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The Rodent Parvovirus H-1 and its Potential in Combination with Tien Hsien Liquid as a Treatment for Breast Cancer

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Breast cancer is responsible for 12% of cancer diagnoses each year, and it is the leading cause of cancer-related mortality in women worldwide. Current treatments have provided some success in combatting the disease but are not considered a final solution. The framework for how researchers approach cancer has continued to change, and this includes the introduction of oncolytic viruses as novel therapeutics for cancer. The rodent parvovirus H-1 has shown strong potential in clinical and subclinical trials, but its S phase dependency limits its usefulness against cancer stem cell populations. Tien Hsien Liquid is commerically available, nontoxic, and has shown selectiveness for cancer stem cells as well as additional oncosuppressive properties. Because of their unique characteristics, there is evidence that the rodent parvovirus H-1 and Tien Hsien Liquid have potential as a novel treatment in combination for breast cancer.

Keywords: *H-1PV, rodent parvovirus H-1, oncolytic viruses, Tien Hsien Liquid, breast cancer, cancer stem cells*

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INTRODUCTION

Imagine a room. Four walls, one door, and no windows. In the center of the room, there is a table with a giant box sitting on top. There is a lock sealing the box shut, and it is your job to find a way around the lock in order to open the container. You have no experience in picking locks and no tools to help you. Regardless, you get to work. Occasionally, someone will open the door and drop off a new tool to help you. Finally, the lock clicks and the box is opened. At last, you open the lid. Inside, you find fifty more boxes, all with new locks that are far more sophisticated than the original.

Our first instinct may be to give up. Why not? The box represents cancer.

Cancer has become a motif for healthcare fears in the past few decades. Worldwide, cancer caused 8.2 million deaths in 2012, and over 14 million new cases were diagnosed.[1,2] Twelve percent of these new cases were breast cancer diagnosis in women.[2] The problem is already astronomical, and it is only expected to grow worse due to an aging global population, a larger global population, and new/exacerbated environmental factors.[3] In developed countries such as the United States, the incidence rate of cancer is twice as high as those in developing nations.[4] By any given standard, cancer is a medical obstacle that has far reaching consequences. Breast cancer specifically makes up a large part of the issue, and it is addressed in more detail in the upcoming section.

The above scenario is not an argument that attempting to cure cancer is impossible or fruitless. The attempt is, however, perhaps the most complex medical problem of the twenty-first century. Cancer is unique from other diseases, because it is a very broad term. “Cancer” encompasses any type of mutation, misstep, or series of events that leads to uncontrolled cell growth.[5] Two people can be diagnosed with the

same disease but have radically different mutations. Different mutations will make treatments more or less affective.[6,7] A mutation refers to a change in a cell's DNA.[8] Because DNA is responsible for providing the outline needed to make a cell's proteins, these mutations have the ability to destroy or morph a protein product into something new.[8,9] If a mutation affects the right gene, called tumor suppressor genes, it can cause the cell to lose its ability to control cell growth.[9]

The human body is comprised of an amazing, complex series of control mechanisms intended to keep the whole organism healthy. Cancer is the result of these systems failing.[9] Ironically, it is these precise, complex mechanisms that make treating cancer so difficult. Each step in these long pathways is an opportunity for failure, resulting in the emergence of transformed cell populations.[6] The mistakes could happen anywhere at almost any time.

While scientific research has grown in its ability to recognize the nuanced differences between types of cancers, this still leaves the research in a strange position. On one hand, understanding the specific mutations in an individual's cancer gives researchers a better idea of how to combat the problem.[10] At the same time, however, there are not enough resources available to try and combat cancer on an individual, case-by-case basis, and personalized medicine is still a long shot from being a perfected science.[11,12] Only about five percent of insurance companies cover the cost of genetic testing, which makes patient interaction with these genomic technologies limited at best.[13] Even if the monetary resources were available, the time constraints of researchers would still be a major roadblock to any approach involving personalized medicine. While new innovations and discoveries might change this, for now it remains an unlikely solution.[13]

A better solution would be a cure for cancer. This is an obvious statement, but one worth exploring nonetheless. A cure would be something that has the ability to affect

breast cancer and melanoma and pancreatic carcinoma. Most likely, a cure would need to harness the human immune system.[14] Today, science does not have the ability to design this kind of cure from scratch. No researcher can design and build 2,000 proteins in perfect sequence in order to create some kind of biological anti-cancer weapon. Alternatively, trying to "poison" cancer cells through chemotherapy and radiation therapy has been relatively effective (depending on the type of cancer) but not effective enough to be considered a long term solution.[16] More importantly, there are major side effects to these current treatments.[15] If poisoning tumors or designing a biological weapon targeting cancer are not options, perhaps it would be possible to discover a solution that already exists.

Science has made great strides toward finding new solutions to the cancer disease. In the same way that penicillin was discovered and later revolutionized the treatment of bacterial infections, there is a possibility that the natural world already has its own set of tools for picking the locks of cancer. Recently, there has been a surge in investigating viruses as potential weapons against cancer. In the natural world, viruses exist that are known to selectively target cancer cells and kill them. These viruses are called oncolytic viruses.

Oncolytic viruses are not a perfect solution, of course. Oftentimes, they interact with the host immune system in negative ways, are not selective enough in their targeting, or they are too selective.[17] Regardless, these molecules do give researchers an adaptable starting point for creating a cure for cancer. The rodent parvovirus H-1 (H-1PV) is one of the many emerging oncolytic viruses that shows strong potential in cancer therapy. When examining the most common issues that typical oncolytic viral therapies face, H-1PV seems to naturally defy these issues. H-1PV does not activate the human immune system by itself, but it does activate the host immune system toward tumors in addition to its natural oncoselectivity.[18] More importantly, H-1PV was found to be

nontoxic in human patients, which serves as a drastic difference to the side effects from current cancer therapies.[19]

Unfortunately, there is still one more locked box to address. With the recent discovery of cancer stem cells, scientists now know that not all cancer cells divide rapidly. Cancer stem cells are thought to be responsible for cancer metastasis (spreading from one location to another) and chemotherapy resistance.[20] This drastically changes the approach to cancer treatment. Not only do treatments need to address the new issue, but the nature of the issue is very different from previous diseases. Cancer thrives because of its ability to disguise itself from the rest of the body. Quick proliferation was the scientists' golden ticket to finding a way to target cancer without harming healthy tissue. When cancer stem cells defy this standard, they push a large part of cancer therapy research back to its starting point.

In order to address this new obstacle, research has taken two approaches. First, it seeks to understand how cancer stem cells work and their characteristics.[20,21] With this information, it would be possible to search for treatments that can directly target the cancer stem cell population. However, similar to the latter discussion regarding personalized medicine, this approach could very easily become a long, narrow road with no guarantee of finding a designable solution.[22] Alternatively, there have been attempts to identify compounds that could selectively target these types of transformed cells.[23] This becomes increasingly important in the context of cancer stem cells.

While cancer stem cell research is a relatively new branch of oncology, there have already been some promising results, including those seen with Tien Hsien Liquid (THL). THL itself has proven to have impressive anti-cancer properties, such as anti-angiogenesis, anti-metastasis, and immunomodulation.[23,24] It has also been suggested that the solution may directly target cancer stem cells.[25] The difference between THL and other research aimed at targeting cancer stem cell populations is the research

supporting its use. While other therapies work at identifying specific biomarkers for cancer stem cells, followed by creating compounds to target these biomarkers, this method has several flaws.[22] THL has already shown positive oncolytic potential and is known to be nontoxic in humans.[25] In order to compare H-1PV and THL, there needs to be some common ground between the therapies. Among the many types of cancer, breast cancer is not only relevant in its incidence rate, but it also has the largest research crossover with both H-1PV and Tien Hsien Liquid.

In a room of locked boxes, research has often aimed at trying to create perfect, individual keys for each type of cancer. Unfortunately, the fight to cure cancer is one of the most complicated endeavors that medical science has ever undertaken. The disease is incredibly complex, which lends itself to the need for a complex solution. There is potential, however, in the exploration of compounds and viruses that have shown naturally oncolytic properties. Perhaps, instead of trying to create a key, the focus should be on modifying those that already exist.

CHAPTER 1: BREAST CANCER

Breast Cancer Today

As defined by the World Health Organization (WHO), cancer is caused by uncontrolled cell proliferation.[26] In 2012, there were 14 million new cancer cases around the globe, and the rate of new cases is rising exponentially.[4,26] Within the next two decades, the number of new annual cases is expected to grow to 22 million.[27] Economically speaking, the global cost of cancer, because of death, sickness, or disability in the population, is estimated at $895 billion dollars per year.[28] While cancer may be caused by preventable dietary and lifestyle risk factors, these cases amount to less than one third of diagnoses.[3,26] Lifestyle and dietary factors include exercise, diet, and tobacco use.

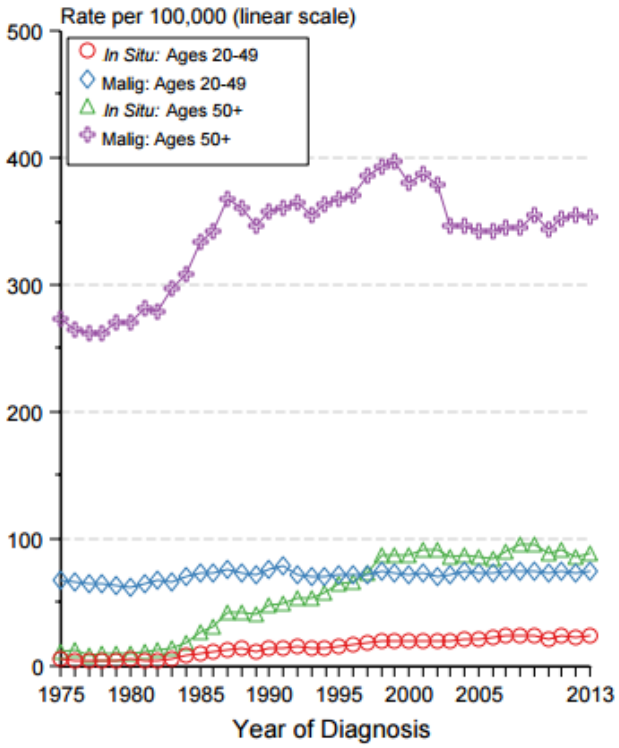

Figure I: Incidence rate of breast cancer (malignant and in situ) for separate age groups from 1975-2013.[30] Notably, the chart looks at the rate of cancer incidence within the population, per 100,000 people. This scale does not account for population growth or an increase in lifespan.

Because cancer is often caused by repeated exposure to both controllable and uncontrollable environmental factors, there is a direct correlation between age and cancer incidence.[3,6] As the average life expectancy grows higher, the frequency of cancer incidence continues to rise, particularly in first world countries.[4] According to the CDC, breast cancer is, by large, the most common cancer diagnosis among women.[29] Every year, 230,815

women in the United States are diagnosed with some form of breast cancer.[29] Despite its high incidence rate, breast cancer is not the deadliest cancer for American women. In terms of mortality, lung cancer causes more female deaths per year than breast cancer.[6,26] However, on a worldwide scale, breast cancer is the second leading cause of cancer-related death in developing countries, beaten only by cervical cancer.[29,30]

Altogether, this makes breast cancer the leading cancer-related cause of death for women globally.[30,31] For these reasons, breast cancer is significant in its impact on American health and healthcare needs around the world. Figure I shows the relative increase of breast cancer incidence over the past forty years. Overall, the trend shows rates increasing slowly over time, with some fluctuation. This is most likely due to the removal or introduction of widespread environmental factors and behaviors.[3,26] Figure II outlines the history of breast cancer treatment and how these treatments have affected the overall mortality rate of breast cancer in American women.

Given these statistics, there is little doubt that breast cancer is a major health concern. In the present day, the need for more effective treatment methods is immense. As discussed, this need will continue to grow as the elderly population expands and the average human lifespan increases.[4] Before looking into these current methods, however, it is first important to take a step back and examine the evolution of the disease epidemiology and its treatments throughout the years.

Radiation and Chemotherapy

X-ray technology was discovered by Wilhelm Conrad Roentgen, and radiation was introduced as a treatment for cancer within the following five years.[32] Soon after, unfortunately, it was discovered that radiation could cause cancer just as easily as it could treat the disease.[33] Many early radiologists developed leukemia because of the

constant exposure to X-ray radiation.[34] In fact, there remains a strong correlation between pregnant women who are exposed to radiation and the rates of cancer in their children.[35] Children who are exposed to radiation have also shown to have much higher rates of cancer later in life.[36] Despite these early risks, radiation is used in the United States today to treat 50% of new cancer cases.[37]

Radiation works, from a medical perspective, by damaging DNA. Because cancer cells are hallmarked by their ability to proliferate quickly, damaged DNA will cause immediate harm to tumors.[38] On one hand, this damage can cause the tumor cells to destroy themselves. If these apoptotic pathways are not resolved or have become too mutated to function properly, damaging the tumor DNA can still halt the replication process and prevent further tumor growth.[39] Regardless, the radiation will damage DNA in any tissue that it comes across, including healthy tissue. While tumor cells may

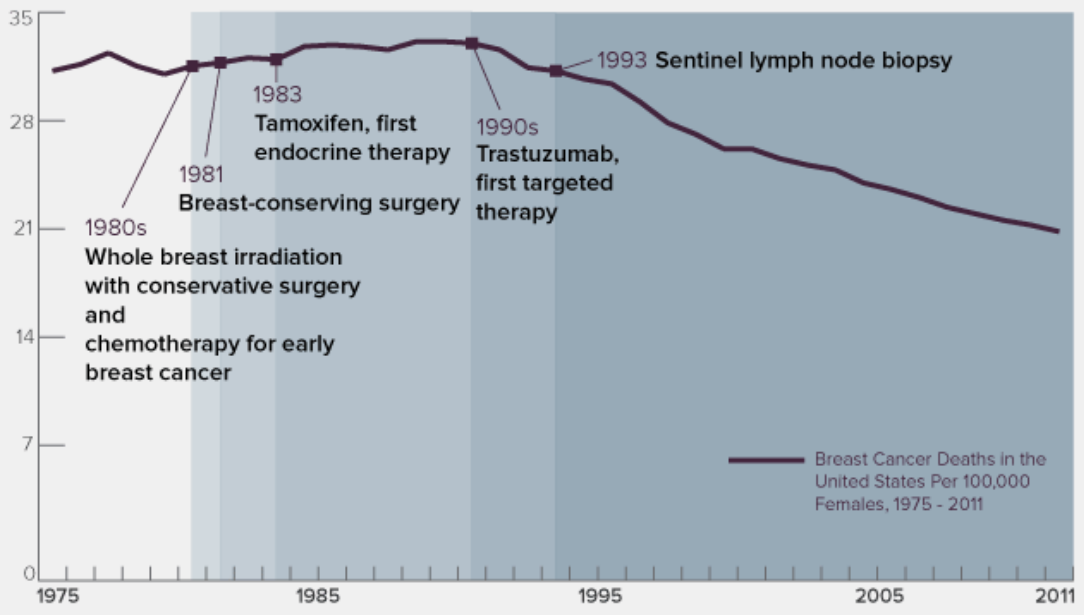

Figure II: This graph is a depiction of how breast cancer treatment methods have progressed over the last forty years. More specifically, it focuses on how these treatments correlate with the number of deaths per 100,000 people in the United States.[35] While anesthetics did allow initial exploration of surgical methods for tumor removal, the introduction of targeted therapy and lymph node biopsies had the largest impact on the mortality rates of breast cancer.

appear have the most immediate and dramatic response to radiation exposure, normal cells are still at risk for destruction following DNA damage as a result of radiation.[39] It is the targeting of these healthy cells, albeit unintentionally, that causes the side effects associated with radiation.

To minimize this problem, researchers have worked toward controlling the direction and intensity of radiation. Conformal radiation therapy (CRT) involves making three dimensional images of a patient's tumor, creating a cast to hold the body still during the treatment, and shooting the radiation directly into the tumor at multiple angles.[40,41] Another technique, called conformal proton beam radiation therapy, uses protons instead of x-rays in order to damage tumor DNA.[41] One new option, intraoperative radiation therapy (IORT), involves administering radiation during surgery. In this method, nearby tissue can be shielded directly via an opening in the body cavity, and doctors can channel the radiation more directly toward the tumor.[42]

All of these types of radiation, while different, have several significant commonalities. First, their purpose is to damage tumor DNA in hopes that the damage will lead to cell death. Second, they run the risk of affecting nearby healthy tissue. Lastly, even when radiation directly targets a tumor, the tumor rarely shows complete regression. More often than not, radiation helps in tumor reduction but is not a complete solution. Most importantly, radiation does not target cells in the G_0 phase of cell growth.[39] The relevance of this will be discussed later in relation to cancer stem cells.

Chemotherapy, following radiation and surgery, is a third common treatment for cancer.[43] Chemotherapy is based on the administration of cytostatics. Similar to the theory behind radiation therapy, cytostatics are meant to specifically target rapidly proliferating cells and stop their division process.[44] Because chemotherapy treatment is usually systemic and less directed than radiation therapy, it effects rapidly dividing cells

from any part of the body. Often, these include red blood cells, hair follicles, and the GI track.[45]

Furthermore, even in cases where chemotherapy and radiation are successful in treating cancer, the side effects are far-reaching. They include anemia, burns, nausea, vomiting, hair loss, heart damage, nerve damage, and infection, among others.[43,44] Of course, the nuances of radiation therapy, chemotherapy, and their combinations (radiochemotherapy) easily contain enough material to require reviews within themselves. However, for the sake of brevity, there are several clear points in this chapter that have been addressed in order to give proper context to the following discussion.

Despite the relative success of both chemotherapy and radiation, breast cancer mortality and disability are still major problems in the United States. While some of these cases may be preventable, the large majority of breast cancer diagnoses are environmental or genetic. Both types of current treatments focus specifically on cells that are rapidly dividing and, the majority of the time, have no other oncoselective properties. Because of this, the methods have side effects that draw further attention to the need for alternative therapies. Moreover, selection of actively dividing cells leaves cancer stem cells relatively untouched with these treatments.

CHAPTER 2: H-1PV

History of viral therapies and their clinical applications

The idea to use viruses as a therapeutic treatment for cancer began with a series of observations in the early-mid 1900's. Leukemia and lymphoma patients who contracted viral infections occasionally went into remission.[17] This sparked well-intentioned, but ultimately ill-fated clinical trials. More often than not, treating leukemia patients with active viruses, such as influenza, did not affect tumor growth because the host immune response destroyed the infection before it could have any oncolytic effect. In cases of immunodeficient patients, the effects of the virus itself generally resulted in more damage than benefit.[46]

Ironically, despite this long lineage of viral therapy for cancer, the study of viruses was in its infancy throughout the twentieth century. The concept of a virus did not appear in full until its description in 1898, when Martinus Beijerinck discovered a pathogen that could not be removed through a filtration process that was known to catch bacteria, called Chamberland candle filtration.[17,47] The existence of viruses was 'proven' in 1917 by a French scientist named Felix d'Herelle, but the first virus was not visualized until 1939.[17,48] From this point onward, understanding of viruses has continued to grow at an exponential rate.

Although research in the field of virotherapy has fluctuated in the past century, it has seen a large resurgence in the last few decades.[49] In part, this is due to a medical gap in safe and effective cancer treatments (as previously discussed in relation to breast cancer). Because traditional methods have not shown sufficient results in the fight against most cancers, oncology has branched into more creative methods for attacking the disease.[50] In addition, this growth has been facilitated by advances in genetic engineering.[51] Through genetic engineering, researchers have created viral strains that

are non-pathogenic but still retain oncolytic abilities.[52] This discovery is the key difference between the failed viral therapies of the early 20th century and the way that these treatments are explored today. The viral DNA/RNA can now be modified to change the protein products of viruses.[17] These changes have allowed for increased oncoselectivity and other attributes (such as replication competence and immune activation) aimed at perfecting the oncolytic properties of viruses.[52]

In general, viral therapies do appear to have the potential to function as effective alternatives to current cancer therapies.[17,52] A wide body of literature including subclinical and clinical trials supports the efficacy of viral therapies *in vitro* and *in vivo*. Notably, appearing in a review published in 2015, over 1,000 patients have been treated with oncolytic viruses in various stages of clinical trials.[51] In general, these current clinical trials have reported few side effects and extremely high or nonexistent toxicity dosages.[51]

Among the oncolytic viruses studied so far, the H-1 parvovirus (H-1PV) is particularly notable. It belongs to the family *Parvoviridae*, and the species name is Rodent parotoparvovirus 1 (RoPV1).[17,51]

The oncolytic properties of H-1PV have been studied in a number of *in vitro* and *in vivo* models, including glioblastoma/glisarcoma, neuroblastoma, medulloblastoma, breast cancer, pancreatic carcinoma, and more.[17,51-57] The first clinical trial for H-1PV (Phase I/IIa, named ParvOryx 01) began in 2011 as a treatment for glioblastoma.[58] The details of these H-1PV studies are discussed in further detail below. First, however, it is of interest to examine the molecular makeup and mechanisms which make H-1PV unique in its ability to target and destroy cancer cells.

The Fundamentals of H-1PV (structure, life cycle, and oncoselectivity)

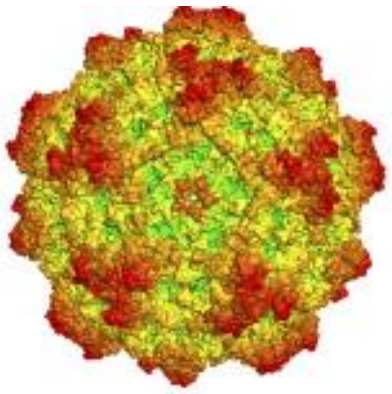

Figure III: Hypothesized structure of H-1PV capsid using MVM parvovirus model[72]

The H-1PV genome is made up of single-stranded DNA (ssDNA), and the viral DNA codes for four major proteins.[59] NS1 and NS2 are non-structural proteins responsible for the main viral activity within a cell.[60] VP1 and VP2 (viral proteins) make up the capsid, or external structure, of the virus.[59] Although other protein products have been identified (SAT and VP3) they exist in small quantities.[17] SAT has been implicated in MHC I processing or ER stress-related apoptosis, and VP3 is created by a 25 amino acid cleavage of VP2. VP3 does not have any known function.[17] Figure III shows a hypothesized three dimensional structure for H-1PV.

H-1PV has two promoters in its DNA sequence, P4 and P38. P4 includes the NS1 and NS2 sequences, and P38 contains VP1 and VP2.[60] Figure 3 is a diagram of these relationships. The NS1 protein is responsible for activating the P38 promoter, resulting in VP1 and VP2 synthesis.[61] The green regions in Figure 3 represent the palindrome sequences on either end of the ssDNA. These sequences play an important role in H-1PV replication.

NS1 is the most widely-studied H-1PV protein, and it is essentially responsible for viral cytotoxic activity and replication.[62] NS1 is a phosphoprotein found predominantly in the nucleus, because its sequence contains a nuclear localization signal.[63] The NS1 protein is responsible for binding to the H-1PV promoters and initiating the process of viral replication.[64,65] It has also been shown to create reactive oxygen species (ROS).[17] While direct host-DNA interaction with ROS can lead to cell death, ROS have also been

found to interact with, and cause malformation of, other host macromolecules, such as lipids and proteins.[66-68]

NS1 expression causes the host cell to arrest before the G_2 phase of the cell cycle, and eventually induces apoptosis.[69] p21 and p27, two cyclin kinase inhibitors (CKI), are upregulated by NS1, which causes the cell cycle to stop before the G_2 phase of mitosis. Specifically, these two CKIs have been shown in inhibit CDK2/Cyclin A/E.[70] This relationship is represented and described further in Figure IV. The lifecycle of H-1PV is dependent on cellular factors only present in the S phase. Thus, by freezing the mitotic process in the S phase through cyclin complex inhibition, the virus essentially creates a cellular atmosphere perfect for viral replication.[71]

Because of the H-1PV S phase dependency, H-1PV has powerful and natural oncoselective properties.[72,73] Only actively mitotic cells will ever present the S phase

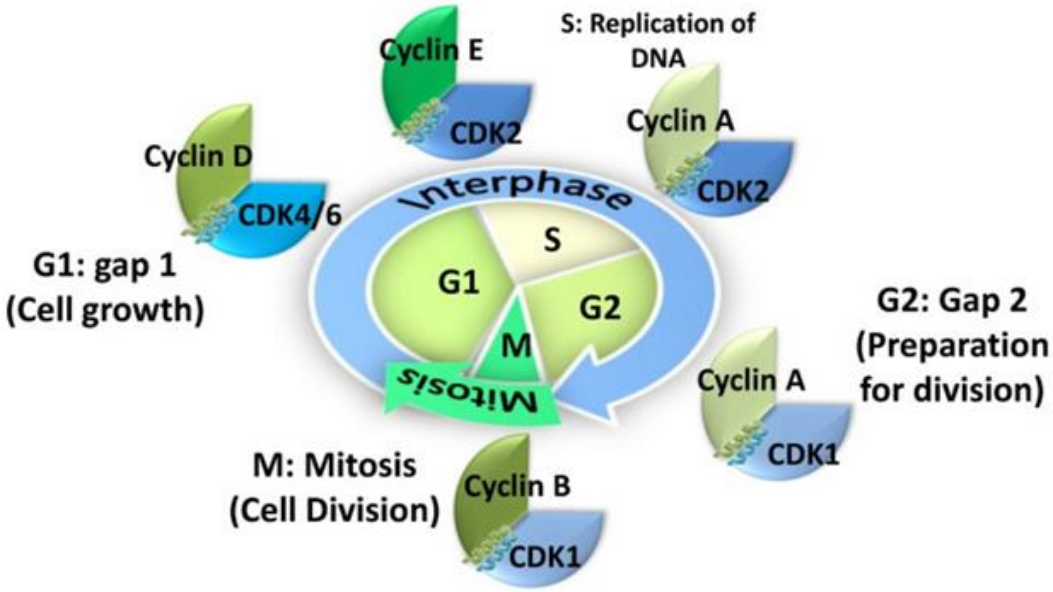

Figure IV: This image is a depiction of the role that different cyclin/CDK complexes play in the cellular replication cycle. The NS1 protein activates p21 and p27. These two CKIs, in turn, inhibit the CDK2/CyclinE complex. The resulting mitosis-freeze leaves the cell stuck before it can reach the G2 phase. Because the viral life cycle depends on factors from the S phase, freezing the cell cycle at this point is ideal for maximum H-1PV proliferation.[70]

factors needed to activate H-1PV, resulting in host-cell death.[17] In comparison, other oncolytic viruses have the ability to push quiescent cells into the S phase, which allows them to then begin replicating.[68] H-1PV, however, does not have this property. This means that, while H-1PV can infect quiescent cells, it will never propagate once inside these types of cells.[71] Altogether, this attribute is a major contributor to H-1PV's non-toxic properties.[68] While this natural phenomenon is important, oncoselectivity can still be increased through genetic engineering, which is discussed further in the subsequent section.

In addition to activating CKIs, parts of the NS1 domain are known to function as a DNA helicase and ATPase.[69.74] While all of these mechanisms are important in inducing apoptosis, H-1PV oncolytic success is not entirely dependent upon cell-mediated apoptosis pathways. Typically, the production of reactive oxygen species, cycle inhibition, helicase and ATPase functions, and macromolecule interruption only cause apoptosis if the cancer cell still has the ability to pursue apoptotic pathways.[75] However, in addition to normal cell-mediated apoptosis mechanisms, research has suggested that

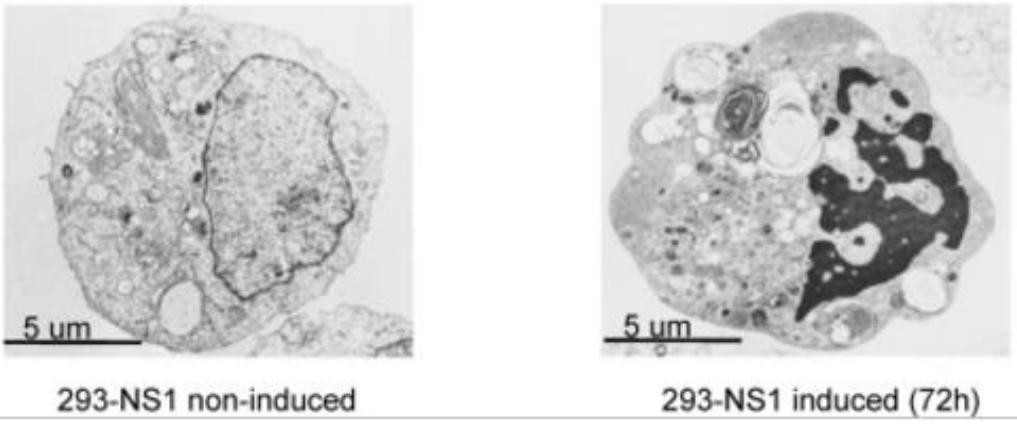

Figure V: *The above photos show two cells in an NS1 expression experiment. The cell on the left does not have NS1 protein expression, and the cell on the right does have NS1 activation. The cell on the right shows signs of apoptosis, which was found to correlate directly to NS1 expression.*[4]

H-1PV may kill cells by means of necrosis.[76] Figure V shows how NS1 upregulation causes apoptosis, compared to a control with a downregulation of NS1 expression.

At first glance, the multitude of functions that NS1 performs may appear contradictory to our understanding of protein specificity and resulting functional limitations. However, post-translational modifications of NS1 result in protein products with minor structural differences yet major functional changes. For example, although NS1 has been classified as a phosphoprotein and is regulated by phosphorylation, it had been discovered that NS1 undergoes acetylation as well as phosphorylation.[77,78] The acetylated form of NS1 performs its own novel functions, such as DNA binding and p48 promoter activation.[79] So far, two acetylation sites have been identified at positions K85 and K257 on the NS1 protein.[79]

Of course, NS1 also undergoes phosphorylation at a few different sites. The protein is phosphorylated by protein kinase C (PKC) produced by the host cell.[73] Phosphorylated NS1 plays a few different roles. It is responsible for ATPase, helicase, protein binding, and DNA binding abilities.[73,80] The big-picture life cycle and cytotoxic properties of H-1PV are outlined in Figure VI. The key points include cellular transcription factors regulating initial H-1PV promoter activity, viral DNA amplification, and the multiple oncolytic pathways that are engaged during viral proliferation.

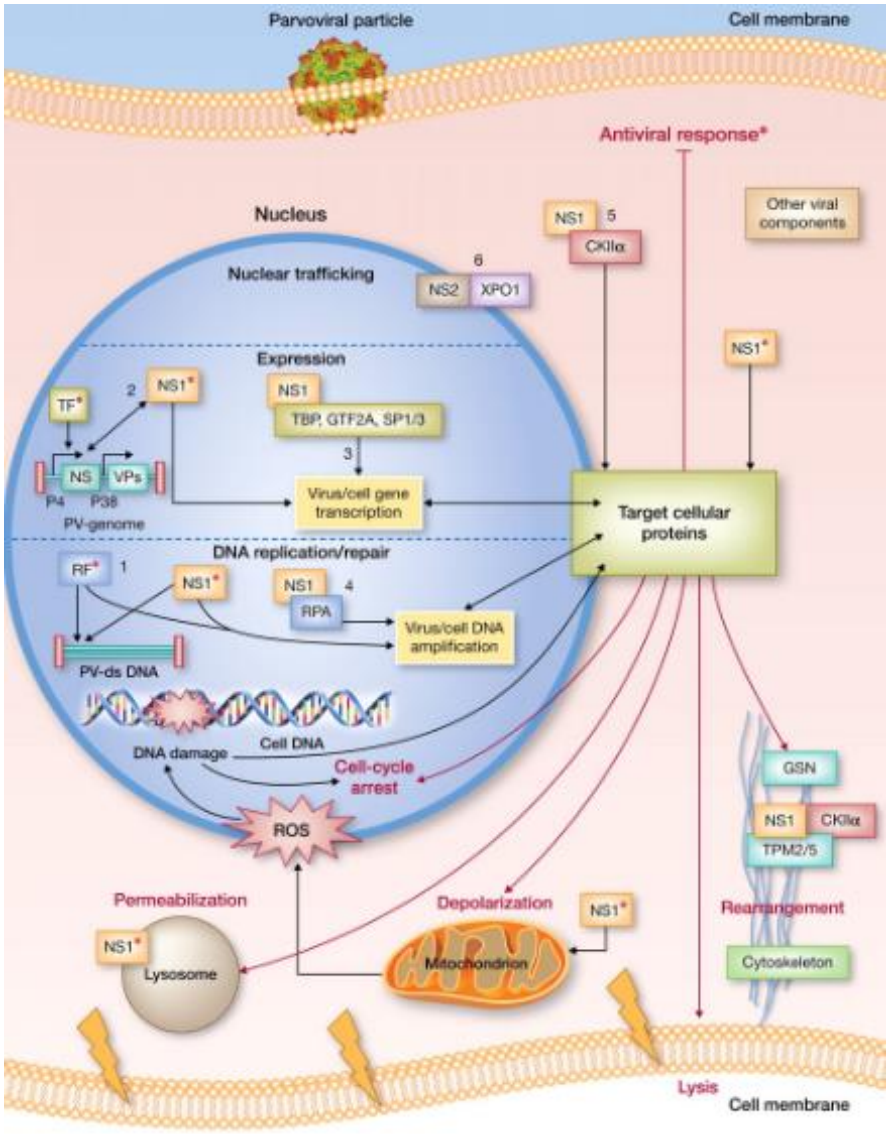

Figure VI: This image depicts the many processes involved in H-1PV replication and oncolytic life cycle. Marker 1 shows the conversion of the ssDNA of H-1PV into dsDNA (where RF = replication factor). Marker 2 shows the original host transcription factors (TF) activating the P4 promoter to incite the initial NS-protein transcription. Once NS1 has been translated (markers 3-6), NS1 functions in a multitude of pathways, as previously discussed. These include viral transcription amplification and host-cell destruction through many apoptotic pathways, such as ROS creation, increased lysosome permeability, and cell cycle arrest through CKIs. H-1PV cell entry is not well understood and thus remains ambiguous in the figure.[73]

H-1PV and the Immune Response

While direct oncolytic properties of H-1PV have garnered the most research interest, recent studies have focused on a secondary method of tumor destruction caused by H-1PV infection: activation of the host immune system.[81] When testing the ability of H-1PV to completely eradicate tumors in mouse models, it was found that immunocompetent mice had a radically higher success rate when compared to immunodeficient mice.[82] This discovery presupposes the conclusion that H-1PV does not only work through direct oncoselectivity and resulting oncolytic activity.

Research has long looked for successful cancer immunotherapies, which adapt the host immune system in order to make it recognize tumors.[81] In order for cancer immunotherapy to be successful, antigen presenting cells (APCs) must first present a tumor antigen to the appropriate T-cells.[83,84] Simultaneously, these APCs (typically dendritic cells) must have the appropriate microenvironment in order for their presentation to be successful. However, tumor cells block the APCs from successful presentation, effectively causing tumor antigen immunity similar to self-antigen immunity found throughout the body.[83-85] This is represented in Figure VII below.

Viruses are known to be highly immunogenic, and immunogenic viruses elicit an immune response.[86] This is because they have the ability to activate all three steps of the APC activation pathway.[87] Broadly speaking, these steps are referred to as antigen presentation, co-stimulation, and inflammatory cytokine release.[87,88] When cancer cells are infected with an oncolytic virus, the immune system not only recognizes the viral particles as foreign, but there is also a co-stimulation targeted toward infected cancer cells.[16] This co-stimulation has the ability to reverse the suppressive effects of the tumor microenvironment.[16] The resulting immune response is neither characteristic of a

typically oncolytic virus immune reaction or a tumor immune reaction. Instead, it has characteristics independent of both pathways.[89]

One study found that when tumor cells are infected with oncolytic viruses, levels of type I interferons are increased.[83] Co-stimulatory molecules are found more abundantly surrounding APCs, and dendritic cells begin secreting a wide variety of stimulatory molecules, including multiple types of interleukins.[83] The viruses cause an increase in factors associated with the Major Histocompatibility Complex I (MHC I), which is responsible for presenting foreign peptides to cytotoxic T-cells.[83,89]

By causing the re-activation of dendritic cells and other APCs despite the adverse tumor microenvironment, and by stimulating the MHC class I synthesis pathway, H-1PV and other oncolytic viruses have immunotherapeutic functions. Through teaching the host immune system to recognize and target the tumor cells, two medicinal benefits emerge. First, activating the host immune response improves the direct oncolytic effect of H-1PV on infected tumor cells.[90] Simultaneously, because the human immune system works in circulation, immune activation will target tumor cells even if they have not been infected by H-1PV and are located in a distant part of the body.[83,85]

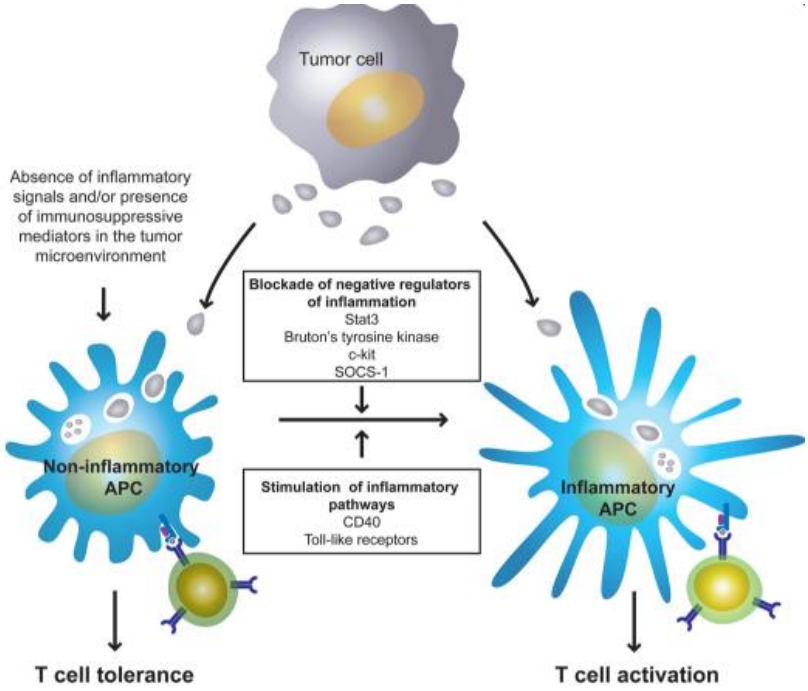

Figure VII: The tumor microenvironment blocks various inflammation regulators and stimulatory pathways. This causes the APC presentation to T-Cells to fail, resulting in T-cell tolerance to the presented antigen. When these pathways are re-stimulated, T-cell activation can occur in response to tumor antigens.[85]

H-1PV research modifications, synergisms, and subclinical trials

The oncolytic effects of H-1PV have been tested in numerous cell lines derived from various cancers. These include, as previously mentioned, glioblastoma/glisarcoma, neuroblastoma, medulloblastoma, breast cancer, and pancreatic carcinoma.[17,51-57] Without room to discuss each of these trials in detail, several conclusions have been drawn from their generalized results. The only cell line that appeared resistant to the major oncolytic effects of H-1PV was colon cancer.[91,92] A study found that, because the H-1 parvovirus cannot induce S phase in its host cell, colon cancer cells infected with H-1PV remain largely unharmed because of colon-specific cellular regulators.[91,92] Outside of this specific example, however, H-1PV infection has shown remarkable oncolytic abilities in cell line cultures. *In vitro* cell lines have shown anywhere from 20-100% cell death.[17]

In vivo studies have shown similar results. Glioblastoma, Burkitt's lymphoma, gastric tumors, and pancreatic carcinoma are among the trials conducted with mouse models.[51,53,57] In each of these experiments, the researchers observed a significant tumor regression. In some cases, inhibition of tumor formation was also observed.[17] *In vivo*, H-1PV delivery has taken several routes. Intratumoral injection, intravenous injection, and intranasal injection have all been observed as capable routes of administration.[93,94]

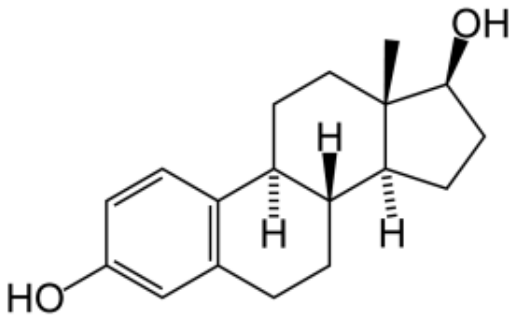

Figure 8: The molecular structure of estradiol[105]

So far, there have been two approved clinical trials using H-1PV. First, for the treatment of glioblastoma, there was a trial called ParvOryx01.[58] The study was completed in May 2015, although the confirmed results have not been reported on ClinicalTrials.gov.[97] Another trial by the same company, called ParvOryx02, aims at using H-1PV to target pancreatic carcinoma.[98] In unofficial forums, it has been stated that the ParvOryx01 trial was highly successful. H-1PV showed no toxicity and led to tumor reduction. Several trial patients were given a follow-up dose of H-1PV as a part of compassionate use standards after their tumors reemerged following the trial.[99]

Because H-1PV can enter any human cell (although it only replicates and lyses tumor cells), work has been done to increase the specificity of H-1PV. In one study, this included detargeting the H-1PV capsid to normal cells and inserting a cyclic RGD-4C peptide into the H-1PV capsid.[72] The RGD-4C peptide is known to bind two integrins that are over-expressed on tumor cells.[100] As a result, the specificity of H-1PV to cancer cells is greatly increased.[72] If H-1PV is more oncoselective, then lower dosages of H-1PV will be needed to achieve similar levels of oncolytic activity.

In addition to tests looking at *in vivo* and *in vitro* administration of H-1PV independent of any other drug, H-1PV has been found to work synergistically with several different compounds.[101] Glioma cell lines exposed to ionizing radiation showed increased levels of S phase factors, which caused H-1PV cytotoxicity to increase dramatically.[95] Valproic acid independently causes tumor regression, and it was recently found to work synergistically with H-1PV *in vitro* and *in vivo*.[79] Traditional chemotheureptic agents have also been found to amplify the oncolytic effects of H-1PV.[96]

H-1PV Research Specific to Breast Cancer

While the general effects and potential of H-1PV are relevant, it is important to isolate how these trends translate to the study of breast cancer. In breast cancer specifically, Parvovirus H-1 has been shown to prevent tumor formation in nude mice by more than 80%.[56] Notably, in this study the implanted tumors were derived from human cell lines, which increases the relevance of the results. The cytotoxic effect of H-1PV has been shown to be specific to transformed mammary tissue. In a comparative study, H-1PV did not inhibit healthy mammary cells compared to their cancerous counterparts.[102]

Another study found that the susceptibility of different types of mammary carcinomas to H-1PV infection correlated to the level of oestrogenic receptors present on the cells.[103] Oestrogenic receptors are estrogen receptors. Cells with high levels of oestrogenic receptors were more susceptible to H-1PV lytic activity than cells without oestrogenic receptors. Interestingly enough, the infection rates between all of the cell lines were around average. The only difference the oestrogenic receptors made was the likelihood of cell lysis. Oestradiol, one of the main types of estrogen produced by the body, was found to activate the oestrogenic receptors, which in turn made the cell lines

more sensitive to H-1PV.[103] The molecular structure of oestradiol is shown in Figure VIII.[104]

Altogether, the effects of H-1PV on human breast cancer models *in vivo* and *in vitro* are similar to those seen in other types of cancers. H-1PV induces cytotoxic effects in human breast cancer tissues.[105] Because of this, H-1PV does appear to be a valid avenue of exploration for future breast cancer treatment research. However, there is one major factor that must be addressed before any conclusive arguments are made regarding H-1PV treatment of breast cancer: cancer stem cells.

CHAPTER 3: CANCER STEM CELLS

The Discovery of Cancer Stem Cells

For many years, there was a question of how cancer could reemerge after a patient had spent months or years in remission. Completely new mutations causing cancer seemed unlikely. One suggestion was that treatment methods may not completely eradicate the disease and leave a few cancer cells in the body.[106] While this can be true, this line of thinking does not explain long periods of remission, particularly if cancer is characteristically defined as abnormal rapidly dividing cell populations.[4] The answer to this question, it turns out, revolutionized the way researchers today view cancer. Tumors are not homogenous masses of cells. Instead, they are heterogeneous populations.[107] This explains why old therapies have not been effective. These therapies, including radiation and chemotherapy, were made to target only a subgroup of the cells that make up tumors.[108]

In the scientific community, there is not a universal acceptance of cancer stem cell theory. Some favor the idea of clonal evolution.[109] Clonal evolution theory is similar to CSC theory in the sense that it hypothesizes that there is a hierarchical system in the creation and differentiation of cancer cell populations.[108,110,110] While clonal evolution and CSC theory are different, they share the common principle of heterogeneity within tumors and explore the way that this affects treatment of cancer cell lines.[109,111]Because of this common ground, the argument between the two theories will remain untouched. Instead, the research will be explored, regardless of which theory it is believed to support. For the sake of clarity, the slowly proliferating tumor sub-populations will be referred to as cancer stem cells.

The initial, revolutionary discovery of tumor heterogeneity was made by Dominique Bonnet and John E. Dick and published in 1997.[112] The researchers found

subpopulations of tumor cells, which were later dubbed cancer stem cells (CSCs), that have different properties than previously characterized cancer cells.[113] Specifically, these properties include the ability to further differentiate, unique microenvironments, and a lifecycle that includes prolonged periods of the cellular lifecycle spent in the G_0 phase.[114] Notably, the microenvironment of CSCs is very similar to healthy human stem cells.[115] While slightly oxymoronic, the environment is both perivascular and hypoxic.[114]

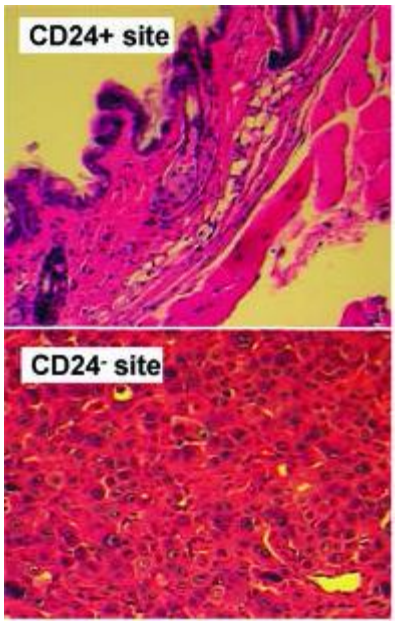

Figure IX: $CD24^+$ sites were not able to initiate new tumor growth. $CD24^-$ tissues, on the other hand, showed massive in vivo *proliferation and tumor formation. This lead to the identification of $CD24^-$ cells as CSCs.[119]*

Because CSCs are not rapidly proliferating, any cancer treatment aimed at completely curing the disease cannot focus solely on rapid division as a means for targeting tumor cells.[116] In fact, CSCs have been identified in most types of cancer.[117] Moreover, most types of CSCs have been found to be resistant to both chemotherapy and radiation.[108] Given the mechanism of both of these treatments, this is not particularly surprising considering the slow growth of CSCs. Outside of discovery, recent research has largely been aimed at trying to learn more about CSCs.[117] While in many ways this has been a successful endeavor, it does leave science today at an impasse. On one hand, scientists now know that CSCs exist, yet at the same time there is now a need for current treatments to reconsider their approach for something inclusive of CSC targeting.[116]

Limitations and Biomarkers of CSCs

Several biomarkers for breast cancer stem cells (BCSCs) have been identified/suggested. These include $CD44^{+}/CD24^{-/low}$, Her^{+}, the Wnt pathway, the Notch receptor, and aldehyde dehydrogenase.[118] All of these biomarkers are involved in very different pathways, but were able to selectively separate cancer cells that could recreate tumors versus those that could not initiate new tumor growth. The results for one of these studies ($CD44^{+}/CD24^{-/low}$) are shown in Figure IX.[119]

The goal of CSC research, ultimately, is to find a way to target cancer stem cells and reduce cancer mortality rates.[116,117] Before delving into the status and findings of the current literature, this end-goal is further outlined in Figure X. Due to the elusive nature of CSCs, researchers so far have been unable to identify the frequency of CSCs *in vivo*.[113] In addition, their precise origin is still under debate.[120] As such, research looking into CSCs remains largely speculative regarding how well animal models and cell lines are able to represent the nature of CSCs *in vivo*.[113]

Finally, one last issue makes researching BCSCs difficult. It has been proposed that CSCs have an ability called phenotypic switching.[121] This means that, while an initial separation between tumor cells with a specific biomarker may accurately divide the two subpopulations of cancer cells, these cells have the ability to switch phenotypes.[118] To rephrase, differentiated cancer cells have the ability to revert back to a CSC state, as shown in Figure XI. This process was suggested when cells separated between CSCs and normal proliferative tumor cells presented strange results. A small number of the separated cells in the non-CSC category were found expressing a previously-identified

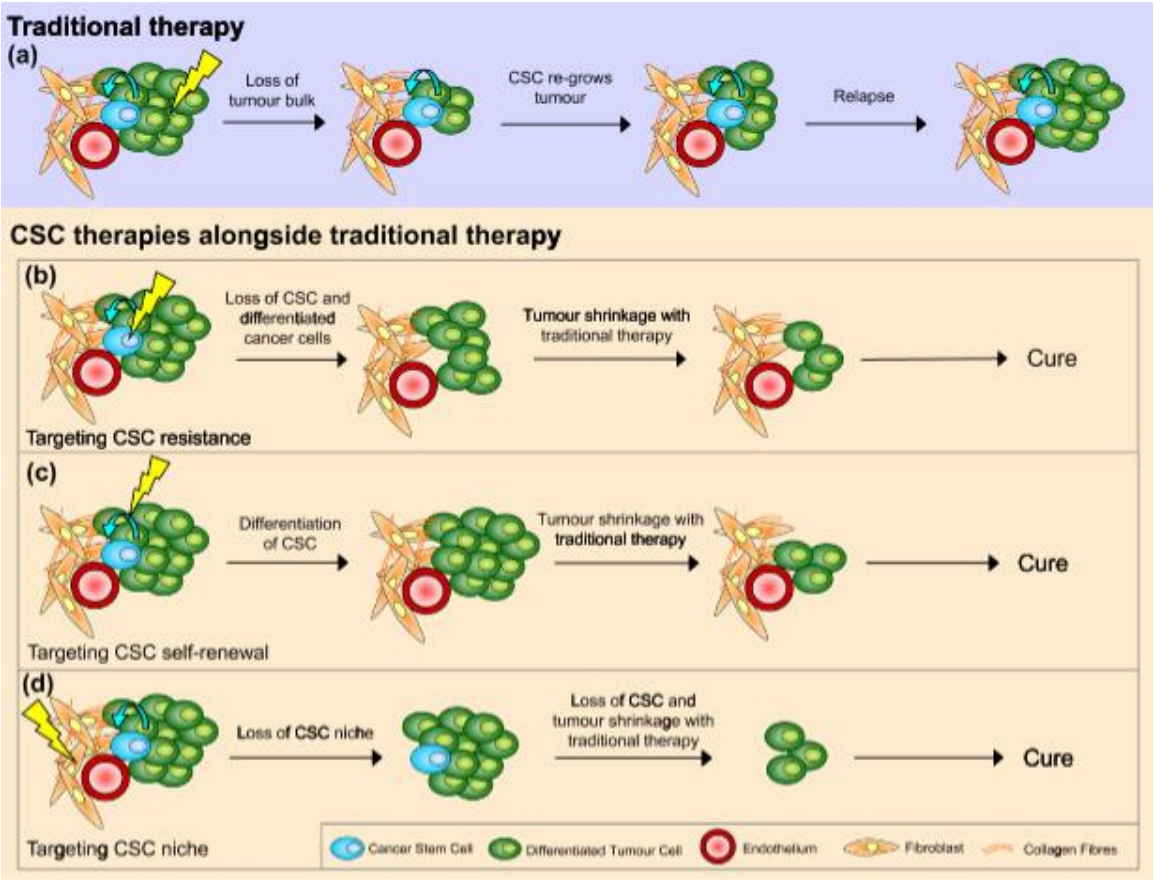

Figure X: This diagram focuses on the way in which a cancer therapy targeting cancer stem cell populations has the potential to revolutionize the outcome of cancer diagnoses.[108] Currently, traditional therapies only target rapidly proliferating cells. While these cells do make up the bulk of tumors, remaining CSCs can repopulate the tumor area once the treatment is over. This is believed to be one of the major factors causing cancer relapse. Alternatively, if treatments target CSCs as well as the general tumor population, cancer could (theoretically) be completely cured. There are three potential avenues for targeting CSCs. These include targeting CSC resistance to current treatments, targeting their ability to act as stem cells in a self-renewal capacity, or targeting the microenvironment that allows them to thrive in standard conditions.

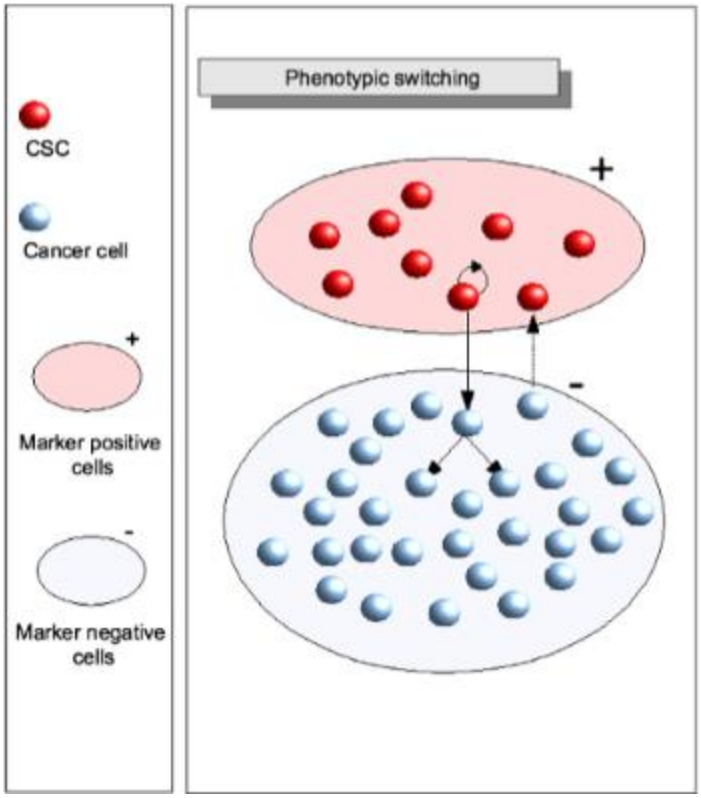

Figure XI: The figure to the left is a depiction of phenotypic switching theory. Non-CSCs (in blue) testing positive for a CSC biomarker indicate a phenotypic switch between a normal tumorigenic cell and a cancer stem cell.[5]

CSC biomarker on their surface.[122] Phenotypic switching is not well understood, and it adds another level of complexity to how BCSCs fit into therapeutic strategies for breast cancer. In fact, it has been alternatively hypothesized that all CSC biomarkers can be expressed on a small subgroup of normal tumor cells.[118] This ideology, if true, takes away some of the legitimacy of CSC biomarkers as a means for therapy discovery.

Altogether, CSCs are still a highly debated topic. While the idea behind CSC-directed therapy makes sense in theory, its actualization has been complicated by a limited pool of knowledge, issues with biomarker identification, phenotypic switching, and the final argument of whether CSCs are an accurate theory at all.

CHAPTER 4: TIEN HSIEN LIQUID

The Direct Tumor Targeting of THL

For the past twenty years, Tien Hsien Liquid (THL) has been used as a supplement for cancer patients. The formula is made up of extracts from fourteen different herbs. These herbs are *Cordyceps sinensis, Oldenlandia diffusa, Indigo pulverata levis, Polyporus umbellatus, Radix astragali, Panax ginseng, Solanum nigrum L., Pogostemon cablin, Atractylodis macrocephalae rhizoma, Trichosanthes radix, Clematis radix, Margarite, Ligustrum lucidum Ait, and Glycyrrhiza radix*.[123]

While THL remains underexplored, the body of research supporting its use continues to grow, with several findings that are of particular interest. Altogether, Tien Hsien Liquid has been observed to produce immunomodulary effects and tumor metastisis inhibition.[24,124] Most importantly, THL has been implicated in cancer stem cell inhibition.[24]

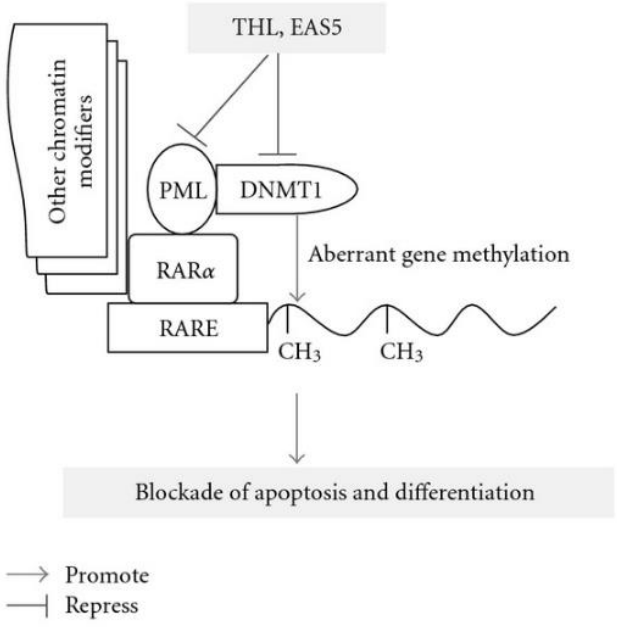

Figure XII: THL represses the DNMT1/PML-RARα complex. When this complex is deactivated, apoptosis occurs.[123]

In a study examining the effects of THL on a promyelocytic leukemia cell line, researchers found that THL decreased levels of cyclin A and cyclin B1.[123] This resulted in cell lines arresting at the G_2/M phase and eventually going through apoptosis. Specifically, the researchers found that apoptosis was initiated by a THL-associated decrease in PML-RARα and DNMT1 proteins. In cancer cells,

DNMT1 (DNA methyltransferase 1) is a chromatin modifier involved in DNA methylation. Hypermethylation caused by these modifiers has been shown to downregulate tumor suppressor genes.[124] In turn, the downregulation of DNMT1 and its recruiter protein, PML-RARα, causes cell death via apoptosis once the hypermethylation pathway is shut down. This relationship is displayed as a diagram in Figure XII. The researchers in this study also identified a specific part of THL, named EAS5, that contained the part of THL responsible for the compound's oncolytic activity. A follow-up study found that THL worked in a nearly identical regulatory way when breast cancer cell lines were tested instead of promyelocytic leukemia.[124]

Additional research has found similar results. One study on cell lines found that, while THL was able to induce apoptosis in fifteen cancer cell lines, it did not induce apoptosis in healthy human cells.[125] Experiments have also uncovered that THL inhibits tumor migration and invasion, inhibited tumor growth, and prevented angiogenesis in various models.[23] THL also inhibited the hypoxic microenvironment in breast cancer cells. This resulted in a reduction of factors known to cause tumor proliferation.[23]

THL Activity in CSC Populations

Recent cancer research has looked to the DNMT1 pathway as a way of targeting and identifying cancer stem cells.[126] It has been found that DNMT1 regulation is a key player in the transition from actively mitotic tumor cells to CSCs.[127,128] In fact, methylation inhibitors are currently being studied as novel anticancer agents.[128] Specifically, high rates of methylation are associated with cancer metastasis.[127] One study found that DNMT1 reduction directly correlates with an increase in the cancer stem cell phenotype.[128] These studies provide strong evidence that THL plays a legitimate role in

combating cancer through methylation pathways. It has also been identified through direct experimentation that THL has the ability to suppress cancer stem cell genesis.[24,129]

Indirect Tumor Targeting of THL and Toxicity

Outside of the direct role that THL plays in targeting cancer cells, the solution has also been shown to affect the immune system.[130] In patients with recurrent aphthous ulcerations, THL has been found to increase proliferation of peripheral blood mononuclear cells.[23] In addition, THL has demonstrated immune system activity in cancer models.

One study identified that THL possess immunomodulary capabilities in cell lines and animal models.[130] Most notably, the researchers found that THL increased NK cells and their activity as well as CD4+ T-cell levels. Complementary to this, the mouse models were also found to have increased levels of several major immune system components: IFN-γ, IL-2, and TNF-α. While all of these molecules have a number of different functions, it is sufficient to say that increased levels of cytokines, including interleukins and necrosis factor, implicate a positive immune system arousal because of THL administration.

In terms of safety, THL has no reported side effects. THL was tested in one clinical trial, where it was found to be non-toxic, although mild constipation and itching were reported.[26] While the trial was for patients diagnosed with refractory metastatic breast cancer and did show promising results, the small size of the trial prevents the data from being accepted as anything more than a positive indicator.[24]

Altogether, Tien Hsien Liquid has properties that make it novel for the purpose of cancer therapy. It displays direct anti-tumor activities in a multitude of pathways. In addition, it has been found to be nontoxic *in vitro* and in a human clinical trial. Most

importantly, however, is the ability of THL to directly target cancer stem cells. Given the complexity of CSCs, this property makes THL particularly striking.

DISCUSSION

One of the greatest difficulties in the fight against cancer is the vastness of what remains unknown.[131] This puts cancer research in an interesting position. On one hand, until every form of the disease is accurately documented and explored on a subcellular level, therapies will always, in some way, be taking a shot in the dark. Historically, the attempt to cure cancer has focused on identifying specific aspects of cancer and then using those identifiers to target the disease. This has been a tricky balance to find.[132] Treatments like radiation therapy and chemotherapy are not specific enough, and this results in damage to healthy tissue.[1,15,45,133]

The problem now relates back to the key metaphor. If every type of cancer is a box with a different lock, understanding every aspect of one lock will not solve the entire problem. Making a very specific key might be the best way to open one box, but it will also, in turn, guarantee that the key cannot open any other box because of its specificity. Today, a universal biomarker for all cancer cells has not been found.[134] Given the heterogeneity of tumor cells, a solution will most likely require a multi-pronged approach, with different therapies targeting different subgroups of cells.[135,136] In curative chemotherapy, for example, a minimum of 2-3 different types of compounds are used.[135] Because of the radical difference between CSCs and rapidly proliferating cancer cells, an approach with multiple fronts seems much more feasible than attempting to find common ground between the two groups of tumor cells that still remains unique from healthy cell populations.

Looking at the scientific literature, H-1PV appears to fill the gap of targeting rapidly dividing cell populations. While other oncolytic viruses also have the ability to target tumor cells and cause apoptosis, the majority of these viruses include S phase inducing mechanisms. This means that other oncolytic viruses, such as the

adenoviruses, have the ability to inflict harm on normal human cells by forcing them to enter into the S phase and then beginning viral proliferation.[68] H-1PV, alternatively, can only proliferate in cells that enter the S phase of their own volition.[71] While other oncolytic viruses have been genetically modified to be cancer-specific in an attempt to solve this problem, this introduced specificity presents a new issue that is two-fold. First, the cost of production increases by making manufacturing more laborious. While production costs are typically low, the added research and development cost associated with making viruses that are cancer-specific translate into higher treatment cost.[137,138] Second, any introduced cancer specificity typically takes the form of proteins binding to specific extracellular biomarkers that have been determined to be unique to cancer cells.[139] Once again, by genetically modifying a virus to increase specificity, the research creates a narrow window for therapeutic use, because the specific treatment runs the risk of losing the ability to recognize parts of the heterogeneous tumor population.[139]

In this regard, H-1PV is unique in its ability to selectively target cancer cells and induce apoptosis. Moreover, unlike other oncolytic viruses, H-1PV is particularly special in its relationship with the immune system. Because H-1PV is native to rodents, it does not illicit a human immune response against itself.[17] It does, however, activate an immune response directed toward cancer cell populations.[90]

Furthermore, the first clinical trial involving H-1PV showed promising results on a clinical scale (Summary of results).[58] Although the results have not been directly published, a subsequent article stated that patients remained healthy after receiving repeated doses of H-1PV (following the trial) in combination with bevacicumab.[99] Despite the results being unpublished as of yet, Dr. Bernard Huber, the CEO of the company running the trial, publically declared the trial a success.[140] More importantly, it was identified that H-1PV is nontoxic, which is how the drug met the compassionate use standards for re-administration.[99]

This collection of characteristics, in total, makes H-1PV remarkable. It does not have the specificity complication plaguing current avenues of cancer therapy, there is no immune response concern, and even if H-1PV is ineffective, it lacks the side effects characteristic to traditional treatment methods. Of course, H-1PV is not going to cure cancer on its own. In fact, it is well agreed upon in today's literature that cancer therapies need some multi-pronged approaches in order to target the heterogeneous tumor population.[135] Because H-1PV is dependent on S phase factors, it will never effectively destroy CSC populations. However, Tien Hsien Liquid does shown promise in this area.

There is a tendency, with new advancements, to compare them or offer them in conjunction with standard treatments. In fact, H-1PV has been tested in combination with traditional chemotherapy treatments and was found to be very effective.[96] However, beyond this, there is a gap in cross application between new innovations. At this point, most CSC-specific treatments are still in the process of development, although a few drugs have reached pre-clinical and clinical trials.[141] At this point, the major roadblocks for current therapies aimed at targeting CSCs are a lack of drug specificity and effectiveness.[141] As a result, H-1PV must either wait on these therapies to catch-up, or H-1PV can enter subclinical and clinical trials as a standalone treatment.

In this respect, Tien Hsien Liquid plays a unique role. THL has shown strong promise in anti-tumor activity.[126] Because THL has been used for centuries, has no known side effects, and is currently market-approved (albeit on a naturalistic scale), it is a unique candidate for combination therapy with H-1PV. It's role in the DNMT1 pathway suggests that, not only does THL target cancer cells, it does have a specific mechanism for targeting CSCs.[129]

For the sake of crossover, breast cancer is the best place to start when looking at these therapies in combination. Both H-1PV and THL have been studied extensively in

breast cancer models, and both have been shown to be effective independently.[26,56] With this type of background, it is possible to justify research that looks at the two treatments in combination. More importantly, the huge impact of breast cancer in terms of diagnoses, mortality, and cost, makes it a prime target for innovative therapies. As previously discussed, breast cancer is the second leading cause of cancer-related mortality in women worldwide.[29] The economic cost is massive, and current treatments are largely ineffective.[45] Even when treatments are effective, the side effects of chemotherapy and radiation are unacceptable.

The advances with H-1PV give cause for some optimism, however. The virus has shown promising results, it translates well in human models, it has no major side effects, and its mechanisms have strong, naturally oncolytic properties. Although this is a definite step up from current treatments, H-1PV will never be a full cure for breast cancer by itself because of cancer stem cell populations in heterogeneous tumors. THL, given its current place in the market, is an optimal choice to begin exploration between H-1PV and cancer stem cell-targeting combination therapies. Combination therapy with drugs aimed at targeting CSCs will give H-1PV an opportunity to fully attack tumor populations, instead of only focusing on rapidly proliferating cells providing S phase factors. Because other CSC-targeting drugs tend to show low cancer specificity or specificity excludes part of the tumor cells, THL remains a strong contender for this combination therapy. Moreover, both of these drugs have negligible side effects. Altogether, their characteristics and the available research warrants further exploration of a combination approach between H-1PV and THL for the treatment of breast cancer.

In a room of boxes, H-1PV and THL are likely candidates for creating a more universal key. While the relative newness of both of these therapies in the realm of cancer research has prevented them from being explored in combination thus far, it is

these types of multi-front approaches that have become increasingly necessary in the fight against cancer as more is discovered regarding heterogeneous tumors.

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