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Screening and evaluation of small molecules targeting mutant p53 for potential cancer therapy

A Thesis in Biology

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Abstract

Cancer is the leading cause of death worldwide. Although cancer is a common disease, there are no commonly used therapies that can kill cancer cells without also harming normal cells. Cancer is a disease caused by the uncontrolled growth of cells due to mutations in their DNA. Tumor suppressor proteins in cells can prevent transformation: if the tumor suppressor protein is mutated, it cannot prevent cancer cells from multiplying. P53 is a tumor suppressor protein that is mutated in 50% of all cancers and signaling through pathways involving p53 is inactivated in almost all human cancers. It has been suggested that restoration of the tumor suppressor activity of the mutated p53 proteins by small molecules could be a significant cancer therapy; however, small molecules with desirable efficacy and safety have yet to be identified.

This project involves the screening and evaluation of small molecules that were synthesized in the RISE laboratory to find potential anti-tumor agents to target mutant p53 and restore tumor suppressor activity. One tumor suppressor activity of p53 is its ability to induce apoptosis in cancer cells. We have developed an assay to screen a large number of small molecules to evaluate their ability to induce p53-dependent cell death. This assay measures the growth inhibition activity of small molecules in two transformed cell lines, one with mutant p53, the colon cancer cell line DLD1, and the other without p53, H1299, a lung cancer cell line. This is a 96 well plate–based assay and has the capacity for large-scale compound screening. Using the this assay, we have screened 100 compounds from the RISE library of compounds and found 20 compounds with increased growth inhibitory activity against the p53 mutant cells compared to the p53 null cells, indicating p53-dependent growth inhibition. These potential lead molecules will be tested over a larger range of

concentrations to confirm their activity to determine their therapeutic index and to induce p53-regulated gene expression in future experiments.

Dedication

I would like to dedicate this work to the many people who have supported me and helped me throughout the entire research and writing process. First and foremost, I would like to dedicate this thesis to my loving and supporting parents, Gita Patel and Mukesh Patel. They are the reason why I was able to get through the four years at Drew University. This thesis is also dedicated to my grandmother, Dhiraj Patel whom I have learned my work ethic from and my sister, Avni Patel, who was a constant reminder that I can do anything I want to, this being one of those things. I would also like to dedicate this work to Anand Shah and Megan Chellew for the constant encouragement to continue my work in biology and for being loyal friends over the past four years. I would like to thank Joshua Hsu, Kaushaly Patel and Allison Nadler for always believing me and making me smile during my writing process. Last but not least, I would like to dedicate this thesis to all of the influential people I have met along the way who have encouraged me to always try my hardest, family and friends at my temples, BAPS and ISSO, my teachers from Burlington Township High School, and the faculty and staff from RISE, Spanish and Biology Departments at Drew University.

Screening and evaluation of small molecules targeting mutant p53 for potential cancer therapy

Honors Thesis in Biology by Runi Patel

Table of Contents

1. Introduction

1.1 Epidemiology of Cancer

Cancer is a disease that affects a wide range of people regardless of their gender, race or age. Since the development of medicine, the medical field has been interested in dealing with infectious diseases that can be treated with vaccines and antibiotics; however, medicine is now moving to treat more chronic diseases such as cancer, autoimmunity, and cardiovascular disease. Effective treatments or cures for these diseases have not yet been found. Cancer is a complex disease that does not have a cure in which the disease is totally eliminated; however, some treatments have been described and they are now being improved by researchers. Cancer garners a great deal of interest because it is one of the leading causes of deaths in the United States and around the world.

Cancer is a group of complex diseases caused by the uncontrolled growth of abnormal cells that are able to invade and grow in neighboring and distant tissues (American Cancer Society 2014). There are over a hundred known types of cancer. Many of these cancers are commonly named after the area or organs invaded. There are multiple stages of cancer that describe the growth of the tumors created by the uncontrolled growth of transformed cells. Transformed cells are those that are different from normal cells because they have mutations in their DNA that have uncontrolled growth properties. The staging of cancer indicates the severity of the tumors in the body. The higher the stage, the larger the tumor and the more advanced the disease.

The different types of cancers fall into specific subtypes. The five subtypes of cancers are carcinoma, sarcoma, lymphoma and myeloma, leukemia, and central nervous system cancer (National Cancer Institute 2014). One of the most common

types is carcinoma, which begins in skin or tissue cells that cover the lining of the internal organs and lead to tumor growth in epithelial cells; this type of cancer includes breast, colon, and lung cancer (National Cancer Institute 2014). Adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma are types of carcinomas that can occur. Sarcoma begins in supportive tissue like the bone, cartilage, fat, muscle, or blood vessels (National Cancer Institute 2014) and includes leukemia, a cancer of blood cells from the bone marrow that enters the blood stream (National Cancer Institute 2014). Both lymphoma and myeloma originate from the cells of the immune system as does leukemia; however, they represent transformation at a different stage of immune cell differentiation. Cancers of the central nervous system start in the tissues of the brain and spinal cord (National Cancer Institute 2014).

There are two types of tumors that are associated with cancer. A benign tumor is an overgrowth of transformed cells that cannot spread to neighboring tissues. Most of the time, benign tumors can be surgically removed and do not have any effect on the rest of the body. A malignant tumor is an overactive growth of transformed cells that are able to spread; moreover, the spreading of a malignant tumor into other areas is called metastasis (National Cancer Institute 2014). Once a cancer metastasizes, the patient is nearing a late stage of the disease, and metastasis usually leads to a loss of life. In many cases, people learn about their diagnosis in the late stages of cancer when the tumor has already spread to other anatomic locations, making it difficult to treat,as cancer has already taken its toll on the body.

Cancer is one of the leading causes of death in the United States. One out of two men and one out of three women is likely to be diagnosed with cancer (American Cancer Society 2014). Cancer can occur easily due to exposure to everyday

substances; therefore, it is said that everyone is at risk of getting cancer because of the high and increased risk of exposure to carcinogens that are in the environment (Cunningham & Cunningham 2012). According to the American Cancer Society, there were 13.7 million people who had survived cancer or were fighting cancer in January 2012 (American Cancer Society 2014). In addition, by the end of 2014, it is expected that about 1,665,540 people will have cancer (American Cancer Society 2014; Figure 1). About 584,720 people will have died in the year 2014 due to cancer, which is about 1,600 people a day (American Cancer Society 2014).

Figure 1 obtained from: American Cancer Society. 2014. Cancer Facts and Figures 2014. Atlanta, GA.
Titled: Estimated numbers of new cáncer cases for 2014

Figure 1: A map showing the number of the cancer cases by state in the United States. This source predicts a total of 1,665,540 people having cancer by the end of 2014 (American Cancer Society 2014).

1.2 Causes of Cancer

Cancer can be caused by multiple factors that affect the body like mutagens and carcinogens. Mutagens are agents that cause genetic damage and alterations, while carcinogens are agents that cause cancer (Cunningham & Cunningham 2012). In the twenty-first century, researchers and doctors are learning that even the slightest exposure to mutagenic activity can cause cancer and that there are multiple ways that cancer can arise in humans (American Cancer Society 2014; Cunningham & Cunningham 2012).

One of the ways that mutations can occur is via environmental effects like exposure to carcinogenic chemicals (Cancer 2013; Cunningham & Cunningham 2012). Carcinogenic chemicals cause mutations that result in cancer. In the twenty-

first century, the number of people with cancer rose in countries that released large amounts of toxic waste into the air such as the synthetic chemicals that are used in the food industry (Cunningham & Cunningham 2012). Similarly, living in a location where there is a large amount of industrial air pollution being released into the air is likely to cause lung cancer because of the chemicals that are being inhaled. There is also an increased risk of cancer in workers that are exposed to carcinogens in the workplace because of long-term exposure. In fact, according to the European Agency for Safety and Health at Work, 32 million people are exposed high levels of carcinogenic toxins in the workplace (Cunningham & Cunningham 2012). These chemicals cause mutations in the DNA of the cells in the body, causing these cells to divide uncontrollably. Cells are known to be mutated when their deoxyribonucleic acid (DNA) sequence is changed or different from the parent cells from which a cell originated. A transformed cell is a cell that is mutated in a way that it has abnormal growth properties. Transformation means that a cell has these growth properties leading to the uncontrolled growth of abnormal cells. In many cases of cancer, the DNA is mutated due to the carcinogenic chemicals that have an effect on the DNA that cannot be repaired (Ames et al., 1993). Research has shown that the toxins that are encountered when smoking tobacco can lead to mutations in a gene in the body that results in a mutated form of a protein called p53 (Gibbon et al., 2014). P53 normally regulates cell proliferation and survival. When p53 is mutated, it cannot control the growth of cells, leading to increased cancerous activity and overgrowth of cells.

In addition to environmental factors, there are behavioral habits that may contribute to cancer. For instance, many people neglect proper diet and exercise (Cancer 2013). There are certain fruits and vegetable that have vitamins A, C, and E

that may have anticancer effects, and people may not consume adequate amounts of these foods (Cunningham & Cunningham 2012; Danaei et al., 2005). Eating these foods could have beneficial effects in preventing cancer or protecting the body from other cancer-causing agents. Other habits that are closely associated with an increased risk of cancer are smoking and high levels of alcohol consumption (Denaei et al., 2005; Doll & Peto 1981; Gibbon et al., 2014). One-third of the 7 million cancer-related deaths in the world are associated with smoking or significant alcohol intake (Denaei et al., 2005). Thirty percent of diagnosed cancers are thought to be due to tobacco intake by smoking or chewing (Doll & Peto 1981). Viral infections can also cause cancer. When some RNA viruses infect cells, their genetic material is integrated into the host's genome and may alter cell division by altering transcription of nearby genes or may alter other cell processes by inserting in the middle of genes like tumor suppressors (Carrillo-Infante et al., 2007). This causes the overgrowth of virally infected cells to form the viral tumors found in infections such as those with Human Papilloma Virus (HPV), Epstein-Barr Virus, and hepatitis B virus (Carrillo-Infante et al., 2007). In addition, the expressed viral proteins can inactivate tumor suppressors like p53 and Rb as in HPV-transformed cells. The viral proteins can also promote uncontrolled growth in EBV-transformed cells. Viruses that cause cancer are called oncoviruses (Carrillo-Infante et al., 2007).

Increased cancer risks can be associated with race, ethnicity and gender. Women are more likely to develop breast cancers, while men are more likely to get prostate cancer due to the hormones and organs of each gender (Bond et al., 2007; Gann et al., 1996). When there are uncontrollably high levels of the hormone, estrogen and testosterone in women and men, respectively, these people are likely to develop cancer (Gann et al., 1996). Estrogen and testosterone have been shown to

increase the number of cells by influencing cell division (Bond et al., 2007). Race or ethnicity can also make a person more susceptible to cancer (Armstrong & Kricker 2001). For instance, people who have light skin are likely to have UV damage in their skin cells, which causes higher rates of skin cancer, compared to that of darker skinned people. This is because people with naturally darker skin have more melanocytes, which protect the skin from UV damage, while people with lighter skin do not (American Cancer Society 2014). UV damage can cause mutations to important cellular proteins such as p53. Mutations in this protein are especially relevant become they cause this protein to lose its ability to function in protecting the cells from further DNA damage (Armstrong & Kricker 2001).

Lastly and more importantly, mutations can also be passed down genetically from one generation to another, leading to a genetic predisposition to cancer (Bell et al., 1993). When genes are mutated, they can be passed down from one generation to next making that generation more susceptible to mutations (Gibbon et al., 2014). For instance, Li-Fraumeni syndrome is a genetic disorder found in children and young adults that is caused by mutations of p53, which makes the person more susceptible to cancers because the protein cannot function properly (Malkin et al., 1990).

It is possible that all of these factors can influence a person at the same time. When this occurs, mutations are more likely to lead to transformation. It is hypothesized that a single mutation in the somatic cells is not sufficient to cause cancer and that multiple mutations would have to occur to transform these cells in order for cancer to arise. This theory is known as the multi-hit hypothesis (Figure 2).

Figure 2 obtained from: http://www.bcpinstitute.org/reproductive.htm
Titled: Mutated Cells Mutating

Figure 2: This is an example of how breast cancer can develop as mutations are passed from one generation of daughter cells to another and new mutations are accumulated, causing tumors (Breast Cancer Prevention Institute 2007).

1.3 Detection, Current Treatments and Therapies

After extensive research, it has been shown that cancer can be treated with many different therapies; however, a cure has yet to be discovered. Before cancerous cells can be treated or before a physician can determine a treatment plan, a person must go through a series of tests in order to determine whether they have cancer and what kind of cancer they have. It is also beneficial for the physician and the patient to know what stage the cancer is in. This is why early detection of cancer can improve therapy options and frequent testing can help to ensure that people can remain healthy. In many cases, tests like mammograms and colonoscopies are recommended for early cancer detection.

Cancer can be detected in various ways depending on the location of the cancer and its stage of progress. Blood tests can be done in order to test for cancer, checking for elevated levels of toxic materials in the blood like creatinine, which shows that the kidney is malfunctioning (Smith et al., 2001, Stanford Medicine 2014). In a similar fashion, a urine test can be conducted to test for the presence of toxins or access to proteins that are being released into the urine (Stanford Medicine 2014). Tumors in the body can be detected using tumor markers: proteins that are released into the blood once a tumor is formed (Stanford Medicine 2014). These tumor markers can also help show how well a person is responding to treatments as their levels decrease if the treatment is working or increase if the tumors continue to grow.

However, this technique cannot be used to test all cancers because not all cancers will make known markers. Various endoscopies can be performed in order to view the internal locations of where a tumor might accumulate (Stanford Medicine 2014). Endoscopies are pictures that are taken using a small tube with a lens in order to see into small internal canals like the stomach and esophagus (Smith et al., 2001). Another method to look for tumors is through the use of x-rays or computed tomography scans (CAT) to take images of the body to locate unusual masses that could be cancerous (Smith et al., 2001). Once a tumor is found, a tumor biopsy can be taken, which allows for oncologists to determine the type and stage of cancer (Smith et al., 2001).

When a tumor is observed, the fastest and easiest treatment method is to remove as much of the cancerous tissue as possible through surgery after locating the exact region. This procedure works well when the tumor is confined within an organ and in some cases the entire organ is removed. However, the drawback of removing the tumor via surgery is that some cancer cells may be left (Cassileth et al., 1984), which can re-form the tumors that were once there (Cancer 2013; National Cancer Institute 2014). In other cases, tumors are located in tissues that cannot be surgically removed, which results in the need for other treatments.

Radiation therapy can also be used to eliminate tumors. Radiation therapy involves the killing of cancerous cells with the use of high-energy radiation. This radiation causes DNA damage to the cancer cells and results in cell death. One of the drawbacks of radiation is the risk involved as one must optimize the lethal effects on the cancer cells and minimize the amount of damage that is caused to normal cells. Because normal cells are being killed, harmful side effects like fatigue are

experienced. Radiation therapy is frequently given in conjunction with chemotherapy, which increases side effects.

Chemotherapy is a treatment that involves the intake of chemical drugs by injection, oral administration, or topical administration to kill rapidly dividing cells (Cancer 2013; National Cancer Institute 2014). Some examples of chemotherapeutic agents include cisplatin, a platinum drug, that destroys the DNA of transformed cells to prevent future reproduction (Cancer 2013). Nonetheless, chemotherapy can be used to control tumors by limiting cell proliferation and can be used to control the pain or pressure that is caused when the tumor is growing rapidly. One of the major drawbacks is that chemotherapy is nonspecific and will also target normal cells, a similar drawback to radiation therapy.

Some of the most recent therapies that are currently on the market target cancer cells by specifically interfering with specific molecular targets required for tumor growth. This approach is more tumor specific than radio- and chemotherapy and thus is less harmful to normal cells; therefore, the side effects that are experienced during radio- and chemotherapy are not felt as severely. Additionally, this approach enables the tailoring of treatment for individual patients as each patient may have a unique mutation. These targeted therapies such as Gleevec, Irressa, Tarceva, Avastin, and Herceptin are used alone or in combination with chemotherapy.

Two of the approaches can be used to inhibit a specific molecular target for tumor growth inhibition. One involves a monoclonal antibody that binds to the extracellular domain of a growth factor receptor or growth factor and prevents the binding of ligands to the receptor. A second approach involves a small molecule that binds to the intracellular kinase domain of that receptor and inhibits the kinase activity, which is termed a kinase inhibitor (Shawver et al., 2002).The places where

targeted therapies can affect a growth factor are illustrated below using EGFR (Epidermal Growth Factor Receptor) (Figure 3 and 4). An antibody can bind in the area of the receptor where the growth factor, EGF (Epidermal Growth Factor) normally binds to prevent the receptor dimerization and conformational change (Ciardiello et al 2008). Such monoclonal antibodies are highly specific for the extracellular domain of EGFR. Examples of monoclonal antibodies are Vectibix (anti-EGFR), Erbitux, and Herceptin (anti-EGFR). The small molecular approach of targeted therapy involves the inhibition of EGFR kinase activity using a tyrosine kinase inhibitor that hinders the autophosphorylation and transmitting signals from this receptor to downstream proteins (Shawver et al., 2002). Some examples of these therapies are Tarceva (EGFR inhibitor) and Tykerb (HER2/EGFR2 inhibitor).

There are multiple therapies that can be used to treat cancer. Most of these targeted therapies do not kill all cancer cells and in many cases, the tumor develops resistance against these targeted therapies. It would be beneficial to find a therapy that would be able to do away with cancer without unbearable side effects, that kills all cancer cells, and that does not elicit resistance.

1.4 Biology of Cancer

Cancer is the overgrowth of abnormal cells that proliferate and cause tumors due to DNA damage. When this DNA is not repaired, daughter cells receive mutated DNA, which can lead to overgrowth and tumors if cancer-related genes are mutated. Mutations are passed down from one generation of cells to another, which is how cancer cells replicate. Cancer can occur in any given anatomic location if the cells have the proper environment to thrive.

After a reevaluation of the causes of the abnormal growth of cells and the genes that cause cancer, two types of genes were discovered: oncogenes and tumor suppressor genes (Croce 2008). Defects in these genes are responsible for the transformation of normal cells into cancer cells (Croce 2008). After validating the tumor-promoting activity of these genes, scientists can use them as targets for antitumor agents (Croce 2008). Proto-oncogenes stimulate the appropriate cell growth in the body (Croce 2008). In normal cells, a proto-oncogene is switched on and off depending on the cell's need to divide. Figure 3 shows the need for a growth factor needed for the activation of downstream proteins to induce DNA replication and mitosis. Each time a protein is activated and sends a signal to the next protein, the protein is deactivated. The proto-oncogene is on when the cells need to divide and is off when the cells do not divide (Croce 2008). In cancerous cells, proto-oncogenes are constantly switched on, signaling to the nucleus to induce cell division, and these constantly activated genes are now called oncogenes (Croce 2008). The continuous cell signaling for DNA replication is seen in Figure 4, the signal is constantly sent to the nucleus for DNA and cell replication. Tumor suppressor genes, like p53 and Rb can signal the production of checkpoint proteins to contain the growth of the cells. In cancer cells, these tumor suppressor genes are inactivated due to mutations (Croce 2008). The tumor suppressor protein p53, which is also known as the guardian of genome, is inactivated in almost all human cancers.

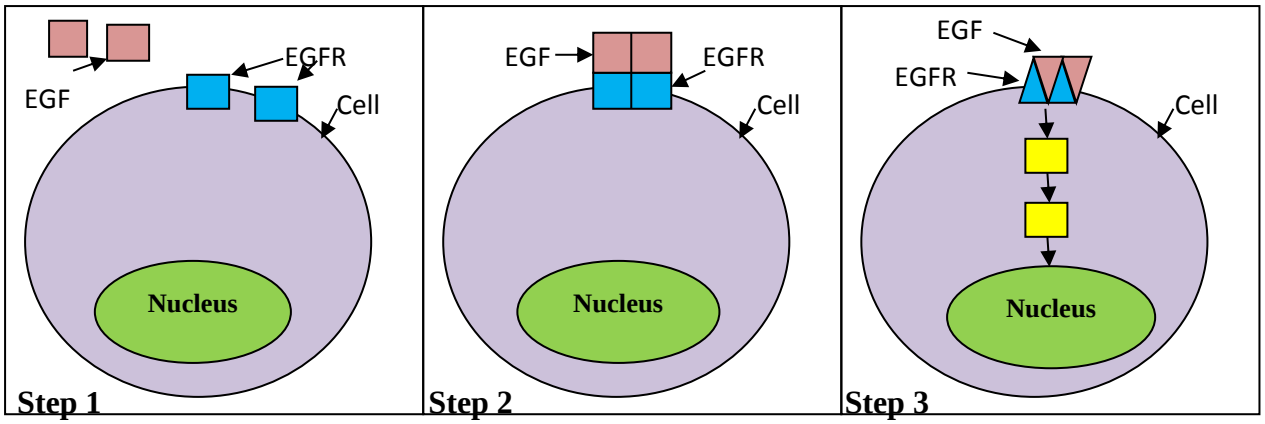


Figure 3: The intracellular signaling cascade that in normal cells. Step 1 shows EGF before it is attached to EGFR; therefore, there is no intracellular signaling to the nucleus. Step 2 shows EGF attachment and EGFR dimerization and Step 3 shows the intracellular signaling cascade down to the nucleus (fewer proteins were drawn for simplicity) (Croce 2008).

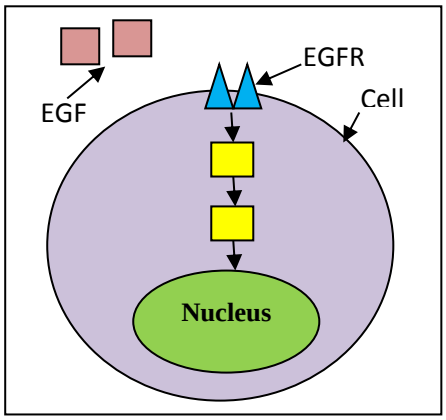


Figure 4: The process of cell signaling in cancer cells is illustrated above. When EGF is not bound, the signal is still transmitted because EGFR is constitutively dimerizing (Croce 2008).

1.5 Function of p53 in Normal Cells and its Role as a Tumor Suppressor in Transformed Cells

One of the most important tumor suppressor proteins is p53, which plays a variety of roles in cellular regulation. P53 is able to regulate cell division and cell death when there are various stresses on the cell (Soussi 2012). When there is DNA damage, p53 is activated and pauses the cell cycle to allow for repair or lead to death

of the damaged cells if the damage is beyond repair (Soussi 2012). In many cancers, evasion of apoptosis occurs due to the inactivation of p53. When this occurs, p53 signals the nucleus to lead to expression of downstream proteins like Bax, PUMA, and other proteins. Bax leads to the release of cytochrome C from the mitochondria, which can lead to apoptosis. When p53 is mutated, a signal cannot be sent to the nucleus for the expression of these downstream proteins; therefore, cell death cannot occur, ultimately leading to cancer (Soussi 2012).

P53 responds to signals from DNA damage, hypoxia, and oncogene activation (Soussi 2012). These stresses include DNA damage causing mutations or breaks, UV radiation, and mitotic spindle damage leading to the release of p53 from MDM2 (Soussi 2012). MDM2 is a negative regulator of p53. In other words, there are low levels of p53 in normal cells because MDM2, which is also a transcriptional product of p53, catalyzes p53 degradation. However, when there are stresses on the cell, MDM2 no longer regulates p53 and the level of active p53 is increased (Momand et al., 1992). When p53 is activated in response to a specific stress, it can transcribe appropriate downstream proteins needed to address the stress. For instance, it can transcribe downstream proteins like BAI-I, which can be used to prevent angiogenesis (Soussi 2012). It can also transcribe p21 in order to initiate growth arrest (Soussi 2012). Furthermore, p53 can transcibe p53 R2 in order for DNA repair or transcibe Bax in order to initiate apoptosis (Figure 5) (Soussi 2012).

P53 is needed in order to control the abnormal growth of cells and to protect the integrity of the cell when damages or stresses are present. Because p53 is inactive in over 50% of cancers, reactivation of p53 is of great interest for biologists and the pharmaceutical industry (Figure 6) (Levine et al., 1991).

Figure 5 obtained from: http://p53.free.fr/p53_info/p53_Pathways.html
Titled: p53 pathways: downstream pathway

Figure 5: The regulation of cells via p53 and downstream proteins is illustrated in this figure, which shows the proteins needed for halting of angiogenesis, growth arrest, DNA repair, apoptosis, and blocking angiogenesis (Soussi 2012).

Figure 6 obtained from: http://p53.free.fr/p53_info/p53_cancer.html
Titled: p53 Mutation- Worldwide distribution of cancers and p53 Mutations

Figure 6: The tumor suppressor protein p53 is mutated in many cancer types worldwide (Soussi 2012).

In cancers where p53 is not mutated, the p53 pathway may still pay a role in transformation. For example, in some cancers, p53 functions are inhibited due to hyper-activation of negative regulators like MDM2 (Kubbutat et al., 1997). When there is an amplification of MDM2, this protein can bind to p53 and ubiquitinate it for degradation. This would allow for the cancer cells to continue growing (Kubbutat et al., 1997).

Another mechanism that can disrupt p53 from being able to function properly is a mutation in the DNA binding domains of the protein; moreover, this leads to unfavorable conformational changes and a loss of function (Ventura et al., 2007). Extensive research has indicated that majority of the oncogenic mutations in p53 are missense mutations and are located in DNA binding domain. There are six spots in particular where p53 is mutated with high frequency in multiple cancers (Figure 7). These mutations induce conformational changes in p53 protein resulting in reduced stability at physiological temperature and loss of DNA binding activity. Because p53 is mutated in many cancers, research is in progress to discover molecules that can restore tumor suppressor activity of mutant p53 for therapy development.

Figure 7 obtained from: Joerger AC, Fersht AR. 2007. Structure-function-rescue: the diverse nature of common p53 cancer mutants. *Oncogene* 26: 226-2242.
Titled: Figure 1 a : Structure of p53

Figure 7: The locations of mutation hot spots in p53 are illustrated in this figure (Joerger and Fersht 2007). These mutations occur in the DNA binding domain of this protein.

2.6 The Six Hallmarks of Cancer

There are six characteristics of cells that lead to transformed cells to result in the disease of cancer are called The Six Hallmarks of Cancer. These characteristics can be seen in Figure 8.

Figure 8 obtained from: Hanahan D, Weinberg RA. 2000. The Hallmarks of Cancer. *Cell* 100: 57-70.
Titled: Figure 1 : Acquired Capabilities of Cancer

Figure 8: The six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion & metastasis, limitless replicative potential, sustained angiogenesis, and evasion of apoptosis (Hanahan & Weinberg 2000).

One of the six hallmarks of cancer is self-sufficiency in growth signals (Hanahan & Weinberg 2000). When cells need to replicate, a signal is sent to their nucleus via receptors on the cell surface that promotes DNA replication and mitosis to form new daughter cells. Cancer cells are able to generate their own growth signals by altering the cell receptors on their cell surface. This makes them hyperactive via mutations that can send nonstop cell growth signals. Alternatively, cancer cells can actively generate the needed growth factors.

The second hallmark of cancer is that cancer cells are insensitive to antigrowth signals (Hanahan & Weinberg 2000). Tumor suppressor proteins are not able to function to hinder the growth of these cells. Tumor suppressor proteins are those that

halt the proliferation of cells and act as regulators of cell growth. These proteins are essential to balance growth and anti-growth cell signaling pathways. However, if a protein like p53 is mutated, inhibitory growth signals cannot be sent to the nucleus in the cell.

The third hallmark of cancer is limitless replicative potential, allowing for a large amount of cell replication and ensuring the growth and spread of cancer (Hanahan & Weinberg 2000). Since there is normally a limit to how many times a cell can divide, this hallmark is especially important. In normal cells, telomeres at the end of chromosomes regulate how many times a cell can replicate; however, in cancer cells, telomeres are re-built in order to avoid limits to the replication process.

The fourth hallmark of cancer is tissue invasion and metastasis (Hanahan & Weinberg 2000). Metastasis occurs when cancer-causing cells spread throughout the body during the malignant stage. The cancer cells can enter the blood stream, carrying the disease throughout the entire body. This allows for the spread of cancerous cells to other regions of the body. There are also mutations that lead to hyperactivation of extracellular proteases that help lead to invasion and metastasis. These proteases allow the tumors to cut into other tissues to grow and spread.

The fifth hallmark is angiogenesis, which is the generation of new blood vessels. As a tumor mass grows, it may become so large that oxygen and other nutrients cannot penetrate to inner cells. These cells need to obtain energy and nutrients to continue their proliferation in the body. Capillaries are located throughout the body to obtain oxygen from the lungs and nutrients from the digestive system. Angiogenesis occurs when cells in the tumor need the proper nutrition to be able to thrive; therefore, cancer cells are able to induce the formation of new blood vessels to bring them nutrients.

The last hallmark that cancer cells adopt is the ability to evade apoptosis, which means that the cancer cells do not undergo this form of programmed cell death (Hanahan & Weinberg 2000). In normal cells, when the damaged DNA cannot be repaired, the cell is able to undergo programmed cell death that is known as apoptosis. When the environment is not beneficial to the cells, such as in nutrient deprivation conditions, they can also undergo cell death via a pathway that leads to the activation of p53. Cancer cells avoid death by inactivating p53 pathway and are immortal.

Many of these properties of cancer cells are regulated by the tumor suppressor protein p53 as seen in Figure 5. In response to various stresses, including DNA damage, oncogene activation, altered telomeres, metabolic stress and many others, p53 is activated and regulates the expression of many genes. This interferes with and inhibits biological properties of cancer cells. Since p53 is inactivated in almost all cancers, restoration of the tumor suppressor activity of p53 will have significant impact on cancer therapy. In fact, a gene therapy approach using adenovirus-mediated expression of p53 has demonstrated potential in treating cancer patients (Hayashi 2005).

1.7 Reactivation of mutant p53 by small molecules

As p53 is mutated in over 50% of human cancers and cancers with mutant p53 are resistant to chemo and radiation therapy, restoration of tumor suppressor activity of mutant p53 should have a significant impact in cancer therapy. Multiple strategies have been pursued in order to reactivate the tumor suppressor function of mutant p53. One is to create a back mutation or reversion that would compensate for the first mutation seen in naturally inactivated p53 (Wieczorak et al., 1996). For instance, Wieczorek and his lab believed that a back mutation could induce a conformational

change creating an interaction between the amino acid side chains of p53 and the phosphate backbone of DNA that would restore p53 to its native function. These researchers were successfully able to restore the transcriptional activity of some mutant p53 proteins by a second site back mutation, clearly suggesting that conformational change can restore tumor suppressor activity in mutant p53 (Wieczorak et al., 1996). This approach, however, is challenging to implement for therapy development.

Reactivating mutant p53 using small molecular compounds is seen as a promising approach for treating cancers (Bykov et al., 2002, Wischhusen et al., 2003). The reactivation of mutant p53 by small molecules restores the function of p53 to its native form. Some examples of small molecules that have been shown to reactivate mutant p53 are PRIMA 1 (Bykov et al., 2002) and the Schering Plough compound, SCH529074 (Demma et al., 2010). Their structures can be seen in Figure 9. These compounds have shown to reactivate mutant p53 in cancer cells and have antitumor activity in appropriate animal models. However, these compounds have not shown the efficacy and the potency required of a potential pharmaceutical drug. The PRIMA 1 compound has been shown to induce apoptosis of cancer cells in mice and does not show any other side effects (Bykov et al., 2002). Although recently, PRIMA 1 was studied in humans as part of phase 1 clinical study, improvements in efficacy and safety are needed for further development. PRIMA 1 acts as an alkylating agent to alkylate cysteine residues in mutated p53 and may have unwanted toxic effects (Kaar et al., 2010).

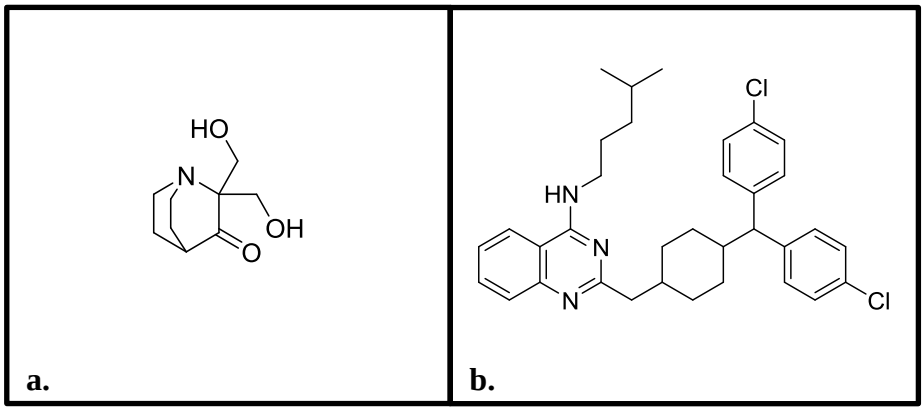


Figure 9: The structures of the two lead compounds discovered that reactivated mutant p53: PRIMA-1 and SCH529074 (Bykov et al., 2002; Demma et al., 2010).

Another compound that has undergone extensive research is SCH529074. This compound was shown to bind to the p53 DNA binding region and reactivate mutant p53 using a chaperone mechanism (Demma et al., 2010). The chaperone theory implies that the small molecule binds to mutant p53, which allows for the new stabilized complex to enter into the nucleus and bind to the DNA (Figure 10) (Friedler et al., 2002), while mutant p53 cannot bind to DNA alone. Mutant p53 is stabilized when the small molecule is bound, allowing it to create a p53-DNA complex to induce transcription, at which time the small molecule is released (Friedler et al., 2002). The potency of SCH529074 was low and this compound had a low therapeutic window. Although other efforts continue to occur to discover small molecules that will restore tumor suppressor activity in mutant p53, novel molecules with superior efficacy and improved safety profiles are yet to be discovered.

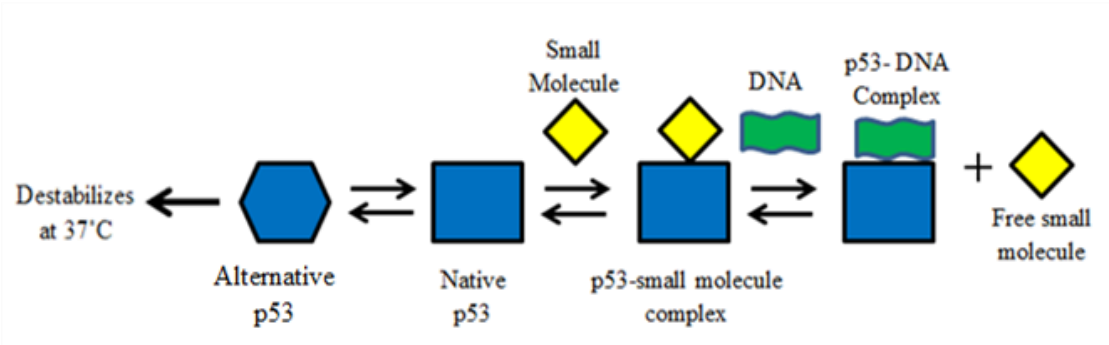


Figure 10: An illustration of the chaperone strategy showing a small molecule that is able to bind native p53 and stabilizing the complex. The alternative p53 degrades quickly at 37°C. The complex can then enter the nucleus and can function as a transcription factor brining to DNA. The DNA will attach to p53 and the small molecule will be expelled allowing it to bind to other p53 in the native form shifting the equilibrium of the inactive conformations to the native forms (Friedler et al., 2002).

1.8 Previous and Current Research: Hypothesis and Goals

It is clear that a small molecule approach to reactivate mutant p53 has potential for the development of cancer therapeutics and many efforts are ongoing at different institutions to identify novel lead molecules. In many cases, pharmaceutical companies screen libraries of compounds for potential anticancer drugs. Our laboratory is using various cell-based assays to follow the reactivation of mutant p53 after the treatment with small molecules. Briefly, tumor cells harboring mutant p53 are treated with various compounds and expression of p53-regulated genes (e.g., p21) via western blot protein analysis is used to show reactivation of mutant p53. Using this assay (p21 western assay) our laboratory, in collaboration with Dr. Ronald Doll, another RISE fellow, is involved in screening a number of compounds related to an angiogenesis inhibitor reported by researchers at Johns Hopkins University (Liu 2010). Many compounds have been shown to induce expression of p21; however,

their efficacy is low. Dr. Doll's laboratory is making various analogs to these active compounds and the evaluation of these compounds is ongoing.

In addition to the p21 western blot assay, a cell viability assay was also developed (MTT assay, Promega Inc) using p53 mutant and p53 null tumor cells to evaluate p53-dependent versus p53-independent tumor cell growth inhibition by these small molecules. Small molecules reactivating mutant p53 would induce apoptosis. P53 null cells have a p53 deletion; therefore, cell death in the null cells is due to factors other than p53 reactivation. The cell viability assay can also be used for large scale screening of unknown compounds for the discovery of novel compounds with the ability to reactivate mutant p53. A library of compounds that was synthesized by former RISE fellow, Dr. Houlihan, that may have potential anticancer abilities was accessible. I screened these compounds using the cell metabolism assay to discover potential novel molecules that inhibit the growth of tumor cells with mutant p53. The library of 100 compounds was given the naming of VG 1 through VG 100 for simplicity.

The goal is to use the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay to find compounds that can kill p53 mutant cancer cells yet do not affect cells without p53, known as p53 null cells. We want a compound that targets the cells with mutated p53 rather than cells that do not have p53 because it would show that reactivation of p53 induced cell death. If both cell lines were killed with a compound, it would not be effective as a potential drug because it is not targeting mutated p53 but may be targeting a factor that they both have in common or may be generally toxic to mammalian cells.

The preliminary screening was performed to find compounds that were only effective against cells with mutated p53 and relatively less against p53 null cells. The

next step will be to examine the therapeutic window by running cell viability assays over a range of concentrations of the compounds to determine the IC_{50}. This would help to determine which compounds are potential anticancer compounds. A large therapeutic window would mean that it would take less of a compound to kill 50 percent of the cancerous cells. The compound that is able to kill 50 percent of the cancerous cells at a low concentration is a more desirable the compound as a drug as high concentrations of a particular foreign compound may be toxic and expensive. After eliminating those compounds that have a low therapeutic window, the p21 western blot assay will be conducted. This will help to confirm whether the compounds that show effectiveness against the mutant p53 cell line and not the p53 null cell line do in fact have an effect on mutant p53. The western blot will help to confirm that p53's function is restored. In this assay, we would screen for p21, which is a downstream protein that is produced when p53 is activated.

The cell viability and the western blot assays can be used to screen and evaluate the library of 100 compounds in order to test their ability to kill cancer cell lines with mutant p53 and p53 null cells. Therefore, a compound has the ability to function as a potential p53 re-activator if it can inhibit the growth of cancer cells with mutant p53 while not affecting cells that have no p53. Compounds inducing cell death in p53 null cells would not be inducing death due to p53 activation since these cells lack p53.

2. Materials and Methods

2.1 Tissue Culture Technique

The cells were handled using aseptic technique using a laminar flow hood. DLD1, a colon cancer cell line, and SF295, a brain cancer cell line, have mutated p53. H1299, a lung cancer cell line, was used as the p53 null cell line. The cells were

obtained from the Cancer Institute of New Jersey. The DLD1 and SF295 cell lines grow in RPMI (Roswell Park Memorial Institute) media and H1299 cells grow in DMEM (Dulbecco's Modified Eagle's Medium) growth media. These medias were supplemented with 10% FBS (Fetal Bovine Serum) and 1% Penicillin/Streptomycin. The cells were frozen at about -196.0 °C in 5% DMSO in a liquid nitrogen tank as stocks. The stock vials were thawed in a water bath (37.0 °C). The 1 mL of cells in the vials was placed into 9.0 mL of the appropriate culture media in a 10.0 cm tissue culture plate. The media was changed after 24 hours with 10.0 mL of fresh media being added. The 10.0 cm plate of cells was incubated at 37.0 °C and 5.0 % CO_2 in sterile conditions.

The cells were fed every 2-3 days by the replacement of spent media with fresh media. When the confluency (coverage of the surface of the plate by cells) of the cells reached approximately 80%, the cells were split by removing the media and adding 2.0 mL of PBS (Phosphate-Buffered Saline) to remove any leftover media on the cells. Trypsin-EDTA (2.0 mL) was added to the plate and incubated at 37.0 °C for 5-7 minutes to dislodge the cells from the bottom on the plate. Media (8.0 mL) was added to the trypsin and the cells were collected in a 10 mL conical tube. The cells were centrifuged for 5 minutes at 3,000 rpm, leaving a pellet of cells at the bottom of the tube. The supernatant was discarded without disturbing the cell pellet and suspended in 10.0 mL of fresh media. The solution (1.0 mL) was placed in a T-75 flask with 9.0 mL of media, giving a 1 to 10 dilution of cells. The cells were then incubated again at 37.0 °C/ 5% CO_2 for maintenance.

In order to create stock cells, healthy cells were frozen in liquid nitrogen at −196.0°C. The cell stock solution was created by taking approximately 2,500 cells in

0.95 mL media and 0.05 mL of DMSO in a vial so that the final DMSO concentration was 5%. The cells were then placed into the liquid nitrogen tank.

2.2 MTT Cell Viability Assay- Growth Curve

A growth curve was generated in order to obtain a number of cells that would be ideal for future experiments using the MTT cell viability assay. The cells were plated into 96-well plate increasing in increments of 500 cells from 1,000 cells up until 5,500 cells. Each column on the 96-well plate was utilized for a particular cell concentration. The first and last columns were used as controls, which contained only media. The cells were incubated for 72 hours to allow for growth and treated with 20 µL CellTiter 96 AqueousOne Solution (MTT dye). The plate was read 3 hours later using a SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices) at 490 nm. The growth curve was obtained after the subtraction of the absorbance seen and control wells as background.

2.3 MTT Cell Viability Assay- Testing the Compounds (Preliminary Testing)

An MTT cell viability assay was conducted to quantify the amount of cells following the treatment with 100 compounds from the RISE compound collection library. These compounds are designated as VG compounds. The compounds (1 mg) were dissolved in 500.0 µL DMSO as the master stock. Stocks A and B were created (See Table 1) to accommodate the various concentrations for screening. The concentrations were plated for each compound using these stocks (Table 2). Two 96-well plates were made for the different cell lines each containing 10 VG compounds at various concentrations in duplicate (see Table 3). The appropriate cells (2,500 cells) were added to their respective wells containing VG compounds and incubated for 72 hours. The cells were treated with Celltiter 96 AqueousOne Solution (20 µL) and plates were read 3 hours later using a SpectraMax M3 Multi-Mode Microplate Reader

(Molecular Devices) at 490 nm in order to quantify the amount of cells after the treatment with the compounds. The average of all the separate concentrations for each compound was generated and the value of the control was subtracted. The zero concentration was taken as 100 percent cell viability since no compound was plated. Each of the other concentrations was divided by the average of zero concentration to get a percent viability. These percent viabilities were graphed.

Table 1: The preparation of the master stock and Stocks A and B that were used to plate the various concentrations of compounds being tested is shown below.

Master Stock: 1 mg of compound in 500.0 µL DMSO (2.0 mg/mL)		
Working Stock	**Stock Solution used to Generate**	**Media Added:**
Stock A (0.10 mg/mL)	20.0 µL Master Stock	380.0 µL
Stock B (0.010 mg/mL)	20.0 µL Stock A	180.0 µL

Table 2: The amount of stock solution that was needed along with the media to make the desired concentration is shown below.

Concentration	**Stock Solution**	**Media**	**Total Volume of Stock Plated into 96-well Dish**
0 µg/mL		50.0 µL	50.0 µL
2.50 µg/mL	25.0 µL of Stock B	25.0 µL	50.0 µL
5.0 µg/mL	5.0 µL of Stock A	45.0 µL	50.0 µL
10.0 µg/mL	10.0 µL of Stock A	40.0 µL	50.0 µL

Table 3: A map of the 96-well plate that was used to conduct the preliminary runs of all 100 compounds is illustrated below. The first and last columns were used as media-only controls to subtract from the other wells. Columns 2 through 11 allowed testing of ten compounds per plate containing concentrations of 10 µg/mL through 0 µg/mL. Rows G and H and Columns 2-11 also served as controls containing cells and media only.

	Media	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Media
A	0 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	0 µg/mL
B	0 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	10 µg/mL	0 µg/mL
C	0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	0 µg/mL
D	0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	5.0 µg/mL	0 µg/mL
E	0 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	0 µg/mL
F	0 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	2.5 µg/mL	0 µg/mL
G	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL
H	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL	0 µg/mL

3. Results

3.1 MTT Assay- Growth Curve

A growth curve was generated to find an optimal number of cells that would be used in the assays when evaluating the VG compounds. The assay was screened using the MTT dye, which is absorbed by living cells and metabolized to lead to a color change. The absorbance data were graphed for all three cell lines: DLD1, H1299, and SF295. The largest number of cells that gave a non-maximal absorbance, meaning that the cells still had the ability to grow cells over the course of three days,was the number of cells that was used in future experiments. According to Figure 11, the number of DLD1 cells that corresponded to these optimal conditions was 2,500 cells. This was similar to the behavior of the SF295 cells (Figure 12). According to Figure 13, the optimal number of H1299 cells that corresponded to 2,500 cells.

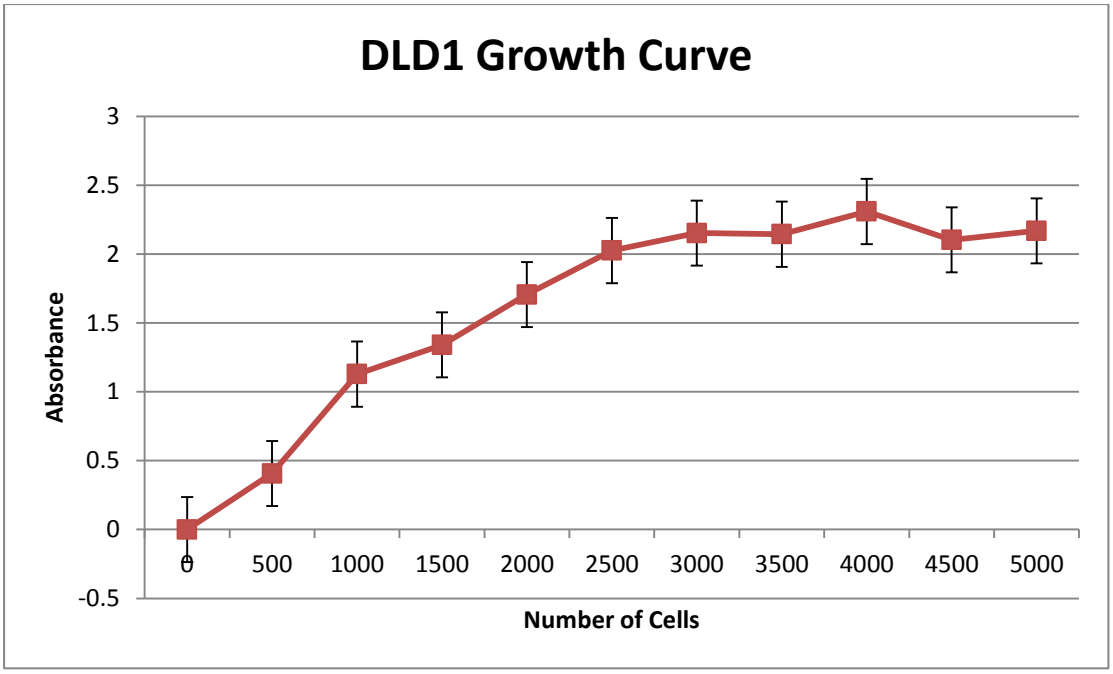


Figure 11: The growth curve of DLD1 colon cancer cells with mutated p53 plated at cell concentrations of 0 cells to 5,000 cells in intervals of 500 cells.

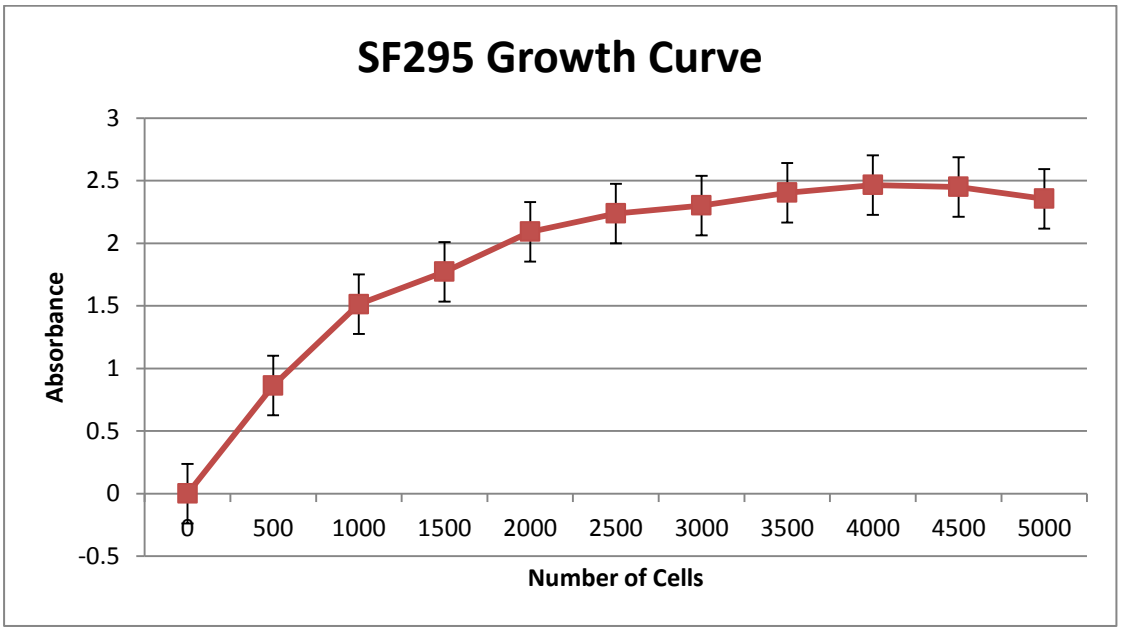


Figure 12: The growth curve of SF295brain cancer cells with mutated p53 plated at cell concentrations of 0 cells to 5,000 cells in intervals of 500 cells.

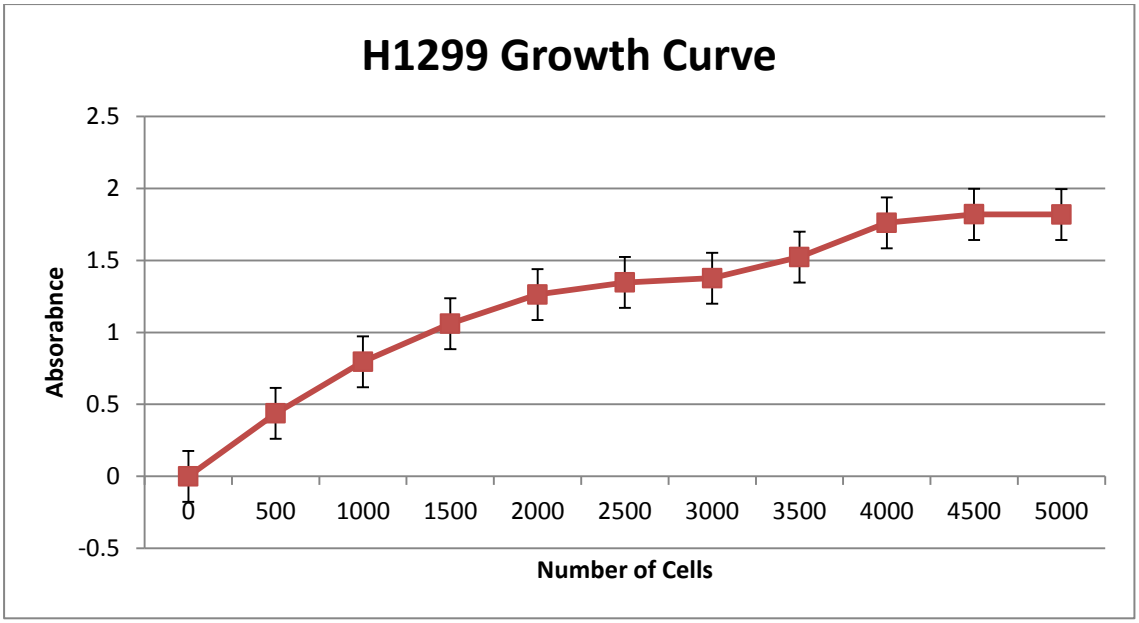


Figure 13: The growth curve of H1299 p53 null cells in lung cancer plated at cell concentrations of 0 cells to 5,000 cells at intervals of 500 cells.

3.2 MTT Viability Assay- Testing the Compounds (Preliminary Testing)

The MTT assay was used in order to measure the viability of the mutant p53 cells and the p53 null cells after treatment with small molecules. This assay is important for screening for growth inhibition due to reactivation of mutant p53 and for measuring the toxicity of the compounds. A desirable compound would be one that led to cell death in DLD1 cells but did not affect H1299 cells. Moreover, since H1299 cells do not have p53, the killing of these cells would mean that there is a p53-independent factor that is causing cell death. If both cells lines died after treatment with the compound, the compound is toxic to both cell lines; nonetheless, if there is an opposite effect, the compound has no effect on the cell lines and is not targeting either cell type.

The effectiveness of the compound to kill the cells with mutant p53, and toxicity, the ability of the compound to kill p53-null cells, were measured after incubation of the compounds with the cells and MTT dye (20.0 µL per well). The

MTT dye is yellow and after absorption by the cells that are living, it is metabolized and turns a violet color. If the cells were living following the treatment with of the compounds, the wells with cells would be purple. However, if the cells are killed off, the dye is not metabolized and remains yellow.

After screening all one hundred unknown compounds in the MTT viability assays, the absorbances were graphed. Based on all 100 graphs, the compounds were grouped as "DLD1 Active," "H1299 Active," "Both," or "Neither." "DLD1 active" indicates that compound led to growth inhibition of DLD1 cells comparatively more than inhibition of growth of H1299 cells. Figures 14, 16, and 17 show the effect of VG 24, VG 55, and VG 58, respectively, on the two cell lines. It can be seen from the graphs that there is a decrease in DLD1 cell number, the p53 mutant cells, while there is little to no effect on H1299 cells that do not have p53. "H1299 active" indicates the compound led to inhibition in H1299 cells comparatively more than the DLD1 cell line. An example of a compound that is H1299 active is VG 17, where there is a decrease in H1299 cells and little to no effect on DLD1 cells (Figure 17). "Both" indicates death of both cell lines. Figure 18 shows an example of VG 57, which is toxic in both cell lines. "None" indicated death in neither of the cell lines or an increase in the number of cells of both cell lines which can be seen in Figure 19, where there is little to no effect on the cell growth. Table 4 shows the results of all 100 MTT viability assays. These data were used as a preliminary measure to determine which compounds had DLD1 activity for further testing. There were 20 compounds that showed effectiveness against the cells containing mutant p53. There were 14 compounds that showed effectiveness against H1299 cells. There were 17 compounds that showed toxicity in both cell lines and 49 compounds that showed no activity in either cell line.

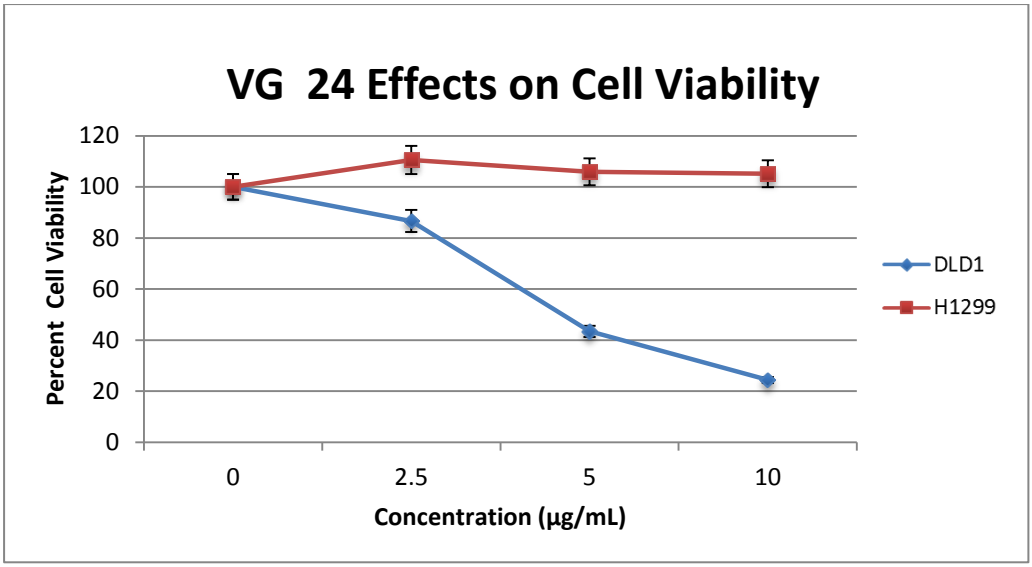


Figure 14: A cell viability graph comparing the activity of cells after treatment of VG 24 after 72 hours. There is cell death in DLD1 cells and little to no effect on H1299 cells labeled as "DLD1 Active" in Table 4.

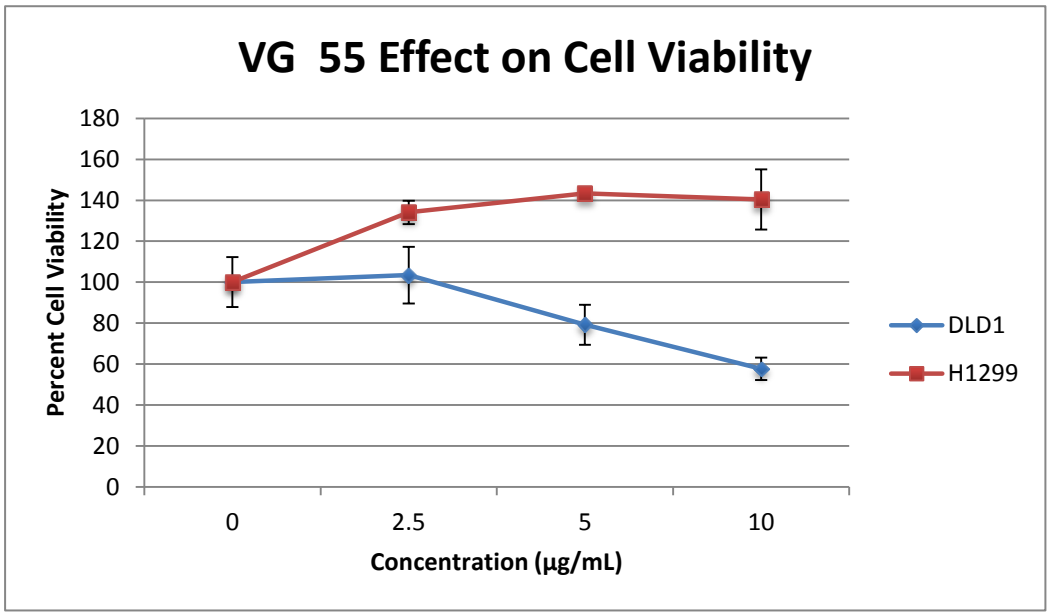


Figure 15: A cell viability graph comparing the activity of cells after treatment of VG 55 after 72 hours. There is cell death in DLD1 cells and little to no effect on H1299 cells labeled as "DLD1 Active" in Table 4.

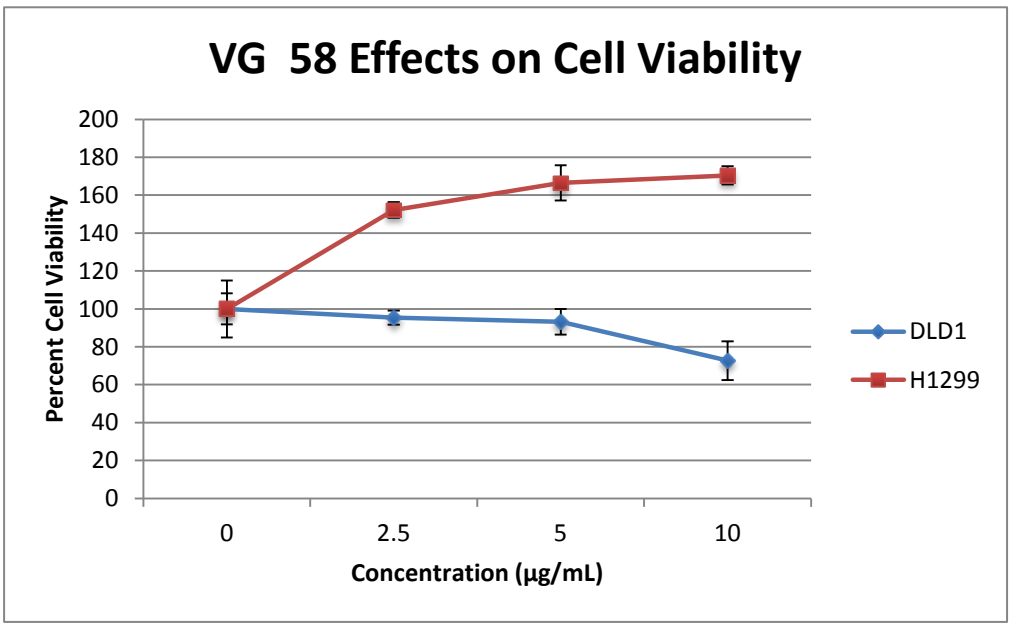


Figure 16: A cell viability graph comparing the activity of cells after treatment of VG 58 after 72 hours. There is cell death in DLD1 cells and little to no effect on H1299 cells labeled as "DLD1 Active" in Table 4.

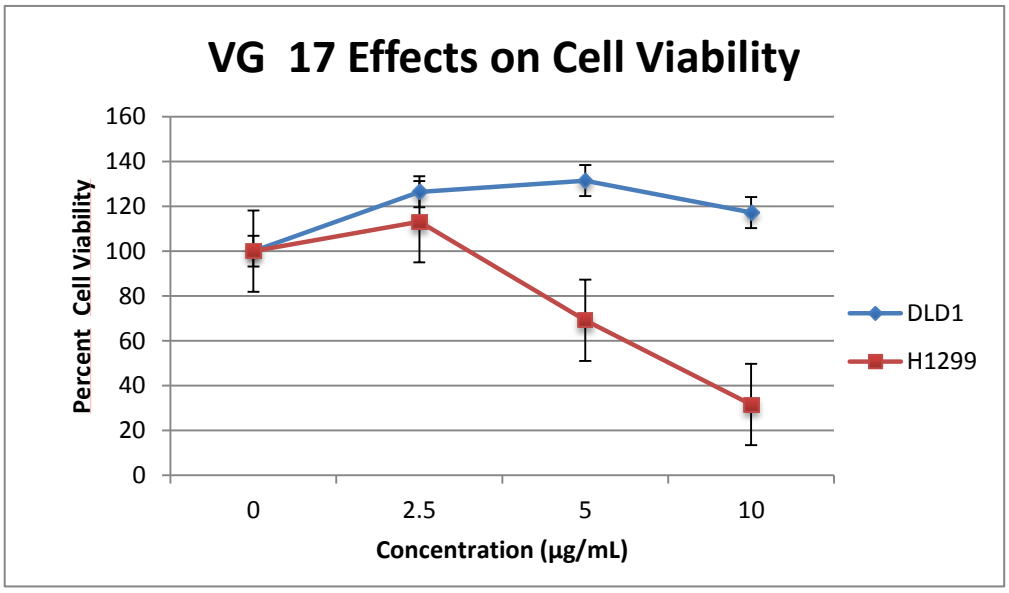


Figure 17: A cell viability graph comparing the activity of cells after treatment of VG 17. There is toxicity in H1299 cells but little to no effect on DLD1 cells, an example labeled as "H1299 Active" in Table 4.

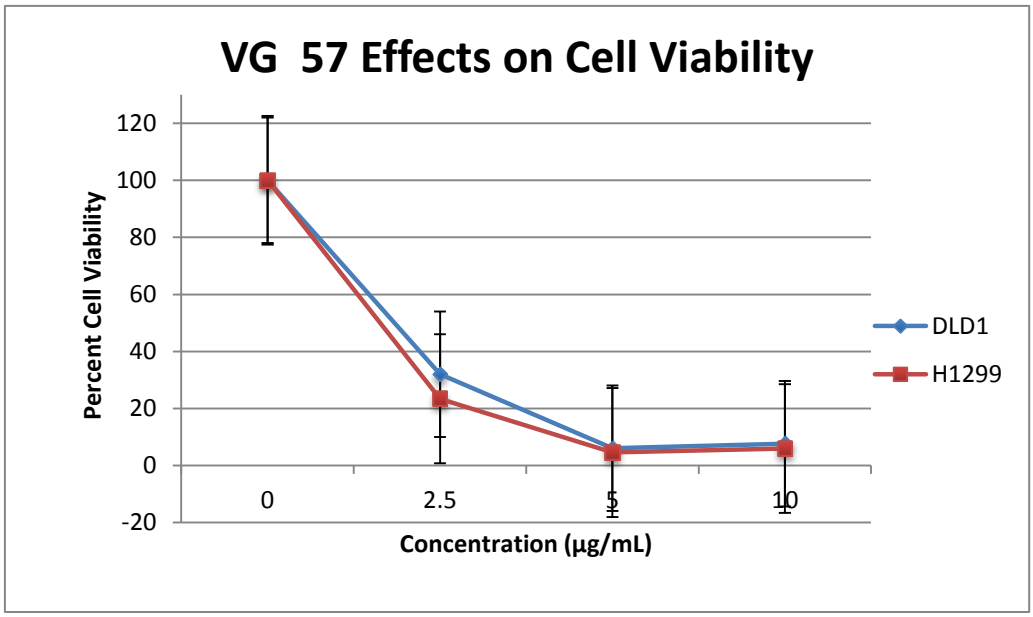


Figure 18: A cell viability graph comparing the activity of cells after treatment of VG 57. There is toxicity in H1299 cells and DLD1 cells, an example labeled as "Both" in Table 4.

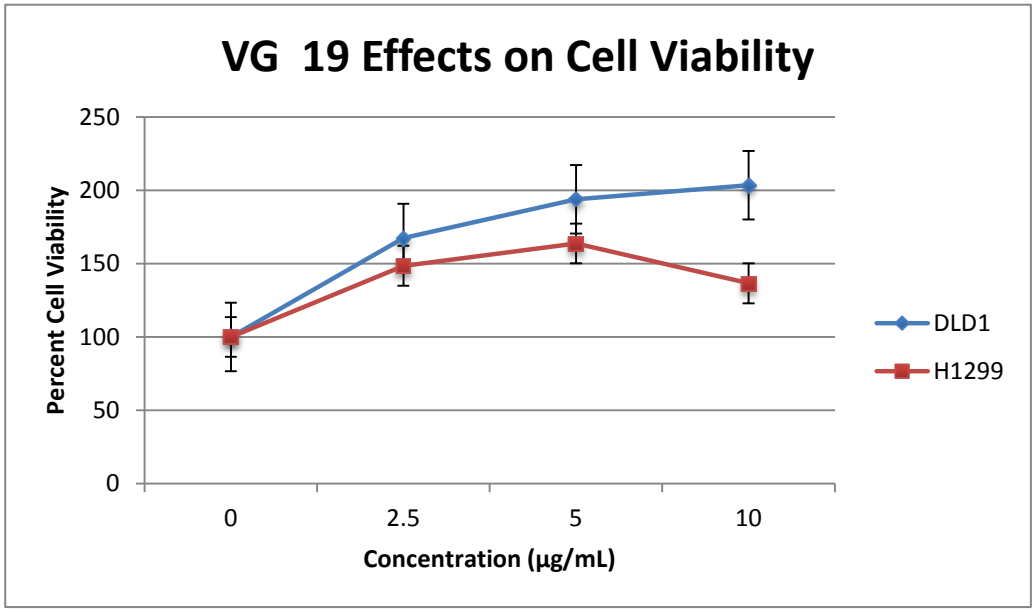


Figure 19: A cell viability graph comparing the activity of cells after treatment of VG 19. This is an example of a viability graph of a compound that has no effect in H1299 cells or DLD1 cells, an example labeled as "None" in Table 4.

Table 4: A table of the library of 100 compounds with the assigned name and the activity against cell lines in the preliminary viability assay is shown below. The yellow highlight indicates the compounds that were effective against DLD1, the green indicates the compounds that were effective against H1299, the purple indicates the compounds that were effective against both cell lines, and the white indicates the compounds that were not effective against either cell line.

DLD1 Active = 20		H1299 Active = 14		Both = 17		None = 49	
VG Number	**Active Cell Line**	**VG Number**	**Active Cell Line**	**VG Number**	**Active Cell Line**	**VG Number**	**Active Cell Line**
VG 1	DLD1	VG 26	None	VG 51	DLD1	VG 76	None
VG 2	None	VG 27	DLD1	VG 52	DLD1	VG 77	H1299
VG 3	DLD1	VG 28	DLD1	VG 53	None	VG 78	H1299
VG 4	DLD1	VG 29	None	VG 54	DLD1	VG 79	None
VG 5	DLD1	VG 30	None	VG 55	DLD1	VG 80	H1299
VG 6	DLD1	VG 31	None	VG 56	DLD1	VG 81	None
VG 7	Both	VG 32	None	VG 57	Both	VG 82	None
VG 8	DLD1	VG 33	None	VG 58	DLD1	VG 83	None
VG 9	Both	VG 34	None	VG 59	DLD1	VG 84	None
VG 10	DLD1	VG 35	None	VG 60	DLD1	VG 85	None
VG 11	None	VG 36	Both	VG 61	Both	VG 86	Both
VG 12	None	VG 37	Both	VG 62	H1299	VG 87	Both
VG 13	None	VG 38	None	VG 63	Both	VG 88	None
VG 14	None	VG 39	None	VG 64	H1299	VG 89	None
VG 15	None	VG 40	None	VG 65	Both	VG 90	None
VG 16	H1299	VG 41	Both	VG 66	Both	VG 91	None
VG 17	H1299	VG 42	Both	VG 67	H1299	VG 92	None
VG 18	None	VG 43	Both	VG 68	Both	VG 93	None
VG 19	None	VG 44	H1299	VG 69	H1299	VG 94	None
VG 20	None	VG 45	Both	VG 70	None	VG 95	None
VG 21	None	VG 46	H1299	VG 71	H1299	VG 96	None
VG 22	None	VG 47	Both	VG 72	H1299	VG 97	SF295
VG 23	DLD1	VG 48	Both	VG 73	H1299	VG 98	None
VG 24	DLD1	VG 49	Both	VG 74	None	VG 99	None
VG 25	None	VG 50	Both	VG 75	None	VG 100	None

4. Discussion

There is increased interest in the search for various cancer therapies because cancer is a major cause of death around the world (American Cancer Society 2014). The guardian of the genome, p53, is mutated in over 50 percent of cancers, and it would be beneficial to reactivate mutant p53 for the development of cancer therapy. When p53 is mutated, it cannot send signals via downstream proteins like p21 and Bax to prevent transformed cell growth. If p53 was not mutated, it could lead to p21 and Bax expression, which would ultimately lead to the prevention of cell division by either causing growth arrest or apoptosis, respectively. One of the ways to restore tumor suppressor function in mutant p53 is by using a small compound to reactivate it. This study entailed screening a library of one hundred random compounds in order to test for these properties. Random screening of chemical libraries is a common strategy used for drug discovery to find small molecules for potential lead molecular structures(Wiman 2006). Once a lead structure that can reactive the function of mutant p53 is identified, medicinal chemistry can be used alter various substituents to find one with the efficacy and potency of a potential drug.

The first approach was to test all of the compounds in the viability assay to evaluate the antigrowth activity of each compound on cells that have mutated p53 and on cells that have no p53. However, before the toxicity assays were conducted on all 100 compounds, a growth curve for each cell line had to be created in order to determine how many cells would be optimal to seed in one well because they would have to be in that well for 3 days. The goal was to find an optimal number of cells that would allow for growth, making sure there would be enough nutrients and room for the cells. Figure 11 shows the growth curve that was created using DLD1 cells. The number of cells had the optimal cell growth for the duration of three days was 2,500

cells. It can be seen that the cells growth starts to plateau in concentrations of cells higher than 2,500 and the cells still have potential to grow with a cell concentration below 2,500 cells. Therefore, 2,500 cells would be the ideal number of cells to use in the preliminary assay to test the compounds. In addition, this was consistent for the SF295 and H1299 cells in which the number of cells that showed 2,500 cells to have room to grow and have an optimal number of cells, shown in Figure 11 and Figure 12, respectively. It also serves as an important control to have the same number of cells in each well for each cell line.

After finding an optimal number of cells that would allow for proliferation, this condition was used in the screening of 100 compounds' activity against both cell lines. Compounds that would be considered to be potential anticancer compounds via p53–reactivation would be those that were active against DLD1 cells, the cells that had mutant p53 and would not be active against H1299, or the cells that had no p53. When the concentration curves were graphed, the DLD1 cells should show a decrease in cell number as the concentration of the compounds increases while the H1299 cells should continue to grow regardless the amount of compound the cells were treated with. According to the preliminary data, there were 20 compounds that showed this type of activity. VG 1, 3, 4, 5, 6, 8, 10, 23, 24, 27, 28, 51, 52, 54, 55, 56, 58, 59, 60 and 97 showed to cause cell death in DLD1 and had little to no effect on H1299 cells. VG 24 was a compound that showed cell death in DLD1 cells and little to no cell death in H1299 cells (Figure 14). As the concentration of VG 24 increases, the percent cell viability drops significantly to about 25 percent at a concentration of 10 μg/mL while the H1299 cells are not affected this way. VG 55 is another compound that has been shown to cause cell death in DLD1 but has no effect on the H1299 cells (Figure 15). As the concentration of VG 55 increases, the percent of cell viability of

DLD1 decreases steadily to about 55 percent at a concentration of 10 μg/mL. It seems as though VG 55 had little effect on DLD1 cells at a low concentration of 2.5 μg/mL. H1299 cells did not decrease in number due to VG 55; moreover, there was an increase in cells that might be due to pipetting error. Similarly, VG 58 showed an effect on DLD1 causing the percent of cell viability to decrease to about 70 percent while the H1299 cells were not affected in a way that the cell viability percentage decreased (Figure 16). It could be hypothesized that VG 58 would have a strong effect on DLD1 cells at high concentrations. The increase in H1299 cells could be explained by errors in pipetting.

There were 14 compounds that showed more activity against the H1299 cell line as compared to the DLD1 (Table 4). VG 17 was a compound that caused a decrease in percent cell viability in H1299 cells and had no effect on DLD1 cells (Figure 17). There was an increase in the H1299 cell numbers at 2 μg/mL of VG 17 and a steady drop to about 30 percent cell viability at 10 μg/mL. There is little effect on the DLD1 cells. H1299 cells have a p53 deletion; therefore, the compound is not reactivating p53 but is causing cells death in a p53-independent manner. There were 17 compounds that were active against both cell lines, which meant that the compound killed both cell lines. VG 57 has almost an identical effect on both cell lines causing a significant of decrease in cell viability; therefore, this compound would not be an ideal cancer therapy since it is toxic in both cell lines (Figure 18). This meant that the antigrowth activity is p53-independent. Lastly, there were 49 compounds that were not active against any of the cell lines meaning that they did not affect the cells. Cells that have been treated with VG 19 have not decreased in percent viability and show an increase in cells numbers may be due to error in plating the cells (Figure 19). It could also be due to the cell growth that may be induced by the

compounds. The cells may be able to intake the compounds and use them in a different manner than expected. There might also be a low concentration of cells in the wells with zero concentration because they did not have DMSO in them. These compounds cannot be used as anticancer therapies because they do not induce cell death in these cell lines.

Since these are preliminary tests, the 20 compounds that were DLD1 active in the preliminary results should be repeated over more concentrations to determine whether these compounds do indeed have significant activity on mutant p53. When repeating the experiment, more concentrations should be plated (0 μg/mL, 0.15 μg/mL, 0.31 μg/mL, 0.625 μg/mL, 1.25 μg/mL, 2.5 μg/mL, 5.0 μg/mL, and 10.0 μg/mL) to determine the IC_{50}. The IC_{50} will indicate therapeutic window to indicate which compounds are toxic in cells. Ideally, a compound that has a larger therapeutic window would be considered a potential anticancer therapy because it would result in the need for less use of the foreign compound. Another approach could be to evaluate the compounds that had an effect on DLD1 cells in a western blot to probe for p21 and BAX proteins. Our laboratory has a standardized protocol for conducting this type of assay. The western blot can show whether the compounds are reactivating p53 by probing for the downstream proteins that are transcribed by p53 when the cell should undergo apoptosis or growth arrest. After the compounds have reactivated p53, their structures can be evaluated and modified based on the lead structure to obtain the best possible compound with a large therapeutic window.

Based on the preliminary results, figures were created to examine the relationship between the 20 compounds's structures and their activity in the DLD1 cells. The 20 active compounds were classified into classes based on structural moieties and their activities in the cell lines. Figure 20 shows the first class of

compounds that were categorized as Class A compounds. These compounds all feature a diphenyl methane with various substituents. VG 10, VG 3, and VG 6 are compounds that are active in DLD1 cells only while VG 18 and VG 11 were inactive in only the DLD1 cells. VG 3 and VG 6 are identical. VG 10 has an imidazole group ortho to the linking methylene and VG 3 has a cyanide group para to the linking methylene; these substituents may allow for better binding to the target protein compared to similar compounds like VG 18 which has an imidazole group at the meta position with a chlorine group placed at the para position. VG 11 also has a chlorine group placed at the para position and an oxazole group placed at the para position.

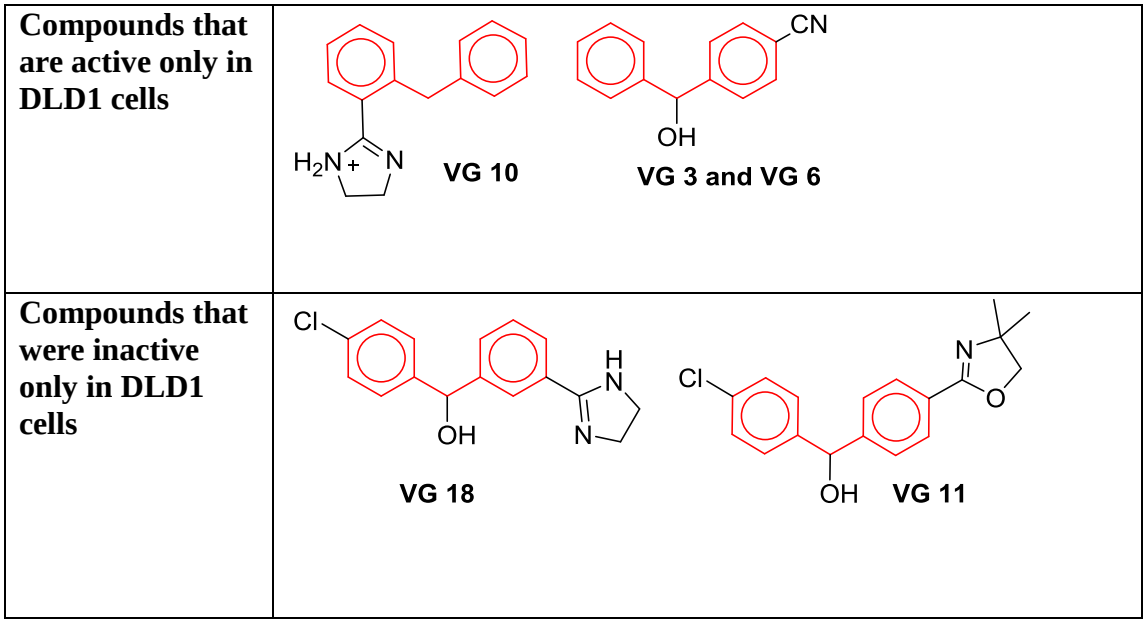


Figure 20: Class A- All of these compounds a diphenyl methane with various substituents. These compounds are both active and inactive in DLD1 cells alone.

Figure 21 shows the second class of compounds that were categorized as Class B compounds. These compounds all have a cyclic pentene ring with either imidazole or oxazole groups attached. VG 5, VG 4 and VG 54, and VG 97 are compounds that are active in DLD1 cells only and VG 13 and 14, VG 96, and VG 95 were inactive in only the DLD1 cells. VG 4/54 and VG 5 have identical structures and VG 13 and VG

14 have identical structures. VG 5 and VG 13/14 have similar structures; however, the oxygen may be beneficial in binding to the target protein compared to a sulfur atom, which is very large. A very large substituent may cause steric hindrance and will not allow for binding to the target protein. VG 4/54 and VG 96 have similar structures with the oxazole group and two methyl groups; however, VG 4/54 have a chlorobenzene while VG 96 has an extra benzene ring and methoxy group which may be causing deactivation in DLD1 cells. Also, VG 97 and VG 95 have similar structures; however, VG 97 has a m-xylene which may allow for high affinity to bind to the target's binding pocket in comparison to the hydroxyl group.

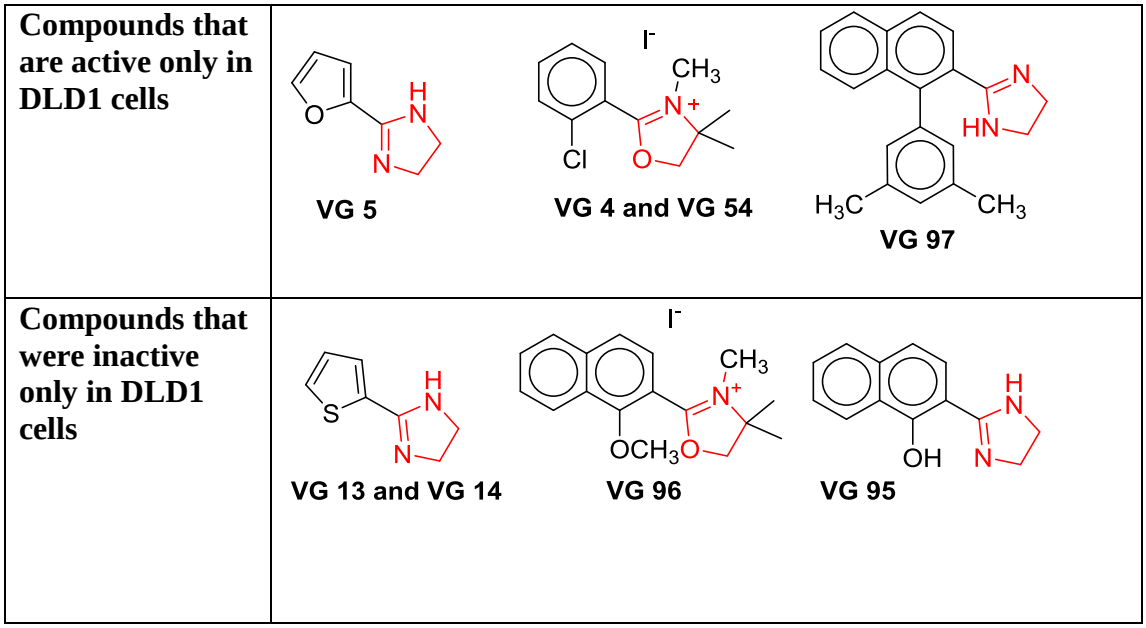


Figure 21: Class B- All of these compounds all have a cyclic pentene ring with either imidazole or oxazole groups attached. These compounds are both active and inactive in DLD1 cells alone.

Figure 22 shows the third class of compounds that were categorized as Class C compounds. These compounds all have a 5,6 spiro-system. VG 24 and VG 27 are compounds that are active in DLD1 cells only and VG 25 was inactive in only the DLD1 cells. VG 24 and VG 25 differ primarily by the cis and trans configurations of the methoxy phenyl substituents; however, VG 24 is active in DLD1 cells and VG 25

is not. This suggests that more than one methoxyl group can be detrimental to binding to the target protein. In addition, VG 27 is another analog of VG 24 but has a hydroxyl group in place of the methoxy groups but has an oxime group in place of the carbonyl.

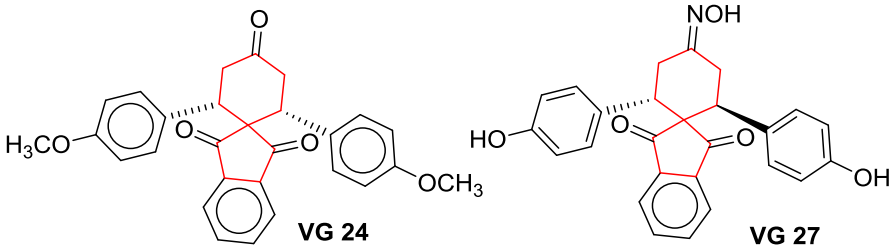
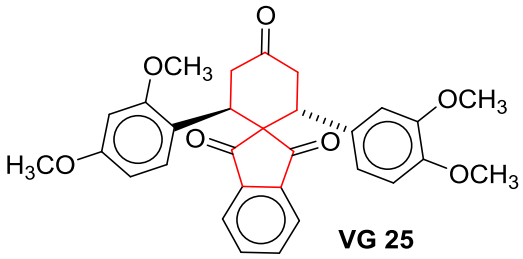
Compounds that are active only in DLD1 cells	
Compounds that were inactive only in DLD1 cells	

Figure 22: Class C- All of these compounds have a 5,6 spiro-system. These compounds are both active and inactive in DLD1 cells alone.

Figure 23 shows the fourth class of compounds that were categorized as Class D compounds. These compounds all have two identical moieties connected by a linker. VG 58, VG 56 and VG 52 are compounds that are active in DLD1 cells only and VG 53 was inactive in only the DLD1 cells. VG 58 and VG 56 look similar to VG 56 except the number of linking carbons are different. VG 58 has 4 linking carbons, VG 56 has 2 linking carbons, VG 56 has 3 linking carbons. Since all three of these compounds are similar yet two of them were active in DLD1 cells, the target protein may be able to bind to molecules that have compounds linked by 2 or 4 carbons. In addition, VG 53 has two moieties that are diphenyls linked by 2 sulfurs. The target protein may allow for target binding due to the large sulfur atoms.2

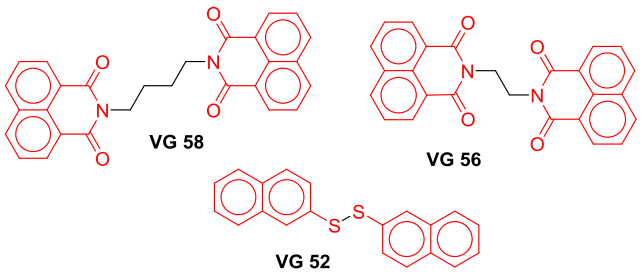
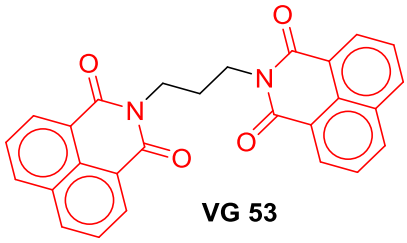
Compounds that are active only in DLD1 cells	
Compounds that were inactive only in DLD1 cells	

Figure 23: Class D- All of these compounds have two identical moieties that are connected by different numbers of bridging carbons. These compounds are both active and inactive in DLD1 cells alone.

Figure 24 shows the second class of compounds that were categorized as Class E compounds. These compounds all have two methyl groups and a terminal hydroxy group. VG 8 and VG 51 are compounds that are active in DLD1 cells only and VG 41 were inactive in only the DLD1 cells. VG 8 and VG 51 are similar in structure except for different halogen groups, which can be helpful in binding to the target protein. VG 8 has a bromine group attached in the meta position and VG 51 has a chlorine group attached to the ortho position. VG 41 has a bulkier substituent, a chloro-benzene with a hydroxyl group coming off the nitrogen which may make the compound too large to fit in the target protein's binding pocket.

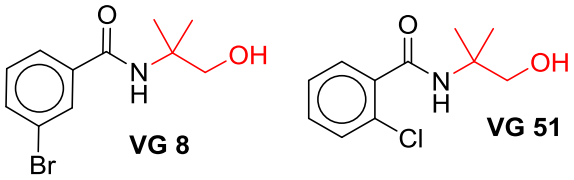
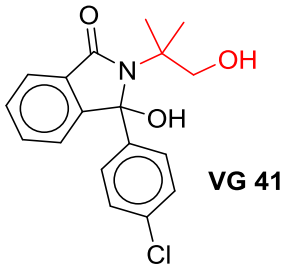
Compounds that are active only in DLD1 cells	
Compounds that were inactive only in DLD1 cells	

Figure 24: Class E- All of these compounds have two methyl groups and a hydroxyl terminal. These compounds are both active and inactive in DLD1 cells alone.

Figure 25 shows the sixth class of compounds that were categorized as Class F compounds. These compounds have a sulfur dioxide group. VG 55 and VG 32 both have a sulfur dioxide; however, VG 55 is active and VG 32 is not. It can be predicted that the attachment of two benzene rings to the sulfonamide in VG 32 maybe be causing deactivation since VG 55 is attached to only one benzene ring.

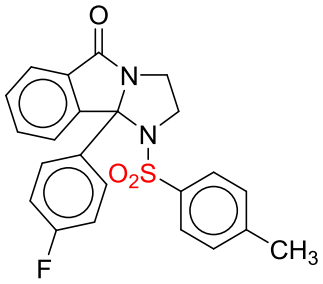
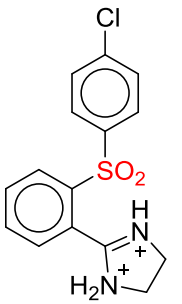
Compounds that are active only in DLD1 cells	 **VG 55**
Compounds that were inactive only in DLD1 cells	 **VG 32**

Figure 25: Class F- These compounds are have a sulfur dioxide group attached to them. The other two compounds were similar in a functional group.

Figure 26 shows the seventh class of compounds that were categorized as Class G compounds. These compounds feature similar functional groups that could be beneficial to binding to mutant p53. VG 28 was seen in the preliminary tests to be active in DLD1 cells but VG 89 was not. VG 28 has an anhydride group and VG 89 features an imide. It could be that the anhydrous is more beneficial in binding to the protein than the hydrazide group. It could be that the bridged ring with the carbonyl could be allowing the compound to bind to the target protein. VG 60 and VG 40 both have methyl piperazine groups, but VG 60 is a planar molecule, while the steric strain in VG 40 forces the carbonyls out of the plan of the central aromatic ring; this may contribute to its inactivity in DLD1 cells. The planarity of VG 1 may also result in its higher activity compared to that of VG 40.

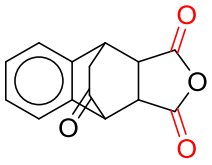
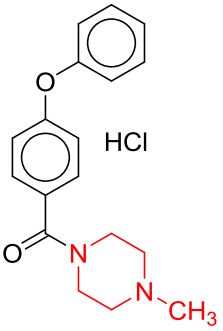
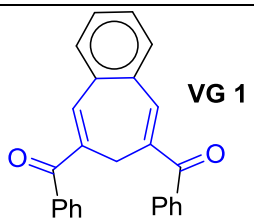
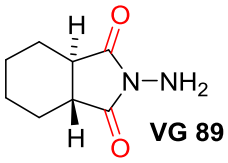
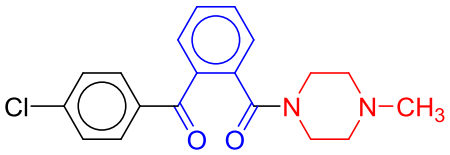
Compounds that are active only in DLD1 cells	 VG 28	 VG 60	 VG 1
Compounds that were inactive only in DLD1 cells	 VG 89	 VG 40	

Figure 26: Class G- These compounds did not fit into any category. However, some of these compounds do have functional groups in common that can be used to observe similarities and differences.

The structures and formula for two of the twenty active compounds is missing: VG 23 and VG 59. Their characterization can be conducted using mass spectroscopy and the NMR. Figures 20 through 26 illustrate the different classes of compounds that were seen to be active in DLD1 cells and inactive in the other cell line. Each of the classes have different properties and substituents. The protein that binds to these compounds to induce cell death in DLD1 cells is unknown; however, observations of the compounds' structures that are active in the DLD1 cells can give some insight on what kind of substituents are favorable and which are not. Predominantly, the active compounds were planar with hydrophilic groups which could suggest the idea that the target protein may be hydrophilic also. The way that the compounds bind to the target protein is unknown; however, this data is based on the preliminary tests. After more testing, a more accurate structure-activity relationship can be determined.

There are a wide range of cancers that can be diagnosed in people; however, researchers are working hard to find therapies and cures for cancers. It is evident that one therapy cannot cure everyone since everyone has a different cancer type and mutation. Because p53 is mutated in over 50 percent of cancers, it would be beneficial to start with attempting to reactivate this protein. As the guardian of the genome and an important check point protein, this is an important protein to reactivate so that the cell can undergo the appropriate control mechanisms. Finding an anticancer agent can help get a step closer to understanding the complexity of cancer and the solution to this disease.

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