Drew University

College of Liberal Arts

Identification and characterization of small molecules that reactivate mutant p53 in human cancer cells

A Thesis In Biology

By

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Abstract

Cancer is a major global public health issue, and the second leading cause of death in the United States. It is a group of complex diseases characterized by the uncontrolled growth of abnormal cells, often caused by the loss of genomic integrity in cells. Cellular genomic integrity is maintained by the tumor suppressor protein p53, which is also known as: the “guardian of the genome”. P53 prevents oncogenic transformation by halting the cell cycle upon cellular stress, and activating protective methods such as DNA repair or elimination of abnormal cells via apoptosis. The tumor suppressor activity of p53 is attenuated in almost all human cancers. In 50% of cancers, p53 is inactivated due to mutations. Most of these oncogenic mutations are missense mutations in the DNA binding domain of the p53 protein, resulting in the loss of DNA binding activity due to a conformational change at physiological temperature. Cancers with mutant p53 are aggressive and often resistant to therapy, making the mutant p53 an attractive target for drug discovery research. We have developed a cell-based reporter gene assay to screen compounds for their ability to restore transcriptional activity of mutant p53. Several small molecules of different chemical structures have been identified through this method. Using conformation specific antibodies, we have also shown that these molecules induce a conformational change in mutant p53 to that of the wild-type protein, underlying the mechanism of reactivation.

Introduction

Cancer Statistics

Many people have experienced some form of cancer, or have a personal connection to someone who has suffered through cancer. Cancer is a group of complex diseases that affects a large percentage of the population. It is the second leading cause of death in the United States, with 155.8 deaths per 100,000 in 2017 (Murphy et al. 2018). It is estimated that deaths from cancer in the United States will number 606,880 in 2019 with the largest percentages of death being breast cancer for women and lung cancer for men (Siegel et al. 2019). Although the overall mortality rates of cancer have decreased from 215.1 deaths per 100,000 in 1991 to 156.0 deaths per 100,000 in 2016, cancer remains a large global public health issue (Siegel et al. 2019). This reduction in mortality rates is largely due to a decrease in tobacco use as well as earlier detection methods. This primarily impacts lung, prostate and breast cancer which have had significant reductions in death rates. However mortality rates for some other cancers, such as liver cancer, pancreatic cancer and brain cancer have risen from 2012 to 2016. It remains difficult to treat many of these types of cancer. There are many different distinct types of cancer, each defined by the type of cell that is affected. For example, three main groups of cancers include carcinomas (cancer of the epithelial cells), sarcomas (cancer of the connective tissue), and leukemias and lymphomas (cancer of the blood-forming cells or cells of the immune system) (Cooper 2000). The many different forms of cancer have become such a common and difficult occurrence that research in both understanding and treating it lies at the forefront of the biomedical sector.

Causes of Cancer

Cancer is characterized by the uncontrolled and rapid growth of abnormal cells (Cooper 2000). These abnormal cells have the ability to metastasize and spread throughout the body to distant sites. Cancer results from an accumulation of mutations in the genome, which disrupts the normal functioning of the cell and causes uncontrolled growth. Many of these mutations occur randomly throughout the lifespan of the cell. The spontaneous mutation rate for humans is relatively low at 8.0×10^{-11} mutations per base pair per replication (Drake et al. 1998). There are mechanisms for the cell to correct any mutations that result from replication and some of these mutations do not result in any harm to the cell. However, most mutations that the cell accumulates may be detrimental.

An important risk factor for the development of cancer is the presence of specific mutations. Certain hereditary mutations can greatly increase the risk for an individual to develop specific types of cancer. For example, for women with a mutation in the tumor suppressor gene *BRCA1*, their lifetime risk for developing breast cancer is 85-90% (Easton et al. 1992). The risk for developing ovarian cancer is also increased. *BRCA1* is a tumor suppressor gene that plays an important role in the response to DNA damage (Zhang and Powell 2005). Specifically, BRCA1 interacts with Rad51 and BARD1 to aid in DNA repair through homologous recombination. BRCA1 may also play a role in the repair of oxidative DNA damage and damage caused by UV radiation (Gowen et al. 1998). Due to the familial nature of several types of cancer, individuals can have a predisposition to develop certain types of cancer.

A number of carcinogens and environmental factors that cause cancer have been described. As early as 1950, people suspected that there might be a correlation between smoking and an increase in rates of lung cancer around the world. Wynder and Graham described the correlation they found between incidence of lung cancer and heavy smoking practices in men (1950). Since then, more than 60 carcinogens present in cigarette smoke have been described (Hecht 2003). Other environmental factors that may play a role in cancer development include alcohol consumption, diet, hazardous chemicals and lifestyle (Parkin et al. 2011).

UV radiation is a particularly harmful factor that is associated with skin cancers. Specifically, UV radiation induces dimers to form between adjacent pyrimidine residues in DNA, which can block transcription and more generally is a harmful mutation (Soehnge et al. 1997). UV radiation is especially harmful when it causes mutations in the genes that are responsible for cell cycle control, such as the p53 tumor suppressor gene.

Some cancers have also been linked to viral causes. For example, infection with the hepatitis B and C viruses significantly increases the risk of developing hepatocellular carcinoma or liver cancer (Levrero 2006). There is also a large amount of evidence supporting the fact that human papillomavirus (HPV) is a causative agent of cervical cancer (Bosch et al. 2002). The viral DNA from HPV is incorporated into the host genome leading to the overexpression of E6 and E7 viral genes (Lazo 1999). E6 interacts with p53 and targets it for degradation, allowing the cell to progress to the S phase. The E7 protein binds to pRB, p107 or p130 allowing E2F to dissociate and promote

progression through the cell cycle. The work of these viral genes can increase the rate of progression through the cell cycle, leading the uncontrolled growth of cells and cancer.

Cellular Biology of Cancer

According to Hanahan and Weinberg, cells must possess several criteria to develop and sustain cancer progression, which they termed the "hallmarks of cancer" (figure 1). These include, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of immune surveillance and programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, deregulating cellular energetics, genome instability and mutation, tumor-promoting inflammation, and avoiding immune destruction (2010). All of these hallmarks are alterations to the controlled growth of a normal cell, which has certain mechanisms to prevent the development of cancer. The development of cancer requires multiple alterations in the genome, resulting in a disruption of cellular mechanisms. It is the accumulation of mutations in the DNA or changes in the gene expression of a cell that drive tumorigenesis.

Some of the most important genes involved in this process are proto-oncogenes (or growth genes) and tumor suppressor genes. When activated, proto-oncogenes have the ability to disrupt the normal function of the cell and promote oncogenesis (Weinberg 1994). Through mutation of the proto-oncogene or increased expression, a gene that normally promotes cell growth and division can become detrimental to the cell. For example, the *RAS* oncogene is found in several types of cancer. The cellular *RAS* gene is involved in the signaling pathway that responds to external growth factors. Upon

mutation of the *RAS* proto-oncogene, it is converted into an oncogene in which its interactions with these external growth factors are altered (Yakum et al. 1985). It can continue to signal for cell proliferation even in the absence of the necessary growth signals. The alteration of the *RAS* gene into an oncogene promotes uncontrolled growth and division of a cell and thus oncogenic transformation.

Cells do have mechanisms to control their abnormal growth and to help prevent their development into a cancerous cell in the form of tumor suppressor genes (Weinberg 1994). In many cases of cancer, inactivation of a tumor suppressor gene prevents regulation of the cell's growth and division. Both the activation of oncogenes and inactivation of tumor suppressor genes play an important role in creating the genomic instability that often leads to cancer. One incredibly important tumor suppressor gene is p53. It plays a large role in maintaining the DNA integrity by either halting the cell cycle for the repair of the damage or leading to apoptosis when there is significant DNA damage.

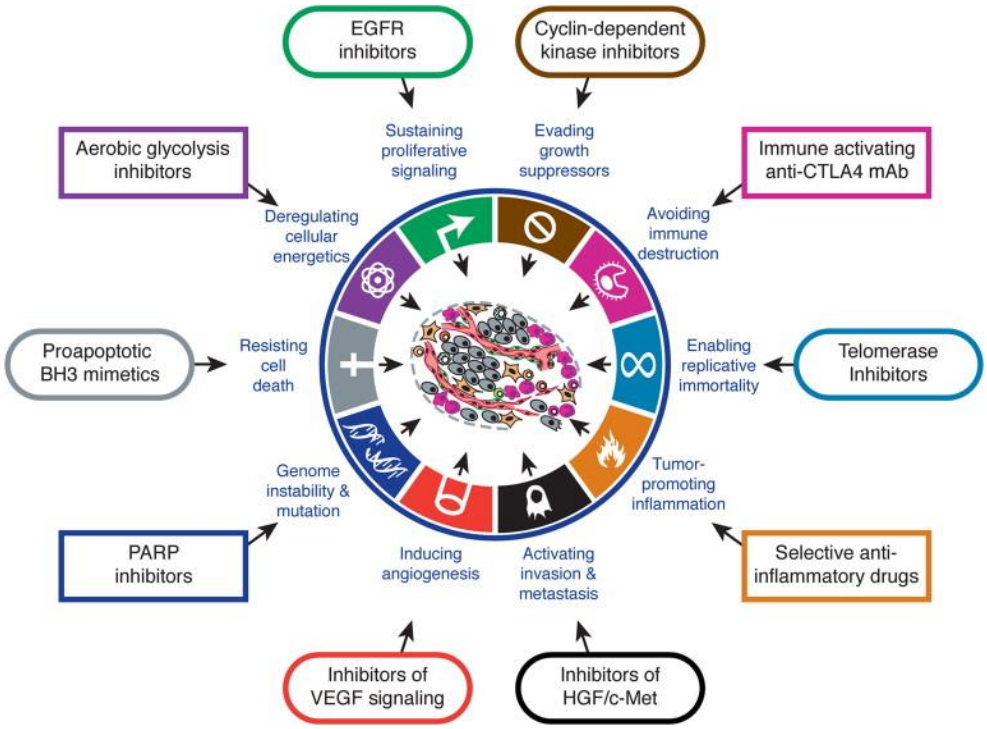


Figure 1: The Hallmarks of Cancer Hanahan and Weinberg, 2010

Current Treatment Strategies

The goal of cancer treatments are to completely remove the tumor or cancerous cells from the body and to prevent any metastatic events, therefore prolonging the patient's life as much as possible. There have been a number of important developments that allow for more effective treatment strategies for different types of cancer. Recently, many of these developments have involved earlier detection of cancer, which greatly increases patient survival. With early detection, there is less of a chance that the cancer has metastasized to other parts of the body. One of the most important forms of treatment

is surgery to remove the tumor. Chemotherapy and radiation are often used along with surgery to completely eliminate all cancerous cells. Both of these methods target the rapidly dividing cancer cells and are largely unselective (Hannun 1997). For example, cisplatin is a commonly used drug that leads to cell death through its binding to DNA and inhibition of transcription and replication (Cepeda et al. 2007). While these drugs are effective in many cases, there are also many side effects. Resistance to the drugs often develops, and there is the possibility of causing transformation in other cells in the body, resulting in relapse. Additionally, many cancers remain difficult to treat through this method.

In recent years, research into treatment strategies has moved more towards targeted therapies. Researchers now look for molecular targets that are specific to tumor cells or inhibit a pathway that is essential for tumor cell survival (Vanneman and Dranoff 2012). These therapies can be very effective for certain cancers. For example, imatinib (a small molecule inhibitor of a tyrosine kinase) has been shown to reduce the number of cancerous cells in patients with chronic myeloid leukemia (Kantarjian et al. 2002). This specific type of cancer is well understood and is caused by the translocation of two chromosomes which brings together the BCR and ABL genes and creates an active protein tyrosine kinase. The drug imatinib (Gleevec) acts as a selective competitive inhibitor of this BCR-ABL protein tyrosine kinase. While many other examples exist, some cancers are not as well understood and patients still develop resistance to these treatments.

Another area that has recently received a lot of attention is immunotherapy. The goal is to activate the body's own immune response against the tumor. T lymphocytes normally function as a part of the immune system to help coordinate the immune response and attack foreign antigens. When tumors are formed, they produce tumor-associated antigens that are recognized by the immune system. However, this process is not always effective given that tumor antigens may be very similar to self-antigens and the tumor can create an immunosuppressive environment (Mellman et al. 2011). The antitumor T cells (killer T cells) are often suppressed in the microenvironment of the tumor due to immunosuppression (Brown et al. 2017). Some drugs (ipilimumab for example) use antibodies to target and block CTLA-4, a receptor on killer T cells, which downregulates the T cell response (Phan et al. 2003). Often used in combination with other drugs, Ipilimumab (antibody to CTLA-4) allows for a stronger T cell response against the tumor. Other drugs are antibodies against PD1 (program cell death receptor) on T cells and its ligand PDL-1 on tumor cells. The interaction between PDL-1 and PD-1 on T cells inactivates the T cell so it is unable to respond to tumors. Blocking this interaction with an antibody with drugs like Keytruda (PD-1 antibody) or Opdivo (PDL-1 antibody) allows the immune response against the tumor to be maintained. However, these drugs often take time to get a response and the body can develop resistance. More recently, one goal has been to induce the production of tumor-specific T cells as well as reverse the immunosuppressive environment of tumors. For example, infection with some viruses has been shown to target human cancer cells and activate immunosuppressed dendritic cells and macrophages (Brown et al. 2017). The infected dendritic cells then

have the ability to induce antigen-specific cytotoxic T cells against the tumor. Thus, infection with the virus induces an adaptive immune response against the tumor.

Both oncogenes and tumor suppressor genes have been a major target for cancer therapies. Several drugs targeting oncogene products are in the market, however they have limitations. Therapeutics targeting tumor suppressor proteins are yet to be developed. Given that the tumor suppressor gene p53 is inactivated almost all cases of human cancer, it is a logical target for cancer therapy. Approaches to restore function in inactive p53 is an important therapeutic strategy.

Cellular Biology of P53

P53 is a tumor suppressor gene and is perhaps one of the most important genes involved in cancer. The protein was discovered in 1979, as a non-viral protein that immunoprecipitated with the SV40 large T-antigen in cells that were infected with SV40 (Land and Crawford, 1979). P53 was initially believed to be a cellular oncogene due to the fact that its levels were increased in many different types of tumors, but was barely present in non-transformed cells. Rotter was able to establish p53 as a biochemical marker of tumors due to its accumulation in transformed cells (Rotter, 1983). The p53 gene was cloned and used in multiple experiments to establish its ability to transform cells. Many groups were able to show that the cloned p53 could transform and immortalize cells (Eliyahu et al. 1984) (Jenkins et al. 1884). This reinforced the idea of p53 as on oncogene, that led to tumorigenesis.

It was finally determined that p53 is actually a tumor suppressor gene when p53 clones were compared and found to have variations in the DNA sequence. These clones

were actually mutated forms of p53 and it is these p53 mutants that are found in high levels in tumor cells. The function of the wild-type p53 is necessary to prevent tumorigenesis and does not have the ability to transform cells (Eliyahu et al. 1988). Mutations in p53 are extremely widespread in cases of human cancer and it is the most frequently mutated gene. In the case of Li-Fraumeni syndrome, individuals have a germline mutation in the p53 gene and there is a high risk for the development of a variety of tumor types at a young age (Malkin D et al. 1990). It has become clear that the function of wild-type p53 is extremely important to maintain genomic integrity and to prevent the establishment of tumors.

P53 acts as a transcription factor and binds directly to specific sequences in human DNA (Kern, et al. 1991). In fact, there are several hundreds of p53 binding sequences present in the human genome (Wei et al. 2006). The protein has a DNA-binding domain where this interaction takes place (figure 2). The transcription of many genes by p53 leads to apoptosis or growth arrest: two important processes for maintenance of cellular integrity. It was found that p53 transcribes p21, which suppressed the growth of a variety of tumor types (el-Deiry WS et al. 1993). P21 is a cyclin-dependent kinase inhibitor and has the ability to arrest the cell cycle when it is present at high levels (Li et al. 1994). P53 also transcribes the bax gene, which is a key part of the apoptotic response (Miyashita and Reed 1995) (Wei et al. 2001).

Under normal cellular conditions, wild-type p53 is bound to mdm-2 and its transcriptional activity is inhibited. (Momand et al. 1992). Mdm-2 also regulates p53 activity by promoting its degradation when there is no cellular stress (Haupt, Maya,

Kazaz and Oren, 1997), so that the protein is absent in normal cells. P53 is degraded through ubiquitination by mdm-2, which acts as a ubiquitin ligase (Honda, Tanaka and Yasuda, 1997). There exists an autoregulatory feedback loop between p53 and mdm-2. Mdm-2 is directly transcribed by p53, then inhibits p53 activity through inactivation and degradation (Wu, Bayle, Olson and Levine 1993). Specifically, Mdm-2 binds to the transactivation domain p53 and inhibits its transcriptional activity. It also facilitates ubiquitination of p53, therefore tagging it for proteasomal degradation. Finally, it promotes nuclear export of p53 to aid in degradation (Michael and Oren, 2003). Therefore, Mdm-2 is a possible target for elevating the activity of p53 in tumor cells. One group of compounds known as Nutlins bind to Mdm-2 and interfere with its interaction with p53(Vassilev et al. 2004). MDMX is also a regulator of p53. It interacts with Mdm-2 to help stabilize it and aid in p53 degradation (Brady and Attardi, 2010). It also binds to p53 and inhibits transcriptional activity. Therefore, it can serve as an oncogene when overexpressed and can be a target for cancer therapy. However, the Mdm-2 and MDMX interaction makes it difficult to design effective drugs that only target one of these partners. Both must be inhibited to prevent the degradation and suppression of p53.

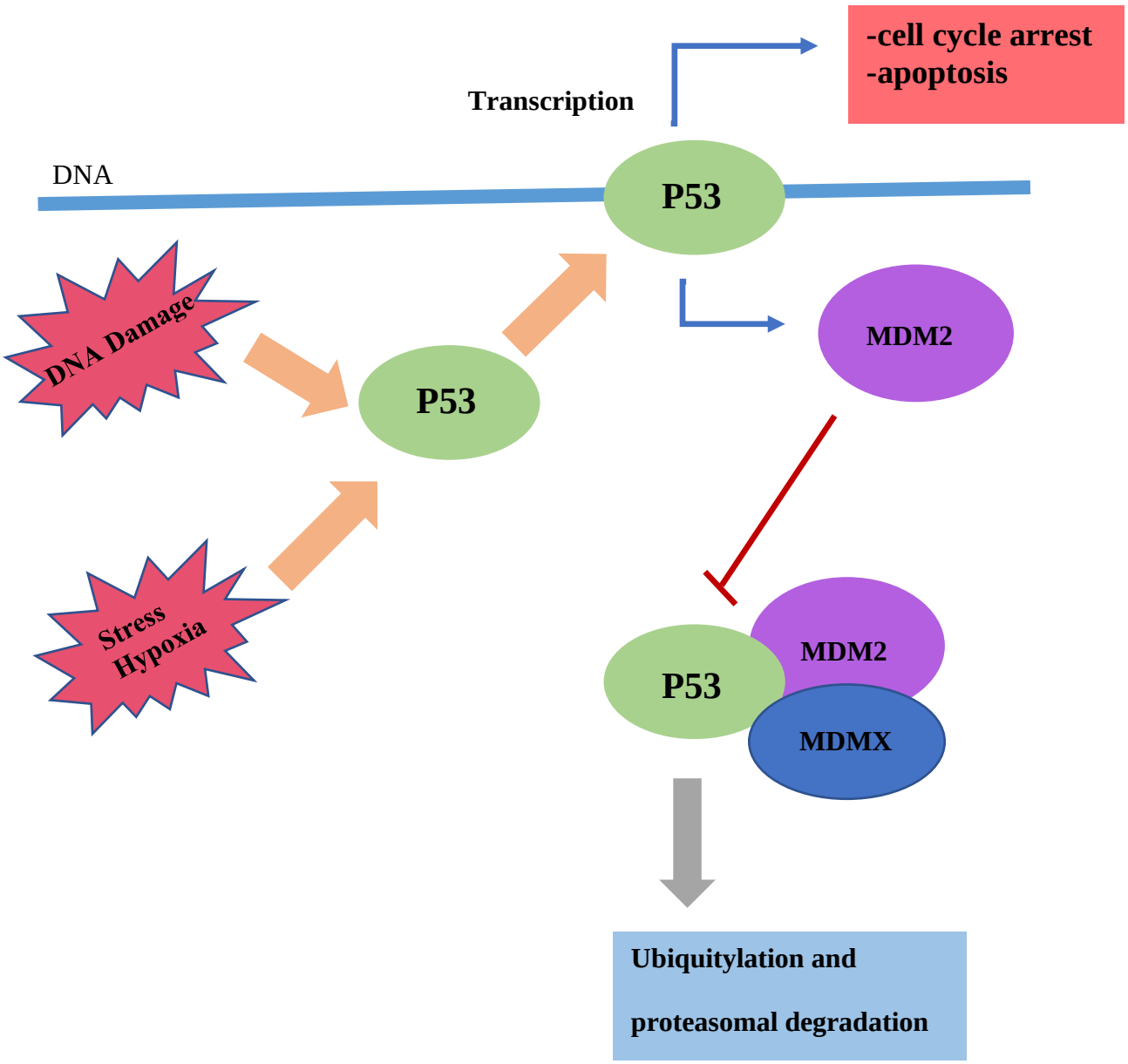


Figure 4: The p53-MDM2 feedback loop. Normally, p53 is inhibited by MDM2 and MDMX, which leads to its ubiquitylation and degradation. In the presence of DNA damage, stress, hypoxia, etc. p53 is activated as a transcription factor.

P53 is inactivated in almost all cases of human cancers. In 50% of cancers, p53 is inactivated due to mutations. Other causes of inactivation include overexpression of MDMX/MDM2, infection with HPV and other viruses, and defects in the signaling pathway. Due to the frequency of p53 mutations in cases of human cancer, mutant p53 has been identified as a promising anti-cancer drug target. The majority of the mutations observed in p53 are missense mutations that occur in the DNA binding domain (Soussi and Lozano, 2005). These mutations abolish the important interaction between p53 and the DNA that is required for transcription to take place. Usually, the mutant forms of p53 with a mutation in the DNA binding domain are thermodynamically unstable. Some of these oncogenic mutant proteins are temperature sensitive and possess reduced transcriptional activity at lower temperatures. It has been suggested that mutant forms are in conformational equilibrium with the wild type p53 and are rapidly destabilized at physiological temperature (Lane and Hupp, 2003). Small molecules stabilizing the conformation of mutant p53 to that of the wild-type protein are reported in the literature. (Foster et al. 1999). Some of these reported molecules have also been shown to restore transcriptional and antitumor activity in cell culture and in animal models (Tal et al. 2016). PRIMA-1 has been found to restore the tumor suppressor activity of mutant p53 and induce p53-dependend apoptosis (Bykov et al. 2002). Due to lack of efficacy and safety issues, many of these small molecules are yet to be developed into therapy. These studies strongly suggest therapeutic potential of targeting mutant p53 for discovering novel and potent small molecules for cancer therapy.

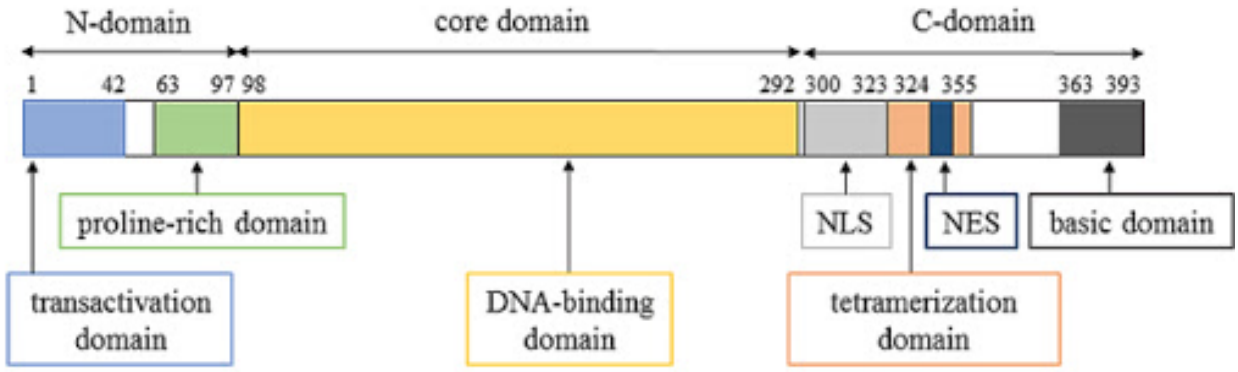


Figure 2: Structural domains of the p53 protein. Image taken from Tanaka, Watanabe and Yamashita 2018.

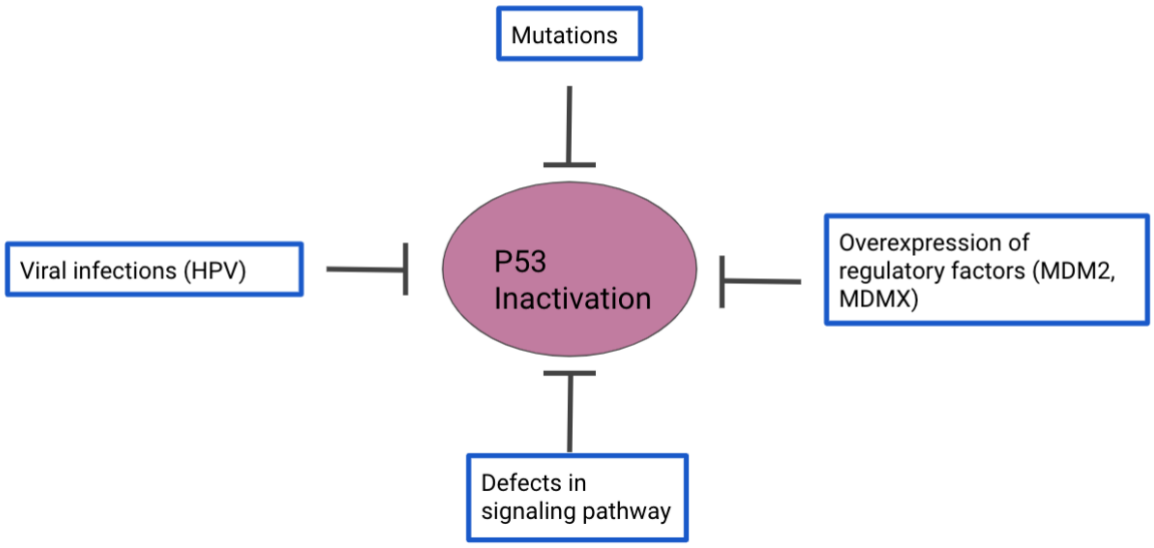


Figure 3: Factors leading to p53 inactivation. There are multiple factors that can inactivate p53 in cancer cells including mutations, viral infections, defects in the signaling pathway and overexpression of regulatory factors.

Goals

Given the need to discover novel molecules that will be effective in reactivating mutant p53, our laboratory at RISE is studying the reactivation of mutant p53 by small molecules. Human tumor cell lines with mutant p53 were transfected with a plasmid containing GFP under a p53 response element. These transformed cell lines were developed for a GFP based reporter assay to screen small molecules for their ability to restore transcriptional activity of mutant p53. Some small molecules were synthesized by a RISE Fellow, Dr. Ronald Doll and his group, named RD molecules. The other class of molecules are from Dr. Vince Gullo's laboratory. My goal was to screen many compounds and evaluate them in other secondary assays. I have worked on developing a reliable screening method, and screened a large number of compounds in the GFP assay. Additionally, I conducted a cell viability assay to determine selectivity of the compounds for mutant p53 as well as an immunocytochemistry assay to examine the ability of the molecules to induce a conformational change in mutant p53. When all three assays are performed, they create a screening method for compounds that reactivate mutant p53 and identify compounds that should be further evaluated for efficacy and toxicity for potential therapy development.

Methods

Cell Culture

The cell lines used include DLD1 (colorectal adenocarcinoma with mutant p53, TP53 p.Ser241Phe), SF295 (glioblastoma with mutant p53, Homozygous for TP53 p. Arg248Gln) and H1299 (non-small cell lung cancer with homozygous deletion of p53). These cell lines were stored at -80℃ in media containing 5% DMSO. To thaw cells, the stocks were warmed at room temperature and 1mL of cell stock was added to 9mL of prepared media in a 10cm cell culture dish. DLD1 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) and both SF295 cells and H1299 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media. Media was supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin then filtered. Thawed cells were incubated at 37℃ and 5% CO_2 and media was changed every 3 to 4 days. Media was changed by removing old media and gently adding 10 mL of fresh media onto the adherent cells.

When cells reached 75-80% confluence they were split into new 10cm dishes which allowed for experimentation, making stocks or the general maintenance of the cell culture. For experimental purposes, media was removed and 10mL of phosphate buffered saline (PBS) was used to wash off any remaining media. This prevents deactivation of the trypsin-EDTA (TE) solution by the media. PBS was removed and 3mL of TE was added to the cells followed by incubation of 5-10 minutes at 37℃ and 5% CO_2. Cells were monitored until they were no longer adherent to the bottom of the plate and 7mL of media was added to inactivate trypsin. After quickly resuspending the cells in the media

and washing the bottom of the plate, this 10mL cell suspension was added to a conical tube and centrifuged for approximately 3 minutes at 3,000 rpm. This created a cell pellet and the supernatant containing media and TE was removed. Ten mL of fresh media was added to the conical tube and the cells were gently resuspended. The cells were counted by adding 10μL of the suspension to a hemocytometer to determine the concentration of cells/mL. If needed, cells were diluted to obtain a concentration of 2-5 $x10^5$ cells/mL. Two new 10cm dishes were prepared by adding 9mL of fresh media to each followed by 1mL of cells. These plates were allowed to sit for a few minutes at room temperature for the cells to adhere before being put back into the incubator.

When making stocks, cells were seeded into T75 flasks and grown until they reached 90-95% confluency. The same procedure was followed and after cells were counted, they were diluted to a concentration of 2-5 $x10^6$ cells/mL. To each CryoTube , 1mL of cells and 50μL of DMSO were added and the tube was immediately put on ice then frozen and stored at -80 ℃.

Stable Cell Lines

Previous students in the laboratory have developed stable cell lines by transfecting SF295 and DLD1 cell lines with a lentiviral vector. This vector contains GFP and luciferase reporter genes under a p53 response element in such a way that expression of these reporter genes depends on transcriptional activity of p53 (figure 5). In addition, the vector also contains a puromycin resistance gene for growth in the presence of puromycin (figure 5). Stable cell lines were grown in media supplemented with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin and Puromycin at 1.5 μg/mL.

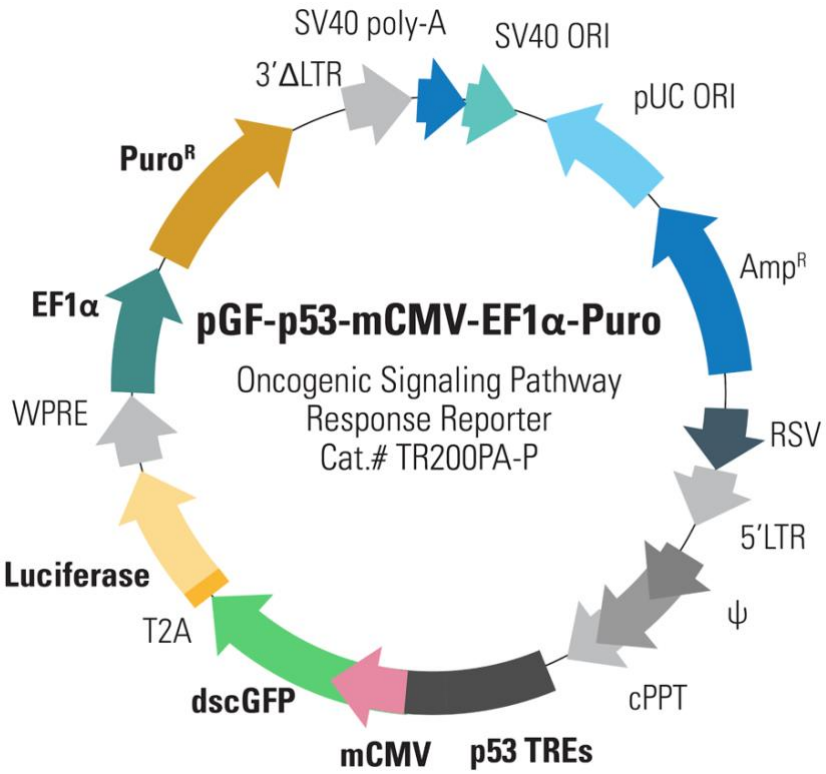


Figure 5: pGF-p53-mCMV-EF1a-Puro Lentivector. Stable cell lines contain this vector with GFP and Luciferase response genes under a p53 response element.

GFP Assay

Stable cell lines will express GFP when mutant p53 is reactivated to wild-type by small molecules. Due to the fact that the GFP reporter gene is located under the p53 response element, GFP will be expressed in the cells when p53 is reactivated and able to act as a transcription factor. GFP expression can be observed under cyan and green fluorescent light. This allows us to determine whether p53 has been reactivated in these cell lines upon treatment with small molecules.

Stable cell lines (SF295-GFP and DLD1-GFP) were grown in puromycin-containing medium up to 75-80% confluence, harvested and counted as before. If needed, cells were diluted to obtain a concentration of 2.0 x 10^4 cells/mL. Each well of a 96-well plate contained 50 µL of cells for a total of 10,000 cells/well.

Fresh media was added to the wells first. With a working volume of 100µL for each well, the volume of media added depended on the compound concentration (see table 1). The two compound concentrations tested were 10 and 20 µg/mL. The compound stocks were prepared by dissolving 0.5mg of the compound in 250 µL of DMSO for a concentration of 2mg/mL. This stock A was diluted 20 fold with the media to create a stock B of 0.1mg/mL, in 5% DMSO. Stock B was added in volumes of 10 or 20 µL for a final concentration of 10 or 20 µg/mL in the well of a 96 well plate with a final volume of 100µL.

Table 1: 96-well plate setup for GFP assay

column	1: No Treatment	3: 10µg/mL	4: 20µg/mL
Volume cells	50µL	50µL	50µL
Volume media	50µL	40µL	30µL
Volume diluted compound	0µL	10µL	20µL

Table 2: 96-well plate map for GFP assay

NT	Compd#1	10µL	20µL	Compd#5	10µL	20µL					
NT		10µL	20µL		10µL	20µL					
NT	Compd#2	10µL	20µL	Compd#6	10µL	20µL					
NT		10µL	20µL		10µL	20µL					
NT	Compd#3	10µL	20µL	Compd#7	10µL	20µL					
NT		10µL	20µL		10µL	20µL					
NT	Compd#4	10µL	20µL	Compd#8	10µL	20µL					
NT		10µL	20µL		10µL	20µL					

In each plate, 8 different compounds were tested and each condition was tested in duplicate. The 96-well plate was incubated at 37℃ and 5% CO_2 and microscopy was done after 24 and 48 hours of incubation. During microscopy, the cells were observed under white, green and cyan light. Pictures of each condition were taken and observations were recorded about the intensity of fluorescence and the approximate percentage of cells that expressed GFP in the well. Compounds were tested in both DLD1 and SF295 stable cell lines.

Cell Viability Assay

The cell viability assay allowed me to determine whether the compounds are specific to the mutant p53 and act through the p53 pathway. SF295 (or DLD1) and H1299 cell lines were both treated with varying concentrations of the compound for 72 hours and viable cells were measured to determine the amount of cell death. Then the cell

death was compared between the two cell lines with the expectation that treatment with the compounds will lead to more death in the p53 mutant cell line (SF295) and will have little effect on the p53 null cell line (H1299).

Growth curves were made for each cell line to determine the ideal number of cells for seeding. Cells were counted and diluted to a concentration of 5.0×10^4 cells/mL. Media and cells were added to a 96-well plate according to the volumes in table 3. Various numbers of cells were plated starting at 500 cells/well and increasing by increments of 500 up to 5,000 cells/well.

Table 3: Volumes in microliters of cell dilution and media added to each of the wells in a column of a 96-well plate.

Column	Cells	Media	Number of cells
1	0µL	100µL	0
2	10µL	90µL	500
3	20µL	80µL	1,000
4	30µL	70µL	1,500
5	40µL	60µL	2,000
6	50µL	50µL	2,500
7	60µL	40µL	3,000
8	70µL	30µL	3,500
9	80µL	20µL	4,000
10	90µL	10µL	4,500
11	100µL	0µL	5,000

Plates were incubated at 37℃ and 5% CO_2 for 72 hours. According to the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS), 20 µL of MTS dye was added to each well and the plate was incubated for a total of 3 hours. The absorbance was read with a spectramax plate reader and percent cell survival at each concentration was calculated.

In order to evaluate the toxicity of various compounds, a cell viability assay was conducted by plating cells and treating them with the compound in increasing concentrations (0µg/mL, 1.25µg/mL, 2.5µg/mL, 5µg/mL, 10µg/mL, 20µg/mL). First, media was added to a 96-well plate, then the cell dilution and the compound following the amounts outlined in table 4. Cells were added from a stock of 2,000 cells/mL for H1299 and 1,500 cells/mL for SF295 and DLD1.

Table 4: 96-well plate setup for cell viability assay, volumes in µL of media, cells and compound added to each well.

Column	Media	Cells	(Compound Stock B)
1	100µL	0µL	0µL
2	50µL	50µL	0µL
3	48.75µL	50µL	1.25µL
4	47.5µL	50µL	2.5µL
5	45µL	50µL	5µL
6	40µL	50µL	10µL
7	30µL	50µL	20µL

Compound dilutions

Starting with 0.5mg, compounds were diluted into 250 µL of DMSO to get stock A (2mg/mL). Stock A was then diluted 20 fold by adding 10µL to 190µL of media to get stock B (100µg/mL). The final DMSO concentration at the highest dose (20 µg/mL) of the compound in the well is 1%, which is tolerable to cells.

Compounds were tested in both H1299 and SF295 or DLD1 cell lines. After plating, the 96-well plates were incubated at 37℃ and 5% CO_2 for 72 hours. MTS dye was added to each well (20µL/well) and after 3 hours of incubation with the dye, the absorbance was read.

Immunocytochemistry Assay

In 4-well chamber slides, 10,000 cells were seeded and grown overnight at 37℃ and 5% CO_2. After 24 hours, the cells were treated with the compound (12 µM) and returned to the incubator for 6 hours. After 6 hours, media was removed, cells were washed twice with 1x PBS and 1mL of paraformaldehyde was added to each chamber to fix the cells. The slides were allowed to incubate for 10-15 minutes at room temperature and the paraformaldehyde was discarded. Cells were washed with 1x PBS three more times and 1mL of 0.5% Triton X-100 detergent was added for five minutes. Cells were washed 4 times with 0.5% Tween-20 in 1x PBS and incubated overnight at 4℃ with 0.5ml of 10% goat serum. The following day, the cells were washed 4 times with 0.1% Tween-20 and 1mL of the p53 conformation specific antibody (diluted in PBS to concentration 1:300) was added to each well. Two wells were treated with the PAB240

antibody which recognizes mutant p53 and the other two wells were treated with PAB1620 which recognizes wild-type p53. The cells were then incubated overnight at 4℃. On day 3, the cells were washed 4 times with 0.1% Tween-20 and 1mL of the secondary antibody was added to each chamber (diluted to a concentration of 1:400 in PBS). The cells were left in the secondary antibody for 2 hours at room temperature, covered with aluminum foil to prevent exposure to any light. The secondary antibody was removed and cells were washed 4 times with 0.1% Tween-20. The chambers were then removed from the top of the slide and cells were treated with Prolong Gold Anti-Fade reagent (Invitrogen) which contains DAPI to stain the nuclei. A coverslip was placed over each condition so that it laid flat with no bubbles, and the slide was incubated at room temperature in the dark for 24 hours. Cells were observed under the microscope under blue fluorescent light (for DAPI) and green fluorescent light (for the GFP-labeled antibodies).

Results

GFP Assay

The ability of various compounds to restore transcriptional activity of mutant p53 in human tumor cell lines has been established through the use of a GFP reporter assay. Tumor cells were treated with the compounds and fluorescent microscopy was conducted to detect the expression of GFP.

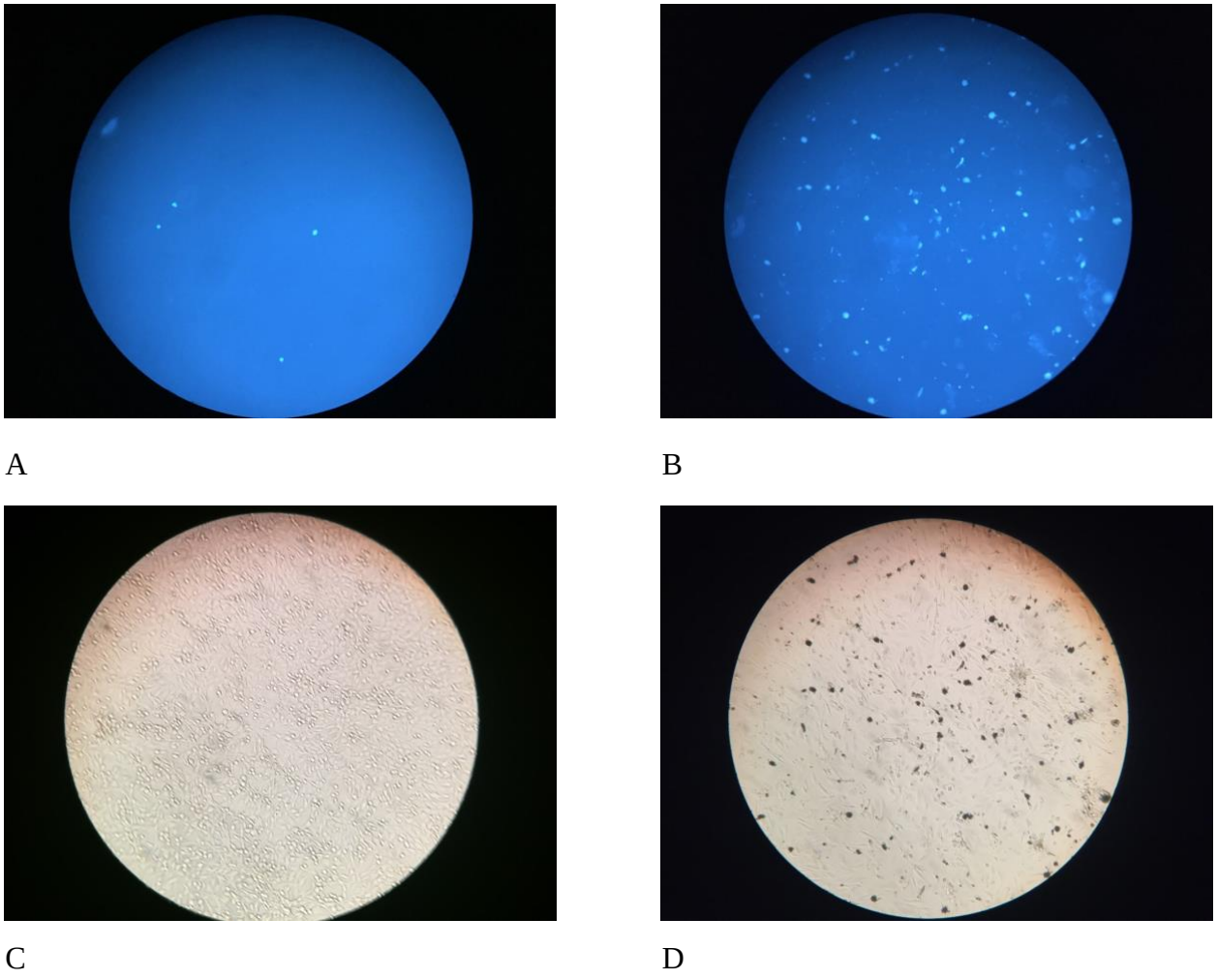
As seen in figure 6, we observe an increase in expression of GFP when cells are incubated with 10 μg/mL of the compound RD 29 for 48 hours. By observing these cells under analytical light as well, we see the presence of cell death upon treatment with the compound.

There is also an increase in expression of GFP when the concentration of the compound is increased (figure 7). GFP expression is dose dependent with RD 29 up to 10 μg/mL. However, GFP expression is reduced at 20ug/ml due to possibly more cell death. Similar effects on GFP expression are observed with compound RD 27 (figure 8).

Both RD 27 and RD29 have also been tested with DlD1 GFP cells (figures 9 and 10). The DLD1 cell line also contains a mutation in the DNA binding domain of p53, but it causes a structural mutation rather than the DNA contact point mutation in SF295. Compared to the GFP expression observed with SF295 cells, the GFP expression when cells were treated with RD 27 and RD 29 is reduced.

Interestingly, RD 30, a close analogue of these molecules (RD29 and RD27) is less effective in GFP expression under similar conditions. The expression of GFP was

about the same for both the control (untreated SF295 cells) and cells treated with the compound (figure 11).



A B C D

Figure 6: Treatment of SF295-GFP cells with RD29 induces GFP expression due to reactivation of mutant p53. A: Untreated SF295 cells under cyan fluorescent light after 48 hours of incubation. B: SF295 cells treated with 10 μg/mL of RD 29 for 48 hours under cyan light. C: Untreated Sf295 cells under analytical light after 48 hours of incubation. D: SF295 cells treated with 10 μg/mL of RD 29 for 48 hours under analytical light.

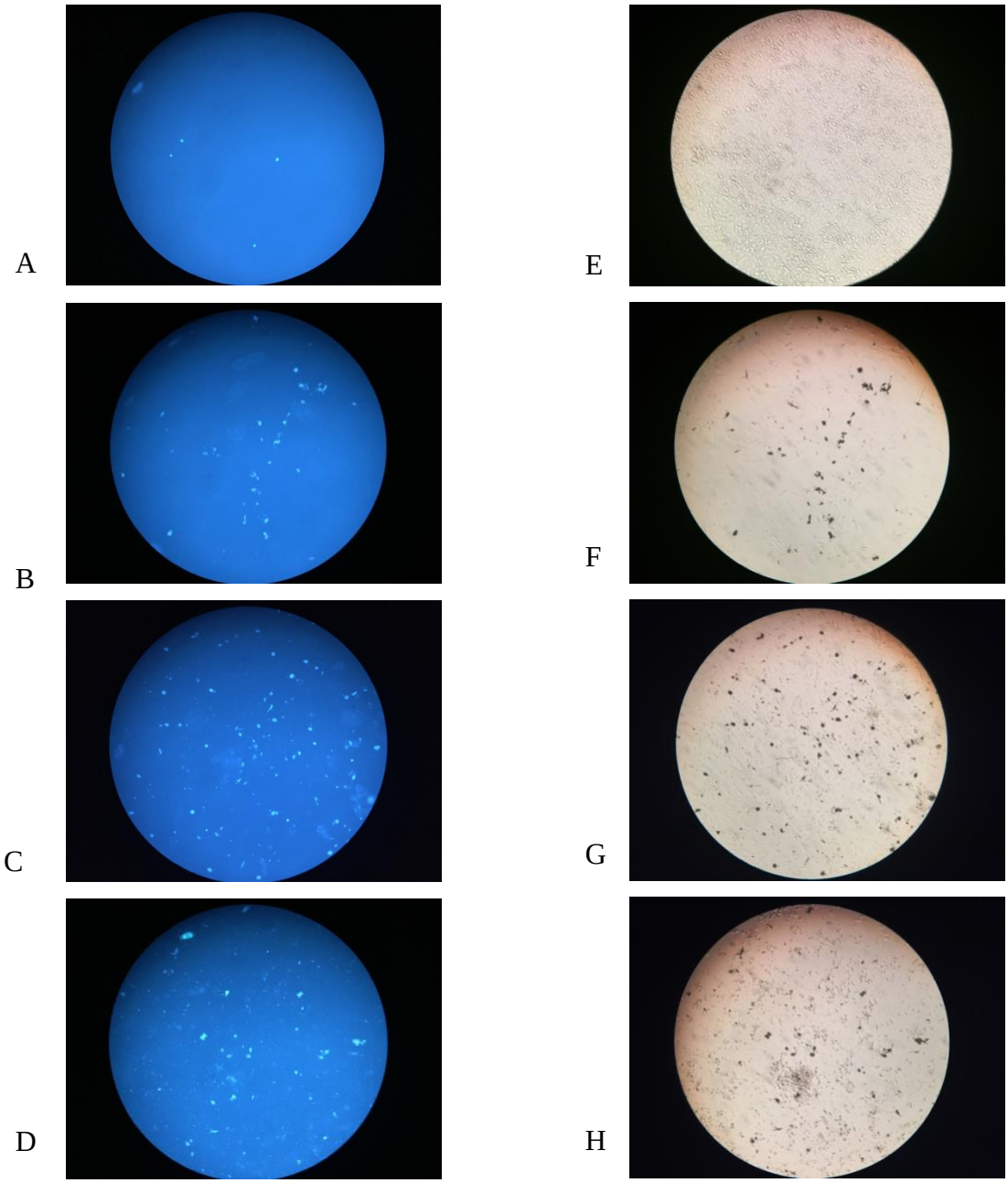


Figure 7: Treatment of SF295-GFP cells with RD29 induces GFP expression in a dose-dependent manner. A: Untreated SF295 cells after 48 hours of incubation under cyan fluorescent light. B: SF295 cells treated with 5 µg/mL of RD 29 for 48 hours under cyan light. C: SF295 cells treated with 10 µg/mL of RD 29 for 48 hours under cyan light. D: SF295 cells treated with 20 µg/mL of RD29 for 48 hours under cyan light. E: Untreated SF295 cells after 48 hours of incubation under analytical light. F: SF295 cells treated with 5 µg/mL of RD 29 for 48 hours under analytical light. G: SF295 cells treated with 10 µg/mL of RD 29 for 48 hours under analytical light. H: SF295 cells treated with 20 µg/mL of RD 29 for 48 hours under analytical light.

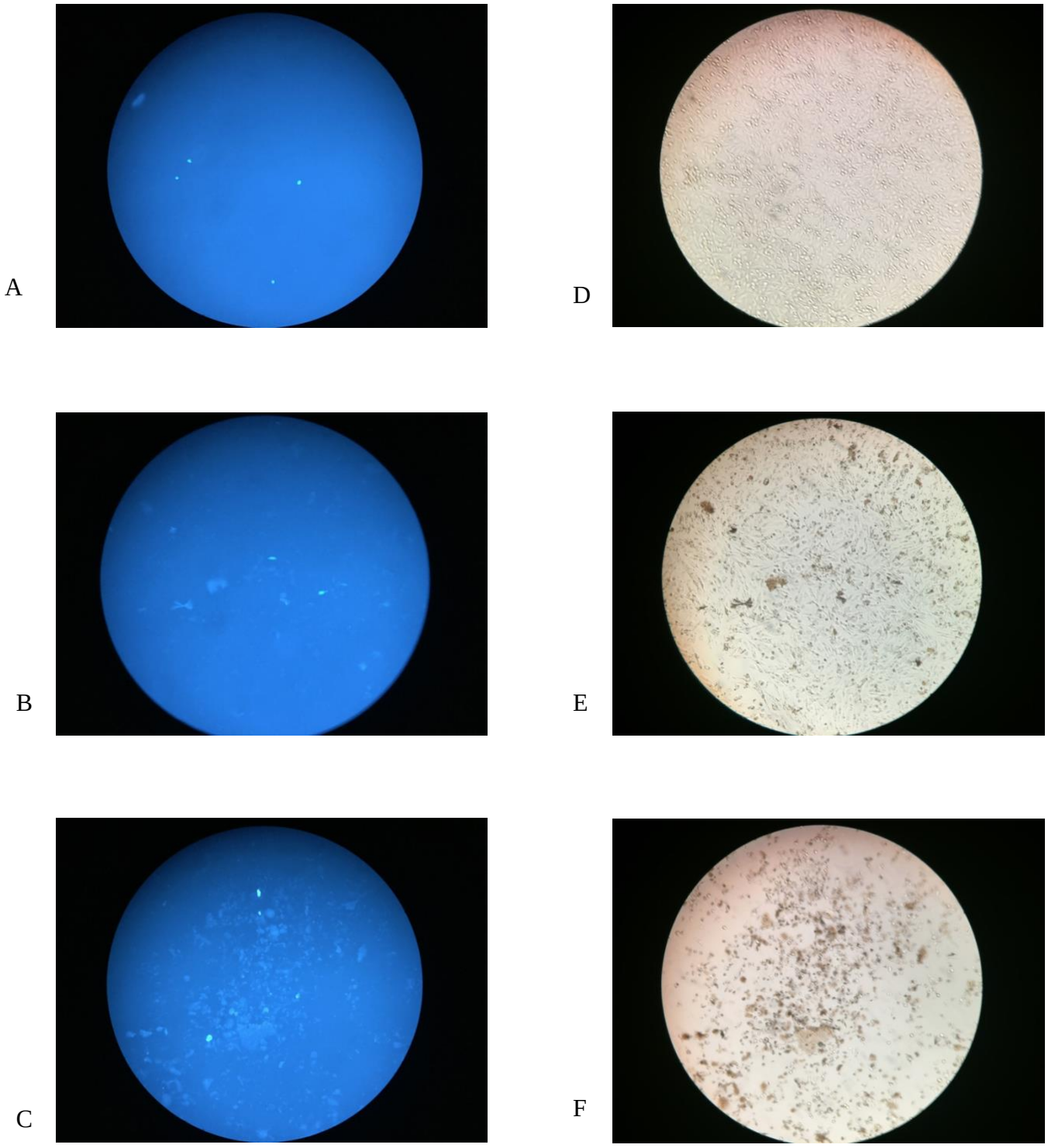
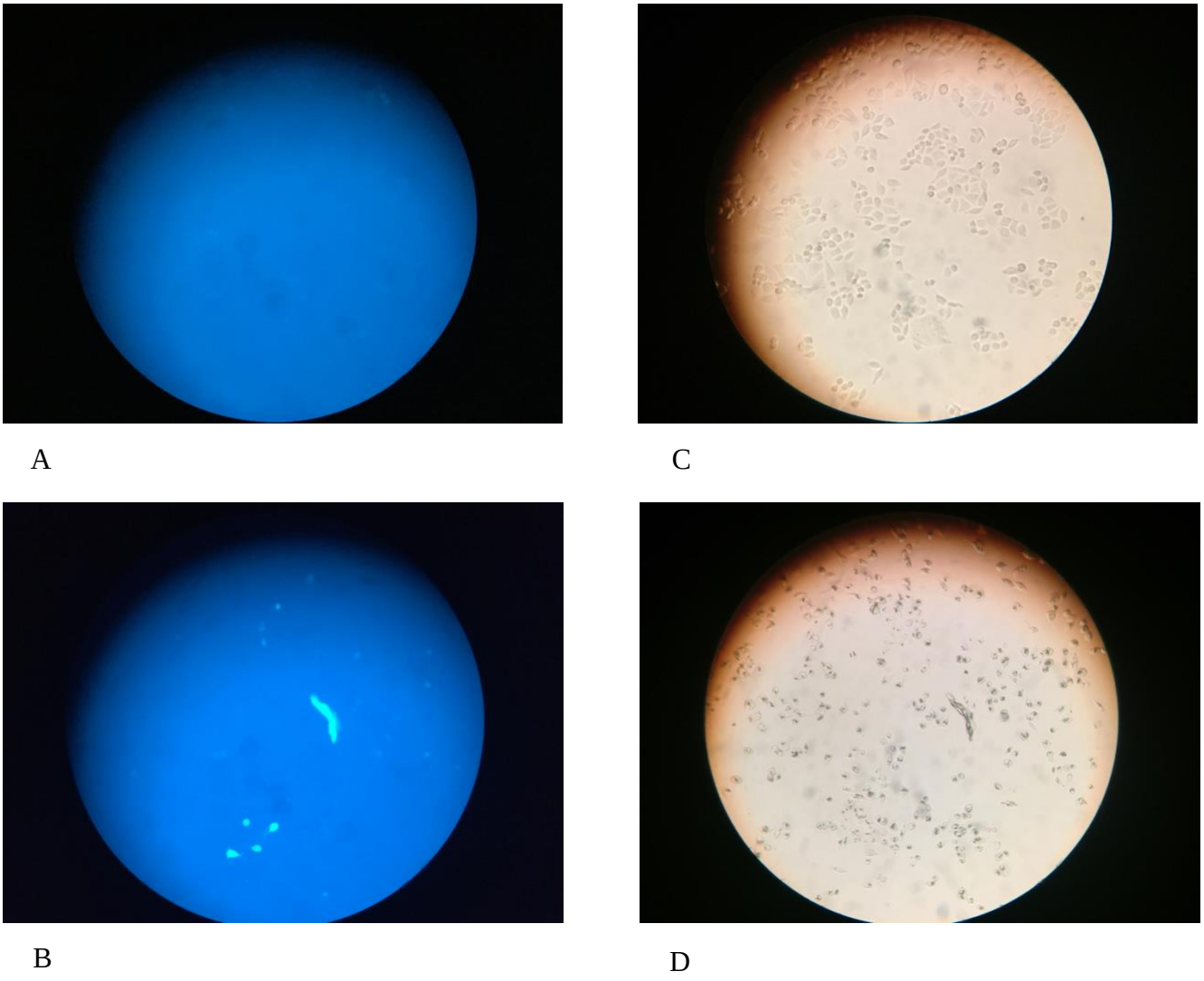


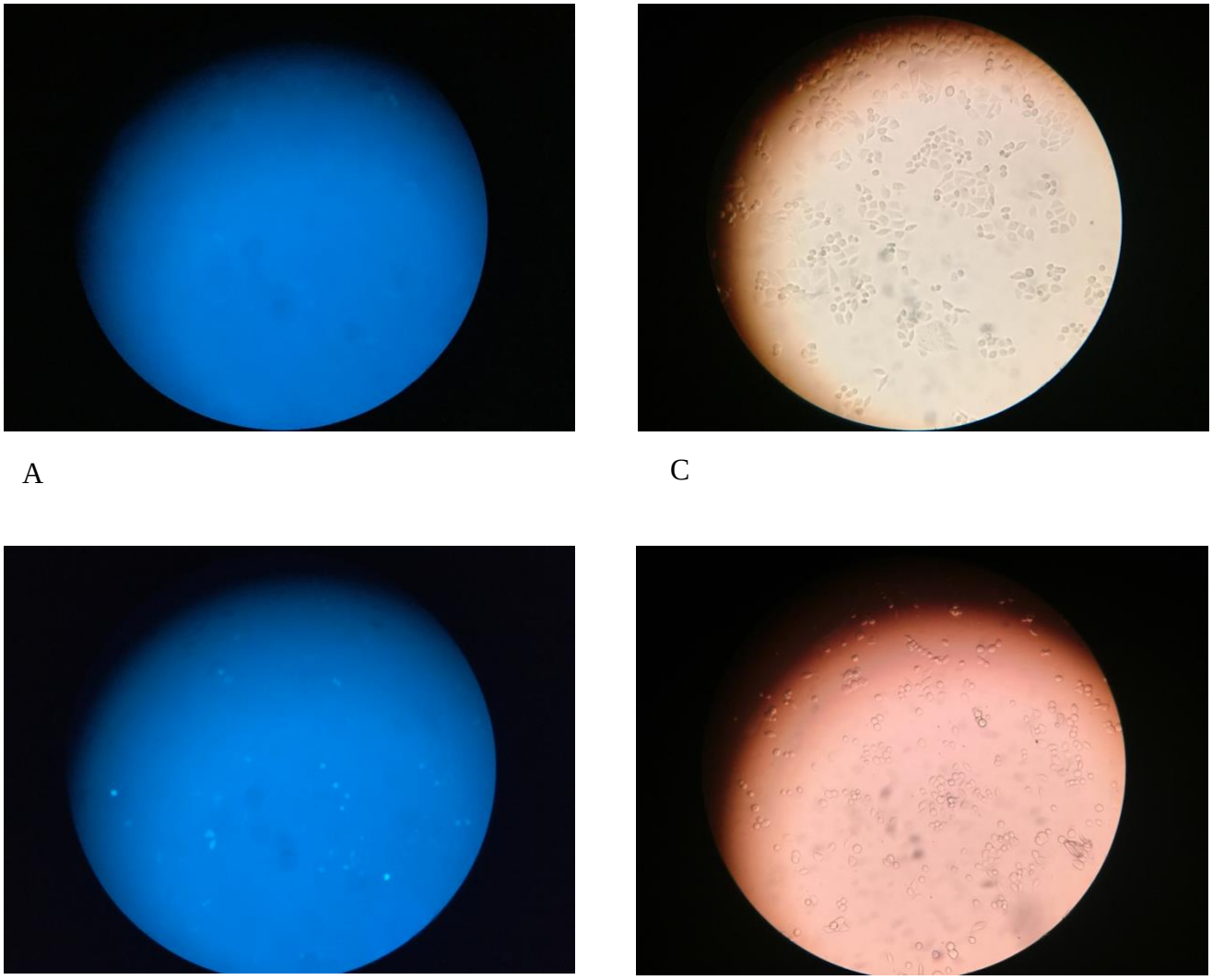
Figure 8: Treatment of SF295-GFP cells with RD27 induces GFP expression due to reactivation of mutant p53. A: Untreated SF295 cells after 48 hours of incubation under cyan light. B: SF295 cells treated with 10 μg/mL of RD 27 for 48 hours under cyan light. C: SF295 cells treated with 20 μg/mL of RD 27 for 48 hours under cyan light. D Untreated SF295 cells after 48 hours of incubation under analytical light. E: SF295 cells treated with 10 μg/mL of RD 27 for 48 hours under analytical light. F: SF295 cells treated with 20 μg/mL of RD 29 for 48 hours under analytical light.



A C

B D

Figure 9: Treatment of DLD1-GFP cells with RD27 induces GFP expression due to reactivation of mutant p53. A: Untreated DLD1 cells under cyan fluorescent light after 48 hours of incubation. B: DLD1 cells treated with 10 μg/mL of RD 27 for 48 hours under cyan light. C: Untreated DLD1 cells under analytical light after 48 hours of incubation. D: DLD1 cells treated with 10 μg/mL of RD 27 for 48 hours under analytical light.



A C

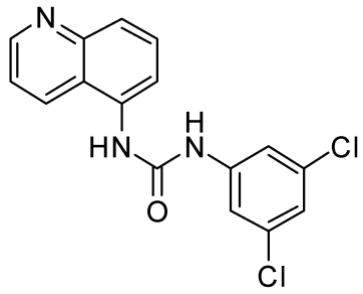
B D

Figure 10: Treatment of DLD1-GFP cells with RD29 induces GFP expression due to reactivation of mutant p53. A: Untreated DLD1 cells under cyan fluorescent light after 48 hours of incubation. B: DLD1 cells treated with 10 μg/mL of RD 29 for 48 hours under cyan light. C: Untreated DLD1 cells under analytical light after 48 hours of incubation. D: DLD1 cells treated with 10 μg/mL of RD 29 for 48 hours under analytical light.

A B C

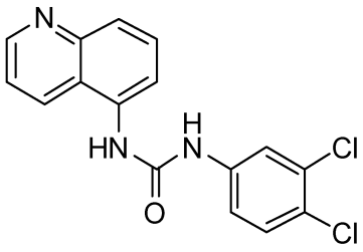
Figure 11: Treatment of SF295-GFP cells with RD30 does not induce GFP expression through reactivation of mutant p53. A: Untreated SF295 cells after 48 hours of incubation. B: SF295 cells treated with 10 μg/mL of RD 30 for 48 hours. C: SF295 cells treated with 20 μg/mL of RD 30 for 48 hours.

A



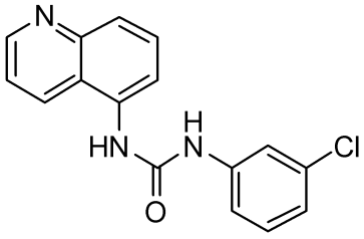
RD 27

B



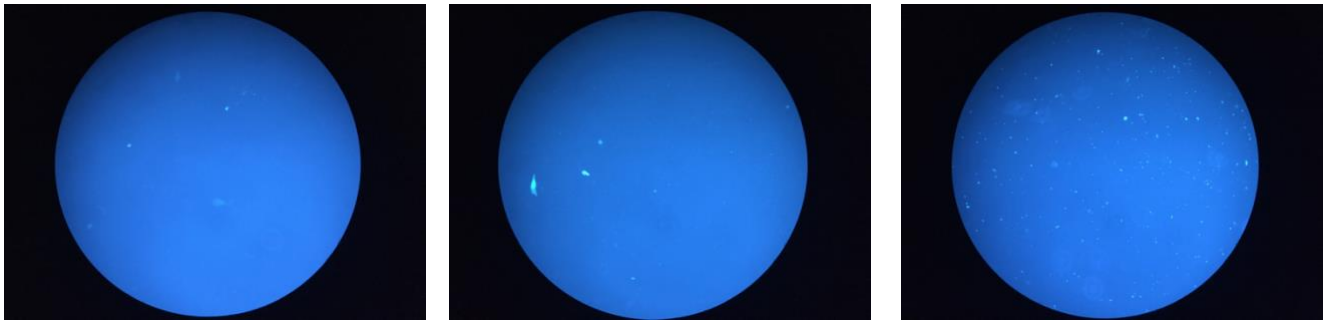
RD 29

C



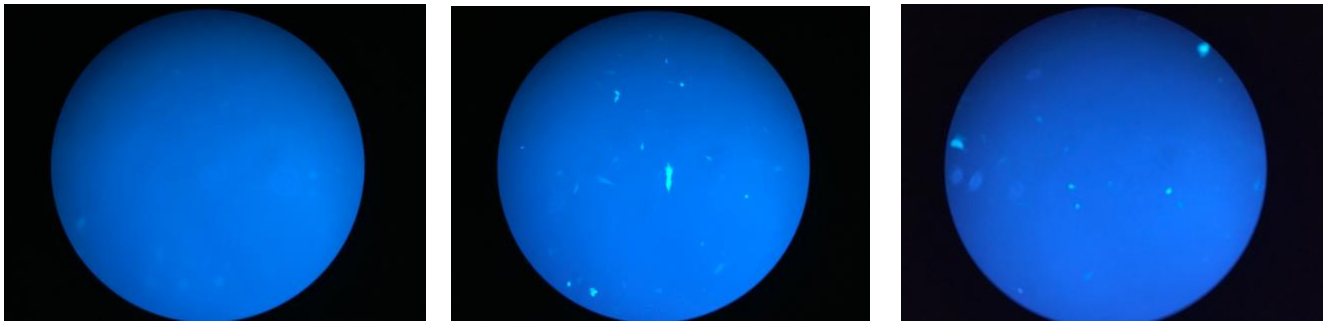
RD 30

Figure 12: Chemical structures of RD compounds. A: RD 27. B: RD 29. C: RD 30.



A B C

Figure 13: Treatment of SF295-GFP cells with WH 263-6 induces GFP expression due to reactivation of mutant p53. A: Untreated SF295 cells after 48 hours of incubation. B: SF295 cells treated with 5 μg/mL of WH 263-6 for 48 hours. C: SF295 cells treated with 10 μg/mL of WH 263-6 for 48 hours.



A B C

Figure 14: Treatment of SF295-GFP cells with RD23 induces GFP expression due to reactivation of mutant p53. A: Untreated SF295 cells after 48 hours of incubation. B: SF295 cells treated with 5 μg/mL of RD 23 for 48 hours. C: SF295 cells treated with 10 μg/mL of RD 23 for 48 hours.

Compounds RD23 and WH 263-6 were also evaluated with the GFP reporter assay. The expression of GFP increases slightly with an increase in concentration of RD23. With treatment of 5 μg/mL, there are a few cells that highly express GFP and with treatment of 10 μg/mL, many cells in the well fluoresce (figure 14). Compound WH 263-

6 is of another class of molecules unrelated to the RD compounds. There is high GFP expression in several cells upon treatment with 5 μg/mL of the compound, but expression decreases upon treatment with 10 μg/mL (Figure 13).

I have screened many compounds using the GFP reporter assay. This assay is the first step of various stages of evaluation. Results for compounds screened are shown in table 5. Each compound was assigned an number on an arbitrary scale to represent relative GFP expression. Various attempts to develop a quantitative GFP assay are yet to be successful. Compounds that test positively in the GFP assay, with an increase in GFP expression upon treatment with the compound, are further evaluated through the cell viability assay.

Table 5: Compounds screened with GFP assay.

RD compound	GFP expression	WH compound	GFP expression
1	2	**255-13**	1
5	3	**258-25**	3
6	4	**259-12**	1
7	4	**259-29**	4
14	6	**263-6**	3
20	3	**265-26**	6
21	4	**270-8**	3
23	5	**274-12**	2
25	6		
27	5		
29	7		
30	0		
32	3		
34	6		
35	5		
36	6		
38	5		
39	2		
40	3		
41	2		
42	2		
43	2		
44	2		
45	3		
46	3		
47	1		
48	3		
49	2		
50	3		
51	3		
52	4		
53	4		
54	2		
55	3		
56	4		
57	1		
62	5		
65	4		
66	2		

67	2		
68	0		
69	1		
70	2		
71	2		
73	5		
74	4		
75	0		
76	4		

Cell Viability Assay

Compounds that tested positive for the GFP assay were then evaluated with the cell viability assay. First, a growth curve was created for both H1299 and SF295 cell lines to determine the correct number of cells to be used for the experiment. These growth curves are shown in figures 12 and 13. For H1299, 2,000 cells per well was chosen to plate, and for SF295, 1,500 cells per well was chosen. This allowed the cells to grow for the full incubation period (72 hours) without reaching the maximum absorbance value for each cell line.

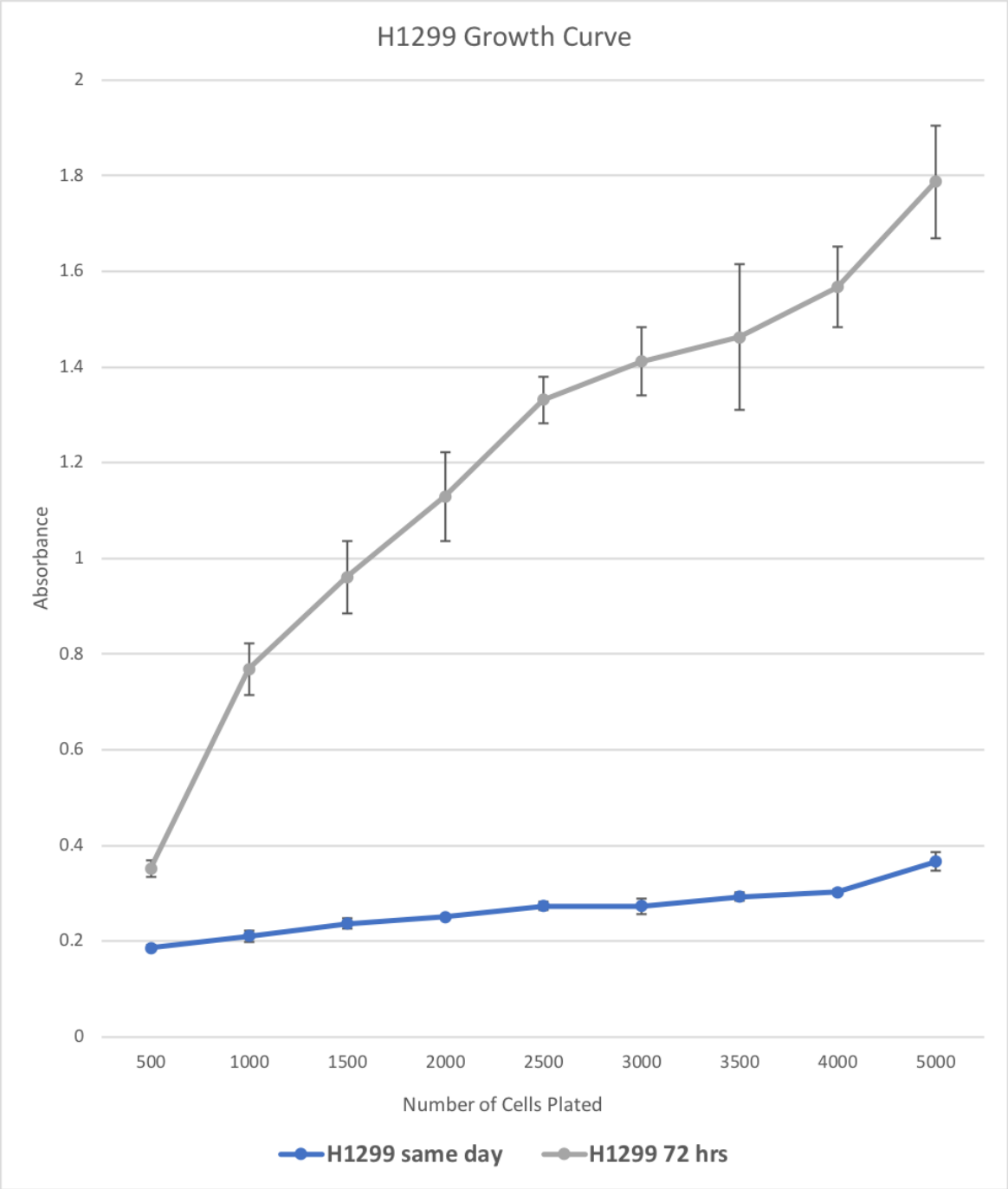


Figure 15: H1299 growth curve. Cells were plated in increments of 500 and the absorbance was read the same day and 72 hours after plating.

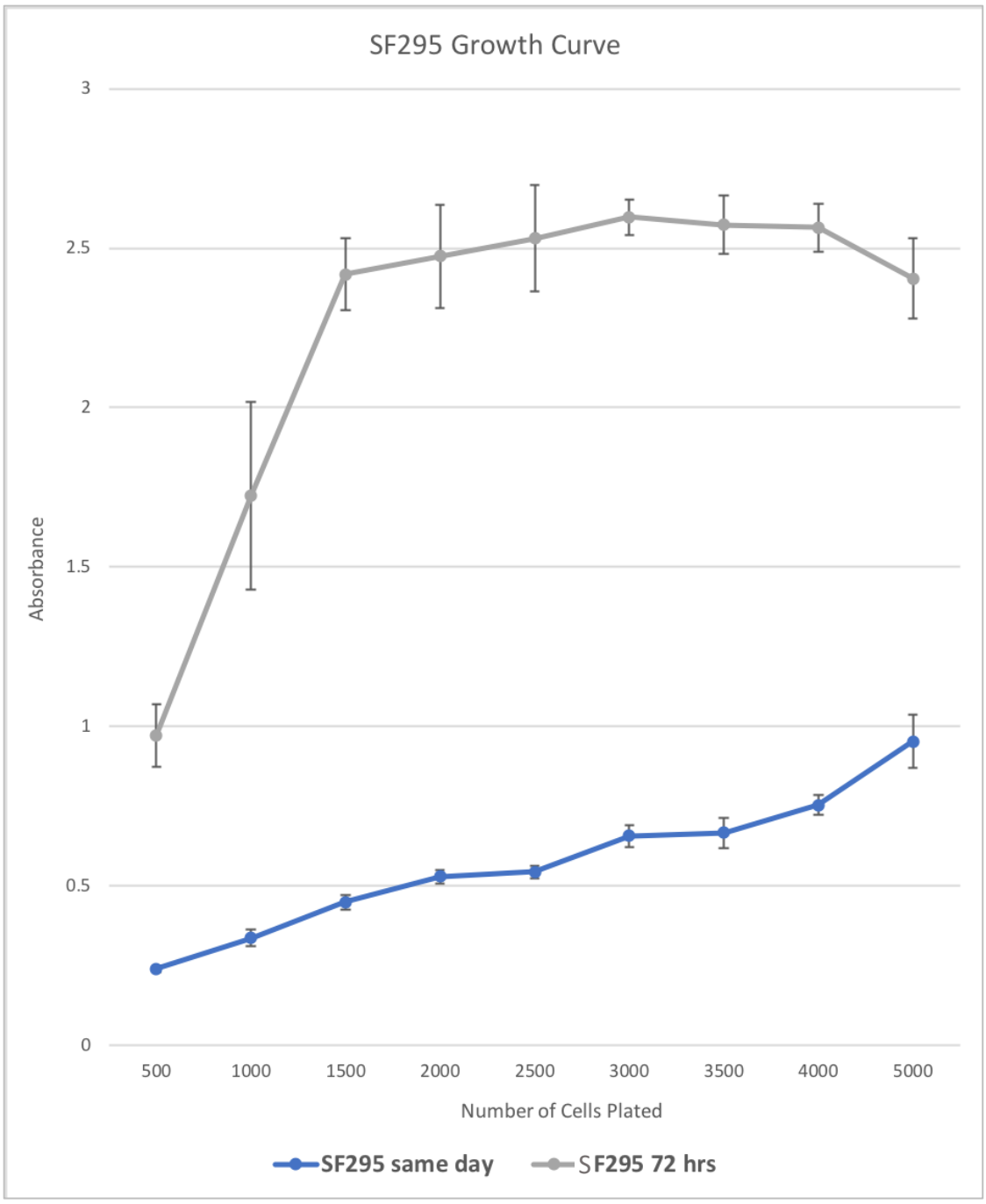


Figure 16: SF295 growth curve. Cells were plated in increments of 500 and the absorbance was read the same day and 72 hours after plating.

The cells were then treated with the compound at various concentrations and incubated for 72 hours. At the end of this incubation, the absorbance was read and percent cell survival was calculated at each concentration of the compound. With the hypothesis that the compounds tested have the ability to reactivate mutant p53, I expected to see a difference in cell survival between the p53 mutant and p53 null cell line. More specifically, I expected to observe a decrease in cell survival in p53 mutant cells with an increase in the compound concentration, but little effect on the cell survival of p53 null cells. For compound 27, there is a difference in viable cells between the two cell lines, being that the p53 mutant cell line (DLD1) is more sensitive to growth in the presence of RD 27 at higher concentrations (figure 17). As seen in figure 18, there is no difference in cell survival between the two cell lines when treated with RD 29. There is no apparent decrease in cell survival with increasing concentration of the compound.

For some compounds, WH 263-6 for example (figure 19), the percentage of cell survival decreases in both cell lines at higher concentrations of the compound, without any difference in cell survival between H1299 and SF295. For compound 23 however, cell survival in both cell lines is not affected by treatment with an increasing concentration of the compound. The cell survival stays around 80-100% until 20 ug/mL where the cell survival of H1299 cells decreases (figure 20).

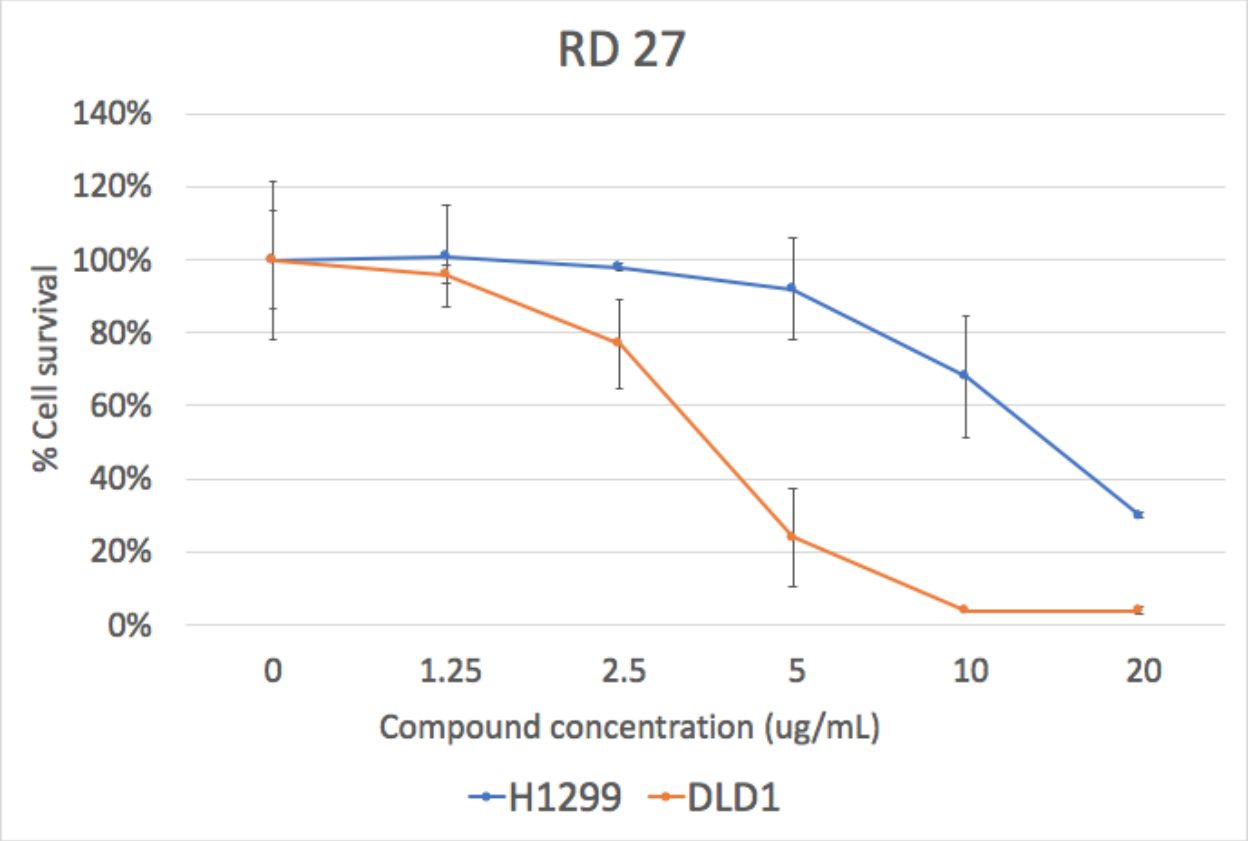


Figure 17: Treatment of DLD1 cells with RD27 induced mutant p53-dependent cell death. Cell viability assay showing the percent cell survival of H1299 and DLD1 cells when treated with various concentrations of RD 27.

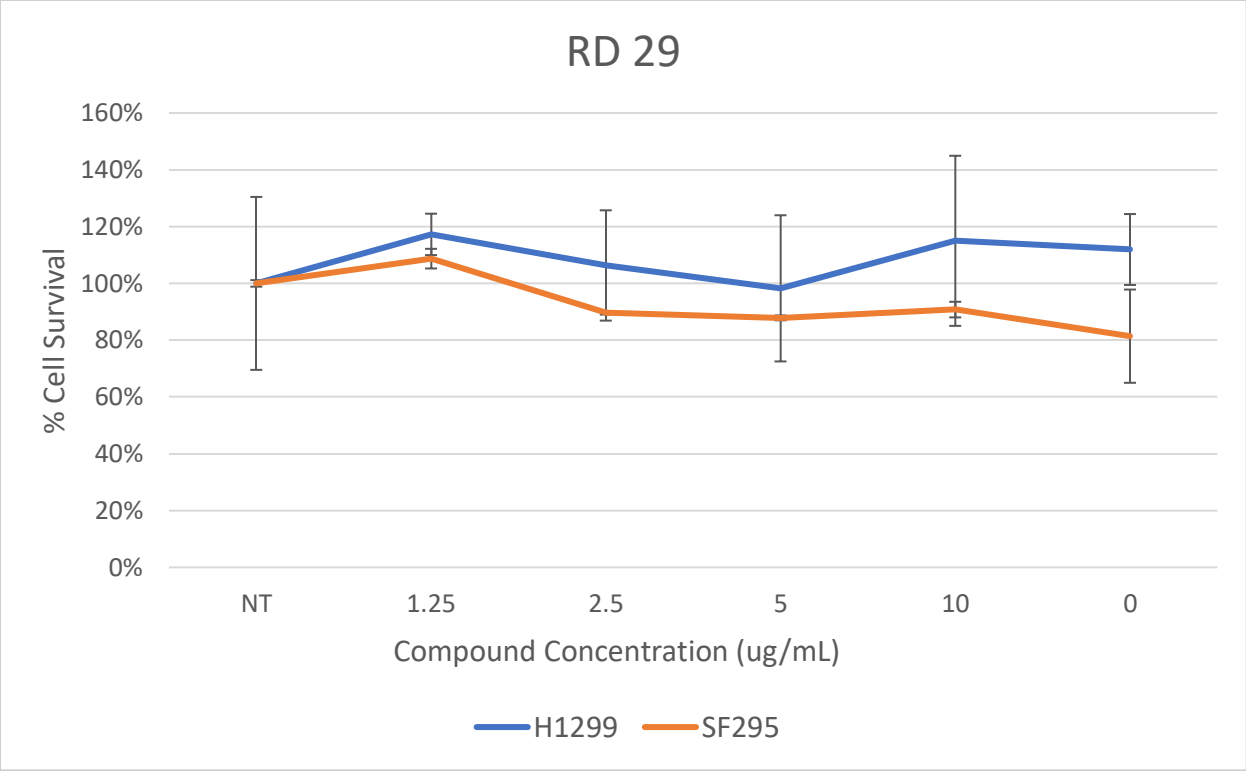


Figure 18: RD29 does not induce cell death in H1299 and SF295 cancer cell lines. Cell viability assay showing the percent cell survival of H1299 and SF295 cells when treated with various concentrations of RD 29.

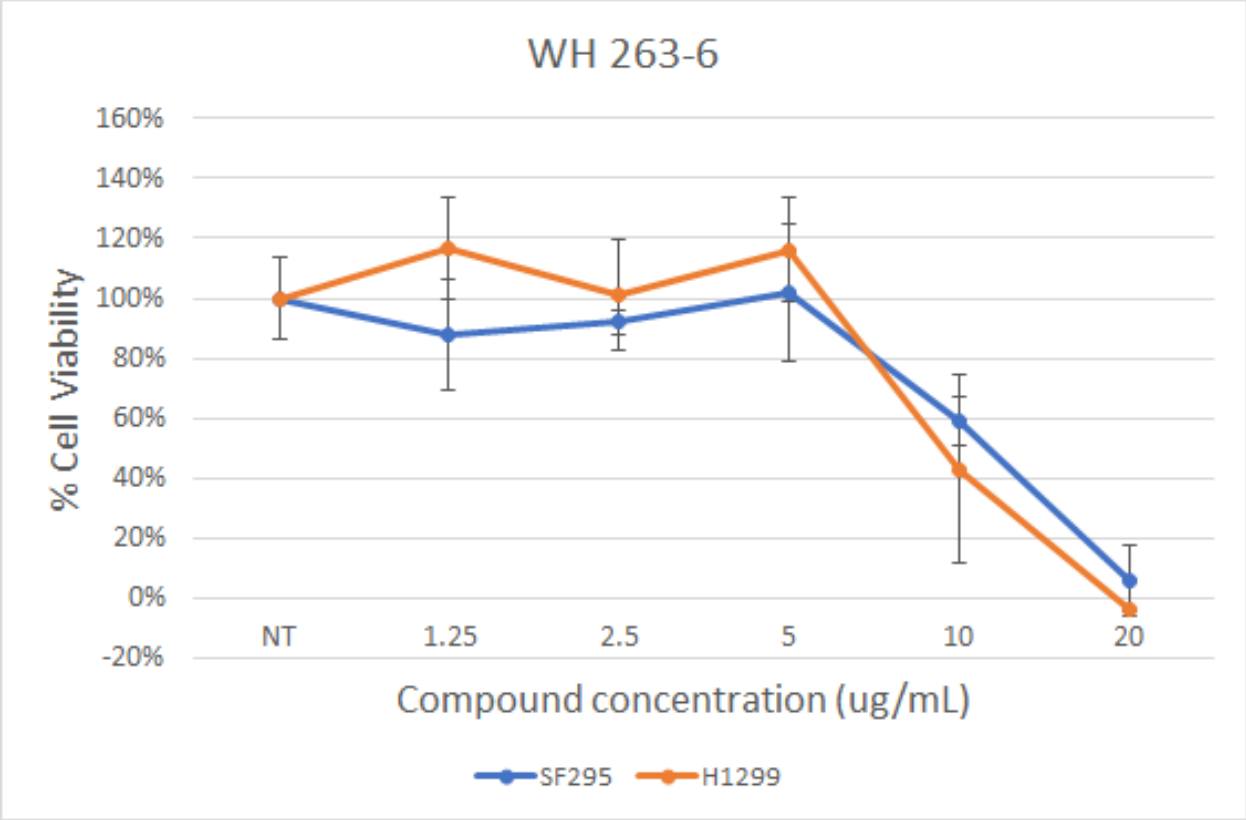


Figure 19: WH 263-6 does not induce cell death specific to p53 mutant cancer cells. Cell viability assay showing the percent cell survival of H1299 and SF295 cells when treated with various concentrations of WH 263-6.

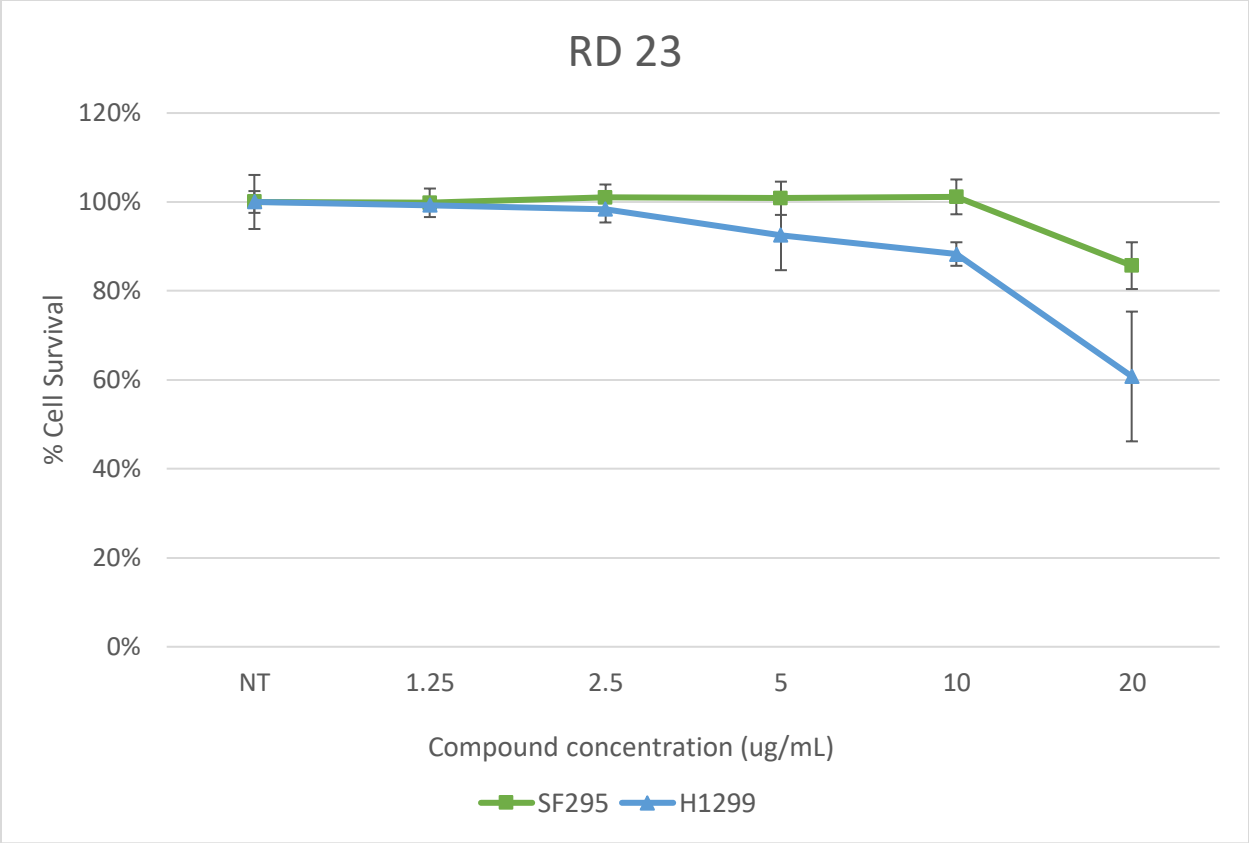


Figure 20: RD 23 does not induce cell death specific to p53 mutant cancer cells. Cell viability assay showing the percent cell survival of H1299 and SF295 cells when treated with various concentrations of RD 23.

Immunocytochemistry Assay

The GFP reporter assay establishes that these compounds reactivate mutant p53 and restore p53 transcriptional activity. In order to evaluate if these compounds restore p53 transcriptional activity by changing the conformation of mutant p53 into that of wild-type p53, we examined them with the immunocytochemistry assay. Cells were treated with the compound and stained with antibodies for wild type p53 (pAB 1620) and mutant p53 (pAB 240). This assay was first done with the H1299 (p53 null) cell line and as can be seen in figure 21, the antibodies do not bind in any of the conditions. Only the blue DAPI (which stains the nucleus of all cells) can be seen. In SF295 cells however, there is

binding of the mutant antibody (green) in the absence of compound. SF295 cells that have been treated with compound show binding to both the mutant and wild type antibodies. This is true for both RD 27 and RD 29 (figures 22 and 23).

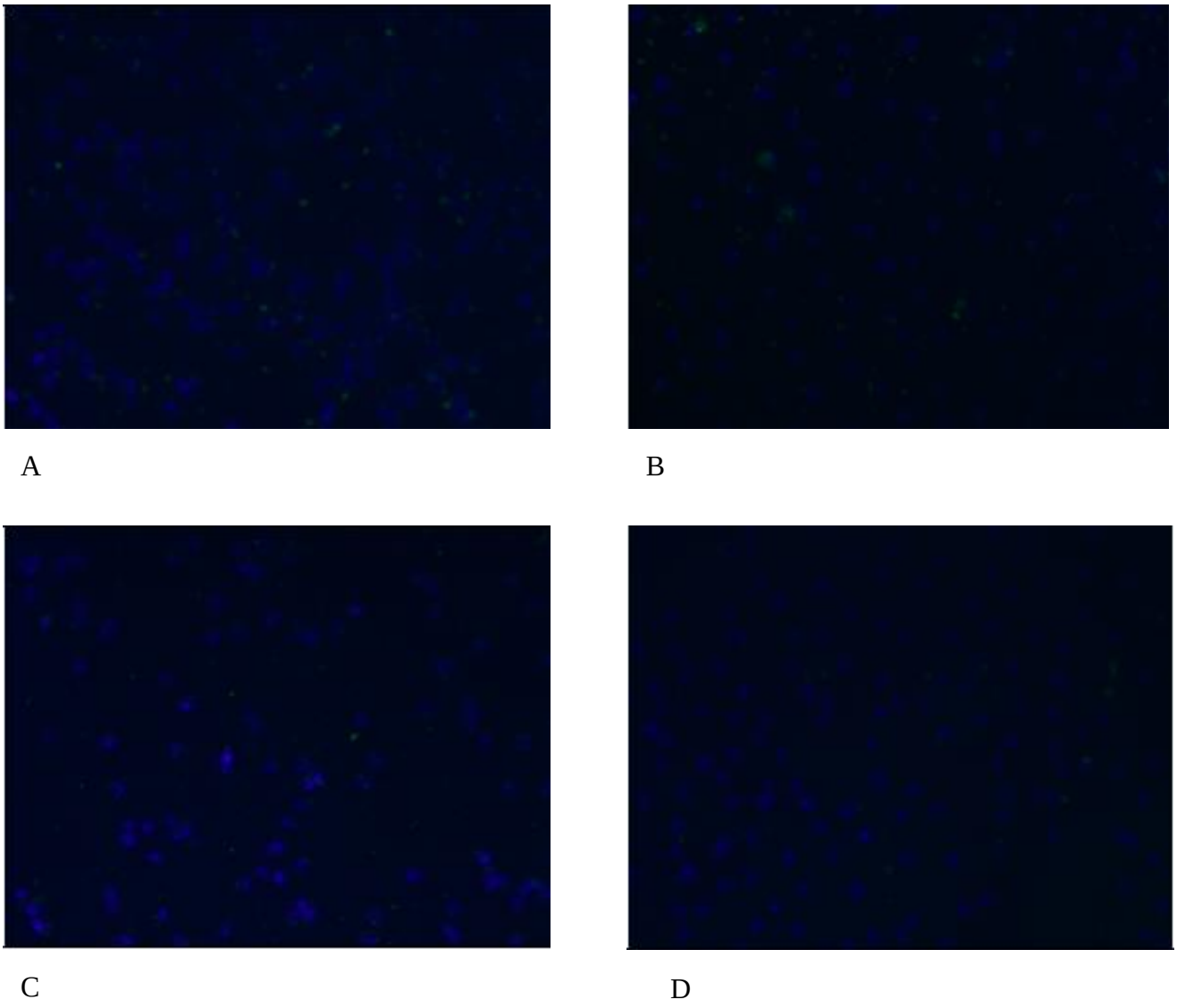
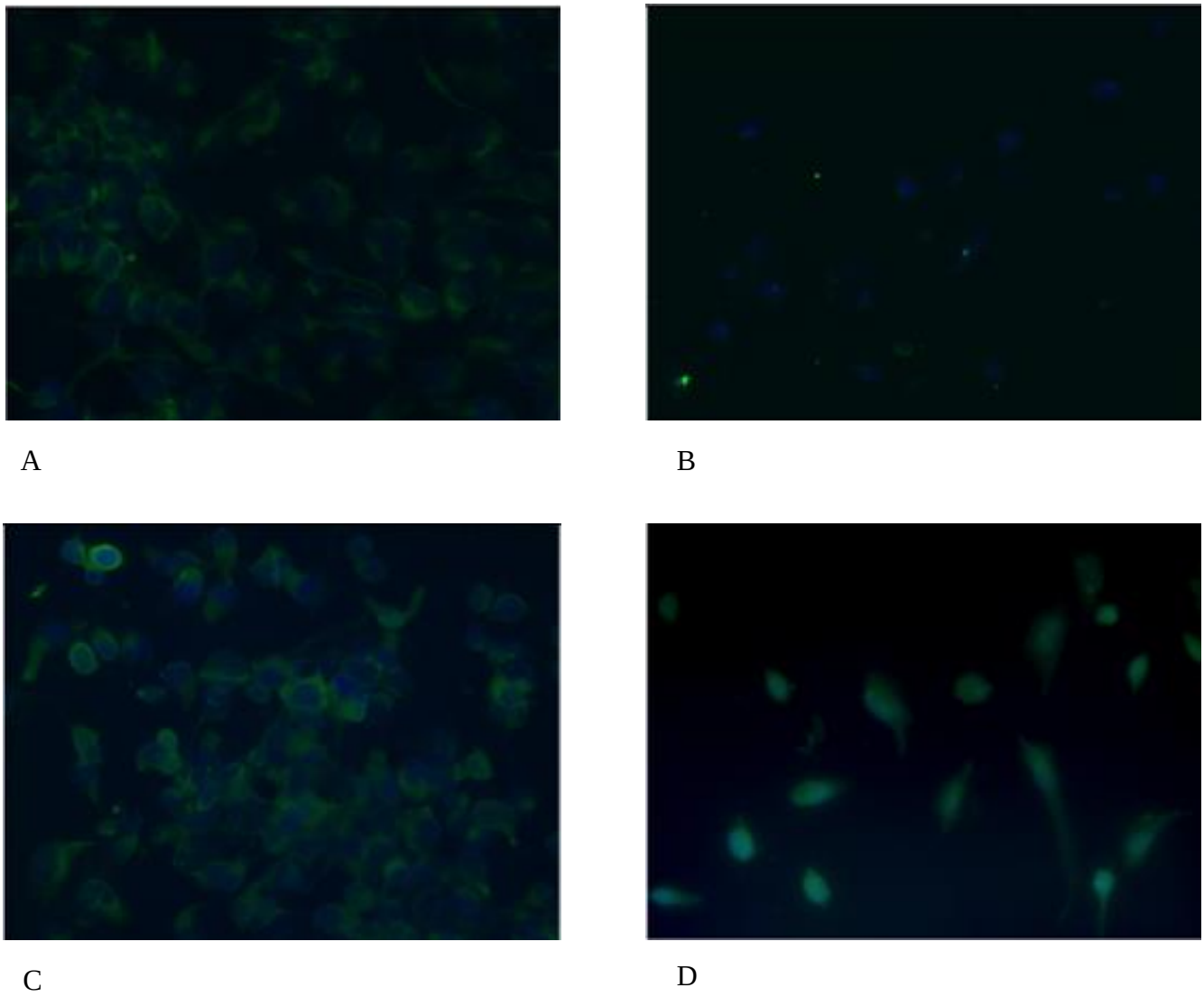


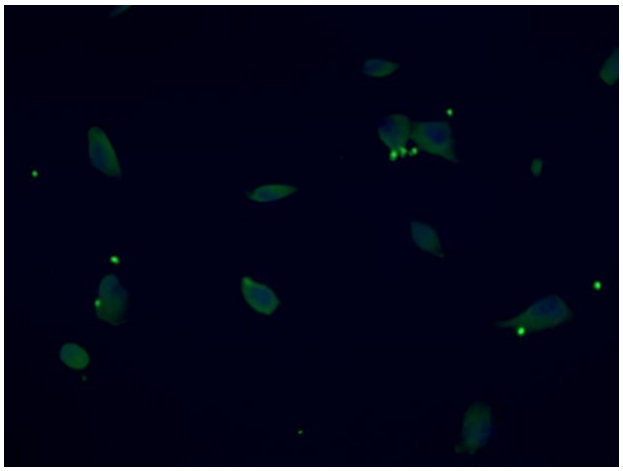
Figure 21: H1299 cells do not show binding to p53 antibodies. Immunocytochemistry assay. A: Untreated H1299 cells with pAB 240 and DAPI. B: Untreated H1299 cells with pAB 1620. C: H1299 cells treated with 12μM RD 27 and stained with pAB 240 and DAPI. D: H1299 cells treated with 12μM RD 27 and stained with pAB 1620 and DAPI.



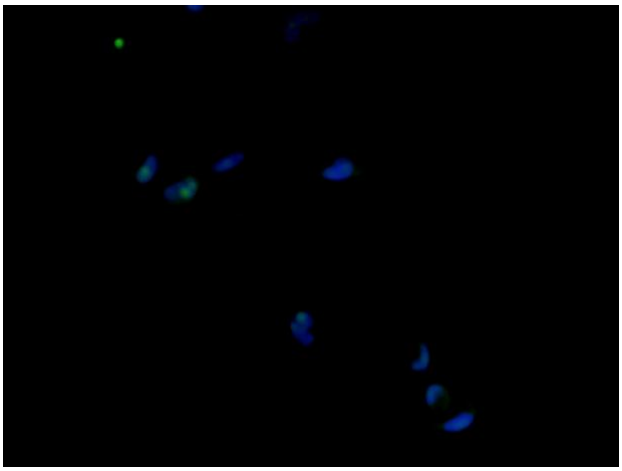
A B

C D

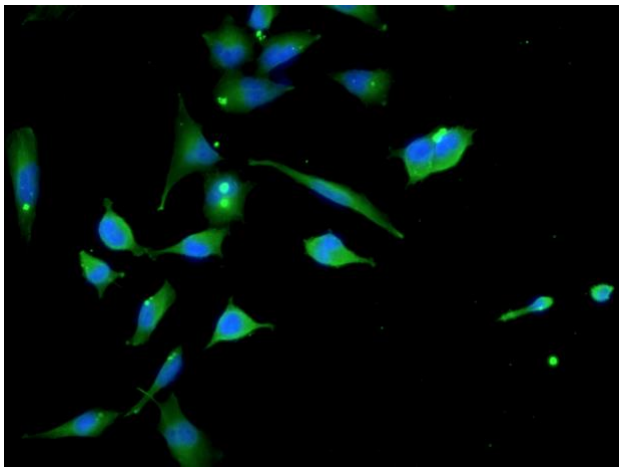
Figure 22: Treatment of SF295 cells with RD27 induces a conformational change from mutant p53 to wildtype p53. Immunocytochemistry assay with mutant p53 and wildtype p53 conformation specific antibodies. A: Untreated SF295 cells with pAB 240 and DAPI. B: Untreated SF295 cells with pAB 1620. C: SF295 cells treated with 12μM RD 27 and stained with pAB 240 and DAPI. D: SF295 cells treated with 12μM RD 27 and stained with pAB 1620 and DAPI.



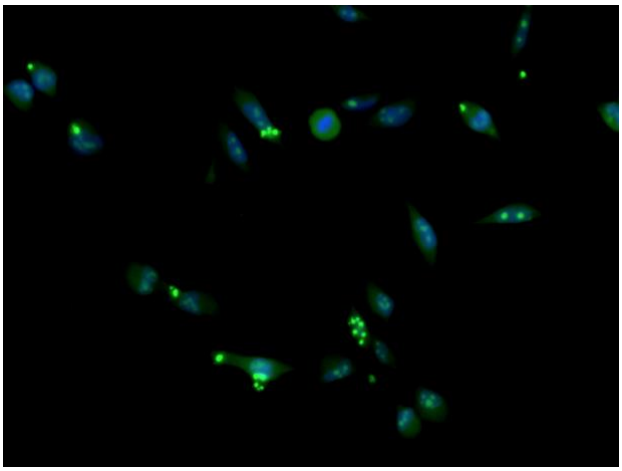
A



B



C



D

Figure 23: Treatment of SF295 cells with RD29 induces a conformational change from mutant p53 to wildtype p53. Immunocytochemistry assay with mutant p53 and wildtype p53 conformation specific antibodies**.** A: Untreated SF295 cells with pAB 240 and DAPI. B: Untreated SF295 cells with pAB 1620. C: SF295 cells treated with 12μM RD 29 and stained with pAB 240 and DAPI. D: SF295 cells treated with 12μM RD 29 and stained with pAB 1620 and DAPI.

Discussion

The tumor suppressor protein p53 plays an important role in maintaining the stability and integrity of the genome and is a critical protein to look at when it comes to the development of cancer. Being that mutations in this gene are involved in about 50% of human cancer cases, it has great potential as a therapeutic target across a wide variety of cancers. When fully functionable, p53 possesses the ability to sense DNA damage and to initiate either cell senescence and repair or apoptosis. Tumor cells with mutations in the p53 gene lack this ability to undergo apoptosis and they divide uncontrollably, resulting in development of resistance to common treatments. Using a GFP based reporter assay in tumor cells with mutant p53 I have screened a number of small molecules and found some that have the ability to restore transcriptional activity of mutant p53. Using a conformation specific antibodies I have also shown that a few of these small molecules change the conformation of the mutant p53 to that of the wild type protein, suggesting the underlying mechanism of reactivation.

RD 27 and RD 29 both show positive results in the GFP assay suggesting that they do in fact restore the wild-type function of p53. The tumor cell lines with mutant p53 that we use have been transfected with a reporter gene plasmid. This plasmid contains the GFP gene downstream of a p53 response element. Using these GFP cell lines in screening compounds, it is expected that upon treatment with compounds, a conformational change to mutant p53 would occur and enable it to bind to the response

element for GFP expression. Reactivation of the mutant protein in cancer cells would allow for p53 to bind to the DNA and transcribe the necessary genes for its antitumor activity. These stable cells were incubated with various compounds for 48 hours and GFP expression was observed under a florescent microscope.

For RD 29, the level of GFP expression was greater for the treated cells than for the control condition in which cells were plated with no compound. In the control, there was very little GFP expression under cyan light and only some background fluorescence in a few cells of the plate (figure 6). In comparison, many cells expressed GFP after treatment with the compound for 48 hours. This is indicative of the ability of these cells to express GFP through reactivation of mutant p53. When I observed the untreated cells under white light, they looked healthy and seemed to be growing normally. Upon treatment with the compound however, the cells look sparse with dead cells scattered throughout the well. These black dots that I believe to be dead cells correspond exactly to the cells that express GFP. This makes sense given the ability of p53 to induce apoptosis in cells when activated. In these specific cells, the compound has reverted the mutated p53 back to the wild-type which allows the functional p53 to induce the apoptosis pathway. Not all of the cells in the well express GFP. It seemed to be only about 25% of the cells. Compared to the other compounds that have been screened, RD 29 showed highest expression of GFP and is thus a potential candidate for further chemical modifications.

I then conducted the GFP assay with RD 29 at three different concentrations and found the GFP expression to increase with the dose for the most part. As seen in figure 7,

The number of cells that express GFP increased in the wells treated with 5 μg/mL and 10 μg/mL. This reinforces my hypothesis that the compound caused mutant p53 to be reverted to the wild-type conformation and led to GFP expression in a dose dependent manner. The amount of GFP expression did not increase when the cells were treated with 20 μg/mL. This may be due to the fact that the compound has some level of toxicity at higher concentrations, resulting in cell death without GFP expression..

Treatment of cells with compound RD 27, a close analog of RD 29, also resulted in an increase in GFP expression (figure 8). This was also accompanied by the appearance of dying cells under white light, as well as a general deterioration of cell health. Compound RD 27 did not induce as much GFP expression as did treatment with RD 29. This compound seemed to restore the p53 function in some cells but it is not as effective as RD 29. However, the cell health in the wells treated with 10 and 20 μg/mL of the compound seemed even worse than the cell death noted in cells treated with RD 29. I believe this compound may be more toxic to the cells, resulting in greater cell death with not as much GFP expression.

Interestingly, the chemical structures of compounds 27 and 29 are very similar. As seen in figure 12, they only differ in the placement of the two chloride groups coming off of the benzene ring. This slight difference in the chemical structure might have been responsible for the difference in GFP expression. Moving forward, changes in the chemical structure may inform how we are able to improve the efficacy of these compounds.

Compound RD 30 also has a very similar chemical structure to 27 and 29. The only exception is that it only has one chloride group on the benzene ring. It was interesting to see that this slight change in the structure resulted in much reduced GFP expression. As seen in figure 11, GFP expression in cells treated with RD 30 was no different than the control untreated condition. We assume that RD 30 is chemically stable.

Compound RD 23 was also tested because it is in the same class of RD compounds but has a different structure to the three previous compounds. As seen in figure 11, there is GFP expression visible upon treatment with the compound. When compared to compounds 27 and 29, there are fewer cells that express GFP, although this expression is intense. Interestingly, there is less GFP expression in the cells treated with a higher dosage of the compound (10 μg/mL). Moving forward, I would be interested in testing multiple lower doses of the compound to test for GFP expression and mutant p53 reactivation.

Another compound that showed GFP expression in the GFP reporter assay was WH 263-63. This compound is of a completely different class of molecules but also induces GFP expression in many of the cells in the well, particularly for the cells treated with 10 μg/mL of the compound. Given that GFP expression was seen in a variety of compounds, it would be beneficial to screen other libraries with this method.

I screened a number of compounds and recorded a number (1-10) for each to represent overall GFP expression. Many of these compounds had relatively low GFP expression compared to control. In the absence of a quantitative GFP assay, I used an

arbitrary rating of 5 or above to define compounds that will be explored further through the use of the cell viability and immunocytochemistry assays. Conducting the GFP assay as the first level of screening is an effective way to determine whether or not these compounds have any ability to activate mutant p53.

Once these compounds tested positively in the GFP assay (with a rating of 5 or above), I evaluated their specificity for mutant p53 in a cell viability assay. Both H1299 and SF295 (or DLD1) cells were treated with various concentrations of the compound and after 72 hours, cell survival was measured with the use of a MTS reagent. I expected cell survival to decrease in the p53 mutant cells with each increase in compound concentration due to the reactivation of mutant p53. If these compounds bind to mutant p53 and restore it to the wild-type conformation, p53 will act as a transcription factor and induce cell death. On the contrary, in H1299 cells I expected no change in cell survival due to the fact that they lack p53. The cell viability assay allowed me to determine if the compound acts through the p53 pathway, based on the differences observed between p53 mutant and p53 null cell lines.

I first produced growth curves for both cell lines to determine the initial concentration of cells to plate in 96 well dishes. The growth curves (figures 15 and 16) show the absorbance measured 3 hours after the addition of MTS dye and 72 hours after the addition of the dye. Cells were initially plated in increments of 500 starting with 500 cells per well. For the H1299 cells, the maximum absorbance for cells after 72 hours seemed to be at 1.8 or perhaps even above that. I chose to plate 2,000 cells per well for the cell viability assay, which corresponded to an absorbance of about 1.1 after 72 hours.

This was a level of absorbance in the middle of the curve which is important so that the absorbance is above the baseline represented by the same day reading and less than the maximum absorbance value. This allows for an effective reading so that there is room to read a lower absorbance if cells are killed. For SF295, I determined the ideal initial cell concentration to be 1,500 cells per well which corresponds to an absorbance of about 2.4. The maximum absorbance for SF295 was around 2.6, so the initial concentration needed to be at a lower absorbance than this. These differences in concentration are due to the fact that mutant p53 in SF295 cells provide a growth advantage over H1299 cells.

Some of the experiments were conducted with p53 mutant DLD1 cells. I have observed that these cells behave similarly to SF295 cells in growth speed and size. Therefore, the initial DLD1 cell concentration used was identical to the SF295 concentration. Ideally, I would have also created a growth curve for the DLD1 cells to determine the precise concentration of initial cells needed and this will be an experiment required in the future.

In cells treated with RD 27, I expected to see a decrease in cell survival due to the reactivation of mutant p53 and the transcription of the necessary factors to cause apoptosis. In DLD1 cells, this decrease in cell survival was observed (figure 17). The cell survival began to decrease at treatment with 1.25 μg/mL of the compound, and continued to decrease until nearing 0% cell survival at 10 μg/mL. The H1299 cells also exhibited a decrease in cell viability when treated with the compound. However, this decrease began at 5 μg/mL. This created a window in which the compound causes a decrease in cell viability in only the cells containing mutant p53, which supports my hypothesis that RD

27 reactivates mutant p53 and leads to apoptosis. However, at higher concentrations, the compound may be toxic as it causes a decrease in cell viability in both cell lines.

Although compound RD 29 showed a high level of GFP expression in the GFP reporter assay, it did not display selectivity for mutant p53 in the cell viability assay. There seems to be no difference in cell survival between H1299 and SF295 cells (figure 18). This goes against my hypothesis based on the positive results of the GFP assay. The GFP expression observed in the GFP reporter assay suggests that RD 29 reactivates mutant p53 in the cancer cells. However, the compound did not cause any change in cell survival for the p53 mutant cells. Therefore, I would like to test higher concentrations of the compound in both SF295 and DLD1 cell lines.

For other compounds tested, there was no substantial difference in cell viability between H1299 and SF295 cells once treated with the compounds. For example, figure 19 shows the cell viability results for WH 263-6. For both cell lines, cell survival remains around 100% until 5 μg/mL when cell survival rapidly declines. At 20 μg/mL, cell survival is around 0% for both cell lines. This suggests that the compound is toxic, since cell death occurred in both cell lines. The compound may be toxic to the cells at the higher concentrations, causing cell death that is not related to p53 activity. For the compound RD 23 (figure 20), the percent cell survival remained at around 100% for both cell lines. At 20 μg/mL there was a slight decrease to about 60% cell survival for H1299 and 85% cell survival for SF295. Moving forward, it is necessary to conduct a cell viability assay with RD23 at higher concentrations to observe cell survival past 20 μg/mL.

I then conducted the immunocytochemistry assay with the compounds that showed GFP expression in the GFP reporter assay. Compound 27 was particularly promising because I also observed a therapeutic index in the cell viability assay. With the use of two different antibodies (one for mutant p53 and one for wild-type p53), the immunocytochemistry assay determines whether there is a change in conformation of p53 due to treatment with the compound. I hypothesized that this change in conformation was occurring since DNA binding function of wild-type p53 is needed to induce GFP expression and cell death. First, H1299 cells were treated with the compound and stained with antibodies for both wild-type and mutant for p53. As expected, there was no binding of either antibody in both the untreated and treated conditions because H1299 lacks p53. The only binding seen is DAPI (blue) which binds to the nucleus of all living cells.

The assay was first conducted with RD 27, which showed positive results in both previous assays. As seen in figure 22, there was binding of the mutant p53 antibody to SF295 cells that were not treated. This was expected because of the mutant p53 genotype of SF295. There was also binding of the p53 mutant antibody to cells treated with the compound. This suggests that not all of the p53 is converted back to the wild-type conformation. Upon treatment with the compound, there was some binding of the wild-type p53 antibody. The addition of the compound is inducing a change from the mutant to wild-type form of p53 which allows the transcription factor function that is observed in the GFP assay. Similar results were observed when I conducted the immunocytochemistry assay with compound RD 29. In this case, there seems to be some unspecific binding of the antibodies. This was possibly due to insufficiently washing off

unbound antibody during the washing steps. However, I can still distinguish between the small specks of unspecific binding and the specific binding of the antibody to p53. Interestingly, for both compounds, the binding of the mutant p53 antibody seemed to occur outside of the nucleus of the cell. The blue DAPI is still visible in the center. The binding of the wild-type antibody however, seems more concentrated to the nucleus. It would make sense that once converted back to the wild-type conformation, the p53 travels to the nucleus to aid in transcription.

While these assays can determine whether these compounds reactivate the wild-type function of mutant p53, there are still many questions that I can ask. First, I feel that the proper control is missing in the cell viability assay. In this assay, I compared SF295, a tumor cell line with mutant p53, to H1299, a tumor cell line that is p53 null. These cell lines are from two different types of cancer with different genotypes (glioblastoma and non-small cell lung cancer respectively) and may respond differently to the compounds. While the use of H1299 cells as a control gives us an idea of how the compound will affect cells that are lacking p53, the proper control would be SF295 cells lacking p53. As a future experiment, I would like to generate a SF295 knockout cell line that lacks p53 using CRISPR/Cas 9 technology. Using this knockout cell line as a control would ensure that experimental conditions are identical except for the presence of the p53 protein.

Improvements can also be made to the GFP assay. When conducting this assay, I visually analyze each of the conditions under the microscope and I also rate the amount of fluorescence in the well on a scale of 1 to 10 (with 1 being almost no fluorescence and 10 being the expression of fluorescence in all cells). While I am able to make some

conclusions about the efficacy of different compounds in this manner, I would like to come up with a way to quantify the amount of GFP fluorescence. This can be accomplished either by the help of a software to quantify GFP expression observed through a fluorescent microscope or the use of flow cytometry to count individual cells that express GFP.

Due to the ability of RD 27 of these compounds to cause cell death in the p53 mutant cells and not the p53 null cells, I have inferred that the addition of the compound leads to apoptosis via the p53 pathway. However, I would like to confirm that these cells are in fact undergoing apoptosis. This can be accomplished with a FAM FLICA Caspase 3/7 assay. This would allow me to quantitatively measure the levels of apoptosis in treated cells by adding a FAM-DEVD-FMK which covalently binds to active caspases 3 and 7 (Lee 2017). The reagent is labeled with green fluorescence and unbound reagent will be washed away. Therefore, apoptosis levels can be measured by the amount of fluorescence, which corresponds to the presence of active caspases 3 and 7 (executioner caspases that are involved in the apoptotic pathway).

With the use of the immunocytochemistry assay I have shown that upon treatment of cells with RD 27 or RD 29 some of the mutant p53 protein is converted back to the wild-type conformation. I hypothesize that the compounds are binding to the protein and stabilizing the wild-type conformation, which allows it to bind to DNA and transcribe the factors necessary to induce apoptosis. However, an experiment is needed to confirm whether or not these compounds are binding to p53 to modify it.

For the compounds that I have shown to reactivate mutant p53 (RD 27 and RD 29), they seem to display some toxicity. When looking at the cell viability assay for RD 27 for example (figure 18), the cell survival for H1299 cells begins to decline at 5 μg/mL of the compound. It continues to decline and at 20 μg/mL, the cell survival is decreased to about 30%. There is a small therapeutic window in which treatment with the compound causes a faster decrease in cell survival of the p53 mutant cells compared to the p53 null cells. However, at the higher concentrations of the compound, survival of the p53 null cells also declines, suggesting that the compound is toxic at these higher concentrations. I would like to work with a chemist to modify these compounds in the hopes of finding one that is less toxic but equally as efficient in reactivating mutant p53. Furthermore, mouse models are needed to evaluate the efficacy of these compounds in a living system.

These compounds have great potential as therapeutic agents to reduce tumors. While mutations in p53 are common to many types of cancer, it is unknown how these compounds will respond to other cancer subtypes. In this case, I used cancer cells with a point mutation in the DNA binding domain. These mutations included R248G in SF295 cells and R241S in DLD1 cells. These are two different classes of mutations. R248G is a DNA contact point mutation, while R241S is a structural mutation. Since these molecules reactivate these classes of mutations I hypothesize that there would be similar results with other point mutations in the DNA binding domain of p53. I would like to look further into the other common mutations of p53 that are found in human cancers, as these compounds may be able to restore loss of activity in these p53 mutants. The use of these

assays also provides an effective screening methods that can be performed with other libraries of compounds.

Here, I have identified and characterized a few compounds that reactivate mutant p53 in cancer cells. These compounds, more specifically RD27 and RD29, display GFP expression which signifies the reactivation of mutant p53 to the functional wild-type protein. RD 27 also selectively targets the mutant p53 cell line and both compounds show binding of the wild-type p53 antibody upon treatment. Much work is needed to examine the exact mechanisms of these compounds. However, they are promising for further modification and the development into potential cancer therapy.

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