Paclitaxel Enhances Oncolytic Potential of Herpes Simplex Virus Type-1 in Cancer Cells

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PACLITAXEL ENHANCES ONCOLOYTIC POTENTIAL OF HERPES SIMPLEX VIRUS TYPE-1 IN CANCER CELLS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Veterinary Medical Sciences - Pathobiological Sciences

by

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ABSTRACT

Taxanes are spindle poisons that bind to and stabilize microtubules resulting in mitotic arrest. Herpes simplex Typ-1 (HSV-1) virions utilize the microtubular network for intracellular transport during both virus entry and virus egress from infected cells. It has been reported previously that taxanes may synergize with oncolytic herpes simplex viruses in the treatment of experimental prostate and breast tumors in mice. Other reports have indicated that taxanes may inhibit viral replication in infected cells. In this study the previously characterized Oncolytic Herpes Simplex Virus type 1 (OSVP), which was constructed in Kousoulas lab was used in conjugation with paclitaxel (taxol) in 4T1 mouse mammary tumor and RM9 mouse prostate cancer cell lines.

The first goal of this study is to examine the effects of taxol on OSVP life cycle. The effect of taxol on the extent of virus entry, replication and number of infectious virus production were measured. The results showed that virus life cycle is not affected by the presence of taxol in these cell lines despite significant stabilization and reorganization of the microtubular network; visual evidence was also provided by immune fluorescence confocal microscopy reflecting that the virus entry was efficiently followed by intracellular transport and finally infection and spread of the virus to the adjacent cells leading to syncytium formation.

The second goal of this study is to investigate the potency of the constructed OSVP virus in combination with taxol in cancer cell cultures. In various combinations of virus multiplicities of infection and taxol concentrations, enhanced cell killing effects were observed. Moreover, a few of the combination ratios lead to a more pronounced effect characterized as synergistic.

Overall, results confirm that OSVP infection in RM9 and 4T1 cell lines are unaffected by taxol. Moreover, the efficacy and potency of OSVP and taxol are improved when combined.
Interestingly, intracellular taxol concentrations and cell cycle analysis results were also affected by virus infection. It is anticipated that taxol-induced leakage of tumors will facilitate spread of OSVP virus within the tumor, producing enhanced tumor destruction and anti-tumor immune responses in addition to the enhanced cellular effect shown in this study.
1. INTRODUCTION

1.1 Statement of Problem

Prostate cancer is the most frequently diagnosed non-cutaneous cancer among males, and the second most common cause of cancer death in males according to Cancer Prevention and Control (2017, June 05)(CDC, 2017b). Although localized tumors can be removed surgically or cured by chemotherapy and radiation therapy, the advanced forms of the disease such as the metastatic castration resistant prostate cancer are still incurable. Breast cancer is the second highest cause of cancerous death in women after lung cancer reported by Cancer Prevention and Control in 2017(CDC, 2017a). Treatments include surgery, radiation, chemotherapy and hormonal therapy, as well as newly discovered targeted therapies such as immune therapy and gene therapy (Chaurasiya et al., 2016; Pusztai, Karn, Safonov, Abu-Khalaf, & Bianchini, 2016). Despite of all these advancement, the survival rates have not changed drastically in the past decades. The high prevalence and the poor prognosis of the disease, as well as the chemotherapy refractory, relapsed and metastatic forms are strong motives to develop new treatment regimens. A relatively new and promising strategy for cancer therapy is the use of genetically modified viruses that have been engineered to selectively replicate within cancer cells, this treatment is known as oncolytic virotherapy(Crunkhorn, 2018). A number of viruses have been explored as tumor-selective replicating vectors, including adenovirus, herpes simplex virus type-1 (HSV-1), vaccinia virus, reovirus, Newcastle disease virus, vesicular stomatitis virus, measles virus, poliovirus and West Nile virus (Bing, Jianru, Yuequan, Shifeng, & Xiping, 2014; Chiocca, 2002; Y. Ding et al., 2017; Kirn, 2003).

Oncolytic replication competent Herpes simplex Virus type 1 (HSV-1) vectors are emerging as a promising strategy in cancer treatment. HSV-1 has been increasingly explored as
experimental therapeutic against a variety of cancers (Ghonime et al., 2018; Latchman, 2005; Mohr, 2003; Akihiro Nawa et al., 2003; Ring et al., 2017; Shen & Nemunaitis, 2006; Todo, 2002; Varghese & Rabkin, 2002; Zhang et al., 2016). There are certain features that makes HSV-1 an attractive choice for cancer therapy (Kirn, Martuza, & Zwiebel, 2001): HSV-1 infects a wide range of cell types; it can be used as a direct cytolytic agent due to its fusogenic and destructive nature; life cycle of the virus is well understood (B. a. S. Roizman, A.E. , 1996); whole genome has been sequenced and the role of different genes are well characterized; additionally, HSV-1 carries a large genome (153 kb) with multiple nonessential genes that can be genetically modified to carry therapeutic transgenes (B. Roizman, 1996). HSV-1 has other pertinent features that make it more promising for clinical application: there are nonessential genes in HSV-1 genome that are associated with neuropathogenicity that can be deleted to eliminate neurovirulence and replaced with therapeutic transgenes (Nishiyama, 1996; B. Roizman, 1996) yet the virus remains competent; it is susceptible to multiple antiviral drugs in case of unfavorable viral replication (Balfour, 1999); various animal models are available for preclinical studies; it can be delivered systemically; HSV-1 genome does not integrate into the host genome; relatively low multiplicity of infection (MOI) can lead to large extent of tumor cell killing.

Human herpes simplex viruses have been genetically modified to ensure their safety by limiting the neurovirulence, establishment of latency and reactivation and to replicate exclusively in cells with deficient apoptotic mechanisms. Deletion of the viral $\gamma$1 34.5 gene, a major neurovirulence gene and an inhibitor of cellular apoptosis, severely attenuates HSV-1 and restricts viral growth to cancer cells due to their deregulated cell cycle and apoptotic mechanisms (J. Chou, Kern, Whitley, & Roizman, 1990; J. Chou & Roizman, 1992; Manservigi, Argnani, &
Marconi, 2010). Also, HSV-1 UL39 gene, which codes for the large subunit of ribonucleotide reductase, an enzyme required for efficient viral DNA replication which is overexpressed in most tumor cells (Boviatsis et al., 1994; Mineta, Rabkin, & Martuza, 1994), can also be deleted to ensure preferential virus growth in tumor cells. Although these genetic changes are important for safety and tumor selectivity of the virus, they lead to inefficient infectivity and very high chance of clearance by the host immune system; therefore, limiting the therapeutic potency of the oncolytic virus. On the other hand, the antiviral immune response in the vicinity of the tumor can help overcome the tumor induced immune suppression and function as an in situ antitumor vaccine (Fukuhara & Todo, 2007; Prestwich et al., 2009). Virus infection in other words can initiate inflammatory and anti-viral response signals to involve more extensive immune responses to the tumor.

Preclinical studies have shown increased efficacy in oncolytic virotherapy when applied in combination with chemotherapy (Aghi, Rabkin, & Martuza, 2006; Bennett et al., 2004; Chahldavi, Todo, Martuza, & Rabkin, 1999; Cinatl et al., 2003; A. Nawa et al., 2003; Petrowsky et al., 2001; Toyoizumi et al., 1999). It has been reported previously that taxanes may synergize with oncolytic herpes simplex viruses in the treatment of experimental prostate tumors in mice (Passer et al., 2009). Other reports have indicated that taxanes may inhibit viral replication in infected cells (Kotsakis, Pomeranz, Blouin, & Blaho, 2001).

1.2 Project Objectives

Our group has shown that oncolytic herpes virus OSVP mitigates immune suppression and reduces ectopic primary and metastatic breast cancer in mice (Walker, Sehgal, & Kousoulas, 2011). The purpose of the current study is to further characterize the novel oncolytic virus OSVP
in presence of taxol, examine the effect of virus infection on taxol internalization, and to explore the effect of combination therapy of breast and prostate cancer using these two agents.

1.3 Overview of Thesis

The first chapter introduces the problems afflicted by prostate and breast cancers, the conventional solutions, as well as the current advancements in treating these cancers.

Chapter 2 describes a more extensive literature review on oncolytic viruses, HSV-1 life cycle, history and usage of taxol, and combination therapy using chemotherapeutic agents and oncolytic viruses.

Chapter 3 presents the materials and methods used in the current study to inform researchers who are interested in repeating or designing similar experiments.

Chapter 4 discusses the results in the context of previous observations by other researchers.

Chapter 5 provides the summary of the work, conclusions from this project and recommendations future studies in taxol-OSVP chemovirotherapy.
2. LITERATURE REVIEW

2.1 Oncolytic Virotherapy

Oncolytic virotherapy dates back to early 1900 upon observations of spontaneous tumor destruction in virus infected patients or in people receiving vaccination. The earliest documented cases of viral oncolysis were early in the last century, describing occasional regression of cervical cancer following rabies vaccination (Dock, 1904) and reduction of Burkitt’s and Hodgkin’s lymphomas after a bout of measles. In 1912 DePace also reported cases of uterine cervical carcinoma tumor regression after inoculation of an attenuated rabies vaccine (DePace, 1912). In 1920 Levaditi and Nicolau started animal models to study these observations in more detail, which lead to the first published oncolytic vaccinia virus study in mouse (Levaditi & Nicolau, 1922). In about 20 years later, in 1940 Pack performed a human study using an attenuated rabies virus against melanomatosis which showed a notable tumor regression (Pack, 1950). Another human trial was performed using different serotypes of wild-type adenoviruses in fifty seven patients suffering from cervical cancer which yielded tumor remission in more than half of the patients, but it did not stop the progression of the disease (Newman & Southam, 1954). Southan in New York was using numerous viruses including myxovirus, paramyxovirus, and arbovirus to treat patients suffering from a number of different types of malignancies, (Newman & Southam, 1954; Southam & Moore, 1952, 1954; Southam, Noyes, & Mellors, 1958); Tumor regression without toxicity was observed in many cases but disease progressed in spite of the treatment. Naturally occurring oncolytic Newcastle disease virus and influenza virus was also observed in the 1940s. In 1965 Cassel and Garrett published another attempt to cure cancer with Newcastle disease virus (Cassel & Garrett, 1965) leading to the same conclusion as before; virus anti-tumor activity was not merely enough to eliminate the disease, persuading scientists to
abandon studying this mode of therapy. With the advent of advanced technologies in cancer biology, molecular biology, molecular virology the modern era was again ready to harness the power of viruses to combat unsuccessful battle of modern medicine in fighting cancer. Oncolytic viruses fall into two categories: natural occurring viruses selectively replicate in tumor cells and genetically modified viruses engineered to grow preferentially in cancer cells.

2.1.1 Naturally Occurring Oncolytic Viruses

Naturally occurring oncolytic viruses utilize molecular and cellular differences in the biology between normal cells and tumor cells. These differences evolve whilst normal cells are transforming into cancer cells, such as up- or down-regulation of certain surface proteins or disruption of molecular mechanisms and pathways. Cancer cells undergo the dysregulated growth by using these alterations in their favor to circumvent the immune system, rapidly grow and multiply or subvert cell adhesion in case of the metastasis. One of these pathways is involved in regulating complement cell killing which protect normal cell against self-destruction. Complement system is a cascade that upon activation by stimuli such as cell membrane glycosylation patterns or antibody-cell complexes leads to assembly of membrane attack complex (MAC). This creates holes in the cell membrane to damage or kill the cell or the pathogen. Inappropriate self-cell destruction is controlled partly by expression of specific proteins on the cell surface to impede the action of complement. Genetic and epigenetic changes in cancer cells increase the probability to activate the complement system. Tumor cells protect themselves through the over-expression of complement-inhibiting proteins such as decay-accelerating factor or membrane cofactor protein (MCP, CD46) (Hofman et al., 1994). Coxsackievirus A21 utilized decay-accelerating factor for entry (Shafren, Dorahy, Ingham, Burns, & Barry, 1997) while oncolytic measles virus uses MCP (CD46) as a receptor to
mediate virus entry into the cells (Anderson, Nakamura, Russell, & Peng, 2004). High affinity laminin receptor is another example where it plays an important role in tumor-cell migration and invasion and it is overexpressed in many types of human cancers (Scheiman, Tseng, Zheng, & Meruelo, 2009). While in mammalian cells high-affinity laminin receptor is a receptor for Sindbis virus entry (K. S. Wang, Kuhn, Strauss, Ou, & Strauss, 1992), this natural oncolytic virus preferentially replicates in cells overexpressing this receptor.

Interferon production due to a viral infection or as a response to a tumor starts a cascade of events in the host cell and the neighbor cells to interfere with the viral replication or activate the immune cells, such as macrophages and natural killer cells (NK) as well as upregulating antigen presentation to T lymphocytes (T-cells) to increase recognition of infection or tumor cells; this leads to destruction of infected cells, cancer cells, and to boost resistance in uninfected host cells. Interferon pathways can also be altered in cancer cells so that these cells lack the ability to respond to interferon signaling in order to disrupt this cell growth suppressor mechanism. Ineffective interferon response in these cells which helps tumor in its uncontrolled growth is also favorable to the virus. The outcome and selectivity of oncolytic virus infection such as Newcastle disease virus (Krishnamurthy, Takimoto, Scroggs, & Portner, 2006), vesicular stomatitis virus (VSV) (Balachandran & Barber, 2004) and myxoma virus (Johnston, Nazarian, Natale, & McFadden, 2005) in many cases is determined by the suppressed interferon response in tumor cells.

2.1.2 Oncolytic Herpes simplex Virus type 1

Oncolytic replication competent Herpes simplex Virus type 1 (HSV-1) vectors are emerging as a promising strategy in cancer treatment. Various mutations have been tested to increase safety and efficacy of the virus in different mouse models; mutations that affect viral
replication, neurovirulence, augment antitumor immune responses or express anticancer genes. There are certain features that makes HSV-1 an attractive choice for cancer therapy(Kirn et al., 2001), such as: HSV-1 infects a wide range of cell types; it is cytolytic due to its fusogenic and destructive nature; life cycle of the virus is well understood(B. a. S. Roizman, A.E., 1996); whole genome has been sequenced and the role of different genes are well characterized; HSV-1 carries a large genome (153 kb) with multiple nonessential genes that can be genetically modified to carry therapeutic transgenes(B. Roizman, 1996).

Moreover, HSV-1 has other pertinent features that make it more promising for clinical application: there are nonessential genes in HSV-1 genome that are associated with neuropathogeneity that can be deleted to eliminate neurovirulence and replaced with therapeutic transgenes(Nishiyama, 1996; B. Roizman, 1996); it is susceptible to multiple antiviral drugs in case of unfavorable viral replication (Balfour, 1999); various animal models are available for preclinical studies; it can be delivered systemically; HSV-1 genome does not integrate into the host genome; relatively low multiplicity of infection (MOI) can lead to large extent of tumor cell killing. Currently several oHSV-1 made their way to clinical trials (Markert et al., 2000; Rampling et al., 2000), still more tumor selective viruses should be designed to further understand the potential of this revolutionary cancer treatment. Talimogene Laherparepvec (T-Vec) from Amgen is the first FDA approved oncolytic virus commercialized under the name Imlygic. T-Vec is a recombinant HSV-1 expressing human granulocyttemacrophage colony-stimulating factor (GM-CSF) to stimulate and antitumor immune response while lacking ICP47 for enhanced MHC-I presentation and ICP34.5 to eliminate neurotropism(Andtbacka et al., 2015).
Several generations of attenuated, replication-competent HSV-1 have been engineered in our lab. First generation HSV-1 OncSyn (OS) carries a deletion in one of the two copies of γ1 34.5 gene, as well as adjacent sequences (One copy of α0, α4); it also carries a syncytial mutation within the UL27 gene encoding gB with another deletion in one of the two genomic regions coding for the latency associated transcripts (LAT) (Israyelyan et al., 2007). OS is extensively fusogenic compared to wild type (F strain) and has shown effective antitumor activity in human breast tumor xenograft model in mouse (Israyelyan et al., 2007). Second generation OSV was constructed by deleting the OS viral host shutoff gene (vhs; UL41) to further attenuate OS virus and to induce antigen presenting cell (APC, dendritic cells) activation to improve immunogenicity of OS. HSV-1 vhs-UL41 gene has multiple roles in suppressing immune response such as degrading viral and host mRNA to reduce the antigen presentation (Becker, Tavor, Asher, Berkowitz, & Moyal, 1993; Kwong & Frenkel, 1987; Pasieka et al., 2008; Sorenson, Hart, & Ross, 1991; Zelus, Stewart, & Ross, 1996), limiting major histocompatibility complex I (MHC-I) antigen presentation and MHC-II expression (Smiley, 2004; Trgovcich, Johnson, & Roizman, 2002) and suppressing the production of cytokines and chemokine, inhibiting dendritic cell functions (Cotter et al.; Smiley, 2004). Therefore, deletion of vhs would trigger immune response against the infected cells acting as an in-situ vaccine and also significantly reduces neurovirulence (Pasieka et al., 2008; Smith, Morrison, & Leib, 2002; Strelow & Leib, 1995). OSV was genetically modified to constitutively express 15-prostaglandin dehydrogenase (15-PGDH); this third generation oncolytic viral vector was named OSVP. Construction and characterization of OSVP was described before (Walker et al., 2011).

Up regulation of prostaglandin E2 (PGE2) in tumor cells has been shown to increase immune suppression (Harris, Padilla, Koumas, Ray, & Phipps, 2002), angiogenesis (Marrogi et
al., 2000) invasiveness (Li et al., 2008), inhibit apoptosis (Leone et al., 2007) and promote multidrug resistance (Liu, Qu, & Tao, 2009) (Figure 2-1). OSVP has been shown to promote tumor suppression by degrading PGE2 through constitutive expression of 15-PGDH which is the primary enzyme for degradation of PGE2 (Walker et al., 2011).

**Figure 2-1.** Schematic of PGE2 role in promoting tumor growth. PGE2 directly promotes proliferation, angiogenesis, metastasis shown with blue arrows and blocks apoptosis (Red T). PGE2 suppresses immune response by directly blocking activation of natural killer cells (NK), Gamma delta T cells (γδ T cells), and Dendritic cells (DC). By activating regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), PGE2 further inhibit T cell activity. Taxol-induced MDSC activation, apoptosis and inhibition of cell proliferation is also shown with purple arrows. Green T represents OSVP’s ability to degrade PGE2.

### 2.2 Herpes Simplex Virus Lifecycle

The herpes virus lifecycle consists of a lytic phase and a latent phase. The virus first infects the epithelial cell located in the mucosa, replicates, and causes epithelial cell death. HSV-
1 most frequently infects oral and ocular epithelial cells while HSV-2 infects the genital areas. HSV can progress to infect the peripheral nervous system, where it establishes lifelong latency in neurons.

In order to enter the host cells, viral surface glycoproteins bind to the host cellular surface receptors such as Heparan sulfate is a glycosaminoglycan (GAG), herpesvirus entry mediator (HVEM), and nectin-1 and nectin-2. Upon entry, virus uses the cellular machinery to reproduce progenies. These new virion particles spread to adjacent uninfected cells as a result of a burst in the infected cells or through virus induced cell to cell (infected and uninfected cells) fusion, which allows the infection to spread without releasing the virions to extracellular space to avoid the immune system. The entire process can be divided into four major steps as entry, viral DNA replication, capsid assembly and virion egress.

2.2.1 Virus Attachment and Entry

Herpesvirus entry is facilitated by glycoproteins on the surface of the viral envelope, which can bind to diverse host cellular receptors. Members of the herpes family evolved to infect different host species and diverse cell types. Therefore, virus tropism and pathology is critically affected by the ability of the virus to attach and enter the cell. HSV-1 entry is very complex and all the strategies, proteins and pathways that the virus utilizes to gain access to a host cell are still not fully understood. Most commonly agreed upon mechanism is Herpes virus binds to heparan sulfate (HS) moieties on cell surfaces using viral glycoproteins gC and gB (Herold, WuDunn, Soltys, & Spear, 1991). Moreover, viral glycoprotein D (gD) binds to different cellular receptors including the herpesvirus entry mediator (HVEM, or HveA), nectin-1 (HveC), 3-O-sulfated HS (Geraghty, Krummenacher, Cohen, Eisenberg, & Spear, 1998; Shukla et al., 1999). After binding
and fusion of virus envelope with the plasma membrane of the cell, HSV releases the capsid-tegument complex into the cytoplasm of the infected cell (Figure 2-1).

2.2.2 Virion Transport to the Nucleus

Following the fusion of virus envelope and cellular plasma membrane, virus tegument and capsid is released into the cytoplasm of the infected cell. Some of the tegument proteins disassociate and remain in the cytoplasm, while others, such as VP16 (aTIF) are transported to the nucleus to pursue their functions. Viral capsids along with the remaining tegument proteins are transported to the nucleus via cellular microtubule network using dynein and actin motor transport system (Figure 2-2). Capsids accumulate at the nuclear envelope and become associated with nuclear pore complexes (NPC) (Ojala, Sodeik, Ebersold, Kutay, & Helenius, 2000). The capsid itself can’t go through the NPC, thus binding to the nuclear pore complex produces a structural change in the HSV capsid, which leads to release of viral DNA into the nucleus (Ojala et al., 2000; Shahin et al., 2006; Sodeik, Ebersold, & Helenius, 1997).

2.2.3 Viral DNA Replication

Herpesviruses show diverse tissue tropism and distinct host cell interaction, but they have one common feature and that is their DNA replication during the lytic infection. Linear DNA of the initial infecting HSV virus is circularized upon entry into the host cell and linear concatemers of tandemly repeated viral genomes are generated via a theta structure or rolling circle mechanism of replication. However, at this stage B genes are already expressed and translated (Quinlan, Chen, & Knipe, 1984).

There are seven HSV proteins that are essential for viral DNA replication in cell culture, viral DNA polymerase (UL30), its accessory protein (UL42), an origin-binding protein (UL9), the single stranded DNA binding protein (ICP8), and the helicase-primase complex that consists
of three proteins: UL5, UL8, and UL52 (Weller & Coen, 2012). Together, these proteins are called replisome. The replisome is loaded on origins of replication by an initiator protein OBP/UL9. Host cell factors such as DNA polymerase, DNA ligase, and topoisomerase II are required in viral DNA synthesis.

![Image: HSV-1 Intracellular Transport](image)

Figure 2-2. Schematic representation of intracellular transport of virion capsids via the cellular microtubular network. The virus enters the cell via fusion or endocytosis and is transported by dynein motors toward the nucleus (retrograde transport), presumably by the interaction of dynein with one or more inner tegument proteins. Kinesin transports the cargo toward the cell membrane (anterograde transport).

HSV DNA replication follows circularization of the DNA in the host nucleus and after IE and E genes are expressed. HSV-1 DNA replication takes place in nuclear foci, which later turn into larger replication compartments (Livingston, DeLuca, Wilkinson, & Weller, 2008). Large number of host cellular proteins localize to replication compartments, which needs active DNA
synthesis to form. Through an unknown mechanism, replication switches from theta form to the rolling circle form of replication. The rolling circle replication forms long head-to-tail concatamers of viral DNA, which become cleaved into individual units during packaging of viral DNA into capsids (B. Roizman & Knipe, 2001).

2.2.4 Capsid Assembly and Packaging

All herpesviruses execute a precise multistep process to assemble capsids which are complicated two-shelled structures with single portals to take up and package viral DNA. Capsids consists of 11 different protein organized in a precise manner as described before (Booy et al., 1991; Zhou, Prasad, Jakana, Rixon, & Chiu, 1994). HSV-1 gamma proteins including capsid proteins are transcribed after DNA replication and the proteins are made in the cytoplasm of the infected cell. The major capsid protein (VP5) is transported into the nucleus along with the triplexes of a scaffolding protein pre-VP22a; VP21, another scaffolding protein, and VP24, a protease that cleaves the major scaffold protein are also transported into the nucleus (B. Roizman, D. M. Knipe and R. Whitley, 2007). two copies of VP19C and one VP23 are transported into the nucleus as triplex already assembled in the cytoplasm(Nicholson et al., 1994). In the nucleus, capsid and scaffolding proteins localize at the replication compartments to complete capsid assembly. Initially, VP5 and pre-VP22 self-assemble into a complex and pentons, hexons and triplexes are brought to form sphere-like procapsids (Trus et al., 1996). Viral DNA is loaded into the procapsids by a three-part enzyme which is the product of the UL28 gene and is called terminase. This enzyme recognizes and cleaves genomic ends in the replicated viral DNA concatamers, accumulated within the nucleus, into single unit monomers. The cleaved genomes are
then inserted into the capsid using the ATPase-driven motor activity of the terminase. During the DNA packaging process, VP24 protease activity is triggered and scaffold is removed. As a result, capsids transform from a fragile and roughly spherical structure to an icosahedral and stable form (Newcomb et al., 1996). There are three types of capsids A, B, and C. They are approximately 120 nm in diameter and share an outer shell made up of hexons and pentons which are linked by the triplex of minor capsid proteins VP19C and VP23 as described before. A-capsids do not contain viral DNA and lack scaffolding proteins and are thought to be an aborted attempt at DNA packaging(Sherman & Bachenheimer, 1987). B-capsids are similar to A-capsids and do not contain viral DNA, but are filled with VP22a and VP21, the cleaved scaffolding proteins, and a viral protease VP24 (Gibson & Roizman, 1972; Newcomb et al., 1993). C-capsids, on the other hand, are mature capsids that contain viral DNA and can proceed to become infectious viruses after egress (Perdue, Cohen, Randall, & O'Callaghan, 1976).

2.2.5 Herpesvirus Egress

To be able to form infectious particles, virus needs to acquire tegument and envelope proteins while exiting the infected cell. Two models have been proposed regarding herpes virus egressone is known as married model and the other one is the separate model. According to the married model, HSV virions acquire tegument and envelope proteins as it buds out of the nuclear membrane and the mature enveloped virus uses secretory pathways to exit the infected cell. The separated model, on the other hand, claims that virions go through primary envelopement and de-envelopement as they move through the nuclear membrane, and the final tegumentation and envelopment occurs in the trans-golgi network (B. Roizman, D. M. Knipe and R. Whitley, 2007).

Both models share the first step that mature nucleocapsids acquire primary envelope via budding through the inner nuclear membrane into the peri-nuclear space. UL31, a nuclear
phosphoprotein embedded in the nuclear membrane of infected cells, and UL34, C-terminally anchored membrane protein present in the inner and outer nuclear membrane, have been shown to be involved in the budding process (Klupp, Granzow, & Mettenleiter, 2000). The viral proteins of UL31 and UL34 can bind, reorganize and destabilize lamin A/C, which is the primary component of the nuclear lamina, and modify the conformation of the nuclear lamina in infected cells (Reynolds, Liang, & Baines, 2004). The two models diverge after this point. In the married model, the virion is considered complete at this point and preserves its envelope and tegument and exits the cell via the secretory pathways, although slight modifications in viral proteins can happen. In the separate model, the enveloped virion goes through deenvelopment and loses some, or all, of its tegument as it buds out of the outer nuclear envelope. Deenveloped virion enters the cytoplasm, where it re-acquires the tegument, mainly through the trans-Golgi network (TGN) (B. Roizman, D. M. Knipe and R. Whitley, 2007). This network is a collection of Golgi-derived endosomes. The final stage of production of an infectious particle is the acquisition of a mature virion envelope, which is believed to happen in the TGN which is not well characterized.

2.2.6 Latent Infection

Herpes viruses have the ability to establish latent infection in cell type different than the primary infected tissue type. The signals necessary to push the virus into latency or reactivate the virus are not fully known, although the phenomenon itself is well characterized clinically. During the initial infection of HSV-1, which generally takes place in mucocutaneous epithelial tissue, virus enters sensory neurons via their termini and infects the neuronal nuclei in the sensory ganglia that innervate the initially infected tissues via retrograde transport. In ganglionic neurons, HSV replicates for a very short time after the infection, but few days later the virus cannot be detected anymore. This is followed by periodic reactivations and subsequent
reoccurrence of clinical symptoms at the tissues of initial infection. This reactivation is mostly thought to be initiated by stimuli such as stress at the site of initial infection or the infected neurons or systemic tension. There are three phases of latency that have been reactivation: establishment, maintenance, and reactivation. Establishment occurs during the period following primary infection. During this phase viral replication is detectable in neurons, but the productive lytic infection leading to cell death is blocked in order to keep the the neurons that are destined to become latently infected alive. The maintenance phase of latency is characterized by life-long retention of viral genome in the host-cell nucleus in a circular episome form, associated with the nucleosomes and near total silencing of gene expression. One region, encoding the latency-associated transcripts (LATs), remains active during latency. Reactivation generally occurs following some type of stress in the neurons or tissue initially infected with the virus. These stress signals include UV radiation, intense heat, or trauma, or a systemic stress especially in immunocompromised individuals (Preston CM, 2007).

2.3 Taxanes

Taxanes are a family of naturally occurring complex diterpene alkaloids that exist in the yew tree. This class of compounds has been known for their toxicity since 1856, when a Lucas, a german pharmacist, extracted a mixture of taxanes. He named the mixture of these compounds taxane as they were obtained genus Taxus. Physical properties of the compounds were inconsistent and lack of modern structural biology techniques and the complexity of the structures halted the characterization of taxines (Zadina & Karmazin, 1950); Later it was shown to be a mixture of at least seven different compounds (Graf & Bertholdt, 1957). It was not until 1962 that taxane nucleus structure was established to be esterified tricyclic polyalcohols (Uyeo, Ueda, Yamamoto, Hazama, & Maki, 1962; Uyeo, Ueda, Yamamoto, & Maki, 1964). In late
The 1960s taxane family became larger by discovering more of non-alkaloidal products of yew tree such as baccatin (Chan & Monteath, 1966). To date more than 300 different taxanes have been isolated and characterized (Mendoza, Ishihara, & Baran, 2011), the most famous of which is Taxol (paclitaxel), a billion-dollar anticancer drug.

2.3.1 Discovery of Taxol

Dr. Jonathan L. Hartwell (1906-1991) was a scientist at the National Cancer Institute who had a vision to explore the plant kingdom for potential anticancer compounds. He extracting podophyllotoxin and several other compounds known as lignans from *Podophyllum peltatum* also known as American mayapple, which ultimately lead to development of chemotherapy drugs. His pioneering work also lead to a contract between USDA and NCI to collect and screen plant materials for potential new anticancer compounds. Over twenty years, samples from about 35,000 different higher plant species were screened for anticancer activity. Scientists could establish reproducible anticancer activity in less than 10% of all these natural derivatives, and only a few were chosen for clinical trials. Among those were compounds from mayapple and yew. Arthur Barkley was USDA botanist at the time, he collected *Taxus brevifolia* in the Gifford Pinchot National Forest in the State of Washington in 1962. Two years later, extracts from the bark of this yew tree were found to be cytotoxic in KB cells, a subline of the ubiquitous KERATIN-forming tumor cell line HeLa. Samples were then sent to Monroe Wall at the Research Triangle Institute (RTI) to isolate and purify the compound which was cytotoxic to KB cells. In 1966 taxol was isolated and a year after it was publicized at the American Chemical Society National Meeting by the Wall group (M. C. Wani, Taylor, Wall, Coggon, & McPhail, 1971). One of the first in vivo trial of taxol was in 1974 on B16 mouse as the human melanoma solid tumor model which was not available before 1970s. Paclitaxel showed significant and
reproducible activity against the melanoma tumor, with a substantial increase in life
span (Suffness, 1995). The structure was discovered by mass spectrometry, X-ray
crystallography, and NMR spectroscopy and was published 1971 by Wall and Wani (M. C. T.
taxol were large, the production was not sustainable; yield from the bark of the yew tree was
about 0.01%, the wood and the needles yielded much less. NCI was unwilling in pursuing taxol
due to the facts that the tree, T. brevifolia was not widespread; only about two kg of bark could
be obtained from each tree and at the time every 12 kg of dried bark yielded only five hundred
mg of taxol. Besides, bark is a limited resource, because stripping the bark of kills the tree.
Matthew Suffness significantly contributed to present taxol as a potential antitumor drug for
development to the NCI Decision Network Committee and paclitaxel was approved as a
development candidate in 1977; following its excellent activity against the then new MX-1 and
CX-1 mammary and colon xenografts in nude mice (Suffness, 1995). In 1979 Susan Horwitz
discovered taxol’s mechanism of action which made taxol the first compound which was able to
promote and stabilize microtubule assembly (Schiff, Fant, & Horwitz, 1979). In the same year
Pierre Potier at Institut des Chimie des Substances Naturelles (ICSN) in France started purifying
taxol from European yew (Taxus baccata). In 1982 toxicological studies on taxol were
completed and NCI approved taxol for Investigational New Drug Application (INDA). Food and
Drug Administration (FDA) the INDA in 1984; taxol entered phase I clinical trials followed by
phase II clinical trials the year after. Two incidents of death due to the hypersensitivity produced
by the surfactant and not the compound itself halted the trials, until in 1987 Wiernik et al. found
an infusion protocol which avoided these hypersensitivity reactions (Wiernik et al., 1987). Over
the next four years phase II clinical trials showed strong evidence of taxol anticancer activity
against melanoma reported in 1987 (Wiernik et al., 1987), in ovarian cancer in 1989 (McGuire et al., 1989) and in breast cancer in 1991 (Holmes et al., 1991). In 1992 Bristol-Myers Squibb marketed the drug as TAXOL® and since it has been used for treatment of refractory ovarian cancer (McGuire et al., 1989), refractory breast cancer and lung cancers and as the second-line treatment of AIDS-related Kaposi's sarcoma, as well as for nonsmall cell lung cancer in combination with Cisplatin (Ramalingam & Belani, 2004) and for advanced breast cancer after failure of combination chemotherapy for metastatic disease. (Holmes et al., 1991; Saville et al., 1995). Paclitaxel is now produced commercially by plant tissue culture technology (Leistner, 2005). This method was developed by German and Canadian biotechnology company phyton Biotech, Inc. In this method taxol is directly purified from a Taxus cell line propagated in aqueous medium. The purification is by chromatography and the compound is isolated by crystallization which eliminates usage of toxic chemicals and surfactants that are used in the semisynthetic production.

Producing Taxol from an excusable source made the urge to find synthetic and semisynthetic processes to produce the drug. In 1986 semisynthetic analog of taxol was developed in France by Potier group (Guenard, 1993; Gueritte-Voegelein, 1986) which is derived from the needles of European yew which makes it a sustainable product. In this semisynthesis the more readily available 10-deacetylbaccatin III is processed into taxol analog Taxotere licenced by Sanofi- Aventis also known as docetaxel with an excellent yield that ended the supply crisis of taxol (Holton, 1995). Docetaxel is approved by FDA for breast, head and neck, prostate and gastric carcinomas (Pazdur, 2011); it is under clinical trials for other types of cancers.
2.3.2 Microtubules

Microtubules are a major component of the cellular structure and tubulin is the principle component of microtubules. Found in almost all types of eukaryotic cell, microtubules are essential in cell division by forming the mitotic spindles; provide platforms for intracellular transport of cellular organelles and vesicles as well as other cellular processes. Microtubules are highly dynamic structures due to polymerization and depolymerization of tubulin heterodimers; they are about 25 nm in diameter and can grow as long as 25 µm in length. Tubulins exist as α - β heterodimers arranging in a polar fashion to form cylindrical polymers along the longitudinal protofilaments that makeup a microtubule. Every 13 tubulin dimers form a ring which is one turn of an imperfect helix formed by association of the protofilaments.

Polar orientation of tubulins gives microtubules a distinct polarity, which is essential for their biological functions. The α subunit of each heterodimer binds to the β subunit of the next tubulin dimer. Thus, different ends of the same protofilament will have either α or β subunits exposed labeled as (-) or (+) ends respectively. Microtubule elongation happens mostly from the (+) end.

A guanine nucleotide-binding site is located at one end of each monomer subunit, situated in a globular amino-terminal domain with a Rossmann fold, which is a protein structural motif found in proteins that bind nucleotides. During polymerization, both the α- and β-subunits of the tubulin dimer are bound to a molecule of GTP. GTP molecule bound to α subunit is trapped between α and β subunits of a dimer, while the GTP bound to the β subunit can be hydrolysed to GDP soon after assembly that makes the structure unstable. Addition of the next dimer with a GTP bound to it’s β end can protect the structure. Since the tubulins add onto the (+) end can play the role of a cap and protect the previous dimers with a GDP on top. Although
GDP-bound tubulins are prone to depolymerization, as long as they are not in the tip of the microtubules they will not dissociate; this process contributes to microtubule elongation and is called “rescue”. When hydrolysis happens at the very tip of the microtubule it causes a chain of depolymerization in the structure as the GDP-bound tubulins are not protected anymore. The rapid disassembly of microtubule is called shrinkage or catastrophe.

It was shown through photoaffinity crosslinking studies that [3H]-3’-(p-azidobenzamido) taxol photolabels the N-terminal 31 amino acids of β-tubulin (S. Rao et al., 1994), 3H-2-(m-azidobenzoyl) taxol photolabels a peptide containing amino acid residues 217–233 of β-tubulin (S. Rao, Orr, Chaudhary, Kingston, & Horwitz, 1995) and 3H-7-(benzoyldihydrocinnamoyl) photolabels amino acid residues 277–293 and more specifically it cross-links to Arg282 in β-tubulin (Srinivasa Rao et al., 1999). Electron crystallography of zinc-induced tubulin sheets has also shown a 3.7-Å resolution structure of αβ- tubulin-taxol complex (Nogales, Wolf, & Downing, 1998). Nogales tubulin map showed GTP attached to α-tubulin while GDP and paclitaxel bound to β-tubulin at S9–S10 loop in β tubulin. Paclitaxel binds to the second globular domain of β-tubulin, on the other side of the core helix from the GTPase domain (Amos & Lowe, 1999). On the other hand research on taxol resistant cell lines also revealed certain amino acid mutations in β-tubulin that are thought to be involved with taxol binding (Giannakakou et al., 1997; Gonzalez-Garay, Chang, Blade, Menick, & Cabral, 1999).

It was shown by Nogales et al. that taxol Paclitaxel binds to the inner surface facing the central hole of microtubules. Years after discovering taxol binding site still computer modeling as well as experimental researches have been conducted to elucidate the kinetics of taxol binding and to find alternative binding sites on microtubules. The accepted model held that taxanes and other microtubule-stabilizing agents (MSAs) would reach their binding pocket in the
lumen of microtubules by diffusing through the fenestrations present on the microtubule wall (Diaz, Barasoain, & Andreu, 2003; Magnani, Maccari, Andreu, Diaz, & Botta, 2009). Moreover, computational analysis has showed that taxol may initially binds to the outer surface and diffuse through the pocket which is formed due to the conformational changes after the initial binding to reach to the inner lumen of microtubules (Magnani et al., 2009).

Apart from microtubules BCL-2 protein is another major binding target for taxol in the cell which was first discovered in 1998 (Fang et al., 1998; Rodi et al., 1999). It was shown that taxol binds to a ∼60-amino acid “loop” domain of Bcl-2 that contains phosphorylation sites that is known to negatively regulate its anti-apoptotic function. It in turn suggests that the apoptotic action of paclitaxel may involve the binding of paclitaxel to Bcl-2 (Cristiano Ferlini et al., 2009; Rodi et al., 1999). It is also mentioned that the binding site on BCL-2 protein is very similar to the one on the β-tubulin (Cristiano Ferlini et al., 2009).

2.3.3 Mechanism Of Action

Taxol has been known as a spindle poison, but the mechanism was not well understood. It was initially thought that its anticancer activity is due to the microtubule destabilization (Fuchs & Johnson, 1978) disassembling and preventing the formation of microtubules, like other anticancer drugs that were known to prevent cell division. In 1979, Horwitz et al. showed that the mechanism of action of taxol was in fact different and unique. Their research showed that taxol bound to polymerized tubulin promoted microtubule formation and prevented disassembly; they also showed that taxol inhibited mitosis which subsequently lead to cell death thus reducing cancer cell growth (Horwitz, 1979).

All dividing cells including cancer cells replicate and divide through a series of coordinated events that compose a “cell division cycle” that can be divided into different
transition periods known as phases or stages of cell cycle; various processes occur during each phase pushing the cell cycle forward leading to replication of genetic materials and cell division. Duplicate DNA precisely and segregate two identical copies and pass it to two daughter cells are the most basic and vital functions of a cell. These processes explain the two major phases of the cell cycle. DNA replication happens during S phase and chromosome segregation and cytokinesis occur during M phase. M phase consists of several different events, it begins with mitosis or nuclear division which in turn starts with chromosome condensation then the nuclear envelope breaks down followed by mitotic spindle microtubules attachment to the sister chromatids. Chromosomes align at the equator of the mitotic spindle which is a signal for segregation this is called metaphase and it is followed by anaphase, during which the sister chromatids are pulled towards opposite poles of the spindle and decondensate to form nuclei of the new daughter cells. Cytokinesis or cytoplasmic division completes cell division. S and M phases take half of the cell cycle time in a normal mammalian cell while M phase occupies much less time than the S phase.

Cells need extra time to grow and synthesize proteins to make organelles and the machinery to be able to replicate their own DNA and divide. Thus, gap phases are present in cell cycle; G1 between M phase and S phase and G2 between S and mitosis. Different phases of cell cycle for G1 phase is more than just a gap for cellular growth, during G1 cells monitor the internal and external environment to ensure that it’s suitable for them to replicate. This can take some time depending on the external conditions and signals that the cell receives, if conditions are not appropriate cells postpone G1 and enter a resting state or a nonproliferative state also known as G0 which can take days, weeks, or even years.
Different cell types divide at different rates, for example replication cycle for human stem cells is about 12 hours, human skin cells is about a day, human sperm takes about 64 days, liver cells and skeletal muscle cells take almost a year and neurons when differentiated don’t divide anymore. Generally at any given time out of approximately $10^{13}$ cells in the human body, there are about 25 million cell divisions occurring; and, the majority of cells reside in nonproliferation state called quiescence (G0)(Alberts, 2002; Pardee, 1989). Normal cells leap back to G1 and continue the cell cycle in case of receiving appropriate extracellular signal molecules, produced by other cells, which are known as growth factors and mitogens such as tissue specific growth factors as well as the internal regulations and controls in the dividing cells.

Two types of cell cycle control mechanisms are recognized: a cascade of protein phosphorylation that passes on a cell from one stage to the next and checkpoints that monitor completion of critical processes(Collins, 1997); in some instances checkpoints delay the progression to the next stage if the event is not completed and if necessary the cell goes through repair or programmed death also known as apoptosis. Cancer cells on the other hand are deregulated and lack the control mechanisms and their checkpoints are disrupted, therefore they can replicate in circumstances that lead to quiescence in normal cells such as the absence of growth signals. Due to the uncontrolled cell proliferation, cancer cells have a higher ratio of cells actively dividing compare to normal cells. Paclitaxel and docetaxel main mechanism of action lies in microtubules that are key components in formation of mitotic spindles which are essential in cell division, intracellular transport, transcription, post-transcriptional modification and etc. (Escuin, Kline, & Giannakakou, 2005; Nogales, 2000; Y. Wang, O'Brate, Zhou, & Giannakakou, 2005). As the cancer cells replicate with a faster pace, adverse effects of microtubule disruption are more featured in these cells. It was thought that both drugs worked by excessive
polymerization or immature depolymerization of microtubules (Calderoni & Cerny, 2001; Fuchs & Johnson, 1978) but later it was shown that taxol binds to B-tubulin and stabilizes microtubules by increasing the rate of nucleation, growth and elongation phases of polymerization (Derry, Wilson, & Jordan, 1995; Derry, Wilson, Khan, Luduena, & Jordan, 1997; S. Rao et al., 1995; Yvon, Wadsworth, & Jordan, 1999). Images of healthy mitotic cells and taxol treated cells with multiple nucleation are shown in Figure 2-3.

![Figure 2-3. Multinucleation of taxol treated cells. Fluorescent confocal microscopy images. A. Normal untreated mitotic RM9 cells. B. RM9 cells treated with 1 µM taxol for 6-8 hours. Microtubules are labeled green and nucleus is blue/cyan.](image)

The correlation between microtubule stabilization, property of taxol, and the mechanism of apoptosis in cancer cells is not certainly clear but it has been shown that taxol can also bind to bcl-2 protein and therefore facilitating the initiation of apoptosis. As mentioned before treatment with paclitaxel blocks the cell cycle at the transition from the metaphase to the anaphase and then activates the intrinsic mitochondrial apoptotic pathway, as shown by the reduction in the mitochondrial membrane potential, followed by the opening of the permeability transition pore channel (PTPC). The subsequent release of proapoptotic factors, such as cytochrome c and apoptosis-inducing factor, leads to the activation of effector caspases and ultimately to apoptosis (Bhalla, 2003). However, the exact molecular pathways connecting interference with
microtubule dynamics to the activation of cell death remain unclear. (Fang et al., 1998; Cristiano Ferlini et al., 2009)

2.3.4 High Vs. Low Concentrations Of Taxol

Intracellular concentration of taxol is dependent on the ability of the cell line to uptake and accumulate the drug but in general very low doses of taxol, less than the IC50 of a 3- to 4-day growth-inhibition assay, do not induce prolonged mitotic arrest but may it may inhibit cell growth; low doses of taxol (10 -300 nM) causes mitotic arrest and lead to programmed cell death; while, high concentrations (3 –100 µM) induce mitotic arrest as well as other immediate effects that are not related to the cell cycle block such as lipopolysaccharide (LPS)-like activity, tumor necrosis factor (TNF) activation and tyrosine kinase–dependent signal transduction activation (Mikhail V. Blagosklonny & Tito Fojo, 1999). lipopolysaccharide (LPS)-like activity includes effects of taxol which are similar to effects of LPS such as TNF-α receptors and TNF-α release (A. H. Ding, Porteu, Sanchez, & Nathan, 1990) IL-1α, IL-1β and IL-8 activation (Burkhart, Berman, Swindell, & Horwitz, 1994; A. Ding, Sanchez, & Nathan, 1993; Lanni, Lowe, Licitra, Liu, & Jacks, 1997; Lee et al., 1996; Manthey, Brandes, Perera, & Vogel, 1992). Taxol also activates AP-1 and NF-κB transcription factors as well as increasing tyrosine phosphorylation of MAP kinase which induces nitric oxide synthase (Das, Rao, & Kashinatham, 1998) production; it also activates protein kinase C and increases manganese superoxide dismutase gene expression (Bhalla, 2003; Derry et al., 1997; Lanni et al., 1997; Manthey et al., 1992; Y. Wang et al., 2005; Wolfson, Yang, & Horwitz, 1997).

Paclitaxel stimulates expression of “early-response” genes, including transcription factors with tumor-suppressor activities (Moos & Fitzpatrick, 1998). For the latter, it has been concluded that the induction was independent of microtubule stabilization because it occurred at 10µM
paclitaxel, a much higher concentration than that necessary for microtubule stabilization (Moos & Fitzpatrick, 1998). Like many other investigators of very high doses of paclitaxel, they suggest that these tubulin-independent effects explain the clinical efficacy of paclitaxel. However, effects observed at high concentrations must be interpreted cautiously since they are not clinically relevant. Furthermore, tubulin independence should be proved since some paclitaxel effects, even at low doses, that initially seemed to be tubulin-independent have been shown to be tubulin-dependent (M. V. Blagosklonny & T. Fojo, 1999). Overall, the specific mechanism of cell killing by taxol has not yet been discovered.

Numerous results clearly indicate that paclitaxel, Vinca alkaloids, colcemid and nocodazole block progression of mitosis. Furthermore, regardless of the binding site, microtubule drugs that depolymerize microtubules at high concentrations have been shown to stabilize microtubule dynamics at low concentrations, with little accompanying microtubule depolymerization (Jordan & Wilson, 1998). Thus, it has been proposed that the common cytotoxic mechanism of paclitaxel and the Vinca alkaloids is kinetic stabilization of spindle microtubule dynamics rather than depolymerization or excessive polymerization of the microtubules. Other studies also indicate that a treatment with a variety of agents active on microtubules can lead to phosphorylation of Bcl-2, Bcl-xL and other proteins (C. Ferlini et al., 2009).

2.4 ChemoViroTherapy

As mentioned in section 2.2, a wide variety of naturally occurring or genetically engineered oncolytic viruses have been investigated for the treatment of cancer. Considering the fact that immunotherapy as one of the main advantages of oncolytic viruses and that these viruses are attenuated for safe treatment, this approach has shown to be less aggressive and more
time dependent compared to the abrasive chemotherapeutics. Thus, successful clinical trials with OVs in rapidly growing tumors have not been reported. While combination therapy with OVs and chemo can stabilize the patient quickly and give the OVs time to produce the desired immunotherapeutic effects. Although, there are examples of successful clinical trial of OVs on single patients large-scale clinical trials of OVs and OVs in combination with chemo are scarce. While there are a few OVs approved outside of the US, as mentioned before, T-Vec, a HSV-1 based OV, is the first and only FDA approved virotherapeutic agent. There are currently many clinical trials to test efficacy and safety of OVs, and a few investigating the combination with other oncotherapeutic agents. Examples include clinical trials of coxsackievirus (Cavatak) and low-dose mitomycin C for bladder carcinoma(Annels et al., 2015), human reovirus (REOLYSIN) and histone deacetylase inhibitors AR-42 or SAHA for head and neck squamous cell carcinoma(Jaime-Ramirez et al.), Toca 511, a nonlytic retrovirus, and 5-Fluorocytosine for recurrent Glioma(Strebe, Lubin, & Kuo, 2016), adenovirus (LOAd703) and nab-Paclitaxel for pancreatic cancer(Rosewell Shaw & Suzuki, 2016).

Numerous *in vitro* and *in vivo* studies have investigated effect of chemo agents in combination with various OVs. To mention a few that included taxol: in 2008 HSV-based OVs (G207 and NV1023) were tested in conjugation with paclitaxel and doxorubicin in the human anaplastic thyroid cancer (ATC) cell lines KAT4 and DRO90-1(Lin et al., 2008). All four agents alone showed dose-response cytotoxicity *in vitro*, but only G207 and taxol combination showed synergistic cell killing. The combination showed successful results in decreasing tumor size *in vivo* as well (Lin et al., 2008). In this study, G207 up to MOI 8 and 32 nM taxol was used in vitro. In another study taxol was used in combination with an oncolytic Rhabdovirus Maraba-MG1 in breast cancer cells as well as in mice. In this study, the results showed enhanced viral
replication both *in vitro* and *in vivo*; according to this study taxol sensitized cells to virus infection by blocking secretion of antiviral factors like IFNβ by infected cells (Bourgeois-Daigneault et al., 2016). In this study, taxol concentrations that were used ranged between 0.5 to 12 µM and virus MOI ranged from 0.01 to 10. In this experiment, 2 µM was selected as the taxol concentration that caused polynucleation in diverse cell lines but did not cause high rates of cell killing in 4 hours. Overall, it is clear that chemotherapy can increase the efficacy of the OV therapy and taxol has shown to be a good candidate for such combination. Although the mechanism of taxol-mediated cell death is still not fully understood it can augment OVs cytotoxicity.
3. METHODS AND MATERIALS

3.1 Cell Lines

African green monkey kidney (Vero) cells, mouse mammary tumor cells (4T1) and murine prostate cancer cell line (RM9) were obtained from the American Type Culture Collection (Manassas, VA) (Rockville, MD, USA). Vero cells and RM9 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad CA), supplemented with 10% fetal calf serum (FCS) and antibiotics. 4T1 cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad CA), supplemented with 10% FCS. The cultures were maintained at 37°C, and 5% CO2.

3.2 Chemotherapeutic Compound

Paclitaxel and Oregon Green® 488 Conjugate (Oregon Green® 488 Taxol, Flutax-2) Paclitaxel was obtained from Invitrogen. Paclitaxel was diluted in DMSO (tissue culture grade; Sigma) and added to media for a final concentration of < 0.1% (v/v) for in vitro studies.

3.3 Virus Entry

Confluent monolayers of 4T1 cells were pre-incubated with Taxol (0, 10, and 100 nM) for 12 and 24 hours. Cells were then infected with OSVP at a multiplicity of infection (MOI) of 0.5 for 1 hour at room temperature. The virus inoculum was subsequently removed, and the cultures were transferred to 37°C. Infected cells were fixed 8 to 12 hours post infection (hpi) and stained with anti-ICP4 mouse primary antibody (Virusys, Inc., Taneytown, MD) and Alexa Fluor 488 goat anti-mouse IgG1(Life Technologies, Grand Island, NY ) secondary antibody for flow cytometry analysis (BD Accuri C6 flow cytometer). The relative efficiency of virus entry was measured as the percentage of cells expressing ICP4 antigen in a population of > 15,000 cells.
Mean values and standard deviations of three independent experiments were calculated and represented as bar graphs.

3.4 Virus Titration and Replication Kinetics

Characterization of OSVP in Presence of Taxol was performed as follows. $2 \times 10^4$ 4T1 cells were plated in 12-well plates overnight and infected with OSVP at a MOI of 0.5. Cells were pretreated with 1–100 nM paclitaxel for 8, 12 or 24 hours prior to infection. To determine the effect of Paclitaxel on the replication of the virus (OSVP) in 4T1 cells, at 6, 12, 24, 36 and 48 hpi cells were subjected to 3 repeated freeze-thaw cycles to release virus from the cells. Cell lysates were heavily vortexed and subsequently used to infect the Vero cells in triplicate wells. Virus suspension was diluted ten folds across wells of 12-well plates. 100ul of each concentration was added to 1ml of serum free DMEM supplemented with 25 mM Hepes to make a ten-fold dilution. Vero cells were incubated with 250 ul of each dilution on the rocker at room temperature for one hour. After 1 h of adsorption, methylcellulose overlay medium (DMEM containing 1.5% methylcellulose and 2% FBS) was added to the infected cell monolayers. Plates were incubated at 37°C for two days to allow for cytopathic effects to develop (until the plaques are easily distinguishable under the phase-contrast microscope) and then fixed with methanol. The cell monolayers were stained either with 0.1% crystal violet or visualized by immunohistochemistry using horseradish peroxidase-conjugated anti-HSV antibody (Dako, Carpinteria, CA) and the Novared substrate development kit (Vector Labs, Burlingame, CA). Images were captured using phase-contrast microscopy; plaques were counted for each dilution, and the number of infectious virus particles was estimated for each treatment group.
3.5 Plaque Morphology and Size Distribution

4T1 cells were incubated in RPMI medium supplemented with 10% FCS containing 10, 100, and 500 nM taxol for 6, 12, and 24 hours prior to OSVP infection at a MOI of 0.0001. After infection methylcellulose overlay medium was added to the infected cell monolayers to allow for cytopathic effects to develop and discreet plaques were formed. Cells were then fixed with methanol and viral plaques were stained with rabbit polyclonal anti-HSV primary antibody (Dako, Denmark) followed by Alexafluor 488 goat anti-rabbit IgG (H+L) secondary antibody (Life Technologies). Fluorescence images were captured using Olympus IX71 inverted microscope. In order to measure size of the plaques, DP2-BSW digital image processing software (Olympus) was used. Area of each plaque was estimated by measuring surface area of a polygon formed around the picture of each plaque. These numbers were then compared between treated and untreated groups.

3.6 Cell Cytotoxicity

Confluent monolayers of 4T1 and RM9 cells were prepared as follows. 10,000 RM9 cells/well and 20,000 4T1 cells/well were seeded in clear bottom, black wall 96 well plates. Cells were treated with taxol (0, 1nM, 10nM, 100nM, 1uM, 10 µM and 100 µM) and, or infected with OSVP (MOIs 0.1, 0.5, 1, 5); virus infected wells were continued to carry the inoculum without washing procedure. Untreated cells served as 100% live cell control and serum deprived cells grown in Phosphate Buffer Saline (PBS) were used as control for 100% dead cells. Wells carried approximately 100 µM of test compounds or vehicle controls.

Cell viability and cytotoxicity were assessed simultaneously by using MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent (Promega). 100uL of the reagent was added to each well and incubate for an hour. Unquenched fluorescent dye was measured by plate reader BRAND at
excitation ~400nm and emission ~505nm. Intensity of fluorescence in relative fluorescence units (RFU) was correlated with the live cell protease activity to compared number of viable cells in between groups. Cell cytotoxicity was calculated by measuring dead cell protease activity at excitation ~485nm and emission ~520nm in relative fluorescence units (RFU). MultiTox-Fluor assay is described briefly as follows:

The MultiTox-Fluor Assay simultaneously measures two protease activities: one is a marker of cell viability, and the other is a marker of cytotoxicity. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate (glycyl-phenylalanylamino fluorocoumarin; GF-AFC). The substrate enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic, cell-impermeant peptide substrate (bisalanyl- alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Because bis-AAF-R110 is not cell-permeant, essentially no signal from this substrate is generated by intact viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously.

3.7 Flow Cytometric Analysis of the Cell Cycle

Confluent monolayers of RM9 cells were treated with taxol (100 nM), OSVP (MOI 0.1, 0.5 and 1) or their combination. At the indicated time points, cells were pelleted, fixed in ice-cold methanol for an hour and permeabilized with 0.5% Triton X-100 for 30 min. Fixed cells were washed twice in phosphate-buffered saline (PBS) containing bovine serum albumin (0.5%) and EDTA (20mM). Cells were resuspended in propidium iodide (100 ug/ml) and RNase (200 ug/ml) in PBS. Samples were analyzed on a FACS (BD Accuri C6 flow cytometer). Cells were labeled with goat polyclonal anti HSV-1 antibody conjugated with FITC (ab20437).

3.8 Immunofluorescence and Confocal Microscopy

Cells were seeded onto glass coverslips (Marienfeld laboratory glassware) at 5x104 cells/ml overnight and were treated with 100nM or 1uM taxol for 12 hrs, and then infected with
OSVP MOI ~1 for 4, 8 and 12 hours. Cells were then fixed with ice-cold methanol for an hour and permeabilized with 0.5% Triton X-100 for 30 min. After three washes with PBS, slides were incubated with polyclonal rabbit anti-herpes simplex virus type 1 primary antibody (Dako, Denmark) for thirty minutes for 30 minutes. Cells were washed three times with PBS and incubated with secondary alexa fluoro 594 goat anti-rabbit IgG (H+L) (Life Technologies) along with mouse anti-alpha-tubulin monoclonal FITC conjugate antibody (Life Technologies) for thirty minutes. Slides were mounted in ProLong Gold Antifade Reagent with DAPI (Life Technologies) and sealed. Confocal pictures were taken with Olympus Fluoview confocal system FV10i, and immunofluorescence pictures were taken with Axio Observer.Z1 (Carl Zeiss) using AxioVision digital image processing software.

3.9 Microtubule Mean Fluorescence Intensity

Confluent monolayers of RM9 cells were infected with OSVP at a MOI 1 and treated with 100 nmole/L taxol concurrently. 18 hpi cells were stained with mouse anti-alpha-tubulin monoclonal FITC conjugate antibody (Life Technologies) for thirty minutes. Amount of polymerized microtubules was determined by measuring mean fluorescent intensity (MFI) of anti-microtubule FITC antibody using BD Accuri C6 flow cytometer.

3.10 Taxol Intracellular Concentration

Confluent monolayers of RM9 cells were infected with OSVP or OSV at a MOI 1 and treated with 100 nM Oregan Green 488 conjugated paclitaxel (Life Technologies) 8 hpi. Control samples were incubated with 20 µM of Indomethacin. Fluorescent paclitaxel in RM9 cells was directly measured by FACS (BD Accuri C6 flow cytometer) at 16 hpi and reported as mean fluorescence intensity.
3.11 Statistical Analysis

Statistical Analysis Systems version 9.1.3 was used to analyze the data. Effects in the models included treatment, time, and their interaction. All comparisons were considered significant at $p \leq 0.05$. When significance was detected, Tukey’s or erronni’s posttest was used. In graphs mean values and the standard error of the mean (SEM) are shown.
4. RESULTS AND DISCUSSIONS

4.1 Cell Cytotoxicity Analysis

In order to establish the base-line cytotoxicity for each agent separately, cell survival in 4T1 and RM9 cell lines was observed for 24-48 hours after treatment. Cells were infected with OSVP (MOIs 0.1, 0.5, 1, 5) or treated with taxol (0, 1nM, 10nM, 100nM, 1µM, 10 µM and 100 µM). Cell survival is reported compared to untreated cells that served as 100% live cell control. Both 4T1 and RM9 cells showed dose-dependent cytotoxicity when treated with taxol and OSVP for 24-48 hours (Figure 4-1). The human breast and prostate cancer cell lines were both highly susceptible to OSVP infection (Figure 4-1A-C). In 24 hours MOI 5 reduced viable cells to less than 20% and 30% in RM9 and 4T1 cells and in 48 hour all cells were killed. Stabilization of microtubules by taxol initiates apoptosis in cells, which can be detected by measuring caspase activity.

In a previous study by Vassileva et al. caspase activation and survival in two human ovarian cancer cell lines were studied, and in the first 24 hours after treating with taxol minimum caspase activity was detected. In 48 hours, however, caspase pathway was activated and was inversely correlated with cell survival (Vassileva, Allen, & Piquette-Miller, 2008). Similarly, in the current work the cytotoxic effect of taxol was also more prominent after 48 hours in both cell lines. In comparison, 4T1 cells were slightly less sensitive to taxol, and OSVP. RM9 cells were particularly more sensitive to taxol treatment at higher doses (1-100 µM), as the cell survival rate at each concentration was lower than 4T1 cells by more than 15%. Moreover, the steepness of the curves show that RM9 cells were more susceptible to cell cytotoxicity induced by OSVP infection. Based on the individual curves EC50, concentration leading to 50% cell death, after 48 hours of treatments was calculated for each agent alone. The EC50 of taxol was 63.7 nM for 4T1
cells, and 87.5 nM for RM9 cells. EC50 of OSVP was measured based on the multiplicity of infection (MOI); EC50 values for OSVP infection in 4T1 and RM9 cells were 1.366 and 0.934 MOI, correspondingly.

4.2 Virus Growth and Replication

In a previous study, Yonezawa et al. showed that Ebola virus entry is susceptible to microtubule stabilizing and disrupting agents. Their observations pre-incubation with taxol (10-20 µM) enhanced ebola virus entry, whereas microtubule (MT) disrupting compounds (MTD), nocodazole and colchicine, significantly impaired it (Yonezawa, Cavrois, & Greene, 2005).
In 2017, Cockle et al. showed that an oncolytic strain of HSV inhibits brain tumor migration by altering cytoskeletal dynamics through stabilizing microtubules. Taxol was used in this study as an internal control and HSV induced MT stabilization was compared to 100 nM taxol (Cockle et al., 2017).

On the other hand, disruption of MT post-virus entry has been associated with higher levels of Adeno-associated virus (AAV) transduction. Interestingly, while using nocodazole (30 µM) to disrupt MTs at 8 hpi, co-treatment with taxol (10 µM) to stabilize the MTs could reverse the effects and lower the transduction levels. In the same study, AAV higher levels of transduction in cells with disrupted MTs are associated with increased trafficking to the nucleus via the RhoA-ROCK-Actin pathway (Xiao, Mitchell, Huang, Li, & Samulski, 2016).

In the current study, cells were pretreated with 1µM taxol for 6 hours and the confocal pictures were taken 4 hpi to investigate the presence of HSV-1 early proteins as a measure for virus entry. Virus entry into 4T1 cells was also measured using FACS as described in the materials and methods section. 4T1 cells were treated with taxol prior to infection or concurrently. Confocal microscopy pictures of HSV-1 infected 4T1 cells, pre-incubated with taxol for 6 hour, are shown 4 hpi (Figure 4-2A). The red color fluorescent shows the early proteins produced by HSV-1, while the green and cyan colors represent microtubules and cell nucleus. The results, presented in Figure 4-2A, clearly shows that HSV-1 early proteins (red) were produced in the infected cells. Additionally, effect of different taxol concentrations as well as pre-incubation periods on successful virus entry, detected by ICP-4 presence, was investigated. Figure 4-2B shows the effect of different taxol concentrations as well as preincubation period on OSVP infectivity in 4T1 cells. Two-way ANOVA followed by Bonferroni post-test compared to 0 taxol at each time point shows that preincubation period by itself and the interaction of
concentration and preincubation time, p-values 0.21 and 0.44, respectively, were not significant. While, the effect of taxol concentration on percentage of ICP-4 positive cells was significant p-value =0.006. Some taxol concentrations significantly affected the extent of virus entry into the cells. 12 hours pre-incubation with 100 and 500nM, and 24 hours pre-incubation with 100nM were significantly different from the control with P-values < 0.05. Overall, OSVP entry into 4T1 was not substantially inhibited due to preincubation period. The higher dose of taxol reduced the percentage of ICP4 positive cells, which can be due to cell death rather than virus entry inhibition (Figure 4-2).

Figure 4-2. Effect of taxol on OSVP virus entry in 4T1 cells. A. Immunofluorescence confocal picture 4 hpi, cells were pre-treated with 1uM taxol overnight; Microtubule (green), Nucleus (Cyan), OSVP (Red). B. Relative efficiency of virus entry was calculated as the percentage of cells expressing ICP4 antigen in a population of 15,000 cells. Bars represent the average percentage of ICP4 positive cells in three replicates and error bars are the SEM.

To determine the effect of taxol on virus replication in 4T1 cells, viral replication was assessed by plaque assays as described in the material and methods. Virus plaque forming units (PFU) counts are presented with respect to taxol pre-incubation at 24 hours post infection (hpi) as well as viral titers measured in various time points. Two-way ANOVA on the effect of preincubation time and different doses of taxol showed that OSVP replication 24 hpi in 4T1 cells pre-incubated with low to high doses of taxol for 8-24 hours did not change significantly (Figure
One-step growth kinetics in the presence of 10-500 nM taxol reinforced previous results and did not show significant changes in virus titers in the course of 48 hours after infection. Higher doses of taxol were not included in this study to avoid cytotoxicity that might affect virus production (Figure 4-3B). Additionally, virus replication 18 hpi in the presence of 100 nM of taxol was captured in the immunofluorescence picture (Figure 4-3C). HSV-1 proteins are labeled with red fluorescent; MTs and cell nucleus are labeled green and blue. Cells with fragmented and altered nucleus that contain the virus proteins show that virus replication was not inhibited in those cells affected by taxol.

In a similar study with HSV-based OV (G207) and taxol in the human anaplastic thyroid cancer (ATC) cell lines paclitaxel did not affect G207 viral entry and early gene expression or G207 viral replication. The study showed that the combination compared with single agents alone significantly affected the cell cycle arrest, increased microtubule acetylation, and apoptosis amongst other effects. The study also showed that combination therapy in mice bearing KAT4 flank tumors was significantly more successful in reducing tumor size compared with single treatments (162).
Figure 4-3. Effect of taxol on OSVP virus replication. A. Standard viral plaque assay showing number of plaque forming units of OSVP cultured in 4T1 cells pre-treated with taxol for 8,12,24 hours. Bars represent mean values in 3 replicates. B. Virus kinetics in the presence of taxol. C. Immunofluorescence picture 24 hpi, cells were pre-treated with 100nM taxol overnight; Microtubule (green), Nucleus (Blue), OSVP (Red). Error bars are the SEM values.
Virus spread was studied by measuring viral plaque sizes in 4T1 cells pretreated 6 hours before infection with different concentrations of taxol. Higher doses of taxol were not included in this study to eliminate cytotoxicity effect of taxol treatment. Plaque sizes were measured according to the number of pixels depicted in them as explained in the methods and materials section. Plaque sizes fall into four major categories 100, 1k, 5k, and 10k pixels, as shown in the immunofluorescence picture (Figure 4-4C). Distribution of plaque sizes for various taxol concentrations are presented in Figure 4A. The mean plaque sizes, shown in Figure 4A, were not significantly different between treatment groups (Figure 4A). To examine the effect of pre-incubation time on plaque sizes, the mean plaque sizes were measured for cells treated for 6-24 with 10 and 100 nM taxol. Significant difference within or between groups was not detected. The plaque sizes were slightly larger when cells were incubated with taxol for a longer time. The fact that cells affected by taxol were generally larger in size due to the lack of cytokinesis as a result of microtubule stabilization in those cells might explain the observation. Mode of plaque size distribution of cells with 10nM taxol also looked slightly different compared to other groups; 10 nm taxol group showed a trimodal distribution while other groups had unimodal plaque size distribution (Figure 4-4A). Kruskall Wallis test did not detect any significant difference between plaque sizes in 4T1 cells treated with taxol for 6 hours prior to infection. Although, two-way ANOVA analysis of the results presented in Figure 4-4B revealed that 12 hours preincubation taxol treatment group had significantly smaller plaques and the 24-hour treatment group produced significantly larger plaques. Taxol dosage was not found to be the source of variation, while the preincubation time and the interaction of time and concentration played a significant role.
4.3 Virus Taxol Combination

Various multimodal treatments of cancer cells have been studied and shown to be more effective than either single agent alone. This combined effect can be smaller, equal, or larger than the sum of the separate agents, which will correspondingly lead to an antagonistic, additive, or synergistic combined effect. Kulu et al. examined oncolytic HSV-1 response in colon and pancreatic cancer cells in the presence of 5-fluorouracil (5-FU), irinotecan (CPT-11), methotrexate (MTX) or a cytokine (TNF-α). In this study HSV-1 MOIs ranging from 0.001 to 10 and chemotherapeutic agents in concentrations ranging from 0-10 µM were used. Kulu et al. reported a significant antagonist interaction when HSV-1 and the chemotherapeutic agents were combined (Kulu et al., 2013). They concluded that reduced of NF-κB activation might be responsible for the inhibition of HSV-1 replication; this is a plausible theory as HSV-1 actively dephosphorylate eIF-2α to activate NF-κB and therefore increase protein synthesis in the host cell. Interestingly, γ134.5 gene that interacts with protein phosphatase-1α to dephosphorylate eIF-2α to promote NF-κB activation has been deleted in OSVP. Therefore, we suspect that this deletion can reduce the unfavorable combination of OSVP and chemotherapeutics.

In a large study McKenzie et al. examined the combination of 73 compounds with an oncolytic myxoma virus against human brain tumor-initiating cells. Drug-virus concentrations used in this study ranged between 0.1-10 µM, and 1-10 MOI, correspondingly (McKenzie et al., 2015). They reported eleven compounds that enhanced the virus oncolysis either additively or synergistically. They found six drug-virus combinations to be synergistic through Chou-Talalay analysis, the same method used in this current study. McKenzie et al. showed that targeting PI3K pathway before the infection may be a key contributor to successful oncolysis. They also concluded that chemotherapeutic agents such as rofocoxib that block or inhibit COX-2 could
lead to synergistic combined therapy. Interestingly, OSVP expresses prostaglandin dehydrogenase, which degrades prostaglandin E2 (PGE2), a major downstream product of COX-2. Therefore, we also suspect chemo agent combination therapy with OSVP to be synergistic as well.

Figure 4-4. Effect of taxol on OSVP virus spread and plaque size in 4T1 cells. A. Virus plaque size distribution in 4T1 cells pre-treated with taxol for 6 hours. B. Mean plaque size of OSVP in 4T1 cells pre-treated with taxol for 6, 12 and 24 hours. C. Immunofluorescence examples of the OSVP plaques grouped by sizes. SEM values are shown with error bars.
To enhance cell killing efficiency in breast and prostate cancer cells, taxol was used in combination with the oncolytic HSV-1 virus OSVP. Cytotoxicity of taxol concentrations ranging 1-100 nM combined with OSVP MOIs of 0.1, 0.5, and 1 was assessed in RM9 and 4T1 cells (Figure 4-5A-B). These concentrations were specifically selected not to considerably surpass the EC50 of each agent alone to ensure that the combined effects are not masked by superfluous cell cytotoxicity from a single agent. Cell survival was calculated based on the percentage of the untreated group. Although low concentrations of taxol (1-10 nM) and OSVP (MOI=0.1) did not show cytotoxicity beyond 10% and 25%, the combination showed enhanced cell cytotoxicity up to 40%. OSVP oncolysis dominated the combined effect when OSVP MOI was increased to 0.5 and 1. However, when higher taxol concentrations were combined with OSVP MOI 0.5 and 1, the combined cytotoxicity reached to 85% compared to 50% in case of each agent alone shown in Figure 5A-B.

Two-way ANOVA was used to analyze the effect of treatment doses as well as combining the agents on cell cytotoxicity. Results showed that both combination of the agents and the different doses used as well as their interactions were significant sources of variation with p-values < 0.0001. Average cytotoxicity between each combination treatment (taxol nM, OSVP MOI) to single agents alone were compared with Bonferroni posttests.

In both cell lines mean cell viability% were significantly different between combinations and taxol alone. While this was not the case when combination and OSVP infection alone were compared. In both cell lines only (100,0.5) and (100,1) in (taxol nM, OSVP MOI) treatments were significantly different from cells treated with OSVP alone. While in RM9 cells, (1, 0.1) combination also showed significantly different mean cell viability compared to OSVP alone. It can be concluded that using OSVP can be very effective in augmented cell killing when
combined with any concentration of taxol. Meanwhile, higher doses of taxol are needed to achieve optimized (synergistic) cell killing compared to both agents alone. As explained before, combination of high doses of OSVP with lower concentrations of taxol led to less significant combined results and showed mainly additive effects; while OSVP and taxol combined both in higher or lower doses the cell killing was substantially augmented (Figure 4-5).

![Figure 4-5. Taxol-OSVP combination cell cytotoxicity. Cell viability was compared between treatments with different taxol-OSVP ratios. A. RM9 cells were treated with taxol (1, 10, and 100 nM) and infected with OSVP (MOIs 0.1, 0.5, and 1). B. 4T1 cells were treated with taxol (1, 10, and 100 nM) and infected with OSVP (MOIs 0.1, 0.5, and 1). Bars represent the average viability in 3 replicate studies, while SEM values are shown by error bars.](image)

### 4.4 Intracellular Taxol Concentration

Various studies have reported diverse mechanisms of action behind additive and synergistic outcomes from combining OVs and chemotherapy. It has been shown that immunomodulation by drugs that can stave off OV clearance by host anti-viral immune response and altered signaling pathways to be a key points in synergistic oncolysis (Bhattacharyya, Francis, Eddouadi, Lemoine, & Hallden, 2011; Nguyen, Ho, & Wan, 2014; Wennier, Liu, & McFadden, 2012). However, in vitro studies rarely address the effect of OVs on the drug internalization, altered mechanism of action, or vice versa.
In section 4.2, effect of taxol treatment on OSVP infection in cells was studied. As shown in Figure 4-4B, viral titers did not change significantly in the presence of taxol. In the current study the effect of virus infection on taxol uptake has been examined to provide new insights in explaining the synergistic outcomes of the combination therapy. Amount of polymerized microtubules was determined via FACS by measuring mean fluorescent intensity (MFI) of anti-microtubule FITC antibody. RM9 cells were infected with OSVP at MOI 0.4, or 1 and treated with 100 nM taxol concurrently. 12 hpi cells were stained with anti-microtubule FITC antibody and mean fluorescent intensity (MFI) was measured by FACS analysis. MFI of infected and uninfected populations in the same treatment group was compared, and results are presented in Figure 4-6. The results show that infected cells contain more tubulin content (Figure 4-6A). In order to elucidate the source of the observed difference, a similar experiment was performed but instead of using the anti-microtubule FITC antibody, oregan-green conjugated taxol was directly measured by FACS (Figure 4-6B). MFI of the conjugated taxol shows that OSVP infected population accumulated three times more taxol than uninfected cells, which explains that the difference in the amount of microtubules which was observed before may be due to the higher intracellular taxol concentrations.

OSVP expresses prostaglandin dehydrogenase, which degrades prostaglandin E2 (PGE2), a major downstream product of COX-2. It has been reported that COX-2 inhibition can affect taxol uptake and sensitize drug resistant cell lines. Thus, in order to investigate if the increase in intracellular taxol concentrations was due to COX-2 inhibition we used a Indomethacin, a COX-2 inhibitor, and the OSV virus, which lacks the PGE2 dehydrogenase as controls (Figure 4-6B). Indomethacin treated cells did not show any difference compared to the negative control, on the other hand a significant two fold increase was observed in the OSV virus infected cells.
Nonetheless, the extent of this increase was less than OSVP virus-infected group. Overall, these results show that virus infection increases taxol uptake in cells significantly and COX-2 inhibition by OSVP is not the only underlying factor.

![Figure 4-6](image)

Figure 4-6. Effect of OSVP infection on taxol uptake and activity. RM9 cells were infected with OSVP and treated with 100 nM taxol concurrently. A. Oregan green conjugated taxol accumulation was directly measured by MFI. Indomethacin (cox2 inhibitor) was used as a control for 15-PGDH. B. Amount of polymerized microtubules (MT) was determined by measuring mean fluorescent intensity (MFI) of anti-microtubule FITC antibody. The average values are represented with bars and error bars show SEM values.

In a previous study taxol was shown to enhance viral production in vivo and in vitro by reduction of antiviral factors such as IFNβ, which is not parallel to the observations made in the current study. On the other hand, it was shown that oncolytic vaccinia virus infection could induce type I IFNs and sensitize tumor cells to taxol (Arulanandam et al., 2015). Similar mechanism has been mentioned in another study using colchicine and VSV co-treatment (Huang, Sikorski, Kirn, & Thorne, 2011). Although not considered in our study, this can be one of the underlying mechanisms of increased taxol uptake by RM9 cells in the current study.

4.5 Cell Cycle Analysis

Taxol mechanism of action to cause mitotic arrest is well studied and is known to be through activation of the mitotic checkpoint. However, a subset of arrested cells will proceed with an abnormal exit from mitosis leading to cells with irregular nucleus. After this slippage,
the fate of the cell is yet undetermined; they can die, arrest again, or resume the cycle with abnormal chromatids. Therefore, it seemed to be interesting to see the effect of virus infection on arrested cells. In this work we examined the cell cycle after single and combined treatments with taxol and OSVP. Cell cycle analysis was initially established on RM9 cells with taxol alone. A temporal profile of taxol concentrations and RM9 cell cycle analysis is shown in Figure 4-7.

![Figure 4-7. Taxol time-concentration cell cycle profile in RM9 cells. RM9 cells were treated with different concentrations of taxol for 6, 9, and 12 hours. Cell cycle FACS analysis was performed after PI staining.]

To further examine the effect of virus infection on the cell cycle, RM9 cells were treated with OSVP and labeled with anti-HSV antibody and PI at 18 hpi (Figure 4-8). FACS analysis data, gated for HSV positive and uninfected cells are shown in Figure 4-8A. In MOI 0.5 and 1 OSVP treated 33.1 and 56.5% of cells were infected. The DNA content in each population labeled by PI is shown as the indicator for the cell cycle. Uninfected cells in Figure 4-8B showed normal distribution, where 50-70% of the cells are in G0/G1 phase and 20-40% are in G2/M. The
S phase, which normally contributes to about 20% of the population, was split in the middle. The infected population however, showed significantly higher levels of G2/M or S/G2/M (Figure 4-8C). Interestingly in 2014, a recombinant oncolytic vesicular stomatitis virus was shown to affect the cell cycle by reducing the G0/G1 populations in human renal carcinoma cells similar to the current findings (Arulanandam et al., 2015).

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Figure 4-8. OSVP infection affects cell cycle analysis. RM9 cells were infected with OSVP; cell cycle profile was compared between infected and uninfected cell labeled with anti-HSV fluorescent antibody. A. Two-way fluorescent intensity distribution of infected and uninfected cells. PI and anti HSV-antibody intensities are shown on y-axis and x-axis, correspondingly. Cells gated based on anti-HSV ab intensity. R5 gates uninfected cells, and R1 shows the infected population. Ploidy analysis in these uninfected and infected populations (B-C). B. PI intensity in uninfected cells. C. Cell cycle analysis in the infected population.

In another attempt, ploidy was analyzed in cells that were pretreated for 6 hours with 100 nM of taxol and then infected with OSVP 18 hpi. The overall effect of OSVP infection to skew the distribution towards the G2/M in untreated cells was similar to the previous observation (Figure 4-9). It was expected that cells pretreated with taxol and infected with OSVP to
accumulate even more in G2/M phase. Surprisingly, introducing OSVP to cells pretreated with taxol reduced the number of cells in the G2/M phase. Compared to the control the percentage in G2/M were almost doubled in cells treated with taxol only, while post treatment with OSVP increased the G0/G1 populations by 35%. In this experiment anti-HSV ab was not used and the effect of infection on the entire cell population was observed. Therefore, the results are not as pronounced as the ploidy analysis in the infected populations shown in Figure 4-9. Nonetheless, this experiment shows that OSVP infection can affect the cell cycle in taxol-induced arrested cells. OSVP infection may press the arrested cells to recycle in spite of the abnormal ploidy state; in this case more cells should have emerged in the far right, which is not the case in this observation. Therefore, it can be concluded that OSVP induced cell death was more dominant in cells that were arrested in M phase after taxol treatment. These results suggest that OSVP infection can affect the fate of arrested cells and create the pressure necessary for cell death.

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Figure 4-9. Effect of OSVP infection on arrested cells. Ploidy analysis was performed in RM9 cells 18 hours post infection with OSVP without taxol pretreatment (top) and with 6 hours of taxol pretreatment (bottom). Untreated cells and taxol treated cells were used as control.
5. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Taxol is a well-known chemotherapeutic agent that has been successfully applied to breast, ovarian, lung, bladder, prostate, melanoma, esophageal cancers and in Kaposi Sarcoma. Unfortunately, due to rapid adaptation of cancer cells to external pressures by genetic and epigenetic mutations and altered pathways, cancer cells often develop taxol resistance.

OSVP is an oncolytic virus based on HSV-1. While OSVP is replication competent, certain deletions were engineered in its genome to prevent it from growing in neurons, promptly elicit an immune response, reduce COX-2 downstream activity, and grow selectively in cancer cells with an extensive fusogenic activity. OSVP clearance from circulation and lower diffusion rate of the virus into the tumor mass are some of the current challenges in oncolytic virotherapy.

Although chemotherapy can be effective for many cancers, in general it lacks selectivity, which can affect healthy cells negatively. Oncolytic viruses can be engineered to target and infect cells that have abnormal cell growth and unregulated pathways but they have limited diffusion rates. Combination therapy using oncolytic viruses and chemotherapeutic agents has proved to be an effective approach in treating cancers and overcoming some of the limitations associated with each agent alone. The combination of the two agents may provide benefits such as, synergistic cell killing, the ability to destruct cancer stem cells and reduce the recurrence rates, need for lower and less toxic chemotherapeutic doses, and enhanced diffusion into tumor mass.

Many studies have shown successful applications of chemovirotherapy before, however, they often ignore to establish the effect of taxol on virus replication and also the effect of infection on taxol uptake by cancer cells. Here, taxol-OSVP interactions were examined with respect to virus life cycle and intracellular taxol concentrations. Interestingly, taxol treatment
showed reduced virus entry but did not affect replication significantly. Preincubation time with taxol was found to be important in virus-taxol combination therapy. OSVP infection in cells that were pretreated with taxol for longer periods produced larger plaque sizes. On the other hand, OSVP infection increased taxol concentrations inside the cells. This is the first time that it is shown that virus infection increases the uptake of taxol. This observation indicates that taxol treatment at the same time or shortly after virus is administered may lead to increased anti-tumor activities, while preincubation with taxol will lead in larger plaque sizes. While previous studies focused on optimizing single agent concentrations, this study shows for the first time that altering the sequence is also very important; in vivo analysis is required to identify an optimized regime based on the administration sequence for the combination therapy.

Cell cycle analysis showed that taxol treated cells that were arrested in the M phase were more susceptible to cell death following OSVP treatment. These results enforced the idea that taxol-OSVP combination can lead to synergistic effects surpassing each agent’s oncolytic capacity if the agents are sequenced properly. Taxol concentrations and virus MOIs used in this study are significantly lower than what is being clinically used making the combination therapy an attractive and attainable solution.

To improve the current work, three dimensional tumor models, cancer stem cells, as well as taxol resistant cell lines are recommended. Animal studies will show the immune response elicited by OSVP better and will shine light on challenges such virus clearance from circulation and OSVP penetration into tumors. Following the steps in the current work, a large study investigating OSVP combination with other chemotherapeutics can be performed. Candidate drugs that would synergize with OSVP better than taxol can be found and effectively of multiple chemotherapeutic agents and OSVP combinations can also be examines.
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VITA

Misagh Naderi, born in Tehran, Iran, attended Sharif University of Technology and received a Bachelor of Science degree in Chemical Engineering in 2008. In the next year he joined the Chemical Engineering graduate program at Louisiana State University, where he earned his Master’s degree in 2012. Misagh lost his grandfather to Parkinson’s and an aunt to cancer, so he was determined to establish his research in human health related topics. Consequently, he joined the Pathobiological Sciences department at the School of Veterinary Medicine at LSU. He researched the application of oncolytic viruses to treat cancers under the supervision of Prof. Konstantin Gus Kousoulas. Concurrently, Misagh joined the computational systems biology group lead by Dr. Michal Brylinski in 2013 to pursue his PhD research in drug discovery for rare and neglected diseases. He earned his Doctoral degree in Biochemistry and Master’s degree in Biomedical and Veterinary Medical Sciences from LSU in 2018. His passion lies at the intersection of science, technology, and communication. He will, therefore, continue his scientifically driven passion to discover cures for human diseases and computational tools to help drug design.