Reactivation of Mutant p53 by Small Molecules: Development of a Reporter Gene Assay for Screening

A Thesis in Biochemistry and Molecular Biology

by

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Abstract

p53 is a tumor suppressor protein that becomes active when DNA is damaged (Levine, 1997). Once activated, p53 induces the expression of many genes capable of halting the cell cycle or causing apoptosis (Levine, 1997). In some transformed cells that have sustained inactivating mutations in the p53 gene, p53 expression is greatly increased (Sun *et al.*, 1993). Most of these oncogenic mutations destabilize the p53 protein, preventing it from being able to bind to DNA and function as a transcription factor (Levine, 1997). Since its expression is greatly increased in some cancerous cells, mutant p53 is an attractive target for developing cancer therapy.

Our lab has previously developed a western blot assay to screen small molecules for their ability to reactivate mutant p53 in human transformed cell lines. We have been successful in identifying compounds with the ability to reactivate mutant p53; however, the potency and selectivity of these molecules towards mutant p53 are very low. In order to screen more compounds efficiently, we are seeking to develop a quick, quantitative reporter gene assay. The assay will be used to screen a large number of random compounds for their ability to reactivate mutant p53 in a transformed cell line with mutant p53 in order to identify possible compounds that reactivate mutant p53 and thus can potentially be used in the development of novel cancer treatments.

In developing this reporter assay, we found that the pGL4.38 plasmid produced a strong firefly luciferase signal when transfected into A549 cells, transformed cells with wild type p53, that were subsequently treated with doxorubicin. Transformed cells with mutant p53 (SF295) were transfected with the plasmid pGL4.38; hygromycin B- resistant

SF295 cells were selected and studied for luciferase expression following treatment with small molecules. Further research is necessary to optimize the treatment conditions to utilize these cells for screening novel compounds for the ability to reactivate mutant p53.

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1. Introduction

Cancer Epidemiology

Cancer affects millions of people worldwide and is the 2nd leading cause of death in the United States (CDC, 2013). Cancer is broadly defined as uncontrolled growth of cells that are capable of invading other tissues in the body. Cancer is not one disease but is rather is a complex group of diseases that are mainly categorized by what type of tissue has been invaded and what type of tissue the cells originated from; for example, an esophageal adenocarcinoma is a cancer composed of cells that were originally epithelial gland tissue that invaded the esophagus.

The prognosis for patients who have cancer varies wildly. Some of the variation in the cancer mortality comes from the type of cancer (Chi *et al.*, 2006), how long the cancer progressed before being detected (Li *et al.*, 2008), the gender of the patient (Hsieh *et al.*, 2012), and the available treatments for that cancer type (National Cancer Institute, 2014). More developed areas of the world may have access to better treatment courses for some types of cancers, such as liver cancers, compared to less developed areas; as such, the mortality rate for certain types of cancers is lower in more developed regions of the world (Schweitzer and Vogel, 2013). There are cancers that still lack effective treatments, such as lung cancers, which is evidenced by the high mortality rate of these cancers in both developed and developing countries (Figure 1).

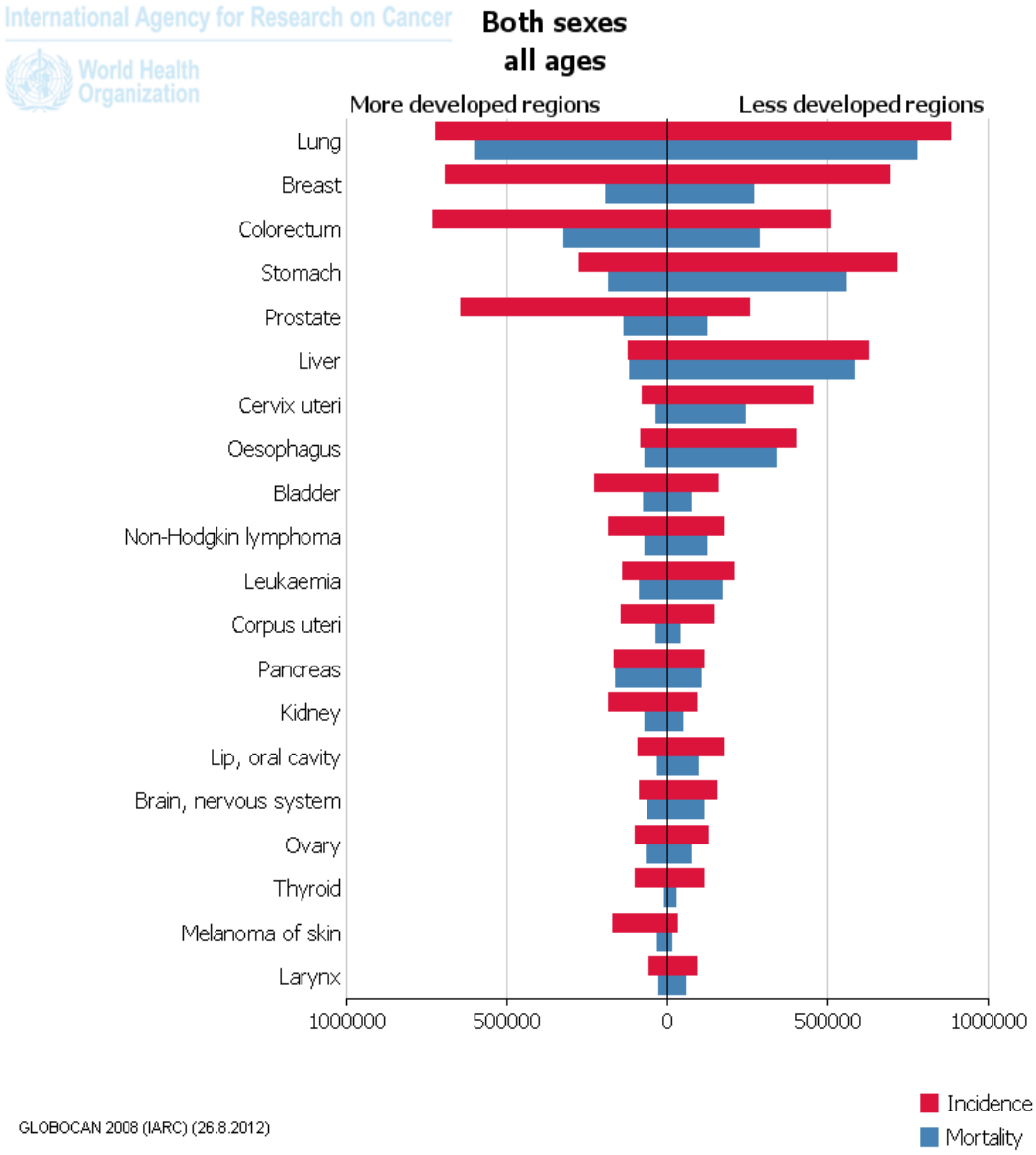

Figure 1: Incidence and Mortality Rates of Various Cancers in More Developed and Less Developed Countries

Different types of cancers are more incident in countries of differing development statuses. The mortality rates of these cancers also vary based on both the economic status of the region and the type of cancer. Figure obtained from Schweitzer and Vogel 2013.

Development of Cancer

Cell Cycle

Cancer is a group of complex diseases characterized by the uncontrolled growth of abnormal cells. Cancer arises when the cell's division cycle becomes dysregulated. In normal cells, each step of the division process is carefully monitored by checkpoint proteins to ensure that each daughter cell receives the correct amount of DNA (Ortmann *et al.*, 2014). In order to enter the division process, the cell must receive growth signals that both cause the cell to grow in size and initiate the production of proteins associated with the division process. After receiving enough growth and pro-division signals from mitogens, the cell enters G1 phase where it grows in size and begins to transcribe proteins necessary for division.

Before the cell can enter the next phase of division, proteins examine the DNA for damage, and the cell checks that it is still receiving the proper amount of mitogen signaling (Ortmann *et al.*, 2014). If the DNA is damage-free and the mitogen signaling is strong, the cell progresses to S phase where it replicates its DNA. Before entering G2 phase, the cell again checks that the DNA is damage-free and has completed replication. During G2 phase, the cell grows in size to ensure that the resulting daughter cells have enough volume. Before entering M phase, the cell once more examines its DNA for damage. The cell then enters M phase, and allots one set of DNA to each side of the cell. Pinching off at the middle, division is complete and two daughter cells are produced.

Every step is monitored for DNA damage (Ortmann *et al.*, 2014). If damage is detected, proteins, such as p53, become active and halt the cell cycle so that the damage

can be repaired (Levine, 1997). In a fully functional cell, cell cycle progression cannot occur if there is damaged DNA. If the damage is too great or mitogen signals are no longer present, the cell will induce apoptosis, or cell death. Cancer can arise when these checkpoints are no longer functional, and the cell proceeds through the cell cycle even when it is not supposed to divide.

Hallmarks of Cancer

Cancer does not arise spontaneously. There are multiple pathways in a cell that need to be altered in order for the cell to become cancerous. These pathways can be altered in a variety of ways, leading to each cancer having a unique set of changes in the cells' control over their replication. While each cancer cell may be unique in the exact mechanisms it uses to continue to proliferate in an organism, the systems these altered mechanisms target are broad in nature and can be used to classify how far along a cell is to becoming cancerous. Six of these major hallmarks of cancer are shown in Figure 2 (Hanahan and Weinberg, 2000). Additionally, many people include the ability to evade the immune system as a final hallmark of successful cancer development as the immune system is very good at killing cells that are not functioning properly (Casey *et al.*, 2014); (Gasser and Raulet, 2006).

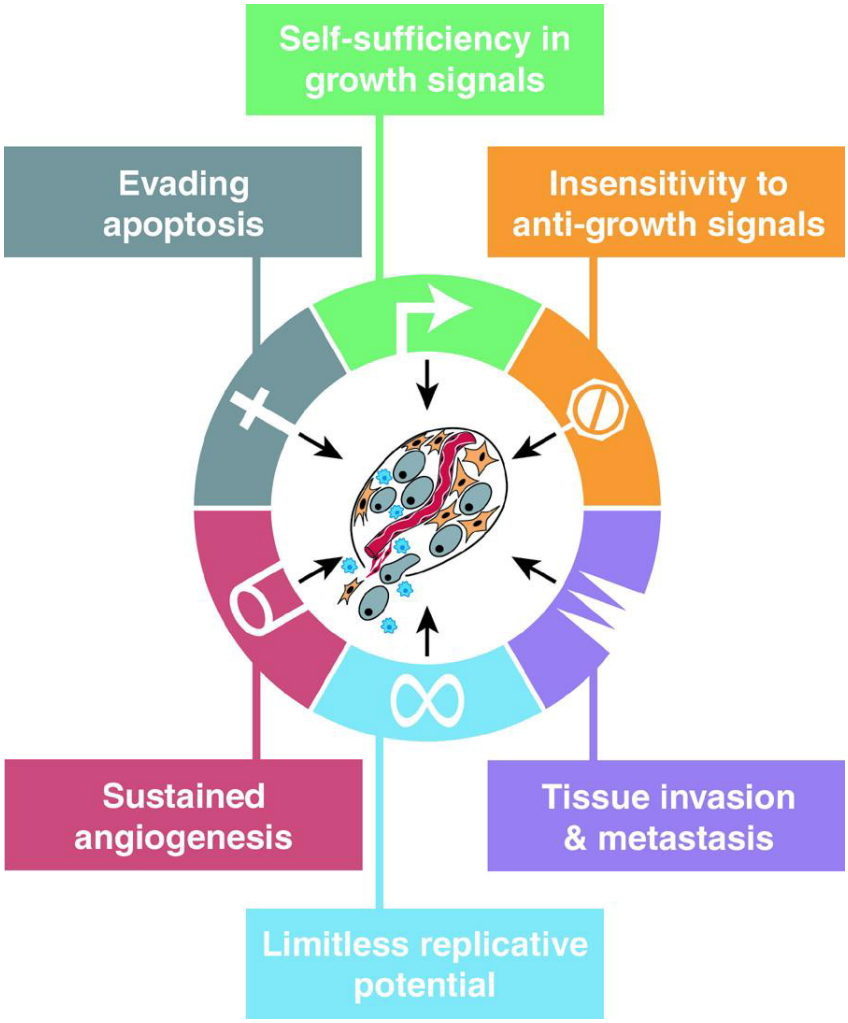

Figure 2: The Six Hallmarks of Cancer

The six requirements of a cell that has the ability to generate a tumor. Figure obtained from Hanahan and Weinberg, 2000.

A cell can begin to push itself through the cell cycle, even when it is not being signaled to divide, due to a mutations in proto-oncogene proteins (Bali *et al.*, 2004). Proto-oncogene proteins are proteins normally associated with pushing the cell into division. If one of these proteins becomes mutated to either lose its ability to be inhibited or becomes constitutively active, the cell can act as if it is constantly receiving mitogen signaling, even if the mitogens are not present.

Similarly, unresponsiveness to anti-growth signals can arise if a tumor suppressor protein becomes mutated (Negrini *et al.*, 2010). Tumor suppressor proteins are proteins that are involved in halting the cell cycle and are a part of cell cycle checkpoints. When active, these proteins prevent the cell from entering the next stage of the cell cycle (Ortmann *et al.*, 2014). p53, a well known tumor suppressor protein, is mutated in 50% of known cancers (Levine, 1997). If tumor suppressor proteins lose their ability to become active, then the cell can continue through the cell cycle even when it is not supposed to proceed.

Limitless replicative potential is the idea that cancerous cells can divide indefinitely (Hanahan and Weinberg, 2000). The number of times that normal cells can successfully divide is fixed. Once cells divide this number of times, the cells cannot be induced to divide again. This limit on replication is known as the Hayflick phenomenon. Normally, cells will either enter senescence or will die (Rubtsova *et al.*, 2012). One hypothesis for why normal cells are limited in divisions is based on their telomeres (Rubtsova *et al.*, 2012). Telomeres are long pieces of DNA that cap the ends of each chromosome. The tail end of the telomere feeds back into itself, forming a loop that

keeps the hydroxyl on the 3' end of the DNA from being free to invade other sections of DNA (Webb *et al.*, 2013). After every division, the telomeres that cap the ends of the DNA can become a little bit shorter due to how the cell replicates its DNA.

The loss of a few nucleotides during the first few cell divisions does not harm the cell (Schoeftner and Blasco, 2009), as no genes are contained in telomere sequences. After many divisions, however, the telomeres become too short and can no longer loop in on themselves. The ends of the cell's DNA are no longer sequestered in protective loops, and can be undistinguishable from a double strand DNA break. As these short telomeres look functionally like a double strand DNA break, the cell can try to fuse the telomeres together, leading to the fusion of different chromosomes (Webb *et al.*, 2013). These fusions can lead to a host of problems for the cell (Webb *et al.*, 2013); thus, when the telomeres are short, the cell stops dividing to preserve its DNA's integrity. Some cells in the body, stem cells for example, need to have the ability to divide indefinitely. These cells use the protein telomerase to replicate the last nucleotides in their telomeres. Telomerase carries a piece of RNA that can be used to act as a pseudo-primer to finish the replication of the DNA (Rubtsova *et al.*, 2012). One way cancer cells can avoid the limitation in the number of times they can replicate successfully is by reactivating the expression of the protein telomerase (Rubtsova *et al.*, 2012). When it is expressed in somatic cells, telomerase can be used to increase the number of times the cell can successfully divide by extending telomeres.

The above hallmarks characterize the uncontrolled growth that is the major characteristic of cancer cells. Since cancerous cells divide aggressively, they consume

more energy than the surrounding blood vessels can provide to the tissue (Morani *et al.*, 2014; Rajaram *et al.*, 2013). One way to increase the amount of nutrients available to the growing tumor is by inducing angiogenesis, the production of new blood vessels. Studies have found that tumors incapable of stimulating angiogenesis cannot grow to be more than a couple of millimeters in size (Folkman and Hochberg, 1973). Angiogenesis is critical in supplying the necessary nutrients for the growth of the tumor.

Functional cells are in constant communication with each other. If a cell is no longer needed in the tissue, the surrounding cells can signal to it that it needs to undergo apoptosis (Park *et al.*, 2013). Additionally, some immune cells can induce apoptosis in cells that are not functioning properly (Barsoum *et al.*, 2014). In order to become cancerous, a cell must not respond to pro-apoptotic signals from either the surrounding cells or from its own intracellular signaling. To do so, the cell may alter its expression of proteins involved in the apoptotic pathway (Dai *et al.* 2004); for example, Apaf1, after binding to cytochrome c, will form the apoptosome that is necessary for the activation of caspase 3. Caspase 3 is the executioner protein that actually induces the apoptotic changes seen in cells. Without functional Apaf1, the cell cannot induce apoptosis, no matter how many pro-apoptotic signals it receives.

The final hallmark of cancerous cells is the loss of a need to be adhered to other cells and the ability to metastasize (Hanahan and Weinberg, 2000). Normal cells require attachment to other cells to ensure their survival. A loss of attachment, or the attachment of the wrong type, results in cell death. Cancerous cells no longer require these adhesions to survive. Notably, cancerous cells can lose or mutate cadherin proteins (Xu *et al.*,

2015), one of the major proteins involved in attaching cells to each other and sorting cells into different tissues (Friedlander *et al.*, 1989). Cancerous cells can alter the pathways associated with adherence by mutating such cell surface proteins in a way that the survival signaling is maintained while the adherence property is lost. After losing adherence, certain cancerous cells can leave the initial tumor site and travel to a new location in the body in a process known as metastasis. The cancerous cell can settle into a new location and survive because it is insensitive to signals from neighboring cells that indicate the cancerous cell is in the wrong type of tissue.

A tumor does not only consist of cells that have developed unregulated cell division, known as transformed cells. Transformed cells have some of the properties of a cancerous cell, but tumor cells have all of the requirements. Cells with all of the above requirements are necessary for a tumor to form, but there is also a well-established microenvironment that is critical for sustaining the growing tumor as well (Figure 3). Cells from the surrounding tissue are employed to alter nearby immune cells, preventing them from killing the cancerous cells (Johann *et al.*, 2010). Tumor cells can also recruit certain immune cells, regulatory T cells, to remain in the tumor site and prevent any other immune cell from becoming active and killing the tumor cells (Domagala-Kulawik, 2015), allowing the growing tumor to remain unnoticed by the immune system.

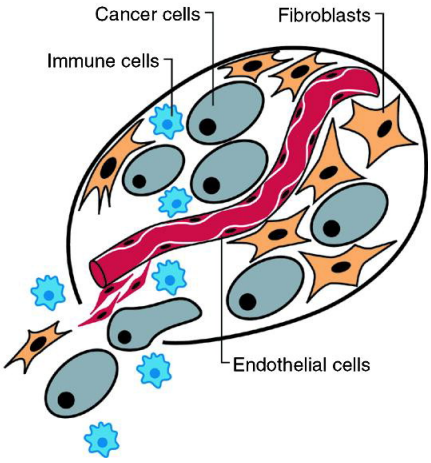

Figure 3: Microenvironment of a Tumor

Many cell types contribute to the growth of a tumor. Nearby regulatory immune cells can inhibit other immune cells from targeting transformed cells. Figure obtained from Hanahan and Weinberg, 2000.

All of these hallmarks can arise from and contribute to uncontrolled cellular division. Tumor cells do not obtain all of these hallmarks at once. Generally, mutations are accumulated gradually that remove the cell's restrictions on its growth. A loss of a tumor suppressor protein is particularly important in facilitating the gradual increase in mutations, further mutations can accumulate due to the dysregulation of checkpoint controls in a tumor suppressor-mutant cell (Figure 4). Studies have found that people heterozygous for a mutant tumor suppressor protein, for example p53, are much more likely to develop cancers earlier in life (de Vries *et al.*, 2002). Loss of p53 function is quite common in cancers (Levine, 1997). This may be due to its ability to regulate each of the hallmarks of cancer during different stages of progression (Figure 4).

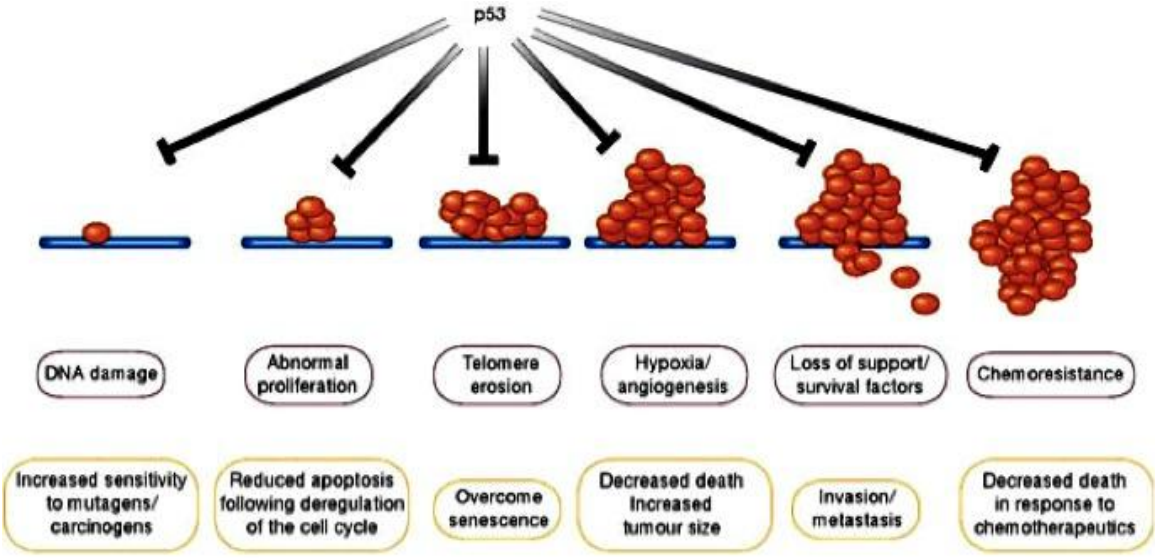

Figure 4: p53 Blocks Cancer Progression at Multiple Stages

p53 is a powerful tumor suppressor with a variety of functions capable of halting the formation of each hallmark of cancer. The text in the purple circles indicates what is occurring in the cells, and the text in the yellow circles indicates the phenotype of the cells. p53 is capable of being activated by either DNA damage or oncogene activation, two of the main drivers in cancer formation. Figure adapted from Evan and Vousden, 2001.

Current Treatments

Current treatments exploit the uncontrolled cell division aspect of cancer. Both chemotherapy and radiation target the cell cycle (Mukhtar *et al.*, 2014; Valerie *et al.*, 2007). Most chemotherapy drugs either halt particular steps during cell division or induce DNA damage. Radiation induces DNA damage, such as double strand breaks, which can halt cell division or lead to cell death (Valerie *et al.*, 2007). Since these treatments focus on the cell cycle, they also affect normal cells that divide quickly, such as those in the gastrointestinal tract or immune cells. This leads to the side effects characteristic of cancer treatments: nausea, hair loss, and immunodeficiency (Rothenburg *et al.*, 2001; Sparano *et al.*, 1997). These side effects are not slight; studies have noted patients dying from side effects of drug treatments such as Irinotecan, which is used in treating colorectal cancers (Rothenburg *et al.*, 2001). Additionally, radiation therapy can induce double strand DNA breaks in the surrounding untransformed cells, further increasing the odds of the patient relapsing or developing another kind of cancer because these double strand breaks can result in mutations (Raissouni *et al.*, 2012). Finally, these treatments are only effective against rapidly dividing cancer cells. In certain cancer types, tumors can be maintained by cancer stem cells (Reya *et al.*, 2001; Singh *et al.*, 2003). These stem cells have sustained mutations that allow them to become tumor cells and often have further mutations to assist in survival, which may allow them to endure traditional cancer treatments such as chemotherapy (Dean *et al.* 2005). While tumors may be surgically removed, some of the stem cells may be missed. These stem cells can then divide and

regenerate the tumor. Because of these issues, researchers are moving toward using cancer-specific targets for new drug development.

One of the first drugs to target a cancer-specific protein was Gleevec, which has been reviewed by Gambacorti-Passerini and collegues (2003). This molecule inhibits the mutant tyrosine kinases Bcr-Abl and c-Kit. Many survival signals induce phosphorylation in the cell by activating kinases. By inhibiting these kinases, which are always active due to mutations, the cancerous cells no longer receive survival signals and die. Compared to traditional treatments, treatment with Gleevec significantly increases the survival rate of patients (Kantarjian *et al.*, 2003), illustrating the promise of targeted therapies against cancers. The success of Gleevec and its derivatives in treating certain cancers with limited side effects for the patient indicates that targeted therapies should be pursued as an option for developing cancer therapeutics. One problem noted with using Gleevec is that Gleevec is incapable of completely eliminating Bcr-Abl function, leading to the cells quickly developing resistance to the drug (Gambacorti-Passerini *et al.*, 2003). Due to the high rate of resistance development, more research is needed to develop drugs to use against these resistant cancers to improve the prognosis for people who have relapsed.

Another possibility for targeted cancer therapy involves using antibodies to target only the cancerous cells in an organism. Antibodies are proteins secreted by the immune system that are highly specific in what they recognize. Some antibodies against antigens on cancerous cells have been developed; the antibodies are conjugated to radiation-emitting particles to specifically deliver radiation to tumor cells while sparing adjacent normal cells (Jurcic *et al.*, 2002). These antibodies have been successful in treating

certain types of cancers (American Cancer Society, 2014), further supporting the idea that treatments geared toward specific targets on or associated with cancerous cells can be effective.

Additionally, some work has been done to develop strategies to reactivate immune cells in order to have them remove the cancerous cells (Yang *et al.*, 2014). In tumors, immune cells can be initially recruited to the site to remove the abnormal cells, but nearby tissue cells or the tumors themselves inactivate the immune cells. Certain antibodies have been developed that bind to receptors on the immune cells in order to restore their function (Zhang *et al.*, 2015). Zhang and collegues found that antibodies against CD40 were able to induce activation of naïve CD4+ T cells and reduce tumor size in mice. Activated T cells express CD40 (Ma and Clark 2010), so treatment with antibodies that bind to and activate CD40 may induce signaling in these cells which could lead to an increase in the strength of the response of these T cells to the cancerous cells.

Similar to using antibodies to activate immune cells, researchers have also attempted to develop antibodies that prevent immune cell inhibition. These antibodies would work by inhibiting proteins that normally halt T cell activation, allowing the T cells to remain active and kill the cancerous cells. CTLA-4 is a protein expressed by active T cells (Walunas *et al.*, 1994). When CTLA-4 is activated, it prevents the T cell from remaining active. Survival rates of patients treated with antibodies that bind and inhibit CTLA-4 activation are significantly greater than patients that did not receive these antibodies (Callahan *et al.*, 2011). This may be due to the antibodies binding to the CTLA-4 on the activated T cells, and preventing the CTLA-4 from sending an inhibitory

signal into the T cells. This would prolong the activation of the T cells, allowing them to kill the cancerous cells.

Unfortunately, immunotherapies have been found to have their own side effects. Current immunotherapies rely on targeting proteins that are normally expressed on many immune cells, leading to effects on the immune system in general and upsetting the balance of immune cell activation that is needed to prevent autoimmune disorders (Kong and Flynn, 2014). Studies have found an increase in the rate of thyroid dysfunction in patients treated with certain immunotherapies (Hamnvik *et al.*, 2011). Additionally, some antibody treatments can lead to side effects such as colitis, inflammation of the gut lining, and hypophysitis, or inflammation of the pituitary gland (Fransen *et al.*, 2013). These limitations further illustrate the need for new targeted therapies that are effective against many types of cancers and have limited side effects. To develop such a therapy, an in depth understanding of the mechanisms underlying cancer's uncontrolled growth is necessary.

Role of p53 in Cells as a Tumor Suppressor

The tumor suppressor protein p53, which is expressed in all cells, is a homo tetramer of a polypeptide consisting of 393 amino acids (Levine, 1997). p53 has four domains: a transactivation domain, a DNA-binding domain, a tetramerization domain, and a regulatory domain (Levine, 1997). p53 is found at very low levels in normal cells due to p53 being rapidly degraded by mouse double minute 2 homolog (MDM2) soon after p53 is translated (Haupt *et al.*, 1997). p53 acts as a transcription factor that induces the expression of many genes and micro RNAs that are associated with halting the

hallmarks of cancer (Levine, 1997). In normal cells progressing through the cell cycle, proteins called Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3 Related (ATR) are constantly scanning the DNA for damage. If damage is found, these proteins phosphorylate p53 and its bound negative inhibitor MDM2 (Figure 5) (Levine 1997). Once phosphorylated, MDM2 can no longer bind to p53, allowing p53 to accumulate in the cell and oligomerize. Phosphorylated MDM2 is degraded by the cell, thus allowing p53 to have a longer half life.

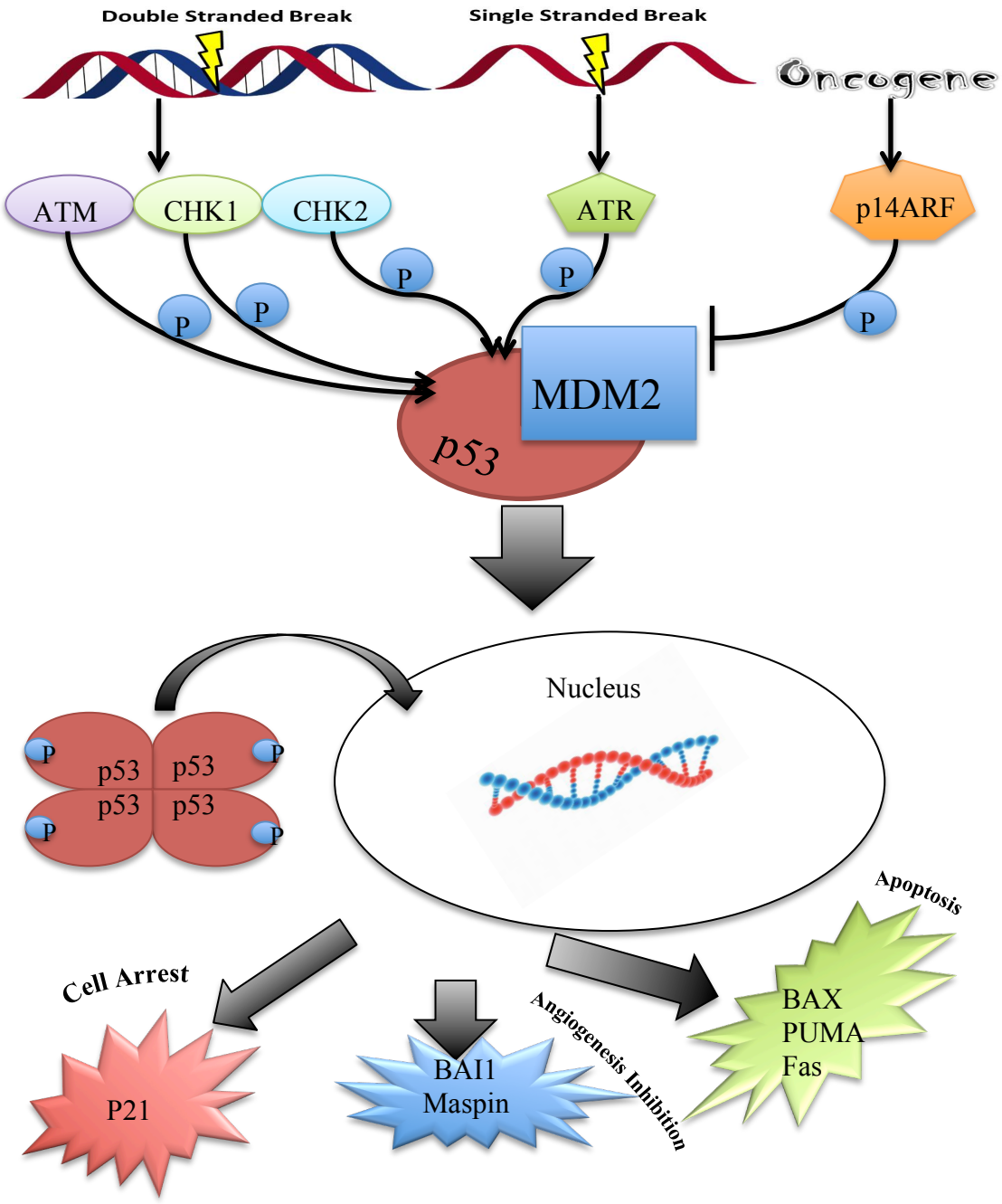

Figure 5: p53 Activation And Downstream Protein Expression

When DNA is damaged, both p53 and MDM2 are phosphorylated to disrupt their interaction, removing MDM2. p53 then oligomerizes and translocates into the nucleus to induce gene expression to halt the cell cycle, induce apoptosis, or inhibit angiogenesis.

Activated p53 translocates into the nucleus to act as a transcription factor (Levine, 1997). The four DNA-binding domains, from four monomers, form fingers that wrap around the DNA sequence known as the p53 response element. By binding to the response element, p53 regulates the expression of many genes, including p21. p21 halts the cell cycle by binding to both cyclin/cyclin dependent kinase 2 (CDK2) complexes to prevent their activation and to proliferating cell nuclear antigen (PCNA) to prevent DNA replication (Abbas and Dutta, 2009). This allows the cell to repair its DNA before proceeding through the cell cycle. Another protein that is expressed after p53 activation is brain-specific angiogenesis inhibitor 1 (BAI1). This protein prevents angiogenesis. In tumors made to overexpress BAI1, there was an observed decrease in angiogenesis in the tumor (Duda *et al.*, 2002). Additionally, Bax expression may also be induced by active p53. Bax is a member of the Bcl2 family (Westphal *et al.*, 2014). It is a pro-apoptotic protein that is present in the outer mitochondrial membrane. In a cell that has received signals to undergo apoptosis, Bax proteins oligomerize and form a channel in the membrane of the mitochondria (Bender and Martinou, 2013). This channel allows cytochrome c to be released from the mitochondria into the cytoplasm of the cell in order to initiate the formation of the apoptosome, which in turn activates caspase 3 to induce apoptosis.

In all known cancers, the p53 signaling pathway has been silenced (Levine, 1997). In half of these cancers, this is due to the *p53* gene having sustained inactivating mutations. Most of these oncogenic mutations occur in the DNA-binding domain (Figure 6).

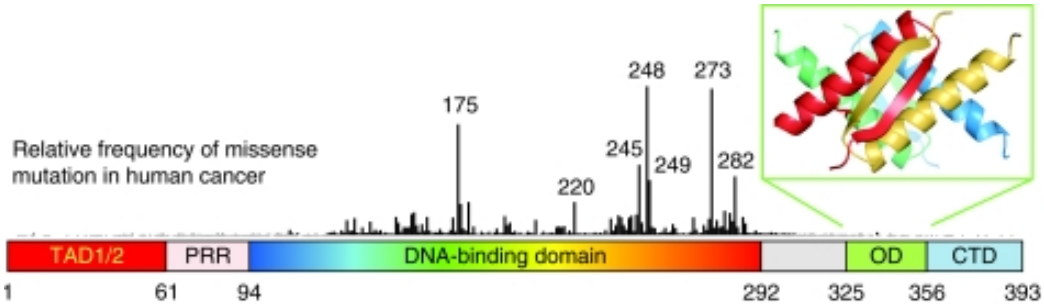

Figure 6: Frequency of Mutations in p53

The frequency of a mutation at a particular amino acid in the p53 protein is indicated by a raised vertical black line. A long line indicates a commonly mutated amino acid. The six hotspot mutations are indicated by their amino acid number. Figure adapted from Joerger and Fersht, 2010.

These mutations result in a protein that is conformationally unstable at physiological temperature, thus rendering it unable to bind to DNA. The six "hotspot" mutations, which are common in many cancers, occur near the DNA-binding surface (Olivier *et al.*, 2010). Five of these six mutations switch one of the positively charged amino acids for a non-positively charged amino acid. These and other mutations in the DNA binding domain induce conformational changes that result in the loss of DNA binding activity. In addition, these mutations allow the cell to acquire growth-promoting activity, making cancers with mutant p53 are particularly aggressive and resistant to therapies (El-Deiry *et al.*, 1992; Dong *et al.*, 2013). Because p53 is mutated in so many cancers, but is not mutated in most normal cells, mutant p53 is an attractive target for the development of a drug that could target cancer cells specifically, while leaving normal cells mostly unharmed.

Compounds have been developed by researchers that are able to reactivate mutant p53. CP-31398 was developed by Pfizer and was found to restore anti-proliferative activity to a variety of p53 mutants (Wischhusen *et al.*, 2003). This compound also binds to DNA (Rippin *et al.*, 2002), which is not desirable because DNA-binding agents may induce mutations by covalently modifying nucleotides (Enoch and Cronin, 2010). PRIMA-1, another compound shown to reactivate mutant p53, works by covalently binding to cysteine residues (Lambert *et al.*, 2009). While it is effective at shifting mutant p53 into an active conformation, there is concern that PRIMA-1 would bind to cysteine residues on other proteins, which could result in toxicity.

Compounds have also been developed that are p53 mutation-specific. NSC219726 is effective at reactivating p53 that contains a mutation in the arginine at position 175 (Yu *et al.*, 2012). This particular compound is able to chelate zinc ions, making it unsuitable for clinical treatments as it can prevent the non-transformed cells of the patient from accessing zinc ions that are necessary for normal cellular function. Because of the undesirable modes of action of currently characterized compounds, researchers are continuing to search for compounds that are able to reactivate mutant p53 while not posing a significant risk to non-transformed cells.

Researchers at Schering-Plough found a small organic molecule, SCH529074, that could bind to mutant p53 and stabilize it in an active form capable of acting as a transcription factor (Demma *et al.* 2010). It is believed that mutant p53 exists in an equilibrium between the native and non-native conformation and these non-native conformations are quickly destabilized at physiological temperature (Friedler *et al.*, 2002). This molecule was shown to bind to mutant p53, but it could be dislodged when DNA containing p53's response sequence was added, suggesting that the compound acted by a chaperone mechanism. Since the compound can be kicked off of the p53 in the presence of the response element DNA sequence, this suggests that the compound is not covalently binding to p53 as PRIMA-1 had. The inability to covalently bind to p53 is desirable because covalent binding of a compound to residues could lead to cross reactivity with other proteins in the cell, as these other proteins could contain similar exposed amino acid residues. Additionally, since it is freed when the p53 is exposed to DNA, this suggests that the compound does not bind to DNA, unlike CP-31398. Because

of these data, the researchers reasoned that this compound is not working through the undesirable reactions of previously found compounds. The researchers suggest that SCH529074 binds to the native conformation and shifts the equilibrium of mutant p53 toward favoring the native conformation, which is capable of binding to DNA and working as a transcription factor.

Since p53 acts as a transcription factor for many genes, it is a particularly flexible protein. Any shift in p53's conformation may move positively charged amino acids that were originally far away closer together where they can have a more favorable interaction with the negatively charged phosphodiester backbone of the DNA. Upon interacting with the DNA, the conformation of p53 shifts again, and the compound is thought to leave to go interact with another mutant p53 molecule (Demma *et al.*, 2010). While this compound may be working through a desirable mechanism, it has not yet been optimized for use as a possible drug candidate. It was found to have low in-cell potency, meaning that a high concentration of the drug was needed in order to see an effect in the treated cells. Additionally, there were still some issues with off-target activity as well, as it was found to be toxic to cells that contained no p53, which indicates that the compound may be able to interact with other proteins in the cell.

Previous Research in the Dasmahapatra Laboratory

Previous work done in the Dasmahapatra lab at Drew University has focused on standardizing a western blot assay to screen novel compounds, synthesized by the Doll lab at Drew University, for the ability to reactivate mutant p53 in transformed human cell lines. The western blot assay measured expression of p21 in transformed cells with

mutant p53 following the treatment with these small molecules that are synthesized by the Doll lab (Lee and Dasmahapatra, 2013). The Doll lab utilized the Schering-Plough compound described above as a framework from which to base their synthesis. Previous students in the Dasmahapatra lab were able to identify multiple novel compounds that were able to induce p21 expression based on the western blot (Lee and Dasmahapatra, 2013).

After identifying compounds that exhibited an ability to reactivate mutant p53 in the western blot assay, compounds were further screened for their ability to specifically inhibit the growth of mutant-p53 containing cells while not affecting cells without mutant p53. These compounds were screened in a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) to verify that they were able to stop the growth of mutant-p53 containing cells but not cells without mutant p53. This assay relies on the use of a dye that is metabolized from a yellow colored-compound to a purple-colored compound by living cells, allowing an indirect measure of cell viability. Brittany Barreto reported that a few compounds were able to preferentially inhibit cell division in mutant p53-containing cells as compared to cells that lacked p53, which indicates that the compounds were affecting the cells in a p53-dependent manner (Barreto and Dasmahapatra 2013).

Many compounds which induced a high level of p21 as measured by the western blot, such as RD 27, were shown to inhibit the growth of a cell line that is null for p53, indicating that the compounds had strong off-target effects. The western blot assay is useful for identifying compounds that are capable of affecting the p53 pathway, but it is a

lengthy process. Additionally, the western blot does not lend itself to easily quantifiable data. Objectively comparing compounds between blots is difficult. An assay that is both quick and easily quantifiable would allow researchers pursuing mutant p53 reactivation to screen many compounds at once, increasing the odds of discovering a possible drug candidate.

Goals

In addition to continuing to screen compounds in our p21 western blot assay, we proposed to develop a reporter gene assay to quickly screen novel compounds. This assay would place a gene that is easily quantifiable under the control of the p53 response element DNA sequence. When transfected into mutant-p53 containing cells, this gene would be expressed when the cells were treated with compounds that reactivate mutant p53 to allow it to bind to its response element. We used the Firefly luciferase gene as our reporter, as the luminescence is easily quantifiable (Lallemand *et al.*, 2011). A kill curve for each mutant p53-containing cell line was determined using the antibiotic hygromycin B, an inhibitor of protein synthesis (Gonzalez *et al.* 1978), because the plasmid containing the firefly luciferase gene under the p53 response element also contained a hygromycin B resistance gene. A kill curve was generated in cells that did not contain our plasmid in order to ensure that we could successfully kill off any cells that did not contain our plasmid of interest. This allowed us to determine an appropriate hygromycin B concentration to use for later selection experiments.

Each cell line was transfected with the plasmid containing the firefly luciferase under a p53 response element and a hygromycin B resistance gene. The cells were then

incubated in the presence of hygromycin B for two weeks to ensure that the only cells able to survive were those that incorporated the plasmid DNA into their chromosome as the plasmid lacks an origin of replication. This allowed us to only use cells that contained our plasmid DNA in subsequent experiments. Colonies were isolated and screened for luciferase by treating them with compounds known to induce p21 expression in mutant p53-containing cells.

2. Materials and Methods

Cell Culture

All cell lines were purchased from the New Jersey Cancer Institute. The DLD1 and SF295 cell lines were used to evaluate compounds for the ability to reactivate mutant p53. DLD1 cells are epithelial cells isolated from a colorectal adenocarcinoma (DLD1). DLD1 contains a missense mutation at amino acid 241 of p53 where a serine has been replaced by a phenylalanine (Soussi, colorectal). The SF295 cell line was derived from a glioblastoma (DTP). SF295 contains a missense mutation at position 248 of p53 where an arginine has been replaced by a glutamine (Soussi, brain). The A549 cell line, derived from a lung carcinoma (A549), contains wild-type p53 and was used to verify that the plasmid was capable of producing firefly luciferase in the presence of active p53. The above cells were cultured in RPMI media supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin and Streptomycin (PenStrep). H1299 cells, derived from a lung carcinoma that had metastasized to a lymph node (H1299), contain a partial deletion in the *p53* gene and do not express p53. H1299 were used as negative controls in the MTS assays due to their lack of p53 expression. H1299 were grown in DMEM media

supplemented with 10% FBS and 1% PenStrep. Cells were incubated at 37°C and 5% CO_2. Frozen cells were stored in liquid nitrogen in a 5% dimethylsulfoxide (DMSO)/media solution.

Western Blot

Cells (200,000 cells/well) were plated in a 6 well dish. After one day of incubation, the cells were treated with 15 μg/mL of compound. Cells were allowed to incubate for 16 to 18 hours in the presence of compound before lysis. Cells were lysed by first washing the cells with 1x phosphate buffered saline (PBS) and adding Trypsin-EDTA. After all cells detached, RPMI media was added to inactivate the trypsin, and the cells were pelleted. The pellet was washed with 1x PBS and re-pelleted. The pellet was suspended in mammalian protein extraction reagent (M-PER) lysis buffer (Thermo Scientific, catalog number: 78503) that was supplemented with 1% ethylenediaminetetraacetic acid (EDTA), 1% protease inhibitor (purchased from Thermo Scientific, catalog number: 87786), and 1% dithiothreitol (DTT; Pierce® catalog number: 20291). Cells were centrifuged briefly and allowed to incubate on ice for 30 minutes to allow lysis to occur. The lysed cells were centrifuged at 13,000 rpm for 15 minutes at 5°C, and the protein-containing supernatant was collected and stored at -20°C.

Both the gel electrophoresis and blot transfer were adapted from the protocol provided by Life Technologies (2010), and all reagents used in the gel electrophoresis and blot transfer were purchased from Life Technologies. Briefly, lysates obtained from treated cells were mixed with NuPAGE sample buffer and reducing agent (catalog number: NP0050). Samples were incubated at 70°C for 10 minutes and then centrifuged.

Samples were loaded into a 12% Novex Bis-Tris polyacrylamide gel (catalog number: NP0341BOX) and run in 1x MOPS buffer (catalog number: NP0050) that had been supplemented with 0.25% of a proprietary antioxidant (catalog number: NP0005) to preserve band sharpness. The Novex® Sharp Pre-stained Protein Standard (catalog number: LC5800) was run in the gel in order to determine the sizes of the proteins in the samples. Samples were run at 200 volts until the dye front began to leave the bottom of the gel. The gel was transferred to a nitrocellulose membrane using NuPAGE Transfer Buffer (catalog number: NP0006) that had been supplemented with 10% methanol and 1% of the proprietary antioxidant. Transfer was run at 30 volts for one hour at room temperature.

The blot was incubated in a blocking solution made of 5% nonfat milk in TBS-Tween (1x tris-buffered saline (TBS) with 1% Tween 20) solution overnight at 4°C. The blot was cut between the 40 and 30 kD bands on the marker in order to allow us to assay p21 separately and utilize a smaller volume of the p21 primary antibody. The piece containing the small proteins was washed with TBS-Tween solution two times and incubated in a 1:100 dilution of purified mouse anti-p21 antibody from BD Pharmingen (catalog number: 556430) for two days at 4°C. All antibodies were diluted with blocking buffer to inhibit non-specific binding. The piece containing larger proteins was washed with TBS-Tween solution two times and incubated in a 1:1000 dilution of mouse anti-p53 antibody from Calbiochem (catalog number: OP43) for one day at 4°C. This larger protein blot was washed with TBS-Tween three times. The blot was incubated with anti-glyceraldehyde dehydrogenase (GAPDH) mouse antibody (Ambion, catalog number:

AM4300) at a 1:2000 dilution for 30 minutes at room temperature. GAPDH is used in our western blot as a loading control, as all cells express GAPDH at consistent, measurable levels (Thiel *et al.*, 2015). Both blots were washed with TBS-Tween four times and exposed to goat anti-mouse alkaline phosphatase antibody (Santa Cruz, catalog number: sc-2008) at a 1:1500 dilution for one day at 4°C.

Blots were developed by washing them with TBS-Tween four times. Blots were exposed to one NBT-BCIP substrate tablet (purchased from Sigma- Aldrich, catalog number: B5665-25Tab) that had been dissolved in 10 mL deionized water and allowed to develop in the dark. Once bands formed, the blots were rinsed with deionized water to halt further development.

A photo of each blot was taken after the blots developed. The blots were analyzed using ImageJ. The protocol for analysis was adapted from lukemiller.org (2010). A photo of the blot was opened in the ImageJ program and each lane was outlined. The optical density of each lane was determined by the software and presented as a series of peaks, with each peak corresponding to a band. The area under the curve was calculated and a percentage of the total area was determined for each band. The band percentages of each condition were compared the compound RD27's levels to calculate the relative density of p21 products. RD27 was used as a positive control in the western blots due to its ability to consistently produce high levels of p21 expression in the western blot. The relative density of the p53 bands for each condition was adjusted by comparing them to the condition's corresponding GAPDH bands. This comparison allowed us to adjust for the varying amounts of protein that were loaded into the gels. This was also done to adjust

the p21 levels by comparing them to the adjusted p53 levels because varying levels of p53 could affect how much p21 could be expressed.

MTS Assay

Both mutant p53-containing cell lines (SF295 and DLD1) and the null p53 cell line (H1299) were examined in the MTS assay. Cells were plated at 50,000 cells/mL into 96-well plates containing a serial dilution of compound (See Table 1). Five compounds were run on one plate using the serial dilutions outlined in Table 1. Each dilution was run in duplicate to account for possible human error. Cells were added to each well, with the exception of the end columns on the plate. These end columns were used to subtract the absorbance of the media during data analysis. The cells were incubated for three days with compound at 37 °C and in 5% CO_2. Tetrazolium dye (purchased from Promega, catalog number: G3580) was added and cells were allowed to incubate for three hours at 37°C. Absorbance was read at 490 nm using a SpectraMax Microplate Reader. The absorbances of treated cells were compared to cells that received no compound to calculate a percent viability. Percent viability was calculated by dividing the absorbance of a sample of cells treated with a certain dilution of compound by the absorbance of the sample of the same cell line that was not treated with compound. This calculation assumes that compound treatment affects cell growth, but does not alter cell metabolism; in other words, a decrease in the calculated viability is assumed to be a decrease in growth rather than a decrease in cell metabolism. The percent viability for each compound dilution was plotted and used to calculate the EC_{50} or the effective concentration at which there is only 50% viability in a cell line. The EC_{50} was determined

by plotting a polynomial trend line and using the resulting equation to determine at what concentration of compound the viability of the cells was reduced to 50%. The mutant cell line EC_{50} values were compared to the null cell line EC_{50} values to generate a therapeutic index using the equation $\frac{EC50\ of\ null\ p53\ cell\ line}{EC50\ of\ mutant\ p53\ cell\ line}$. Therapeutic indices are used to indicate how specific a compound is at limiting cell viability in a p53-dependent manner.

Table 1: Serial Dilution of One Compound for the MTS Assay

10 µg/mL	0.6 µg/mL
10 µg/mL	0.6 µg/mL
5 µg/mL	0.3 µg/mL
5 µg/mL	0.3 µg/mL
2.5 µg/mL	0.15 µg/mL
2.5 µg/mL	0.15 µg/mL
1.25 µg/mL	No compound
1.25 µg/mL	No compound

The above table is a representation of how one compound would be diluted in a plate used for an MTS assay. Each compound requires the use of two columns in the 96-well plate in order to generate a full serial dilution. Each sample was run in duplicate, and five compounds were screened in this manner per plate. Columns at either end of the plate remained open.

Kill Curve

A kill curve for both DLD1 and SF295 in response to hygromycin B (Life Technologies, catalog number: 10687010) was generated according to the protocol provided by Mirus Bio. Briefly, cells were plated at a concentration of 80,000 cells/well in a 24-well dish. Cells were then treated with serial concentrations of hygromycin B according to Table 2.

Cells were assessed every day by examining them under the microscope to determine if cells were being affected by the hygromycin B. Wells were checked to determine if adherent cells were present, whole cell bodies were floating, or if only cellular debris was present. The supplemented RPMI media was changed every four days and fresh hygromycin B was added at that time. The treatment lasted two weeks. The concentration one dilution above the minimum needed to kill all cells after one week was used in subsequent experiments to ensure that cells lacking our plasmid would not grow. It was necessary that all cells not containing our plasmid DNA were killed before the cells are used to screen compounds to generate a stable Firefly luciferase-expressing cell line. If cells that do not contain our plasmid were not all killed, then resulting data indicating low luciferase expression may not have been due to the compound not reactivating mutant p53 but rather due to a lack of luciferase gene present in the well of cells used to test the compound.

Table 2: Kill Curve Hygromycin B Concentrations

0	0	50	50	100	100
150	150	200	200	300	300
400	400	500	500	600	600
700	700	800	800	1000	1000

24-well plate map used to generate the kill curve for DLD1 and SF295 in response to hygromycin B. The numbers are in μg/mL for each well. Table taken from stable cell line generation protocol created by Gen Matsumoto.

Transient Transfection

Transient transfections were used to verify that the firefly luciferase gene-containing plasmid was capable of producing a functional firefly luciferase protein. The protocol for the transient transfection using Lipofectamine 3000 was provided by Life Technologies. A549 or SF295 cells were plated at a concentration of 100,000 cells/mL into 24-well dishes and allowed to incubate overnight. Lipofectamine 3000 (Life Technologies, catalog number: L3000008) incubated for five minutes at room temperature in a solution of Opti-MEM® media (Life Technologies, catalog number: 31985070) with a 1:10 mixture of *Renilla* Luciferase plasmid (Promega, catalog number: E2241) and either pGL4.38 (Promega, catalog number: E3651), or p53luc, gifted by Dr. Mark Demma. Each well of cells received 3 μg of plasmid. Cells were incubated for 24 hours before treatment with either compounds meant to reactivate mutant p53 or doxorubicin.

Stable Cell Line Generation

The pGL4.38 plasmid was linearized in order to facilitate recombination to integrate the plasmid into its genome. The firefly luciferase plasmid, pGL4.38, was cut using 20 units of the *Sal*I restriction enzyme (gifted by Dr. Brianne Barker purchased from New England BioLabs) in NEBuffer 3 for six hours at 37°C. The restriction digest products were run on a 1% agarose gel, and the digested plasmid was extracted using the QIAGEN Gel Extraction Kit. Briefly, the band was cut from the gel using UV light to visualize the DNA. The gel was mixed with QG buffer and incubated in a 50°C water bath to liquefy the agarose. Isopropanol was added to the solution, and the solution was

run through a spin column. The column was washed with buffer. DNA was isolated from the column using elution buffer. The concentration of the resulting DNA was determined using a NanoDrop.

The DNA was dried and isolated using alcohol precipitation in order to concentrate the sample (Ausubel *et al.*, 2002). One volume of sodium acetate was added to the DNA. Ice-cold 100% ethanol was added and allowed to precipitate overnight at -20°C. Ethanol was removed and the DNA was allowed to finish drying at room temperature.

The protocol for developing a stable cell line was adapted from a protocol provided by Dr. Brianne Barker. SF295 cells were plated at a concentration of 300,000 cells/mL and allowed to incubate overnight. SF295 cells were utilized due to their containing a hotspot mutation in the *p53* gene. Linearized pGL4.38 plasmid, 2 μg, was suspended in Opti-MEM® media and allowed to incubate in a solution containing 5 μL of Lipofectamine 3000 for five minutes at room temperature. The DNA/Lipofectamine 3000 complex was added to the cells, and the cells were returned to the incubator for four days.

The transfected cells were then split into a 6-well dish and two 10-cm dishes. The 10-cm dishes were utilized in the hopes of easily isolating colonies that were growing distantly from each other, while the 6-well dish was used in order to group cells close enough to ensure further divisions. Cells were treated with a 150 μg/mL solution of hygromycin B based on the kill curve experiment. Media and hygromycin B were replaced every four days for two weeks. The cells that were growing at the end of the treatment were isolated using trypsin and stored in liquid nitrogen.

Treatment with Doxorubicin

A549 cells were used due to their expressing wild-type p53. A549 cells were treated with doxorubicin after being transiently transfected with either the pGL4.38 or p53luc plasmids in order to damage the DNA and induce p53 activation in the cells. This treatment was done to verify that our plasmids contain functional firefly luciferase that was able to be expressed when active p53 bound to the response element. A549 cells were treated with doxorubicin at concentrations of $2.5x10^{-7}$, $5x10^{-7}$, $1x10^{-6}$, or $1.25x10^{-6}$ M in duplicate for 16 hours (Yokochi and Robertson, 2004). Cells were lysed according to the protocol provided by Promega using the Dual Luciferase Reporter Assay System (Promega ,catalog number: E1910). Briefly, cells were rinsed with 1x PBS. Passive lysis buffer was added and cells were shaken at room temperature for 15 minutes. The lysate was obtained and stored at -80°C.

Luciferase Assay for Transient Transfection

Luciferase activity was measured using a GloMax 20/20 Luminometer following the protocol provided by Promega. A 1:5 ratio of lysate and Luciferase Assay Reagent II (Promega, catalog number: E1910) was placed in the luminometer and firefly luciferase luminescence was measured for 10 seconds after a 2 second delay. The same volume as the Luciferase Assay Reagent II was added of Stop & Glo® (Promega) and *Renilla* luciferase luminescence was measured for the same amount of time. The Relative Light Units (RLUs) were calculated by dividing the reading for the Firefly luminescence by the reading for the Renilla luminescence in order to account for variances in the transfection

rate between conditions. Treated cells were compared to untreated cells in order to calculate a fold change in luminescence.

Luciferase Assay for Stable Cell Line

Stable cell lines from above were treated with compounds RD27, RD20, or CP-31398 as these compounds either induced high levels of p21 in the western blots or had relatively high therapeutic indices in the MTS assays relative to other tested compounds. The compound concentration varied from 2.5 μg/mL to 10 μg/mL. The treatment length also varied from ten hours to 24 hours. Cells were isolated after treatment by rinsing them with 1x PBS and exposing them to trypsin-EDTA to detach them from the plate. After all cells detached, the trypsin was inactivated by adding RPMI media. Cells were pelleted, and the pellet was rinsed with 1xPBS. The pellet was resuspended in lysis buffer (Promega, catalog number: E1500) and vortexed. Samples were spun at 12,000 g for 15 seconds. The lysate was isolated and mixed in a 1:5 ratio with luciferase reagent (Promega, catalog number: E1500) in the luminometer, and the luminescence was measured for 10 seconds after a two second delay (Promega). Each sample was run in duplicate and compared to control samples that received no treatment to calculate the fold change over control.

3. Results

Compound Screening

We screened our compounds in both DLD1 and SF295 cells in order to determine whether the compounds were able to reactivate mutant p53 in these cell lines by examining their ability to induce p21 bands. All samples lysed from DLD1 cells

contained bands that correspond in size to p53 and GAPDH (Figure 7), indicating that our protein isolation was successful and our cell lines expressed their versions of p53. The untreated control sample had no band that corresponds in size to p21, which indicates that the DLD1 cells do not express p21 normally. Because the p21 levels are typically low in treated samples, we loaded the maximum amount of protein possible into each lane, increasing the risk of spillover when the lanes are being loaded. This spillover is evident in the blank lane, due the presence of a small amount of GAPDH. Lysates obtained from DLD1 cells treated with compounds RD 1, RD 14, RD 23, RD 27, RD 33, and RD 35 all exhibited a p21 band with varying intensities. Lysate obtained from cells treated with RD 27 had the darkest p21 band. The RD 23 condition also had a particularly dark p21 band. RD 27 had the highest peak of all of the screened compounds when the p21 bands were measured in ImageJ software (Figure 8). Taken together, these data indicate that our compounds are able to induce p21 expression in cells that do not normally express p21. Since p21 is a downstream protein from p53, these results support the hypothesis that our synthesized compounds are able to reactivate mutant p53 in DLD1 cells. We have also tested some of our compounds using SF295 cells, which contain a hotspot mutation in p53 that is a particularly common in a variety of cancers. Many of these compounds also induced p21 expression in the SF295 cells (Figure 9), which indicates that our compounds' activities may not be mutation-specific.

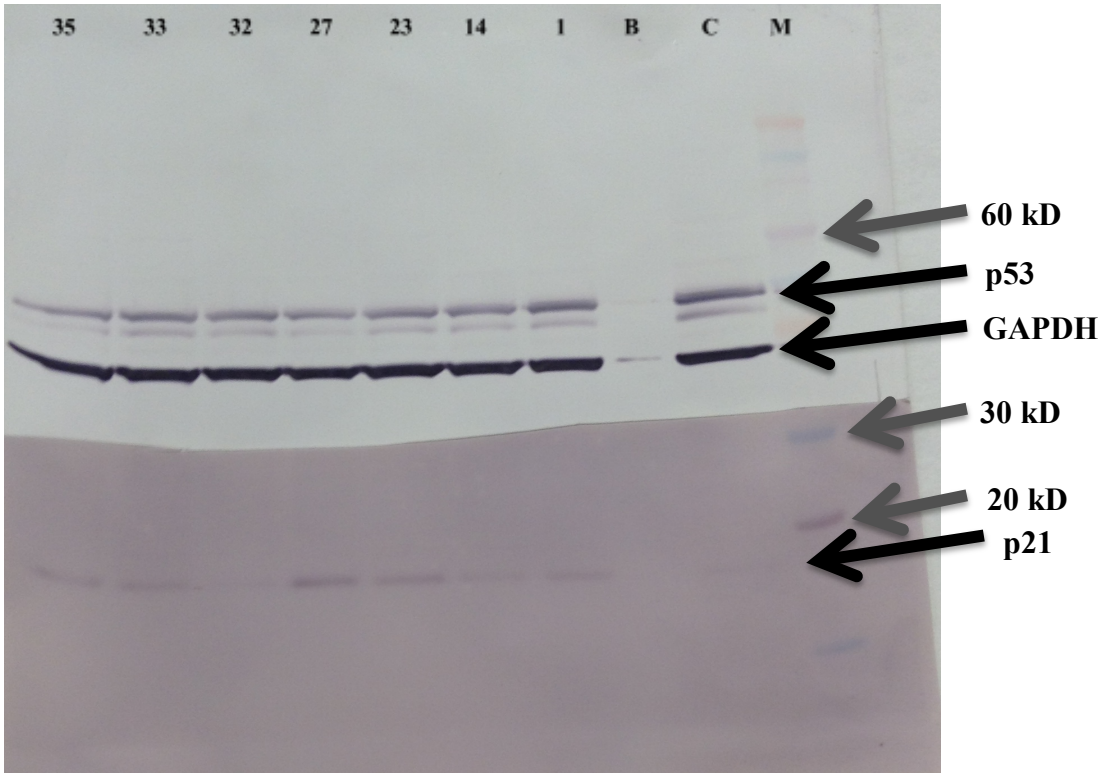

Figure 7: Western Blot of RD Compound-Treated DLD1 Cells

Numbers correspond to the RD numbers, which are assigned by the Doll Lab in the order of synthesis. M is the lane of the Molecular Weight Marker. C is the untreated control. B is a lane that had no lysate added. Bands are as labeled with the black arrows indicating proteins in the samples and the gray arrows indicating molecular marker weights.

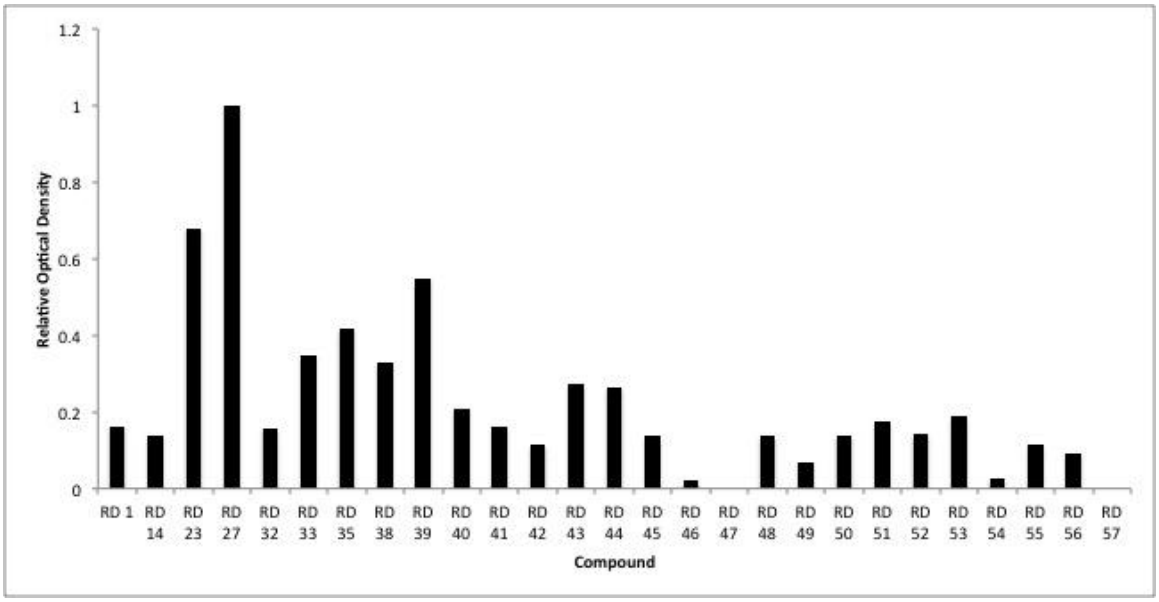

Figure 8: Densitometry of Western Blots for p21 Expression in DLD1 Cells

Data shown represents the relative optical density of the p21 band for each treatment in mutant p53-containing cell lines. Band density was adjusted using the band density of GAPDH and p53 for each sample. RD 27 was used as a positive control in order to compare multiple blots. All compound treatments consisted of 15 μg/mL for 16 hours. n=1 for each compound.

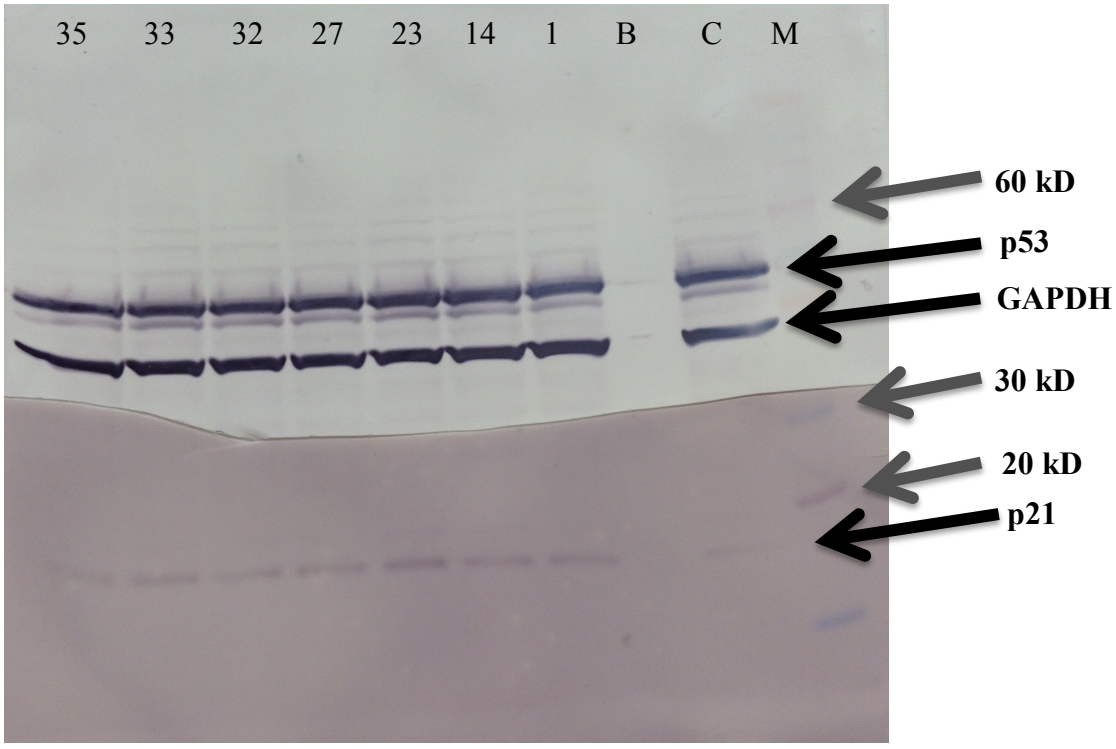

Figure 9: Western Blot of RD Compound-Treated SF295 Cells

Numbers correspond to the RD numbers, which are assigned by the Doll Lab in the order of synthesis. M is the lane of the Molecular Weight Marker. C is the untreated control. B is a lane that had no lysate added. Bands are as labeled with the black arrows indicating proteins in the samples and the gray arrows indicating molecular marker weights.

Given that RD 27 was shown to consistently induce strong p21 expression in both DLD1 and SF295 cell lines, we screened RD 27 in the MTS viability assay to determine if this compound was able to inhibit cell growth in a p53-dependent manner. Both DLD1, a mutant p53 containing cell line, and H1299, a null p53 cell line, cells show a decrease in viability at RD 27 concentrations greater than 2.5 μg/mL (Figure 10). DLD1 had a slightly greater decrease in viability at the 5 μg/mL compared to H1299. The therapeutic index for RD 27 was 1.03, confirming that RD 27 is toxic to all cell lines. These data indicate that while RD 27 is able to induce strong p21 expression, RD 27 may also have strong off-target effects that make it unsuitable as a potential lead molecule for future compound synthesis.

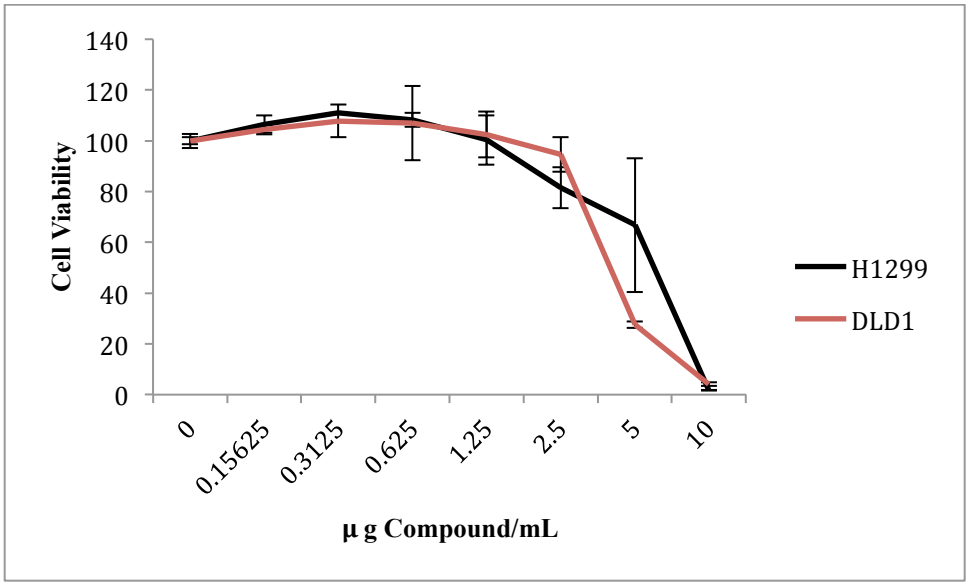

Figure 10: Percent Viability for RD 27

Conditions were plated in duplicate. Data reported as a percent viability compared to untreated control. Error bars represent standard deviation between duplicates. DLD1 has mutant p53. H1299 is a p53 null cell line. n=2.

Stable Cell Line Generation

As the western blot assay cannot screen many compounds at once, we next attempted to develop a reporter gene assay using firefly luciferase that could allow us to examine multiple compounds on one plate. We obtained a plasmid from Promega, called pGL4.38, that contains both a firefly luciferase gene under a p53 response element, and a hygromycin resistance gene. Before we attempted to develop a cell line that had incorporated this plasmid into its genome, we first decided to use this plasmid in a cell line containing wild-type p53 in order to verify that this plasmid contained a functional luciferase gene that could be induced by active p53. A549, a wild-type p53 containing tumor cell line, was transiently transfected with the pGL4.38 plasmid. The cells were then exposed to doxorubicin, a DNA-damaging reagent. Since DNA damage initiates a signaling pathway that activates p53, we hypothesized that doxorubicin treatment would be able to induce firefly luciferase expression in A549 cells. As seen in Figure 11, treatment with doxorubicin at concentrations of at least $1x10^{-6}$ M was able to induce a two-fold increase in Firefly luciferase expression in A549. This indicates that the plasmid contained a functional luciferase gene that can be induced by active p53.

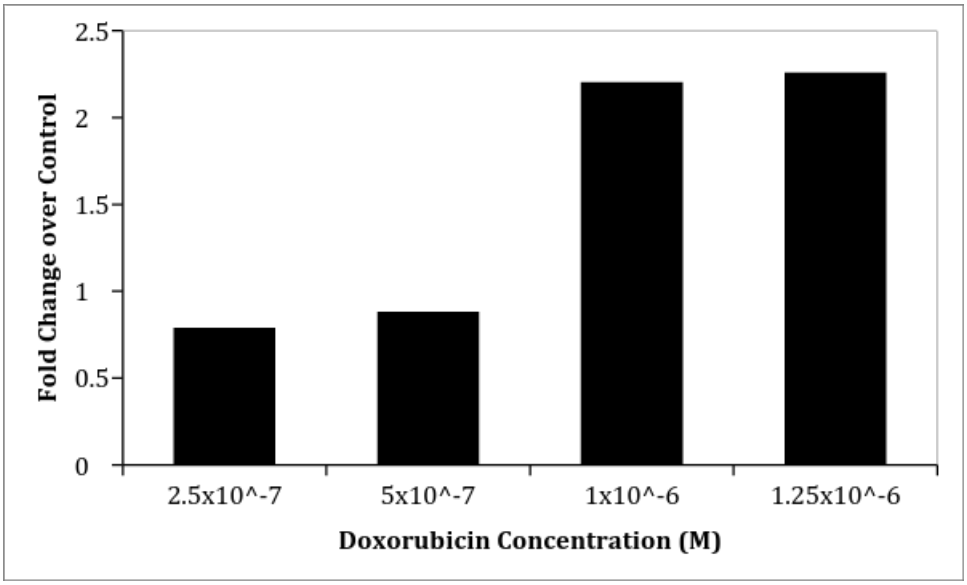

Figure 11: Verification of pGL4.38's Ability to Produce Functional Firefly Luciferase in a wildtype p53-containing cell line (A549)

A549, containing wild-type p53, were transiently transfected with pGL4.38 plasmid and treated with increasing concentrations of doxorubicin, a DNA-damaging reagent. Data were initially recorded as a ratio of Firefly to Renilla luciferase for each sample. The ratios were used to convert data to a fold change over untreated, transfected cells. Fold increase was calculated by dividing the treated condition's luminescence by the untreated condition's luminescence. Samples were run in duplicate and data are presented as the average fold change.

Having confirmed that our plasmid was functional, we began the next step in generating a stable cell line. Before we could transfect the plasmid into our mutant-p53 containing cells, we needed to determine how sensitive each cell line was to hygromycin B. Hygromycin B is an antibiotic that can kill eukaryotic cells (Eustice and Wilhelm, 1984). Since our plasmid contains a hygromycin B resistance gene, any cell that successfully took in the plasmid should be able to survive in the presence of hygromycin B. However, some cells types may be more sensitive to hygromycin B compared to other cell types. Without first knowing how sensitive our cell lines are to hygromycin B when they do not have the resistance gene, we would not know how much of the antibiotic to add to successfully kill all cells that did not take in our plasmid. Being able to kill any cells that do not have our plasmid is necessary, as subsequent experiments rely on comparing resulting luciferase expression in the same number of cells that are treated with different compounds. If some cells survive that do not have our plasmid, those cells would lower the overall luciferase measured for that treatment condition as a portion of the cells in that sample would be incapable of expressing luciferase even if the p53 in the cells is active.

Both SF295 and DLD1 cells died in the presence of hygromycin B (Figure 12; DLD1 not shown). SF295 was more sensitive to hygromycin B; all cells died in the presence of 150 μg/mL. DLD1 cells survived in hygromycin B until treated with 400 μg/mL. SF295 cells were chosen for the subsequent generation of a stable cell line due to their containing a hotspot mutation in p53. SF295 cells transfected with the pGL4.38 plasmid were able to grow in the presence of hygromycin B at concentrations that had

killed the SF295 cells not transfected with the plasmid (Figure 12C). In the case of SF295 cells, these data indicate that the pGL4.38 plasmid's hygromycin B resistance gene is successful in preventing hygromycin B from killing the cells.

Colonies of transfected SF295 cells were visible after two weeks of incubation. After this amount of time, only the cells that were able to incorporate the linearized plasmid into their genome would be able to continue to replicate, as the plasmid does not contain an origin of replication needed for the cells to replicate the DNA. The colonies were collected and screened for firefly luciferase expression (Figure 13). Cells were treated with CP-31398, as this compound was reported to be able to reactivate mutant p53 at lower concentrations than the RD compounds (Foster *et al.*, 1999). We found that cells in Well 1 and Well 5 had expression of the luciferase gene that was higher than the levels seen in controls. Therefore, we concluded that these cell groups had incorporated the plasmid correctly.

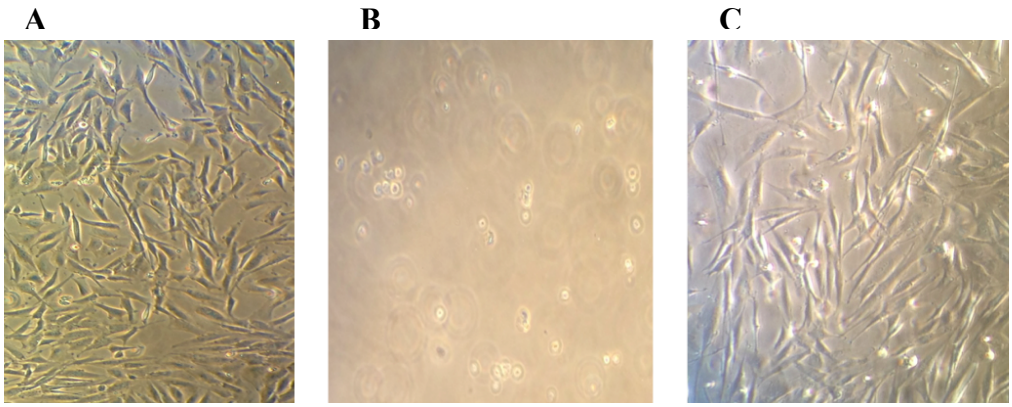

Figure 12: SF295 cells in the Presence of Hygromycin B Before and After Transfection with the pGL4.38 plasmid

A. Untreated SF295 cells are pictured. B. The cells die in the presence of 150 µg/mL of hygromycin B after a 72 hour incubation. C. Cells transfected with the pGL4.38 plasmid are able to grow in hygromycin B. Cells pictured were incubated in hygromycin B for 1 month.

We grew up the cells from Well 1 to use in subsequent experiments, as they exhibited the highest luciferase expression. We used two concentrations of the CP-31398 compound (2.5 and 5 μg/mL) and of RD 27 compound (5 and 10 μg/mL) to determine if our low levels of luminescence (Figure 13) were related to the compounds being tested. We also varied how long the cells were exposed to the compounds by both repeating the 10 hour treatment and performing a 24-hour treatment. We found that the cells that were treated for 24 hours only had a higher luminescence compared to the untreated control when treated with the low concentration of CP-31398 (Figure 14). Cells treated with the high concentration of RD 27 had approximately half the luminescence of the untreated control. These data indicate that both compounds are toxic to the cells, as only the low concentration of CP-31398 had a higher expression than control. This level of expression is also lower than the initial level reported in Figure 13 that was for a 10 hour treatment, possibly due to the toxicity of being exposed to the compounds for a longer time period.

When the experiment was repeated for a 10 hour treatment, there was more luminescence in the RD 27-treated cells, though both of RD 27's concentrations remained below the control's luminescence (Figure 15). These data further support the hypothesis that these compounds are too toxic for these cell treatment times. Additionally, these data also suggest that both CP-31398 and RD 27 are not potent molecules at reactivating mutant p53, as the firefly luciferase expression levels are at best only slightly higher than untreated control levels. Cells treated with CP-31398 at a high concentration for 10 hours had a decrease in luminescence relative to control compared to

the 24 hour treatment at the same concentration. Given the small sample size, this measured difference may not be an accurate reflection of what is occurring in the cells.

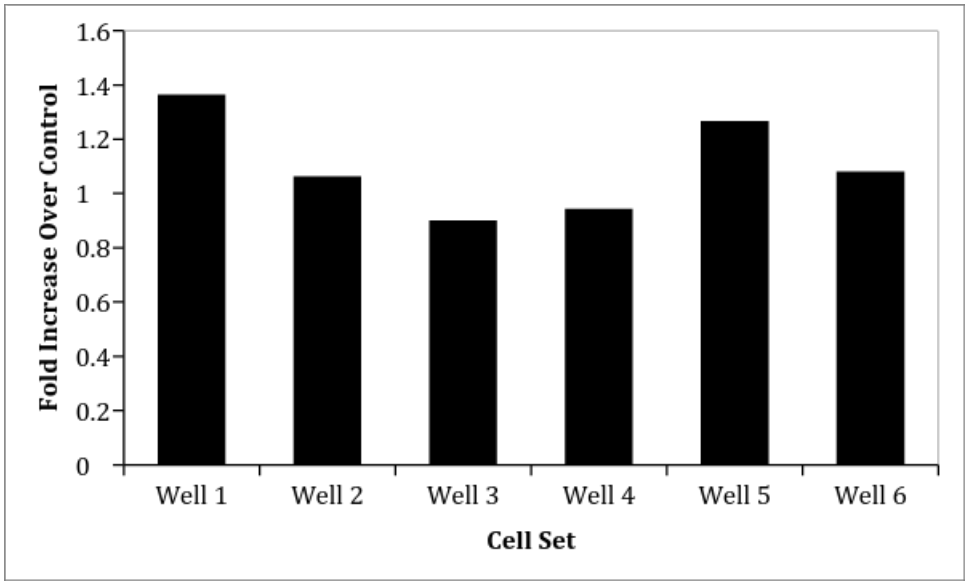

Figure 13: Preliminary Firefly Luciferase Screening

SF295 Firefly Luciferase cells were plated into a 6-well dish and treated with CP-31398 (5 μg/mL) for ten hours. Well refers to a particular group of cells. n=1 for each condition.

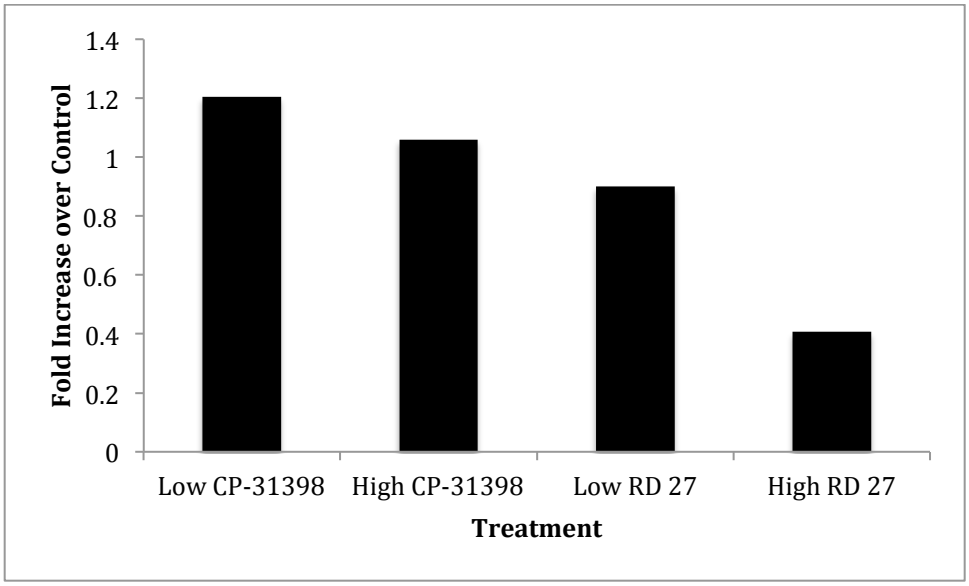

Figure 14: Firefly Luciferase Expression in SF295 Firefly Luciferase Cells after a 24 Hour Treatment

Cells were treated with either CP-31398 or RD 27. Low CP-31398 refers to a 2.5 μg/mL treatment, while the High CP-31398 refers to a 5 μg/mL treatment. Low RD 27 refers to a 5 μg/mL treatment, and a High RD 27 refers to a 10 μg/mL treatment. Cells were incubated with compound for 24 hours. Data represented as the average fold change over untreated controls. Samples were run in duplicate.

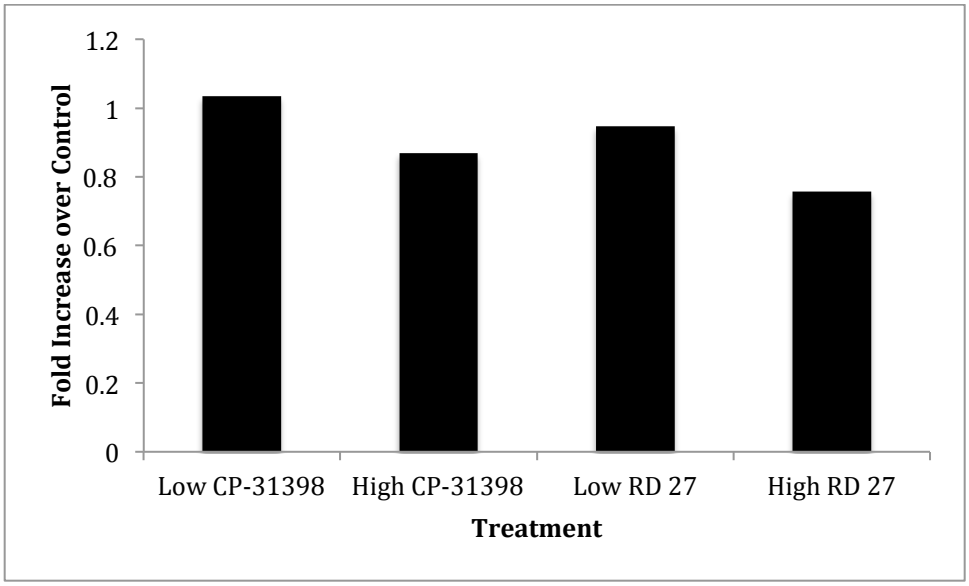

Figure 15: Firefly Luciferase Expression in SF295 Firefly Luciferase Cells after a 10 Hour Treatment

Cells were treated with either CP-31398 or RD 27. Low CP-31398 refers to a 2.5 μg/mL treatment, while the High CP-31398 refers to a 5 μg/mL treatment. Low RD 27 refers to a 5 μg/mL treatment, and a High RD 27 refers to a 10 μg/mL treatment. Cells were incubated with compound for 10 hours. Data represented as the average fold change over untreated controls. Samples were run in duplicate.

We obtained another plasmid from Dr. Mark Demma that has the Firefly Luciferase gene under the control of multiple p53 response elements, called p53luc. We thought that having more places to bind active p53 could lead to an increase in firefly luciferase expression from the plasmid. We transiently transfected either the pGL4.38 or p53luc plasmids into normal SF295 cells. We then treated the transfected cells with either CP-31398 or RD 20 for 10 hours. We chose RD 20 as it had been reported to have a relatively strong therapeutic index in Brittany Barreto's thesis (Barreto and Dasmahapatra 2013). We found that the p53luc plasmid was not able to induce more Firefly Luciferase expression compared to the pGL4.38 plasmid (Figure 16). Additionally, RD 20 treatment resulted in Firefly Luciferase expression that was lower than untreated levels for both plasmids, indicating that the compound is not able to reactivate p53 in a potent manner.

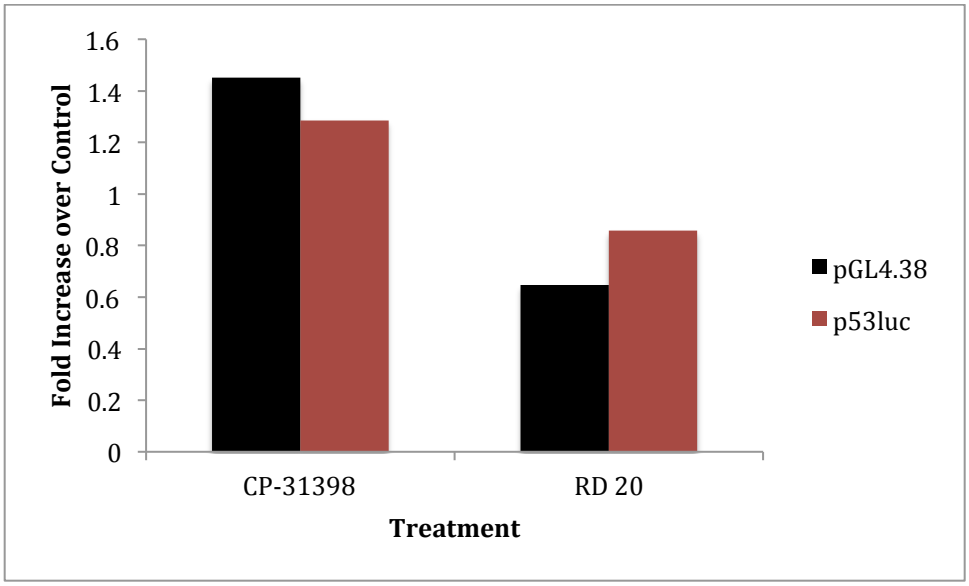

Figure 16: Transient Transfection with pGL4.38 or p53luc Plasmid

SF295 were treated with compounds after an overnight transfection. Treatment with either CP-31398 at 5 µg/mL or RD 20 at 10 µg/mL occurred for 10 hours. Each sample was compared to a control containing the matching plasmid luciferase. Data reported as the average between duplicates for each condition.

4. Discussion

Cancer is a prevalent killer in our society that is characterized by its ability to quickly mutate its cellular DNA. This high mutation rate leads to the cancer developing resistance to administered therapeutics. In order to continue to be able to treat people who have developed these aggressive cancers, new drug therapies must be developed. Mutant p53 is an attractive drug target due to its being mutated in a wide variety of cancers, but it offers a distinct set of challenges. One such challenge is that researchers would need to find a small molecule that activates the function of p53, which is more difficult compared to developing a molecule that can inhibit a protein. Additionally, the crystal structure of p53 when it is not bound to DNA is unknown, so attempting to rationally design a molecule to target the protein is challenging.

By screening random compounds that all share a similar backbone, our laboratory has identified several small molecules using a western blot assay that indicated some ability to reactivate mutant p53 as assessed by their relative activity to induce p21 by Image J software. We are limited with the capacity of the western blot assay to screen many compounds at the same time, so comparing the potency of multiple new compounds is challenging. Moreover the low potency and nonspecific activities of these preliminary active compounds pose additional challenges in reproducibility. In order to improve our primary screening assay, we decided to develop a reporter gene assay. The reporter gene assay is not only quantitative, but also much simpler and less time consuming than our current western blot assay, and can be used for screening a large number of compounds at any given time.

We decided to develop a stable cell line rather than perform transient transfections in order to simplify the resulting reporter assay. The overall goal of this assay was to develop a way to utilize 96-well plates to screen many compounds at once. By generating a cell line that consistently has the ability to express firefly luciferase in every cell, we hypothesized that screening compounds would be simpler. Without a stable cell line, we would have to transfect cells every time before we could use them to screen compounds. This would both add a step to our screening process that we are attempting to simplify, and could potentially introduce variability in our data across plate readings due to variances in DNA uptake during transfections.

In order to develop this reporter gene assay, we first examined our screened compounds to identify likely compounds that could serve as successful positive controls in the assay. Among the many screened compounds, we found that RD 27 induced the strongest p21 band as seen in Figure 6. We also chose to use CP-31398 ,which is available commercially, due to its reported efficacy in the Foster and colleagues paper (1999) where it was used successfully to elicit luminescence from cells transfected with a luciferase gene under a p53 response element.

We found that we were able to see a luciferase signal using the pGL4.38 plasmid in wild type p53 cells exposed to doxorubicin (Figure 11), indicating that the plasmid had been correctly manufactured with the firefly luciferase gene under the control of the p53 response element. Our luciferase signal in the A549 cells, containing wild type p53, was only a two-fold increase over untreated cells. This may be due to our only exposing the A549 cells to doxorubicin for 16 hours. A549 cells normally have low amounts of p53

present in the cells due to its being quickly degraded after synthesis. In order to observe a stronger p53 signal, the degradation pathway must first be inhibited in order to allow the newly synthesized p53 to accumulate in the cell. This process is rather slow. We only wished to verify that our plasmid was capable of producing functional luciferase, so we limited the exposure time of our cells. Since we used the minimum amount of exposure time reported in the literature to give a signal, it may be that we did not expose our A549 cells long enough to doxorubicin in order to observe a stronger signal.

After verification of the plasmid, we examined our cells for sensitivity to hygromycin B, a protein synthesis inhibitor (Gonzalez *et al.* 1978). Both DLD1 and SF295 were exposed to serial dilutions of hygromycin B to determine sensitivity. DLD1 was more resistant to hygromycin B compared to SF295; it was able to grow in over twice as concentrated a hygromycin B solution (Data not shown). SF295 cells were able to grow in hygromycin B after they had been incubated with linear pGL4.38 for four days (Figure 12C). As mammalian cells cannot replicate plasmid DNA due to the lack of an origin of replication in the plasmid, the cells would die in the presence of hygromycin B after a week because they would have lost their plasmid DNA over the course of replication. Since our cells were able to continue to replicate in the presence of hygromycin B for over two weeks, this suggests that our cells successfully integrated the linearized plasmid DNA into the cells' chromosomal DNA. These cells can continue to grow indefinitely while maintaining the newly acquired luciferase gene; as such, these cells could be useful in growing large quantities of cells capable of being used in a high-throughput screening of compounds for the ability to reactivate mutant p53.

Certain wells of cells expressed lower levels of luciferase upon treatment compared to the untreated cells (Figure 13). It may be that these groups of cells integrated the plasmid into an area that was difficult to transcribe, leading to lower luciferase levels. The treated cells may have measured below control levels due to the toxicity of the compound killing the cells, reducing the total number of cells available from which to isolate luciferase. It may also be that our compounds are successfully reactivating mutant p53, leading to apoptosis of the treated cells. The cells should be examined under current treatment conditions in an assay that measures apoptosis, such as the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, catalog number: G7792), which measures how active caspases 3 and 7 are in a sample. If our cells are found to be undergoing apoptosis, then treatment times or the concentration of compound should be lessened.

Altering the compound, concentration, or treatment duration had little effect on luciferase expression (Figures 14 and 15). Our compounds may not potent enough to induce strong firefly luciferase expression in the stably transfected SF295 cells. These compounds are known to be rather toxic to cells and not potent in their action on mutant p53 (Figure 10). It could be that the off-target effects are too strong for the current treatment durations. An example of an off-target effect that may be occurring is that the compounds, in addition to reactivating mutant p53, may also be interacting with other, non-mutated proteins that are essential to cell survival. By altering the functions of these essential proteins, the compounds could be killing the cells without having to rely on p53.

RD 20 was reported in Brittany Barreto's thesis to have the highest therapeutic index of all of the screened compounds to date (Barreto and Dasmahapatra, 2013), but it was unable to generate a robust luciferase signal (Figure 16).

It might also be that the recombination event altered the luciferase gene to generate a protein that has lower enzymatic activity, which might explain why we obtain slightly better readings using a transiently transfected plasmid compared to our stable cell line (Figures 15 and 16). Alternatively, it could be that the transiently transfected cells had more copies of the plasmid than the stable cell line, as Lipofectamine is an effective transfection agent capable of facilitating cells initially taking up multiple copies of the plasmid, but the recombination events that would insert the plasmid into the cell's chromosomal DNA are rather rare in the cell (Zaunbrecher *et al.*, 2008). Our alternative p53-luciferase reporter plasmid was not compatible with our cells, as seen by the low signal in Figure 16. As we did not verify plasmid integrity via restriction digest or gene sequencing before use, it could be that this alternative plasmid had degraded over time and was no longer able to produce a functional luciferase protein.

The next steps in this project involve developing a stable cell line in our other mutant line, DLD1, to determine if the mutation in SF295's p53 is preventing a strong signal. We could also use PCR to determine if the luciferase gene is still intact in our current stable cell line. Sequencing the gene would show if a mutation had been incorporated. Utilizing an apoptosis assay would allow us to determine what treatment conditions do not result in toxicity to the cells. These treatment conditions could then be used on the stable cell lines to determine what treatment conditions result in the highest

expression of firefly luciferase. Once the treatment conditions have been optimized to elicit a consistent, strong expression of firefly luciferase from the stably transfected cells, our laboratory can begin to process numerous compounds with this assay.

Being able to objectively report to our collaborating chemistry laboratory how much better one compound is at inducing luciferase expression compared to another would assist our collaborating laboratory in synthesizing more potent compounds as they could determine how a side group affects the resultant fold change in luciferase expression. We need to identify multiple potent compounds before we can move forward with testing to determine if we have a viable drug candidate, as many compounds that appeared to be promising in *in vitro* studies have been found to be ineffective during animal testing or in clinical trials (Hay *et al.*, 2014).

We were able to discover small molecules that reactivate mutant p53 in human cancer cell lines as evidenced by the appearance of a p21 band from protein samples isolated from mutant p53-containing cell lines. We were also able to initiate the development of a reporter gene assay that utilized the Firefly Luciferase gene by determining the hygromycin B sensitivity of two mutant p53-containing cancer cell lines and developing a stable cell line.

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