the Cell Cycle. Cambridge University Press, Cambridge; 1971

40. Nigg EA (June 1995). "Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle". BioEssays. 17 (6): 471–80. doi:10.1002/bies.950170603.

41. Malumbres M, Barbacid M (March 2009). "Cell cycle, CDKs and cancer: a changing paradigm". Nature Reviews. Cancer. 9 (3): 153–66. doi:10.1038/nrc2602

42. Bertoli C, Skotheim JM, de Bruin RA (August 2013). "Control of cell cycle transcription during G1 and S phases". Nature Reviews. Molecular Cell Biology. 14 (8): 518–28. doi:10.1038/nrm3629.

43. Wang Y, Ji P, Liu J, Broaddus RR, Xue F, Zhang W (February 2009). "Centrosome-associated regulators of the G(2)/M checkpoint as targets for cancer therapy". Molecular Cancer. 8 (1): 8.

44. Mendonça, E. A., Evans, J. A., & Rzhetsky, A. (2011). Conflicting Biomedical Assumptions for Mathematical Modeling: The Case of Cancer Metastasis. PLoS Computational Biology, 7(10), e1002132. https://doi.org/10.1371/journal.pcbi.1002132

45. Klein, C. A. (2008). CANCER: The Metastasis Cascade. Science, 321(5897), 1785–1787. https://doi.org/10.1126/science.1164853

46. Gordaliza, M., García, P. ., Miguel del Corral, J. ., Castro, M. ., & Gómez-Zurita, M. . (2004). Podophyllotoxin: distribution, sources, applications and new cytotoxic derivatives. Toxicon, 44(4), 441–459. https://doi.org/10.1016/j.toxicon.2004.05.008

47. Canel C, Moraes RM, Dayan FE, Ferreira D (May 2000). "Podophyllotoxin". Phytochemistry. 54 (2): 115–20. doi:10.1016/s0031-9422(00)00094-7

48. Hamidreza A, Amir A, Majid G (2017-06-01). "Podophyllotoxin: a novel potential natural anticancer agent". Avicenna Journal of Phytomedicine. 7 (4). doi:10.22038/ajp.2017.8779.

49. Magedov, I. V., Frolova, L., Manpadi, M., Bhoga, U. devi, Tang, H., Evdokimov, N. M., … Kornienko, A. (2011). Anticancer Properties of an Important Drug Lead Podophyllotoxin Can Be Efficiently Mimicked by Diverse Heterocyclic Scaffolds Accessible via One-Step Synthesis. Journal of Medicinal Chemistry, 54(12), 4234–4246. https://doi.org/10.1021/jm200410r

50. Ayres, D. C, & Loike, J. D. (1990). Lignans : chemical, biological, and clinical properties. Cambridge: Cambridge university press.
51. Wilson J. 2002. Treatment of genital warts—what's the evidence? Int J Std Aids, 13: 216-222.
52. Edwards L, Ferenczy A, Eron L, Baker D, Owens ML, Fox TL, Hougham AJ, Schmitt KA. 1998. Self-administered topical 5% imiquimod cream for external anogenital warts. Arch Dermatol, 134: 25-30.