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Honors Thesis

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Abstract

Gliomas, which are brain tumors that arise from glial cells, are some of the most aggressive and lethal types of tumors. These brain tumors are difficult to treat because not enough information regarding the mutations present in these tumors exists. This project studies effects of a p53 mutation on Drosophila glioma progression and then will test to see if this results in resistance to current chemotherapy. The main goal of this endeavor is to investigate the numerous defects occurring at the cellular and biochemical level in gliomas, which will give insight into why these types of tumors are so difficult to treat. Additionally, this document also discusses some promising chemotherapeutic agents found through a drug screen project. The effects of five different Tyrosine Kinase inhibitors on glioma development are presented here.

Acknowledgements

Very special thank you to the wonderful Dr. Kango-Singh for all of her guidance and support with these projects, research program application and help in developing and editing this thesis. Dr. Kango-Singh has been an amazing research mentor these past four years and has helped me realize that I want to incorporate research into my career. Thank you to Dr. Kirti Snigdha and graduate student Karishma Gangwani for all of their help with this project and training me how to do various lab techniques. They welcomed all my questions and helped me become even more interested in cancer research. I am grateful for my fellow undergraduate members and all their contributions to this project as well. Thanks to the University of Dayton Stander Fellowship, Mr. Berry and the Berry Family Foundation and the support of the University Honors Program. Finally, I am grateful to my parents and my nana for always encouraging and supporting me.

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Preface: In September of 2017, I began working in Dr. Kango-Singh's lab as a part of the drug screen team. This project aims to look at the effects of a slew of possible chemotherapeutic agents on glioma development. As a member of this group, I had the opportunity to learn many lab techniques, how to work with fruit flies and gain a better understanding of this type of cancer and its mechanisms. Due to my interest in the subject of brain tumor research, I decided to apply to the Berry Summer Thesis Institute in hopes of working on a project to write my honors thesis about. With the help and guidance of Dr. Kango-Singh, I began working on a stand-alone project in addition to the chemical screen. This new project involved inducing a *p53* mutation in a *Drosophila* glioma model in order to explore how this specific mutation affects tumor progression. We had planned to treat these tumors with various Tyrosine Kinase inhibitors to see if this mutation contributed to the tumor's resistance to therapy. Although the summer provided a successful start to the project, which involved some initial tests, we were not able to complete all of our goals. COVID-19 shut down our lab work, as the university halted in-person undergraduate research. Even though my thesis project was cut short, there were multiple promising findings with respect to the drug screen project, which I will discuss in this paper. Furthermore, I have compiled the findings of numerous papers and articles in order to write a literature review regarding the *p53* mutation and gliomas.

Drug Screen

1. Background

Brain cancer, specifically glioblastoma multiforme (GBM) is one of the most deadly and devastating diseases. Median survival after diagnosis is about 15 months, even after surgery, radiation and chemotherapy. Patients are often plagued by headaches, seizures and other neurological symptoms, along with the side effects of treatments. In order to properly treat this disease, it is necessary to develop and identify more effective, targeted treatments.

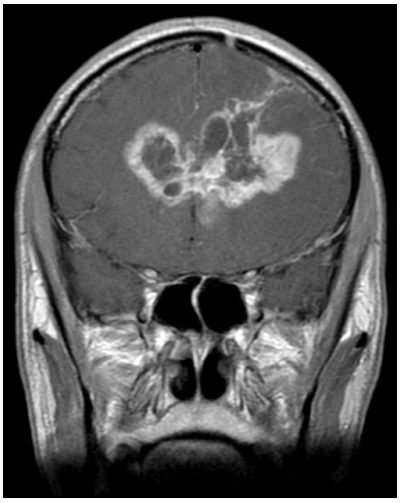


Fig. 1 Coronal T1 C+ MRI of a patient suffering from a "butterfly" glioma (GBM that has spread to both hemispheres of the brain). Patients presenting with bilateral involvement typically have even worse survival outcomes and usually die within a few months following diagnosis, despite treatment.
Image retrieved from: Gaillard, Frank. (2016). Glioblastoma NOS (butterfly morphology). *Radiopaedia*. https://radiopaedia.org/cases/glioblastoma-nos-butterfly-morphology?lang=us

In an effort to identify possible treatment options for glioma patients, the Kango-Singh lab developed a drug screen which involves testing the effects of many different chemicals at varying concentrations on fruit fly gliomas. This involved creating a genetic cross that would induce a feasible glioma model. This was accomplished by inducing the two most frequently occurring human glioma mutations into the *Drosophila* model. The Ras/MAPK signal transduction pathway was altered by inducing a Ras^{V12} mutation,

which is one commonly found in human cancers. *Pten*^{RNAi} was co-expressed with this mutation in an effort to better mimic human tumors. When *Pten* functions normally, it plays a role in growth regulatory pathways as a tumor suppressor. However, since *Pten* was eliminated, its tumor suppressor abilities were lost, and results in tumor development. The combination of this mutation and *Ras*^{V12} drove tumor development and progression to create an effective glioma model. The *Pten*^{RNAi} ; *Ras*^{V12} stock were crossed with the *repo GAL4 UAS GFP* stock, which drives expression of UAS-linked transgenes in the glial cells in developing larvae. The GFP allows for tracking glial cells using fluorescent microscopy imaging, as glial cells glow green due to expression of the GFP (Green Fluorescence Protein). Tyrosine Kinase inhibitors were fed to the *Drosophila* larvae, and tumor progression/growth was analyzed.

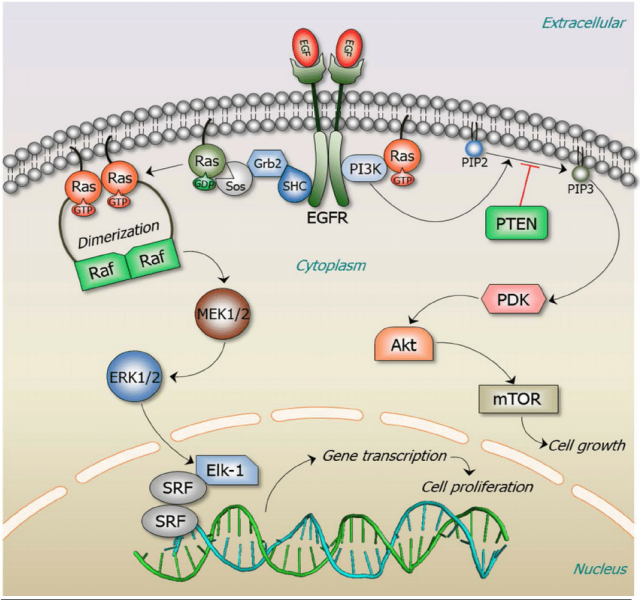


Fig. 2 Mutations within Ras/MAPK and PI3K/ Pten can drive tumorigenesis and tumor growth. (https://www.researchgate.net/figure/Ras-signaling-pathways-Ras-signaling-is-involved-in-numerous-cellular-functions_fig1_266582872)

All of the drugs used in this project are classified as Tyrosine Kinase inhibitors. They are also approved by the FDA for the treatment of certain cancers as well as other dangerous diseases. Tyrosine Kinases are a family of enzymes which function as part of a signaling cascade and are known to mediate cellular processes important to the prevention of cancer. They do this by selectively phosphorylating substrates. Tyrosine kinases play a role in cell metabolism, migration, apoptosis, proliferation and differentiation. Certain mutations can lead to loss of these functions and the development of cancer. Furthermore, cancerous mutations in this cascade can contribute to angiogenesis as well, making tumors more vascularized [21].

Tyrosine kinases and tyrosine phosphatases regulate tyrosine kinase phosphorylation, however, in mutated cells, this antagonistic control becomes dysregulated. Cancers such as glioblastoma, non-small cell lung cancer, multiple myeloma and ovarian cancer are known to result, in part due to mutations within the extracellular domain. Such mutations lead to constitutive activity of receptor tyrosine kinase and, in turn, rapid proliferation of mutated cells. Another way in which tyrosine kinases become over-expressed or abnormally expressed results from autocrine-paracrine signaling. Essentially, this feedback loop becomes overstimulated and there is over-expression of the ligand [21].

A mutation present in about 40 percent of gliomas lies in the epidermal growth factor receptor (EGFR). Although there are numerous mutations present in this type of tumor, this specific one is known to enhance tumorigenesis in humans as a result of amplification. Additionally, it is believed to be a biomarker of resistance in certain types of tumors. In terms of tumor development, EGFR functions in a signaling cascade which

regulates the activation of genes within the nucleus important for cell differentiation, proliferation and survival [28].

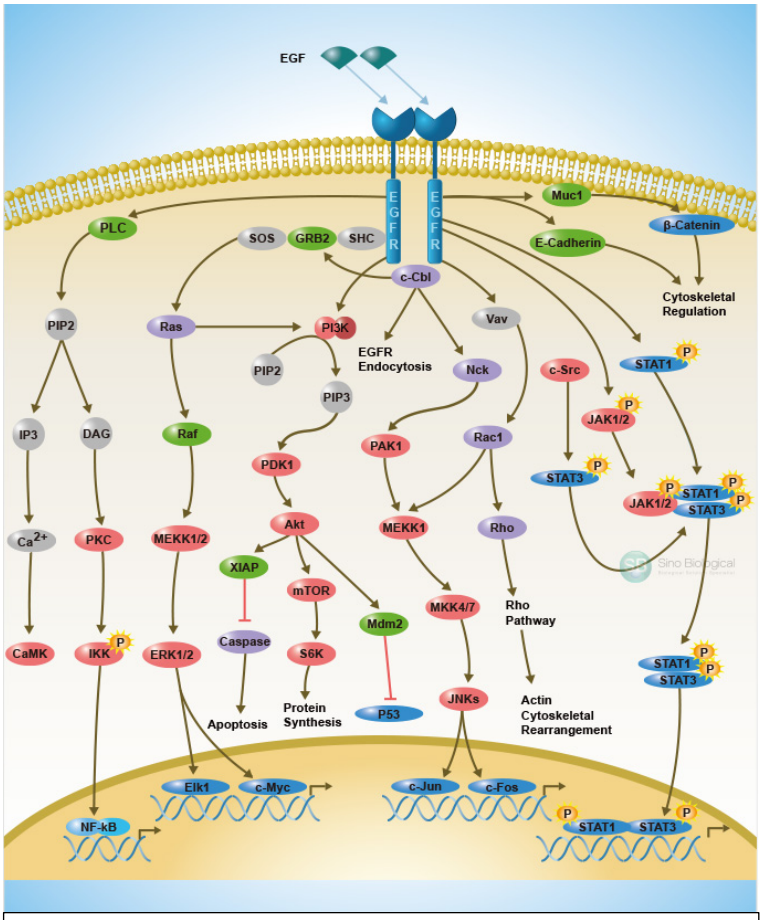


Fig. 3 EGFR regulates numerous pathways and subsequently, many major cellular processes. (https://www.sinobiological.com/pathways/egfr-signaling-pathway)

When a ligand attaches to the EGFR, EGFR protein dimerizes and activates the receptor complex, beginning the signaling cascade. Two important pathways, PI3K and RAS-MAPK are modulated via a downstream signaling cascade by EGFR. These both function to promote cell proliferation, metastasis and the inhibition of programmed cell death. Therefore, amplification or mutations within this gene can have cancerous effects [32]. The reason

that EGFR is important to this study is the fact that many of the drugs tested are known EGFR inhibitors. EGFR inhibitors work to block this protein's activity and slow the proliferation of cancer cells.

Promising Drugs Identified

Saracatinib is an oral chemotherapy drug used for the treatment of chronic myeloid leukemia due to its action as an Abl and Src inhibitor. It was developed by AstraZeneca and is approved by the FDA. These two protein kinases are frequently overexpressed in leukemia cells. This drug is also being tested for the treatment of various bone cancers such as osteosarcoma because it is known to inhibit Src kinase-mediated osteoclast bone resorption [16]. The role Saracatinib plays in modifying various cellular processes is still being investigated. For example, it was recently discovered that Saracatinib inhibits the Fyn Kinase as well, which also falls into the family of Tyrosine kinases and is a known oncogene. Due to the fact that Fyn mediates beta-amyloid toxicity, Saracatinib is being tested as potential treatment for Alzheimer's disease [34].

For the purposes of this experiment, Saracatinib was tested for its effect on gliomas. In one study, it was found that SRC activity is increased in GBM brain samples as compared to healthy brain tissue. Interestingly, the rise in activity is not due to mutations or overexpression, but rather as a result of higher levels of growth factor receptors on the surface of the cells. Another reason for this amplification is the activation of integrins which in turn activate SFKs. SFKs function as tyrosine kinases and mediate signaling within the cell. According to various GBM cell lines, the dysregulation of SFK affects cell adhesion. SFK dysregulation is also believed to play a role in cancers

that metastasize to the brain. In studies where SRC was inhibited, GBM, in addition to numerous other types of cancers, exhibited lower levels of cell proliferation. This finding points to the role that an SRC inhibitor, such as Saracatinib, may play in preventing the proliferation of cancerous cells within the brain [2].

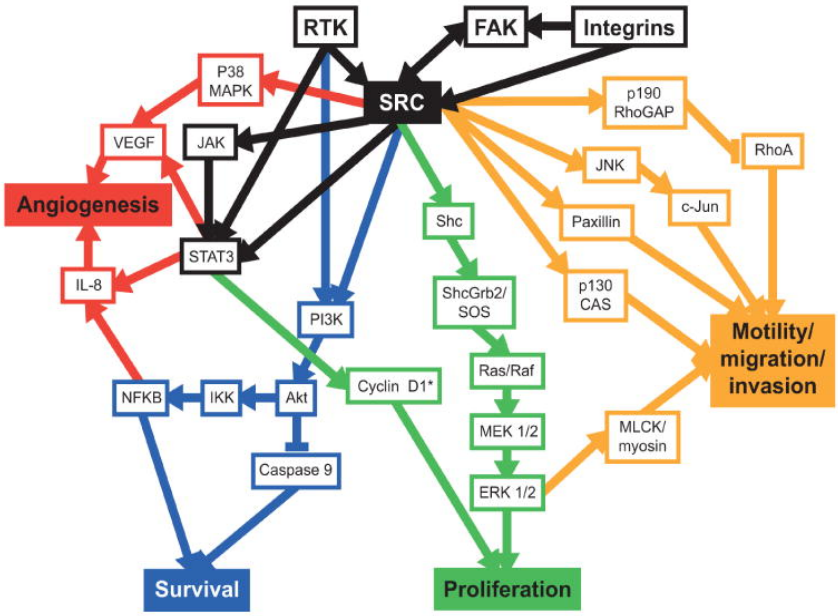


Fig. 4 SRC mediates numerous cellular processes, including angiogenesis, survival, proliferation and motility. If dysregulated, this pathway can contribute to rapid division of cancerous cells, tumor development and metastasis.

Gefitinib (brand name:Iressa) is also approved by the FDA to be used for the treatment of various cancers, specifically those affecting the breast and lung. It was also developed by AstraZeneca as a Tyrosine Kinase inhibitor [18]. Gefitinib inhibits EGFR by selectively binding to its domain, therefore interfering with autophosphorylation and disrupting the signaling cascade. This disruption leads to decreased cell proliferation and increased rates of apoptosis. Additionally, Gefitinib plays a role in preventing angiogenesis, which is the growth of new blood vessels. Tumors responding positively to this drug may become less vascular.

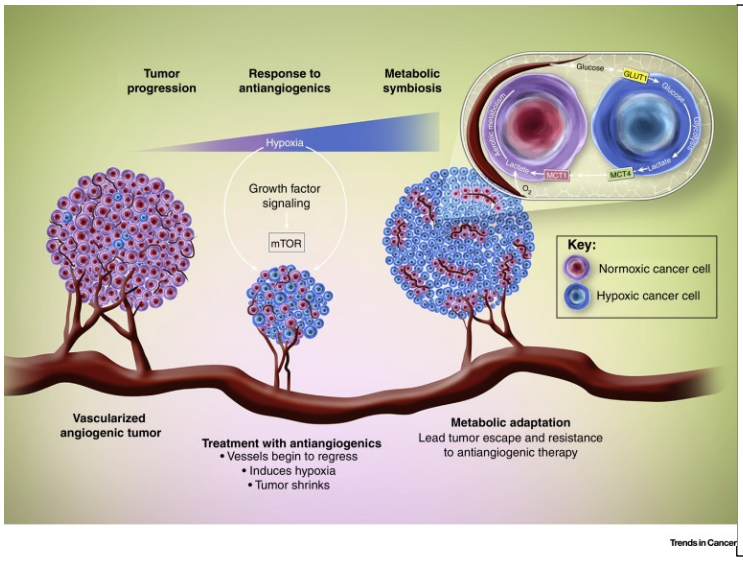


Fig. 5 The process of angiogenesis increases blood supply to tumors and affects tumor growth. Drugs that inhibit angiogenesis may shrink tumors due to decreased blood supply and subsequent hypoxia. Unfortunately, tumors can become resistant to these efforts and continue to grow. (https://www.cell.com/trends/cancer/fulltext/S2405-8033(16)30185-6)

In a study done in 2018, GBM patients with either an EGFR mutation, PTEN mutations or both upon biopsy were treated with Gefitinib. Although side effects were common, patients treated with Gefitinib fared better than others. $EGFR^{+ve}/PTEN^{-ve}$ patients' survival rates were significantly higher after being administered 250–500 mg/day of the drug over a span of several months. After tumor resection, several patients with remaining tumor had stable scans or shrunken tumors after taking Gefitinib [4]. Another study, focusing on recurrent gliomas suggests that Gefitinib in combination with other chemotherapeutic drugs may target EGFR mutations, improving patient survival. However, this same study discussed the need for further research into this drug and the pathways it may affect in order to target specific mutations [24].

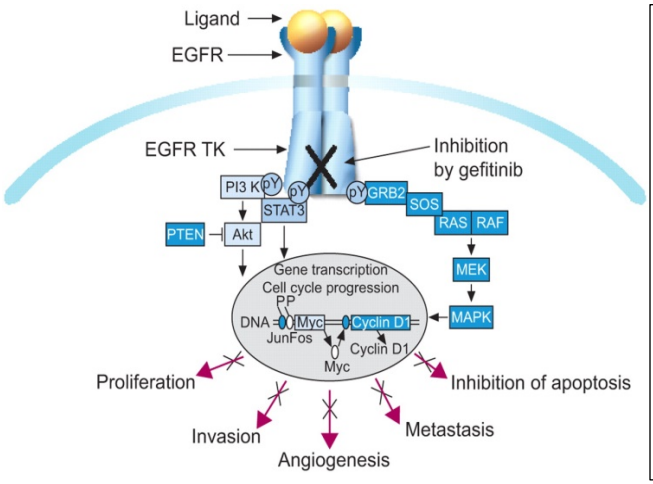


Fig. 6 When Gefitinib binds to the receptor, it can inhibit numerous cellular processes, including cell proliferation, invasion, angiogenesis, metastasis and the inhibition of apoptosis. All of these actions can have anti-cancer effects by preventing the development and migration of cancer cells. (https://err.ersjournals.com/content/19/117/186)

Genistein is a phytoestrogen that exhibits anti-cancer effects. Genistein is commonly found in soy and in countries with soy-rich diets, people are less likely to develop breast or prostate cancer. Although its exact mechanism is unknown, the chemical is believed to target MAPK and PI3K/Akt (important targets for GBM therapy) pathways among a few others [29].

In one study involving GBM and medulloblastoma (brain tumor located in the medulla, typically a childhood cancer), Genistein was found to arrest cell growth in cancerous cells. The growth arrest happened during the transition from G2 to M stages of the cell cycle, which halted mitosis. The drug also inhibited TR- and TERT mRNA, which decreased telomerase activity. Telomerase lengthens telomeres, preventing degradation of the chromosome. Even though Genistein damaged the DNA in cancer cells and arrested growth, it did not induce cell death [12]. Therefore, the addition of radiation therapy or another chemotherapeutic agent known to induce cell death may be a more useful approach than just the treatment of Genistein alone. In a separate study using head and neck cancer cell lines, Genistein was found to arrest the cell cycle by upregulating Bax (pro-apoptotic agent) and p21(regulator of the cell cycle). This mechanism helps prevent the transition to the M phase of the cell cycle as well [3].

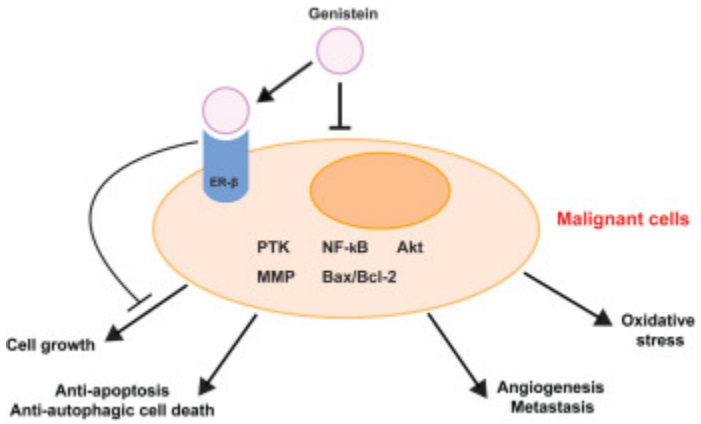


Fig. 7 Genistein can inhibit dysregulated cellular processes that lead to the development of tumors. It does this by binding to ER-B receptors and altering the signaling cascade. (https://www.sciencedirect.com/science/article/pii/S2225411016300827)

Ibrutinib (brand name: Imbruvica) is an inhibitor of Bruton's Tyrosine Kinase, marketed by Abbvie (previously Pharmacyclics LLC). It is used for the treatment of white blood cell disorders such as B-cell lymphoma, chronic lymphocytic leukemia, mantle cell lymphoma and Waldenstrom macroglobulinemia. Bruton's Tyrosine Kinase (BTK) operates in a pathway that regulates B-cell proliferation [14]. When mutations occur in BTK that cause upregulation of the pathway, this can lead to the survival and division of cancerous cells. The binding of Ibrutinib to BTK inhibits NFκB DNA binding. This in turn decreases cell proliferation, DNA synthesis and cell survival. In experiments using models where BTK is knocked down in mantle cell leukemia cells, the NFκB pathway is inhibited, therefore decreasing these cells' ability to continue to grow and migrate [1].

In terms of GBM, Ibrutinib has shown some promise in overcoming therapy resistance by inhibiting BMX-STAT3. This receptor is known to mediate the activity of STAT 3, which is a transducer needed for the maintenance of glioma stem cells. These stem cells are thought to be instrumental to the tumor's ability to resist therapy, whether it be chemotherapy or radiation therapy. Inhibition of this pathway by Ibrutinib decreased gliomas stem cell- induced tumor progression/growth and in turn, was able to shrink tumors in GSC-derived orthotopic xenografts [27]. Case Comprehensive Cancer Center is currently conducting a clinical trial involving the use of Ibrutinib with Temozolomide (chemotherapy commonly used to treat GBM) along with radiation therapy, in hopes of decreasing the growth of these malignant cells [19].

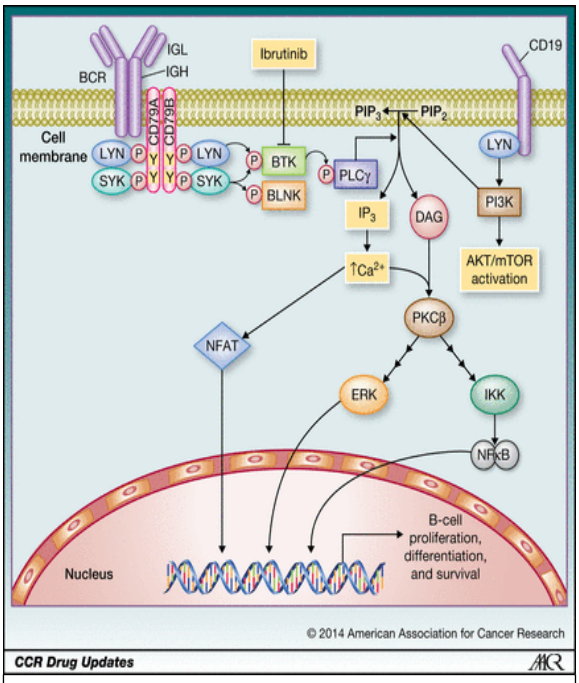


Fig. 8 Ibrutinib binds to BTK, inhibiting the pathway important for B-cell proliferation, differentiation and survival.(https://clincancerres.aacrjournals.org/content/20/21/5365)

Apatinib, also called rivoceranib, is sold under the brand name Aitan by Elevar Therapeutics and was originally developed for the treatment of gastric cancers. Its efficacy is currently being studied in a clinical trial for patients with either advanced or metastatic stomach cancer across twelve different countries. It has also shown some promise for in the treatment of hepatocellular carcinoma, colorectal carcinoma and adenoid cystic carcinoma [7]. Apatinib is a small molecule and is taken orally in order to inhibit vascular endothelial growth factor receptor-2. VEGFR-2 has angiogenic effects because of its autophosphorylation at its kinase-insert region and carboxy terminal tail. The process of angiogenesis is an important step in the development of solid tumors, so inhibiting this mechanism may slow or stop the growth of tumors.

Since Apatinib has shown to inhibit VEGFR-2 and subsequent downstream phosphorylation, it was used in this screen, as angiogenesis is a driver of brain tumor growth. In fact, gliomas are the most angiogenic of all cancerous tumor types. In gliomas, this mechanism is both hypoxia- dependent and independent, so targeting genes associated with hypoxia, in this case VEGF, may be a useful therapeutic approach. Furthermore, stem cells sampled from gliomas tend to have significantly higher VEGF levels, which is hypothesized to be the reason behind the high vessel densities measured in gliomas [23]. In one study, Apatinib was paired with Temozolomide and administered to patients with recurrent GBM. Even though the disease control rate was 90%, the median overall survival was still less than one year (nine months) [36].

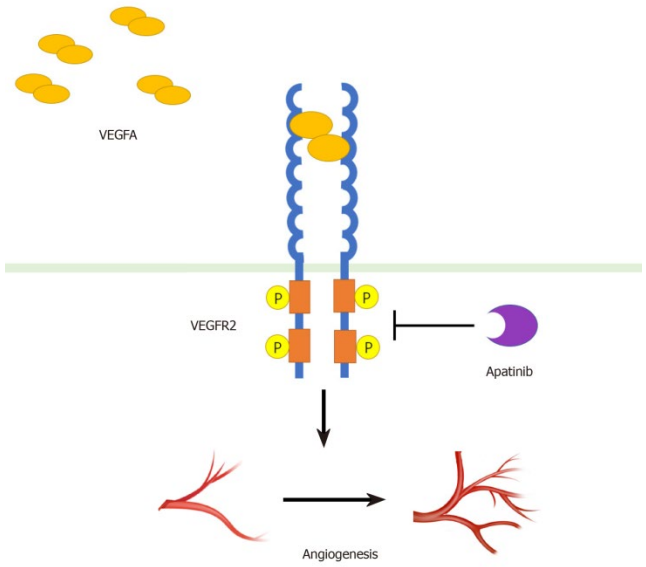


Fig. 9 When VEGFA binds to VEGFR, a cascade is initiated, leading to the development of new blood vessels. This allows for the continued growth of the tumor. Binding of Apatinib to VEGFR-2 prevents angiogenesis. (https://www.wjgnet.com/1948-5182/full/v12/i10/766.htm)

2. Materials and Methods

Drosophila with UAS Pten^{RNAi} and UAS Ras^{V12} mutations were crossed with each other in order to create a new stock [*UASpten^{RNAi}; UASRas^{V12}*]. Female virgins from this stock (as denoted by meconium in the abdomen) were crossed with males from the *repo-Gal4 UAS-GFP* stock. Virgins were collected twice a day, once in the morning and then approximately four hours later. Depending on the amount of flies available, they were placed in the same vial at a ratio of three males to six females or five males to ten females. These flies were placed in a box the incubator regulated at 24 degrees Celsius. Each morning, the flies from all stocks were flipped into new vials and labeled and dated accordingly. The *Pten^{RNAi};Ras^{V12}* and *repo-Gal4,GFP* stocks were stored at room

temperature, while the glioma cross was maintained in the 25ºC incubator during the course of the study.

The Tyrosine Kinase inhibitors were added to vials containing only the fly food (the same type of food present in the vials of all the stocks). This was performed by a graduate student to ensure that the technique was done properly and safely. The drugs were added to the vials at two different concentrations: 10uM and 300uM. Four separate vials were prepared for the 10uM concentration and four for the 300uM concentration. These vials were prepared either the day of or a few days before the addition of the larvae, and then stored in the lab refrigerator immediately. The identity of each drug was unknown to the students and rather denoted by letters and numbers.

The larvae that were added to the vials containing the drug came from the *repo GFP* stock and the glioma stock. Approximately 20 larvae from the *repo GFP* stock were added to the 10uM drug vials and then 20 larvae were added to the 300uM vials. The same thing was done for the glioma stock as well. Additionally, when larvae were not crawling on the sides of the vial, a small amount of water was added to the vial to get the larvae to come up. When collecting the glioma cross, only the non-TM6B larvae were added to the drug vials, meaning these larvae were "non-tubby." All vials were labeled according to the drug, the concentration and which stock was placed in the vial and dated. These vials were then placed in a separate container in the incubator and with one *repo GFP* at 10uM and one glioma cross at 10uM left to develop for five days, one *repo GFP* at 10uM and one glioma cross at 10uM left to develop for six days, one *repo GFP* at 300uM and one glioma cross at 300um to develop for five days and one *repo GFP* at 300uM and one glioma cross at 300uM to develop for six days.

After either five or six days had passed, according to how the vials were labeled, larvae were removed and dissected. The goal was to remove enough larvae in order to have five brains to mount on a microscope slide. Sometimes, there were no larvae available and other times, water had to be added in order for larvae to come up. After enough larvae were collected for dissection, they were placed on dishes in a solution of PBS. One five and one 55 forceps were used to pull the larvae in half to remove the head because only the brains were used for this study.

Once the larvae had been pulled apart, the portions containing the brains were placed in an Eppendorf tube containing 150uL of PBS and 50uL of 15% PFA (well mixed) and left to sit upright for 20 minutes. Next, 1000uL of PBST was added to the Eppendorf tube and it was placed on a rotator for ten minutes. Then, most of the liquid was suctioned up using a vacuum, leaving the sample at the bottom and 1000uL of PBST was added again into the tube. It was then placed back onto the rotator for another ten minutes. This process of vacuuming, putting more 1000uL of PBST back into the tube and placing on the rotator was repeated two more times. After the final rotation step, the liquid was not vacuumed up so that the sample did not dry up.

Once the sample had been properly washed, a slide was prepared for each set of samples, which was properly labeled and dated. The tip of a pipette was cut in order to suction up the sample and place it onto the slide. Then, using two 55 forceps, the sample was delicately pulled apart and the debris was washed off using a Kim wipe so that only the ventral nerve cords and the brain lobes attached were left. Once all the brains were prepared and debris wiped away, they were pushed to the side of the slide so they could eventually be mounted in the center. While dissecting the brains, it was important to keep

the sample moist with PBST so it did not dry out and no longer be usable. In the center of the slide, 10-20uL of Vectashield (depending on how many brains were dissected) was added and the samples were lined up, one by one in the center. Excess Vectashield was wiped away using a Kim wipe. Then, a slide cover was slowly and carefully lowered onto the sample and held down by painting the edges with a small amount of nail polish. The slides were then placed in a folder in the freezer for storage.

The final step of this process involves imaging the brains using fluorescent microscopy. Each slide was placed onto the slide mount and put into focus on each individual brain. The images were saved to a flash drive and placed in a file to study further. The slides were then put back into the folder and placed into the freezer. The images were analyzed according to glia cell density and the shape of the brain.

3. Results

The following images were taken using fluorescent microscopy. The green fluorescence indicates glia, as the GFP makes these cells glow green using this imaging technique. Not all images of samples are included, but rather, images that clearly exemplified the effects of the various drugs. In all cases an example of the most represented effect is presented.

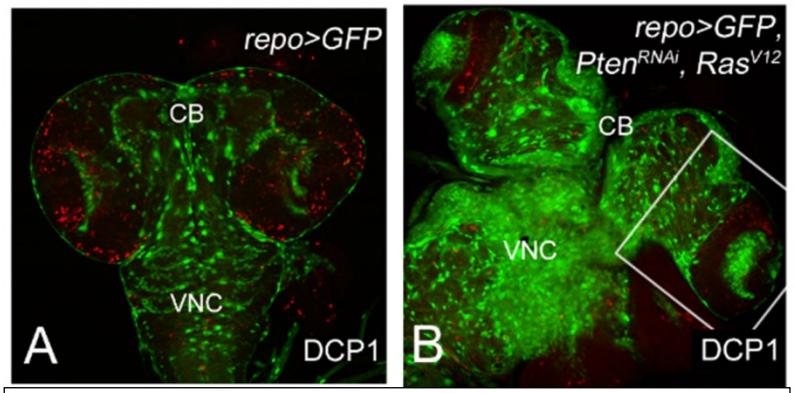


Fig. 10 Image A is a normal brain in which all glia are marked by repo GFP, with the optic lobes that connect together to form the Central Brain (CB) and the ventral nerve cord (VNC). Image B depicts a brain from the glioma cross. There is an increase in glia density and the lobes are deformed.

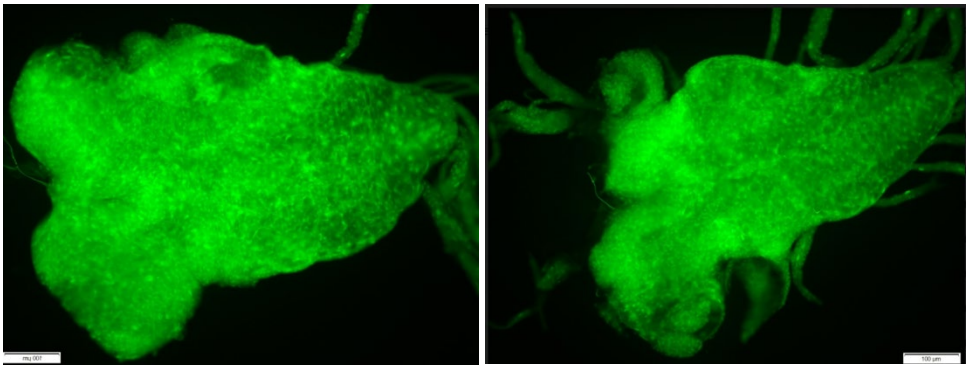


Fig. 11 Glioma cross day 6 treated with Saracatinib

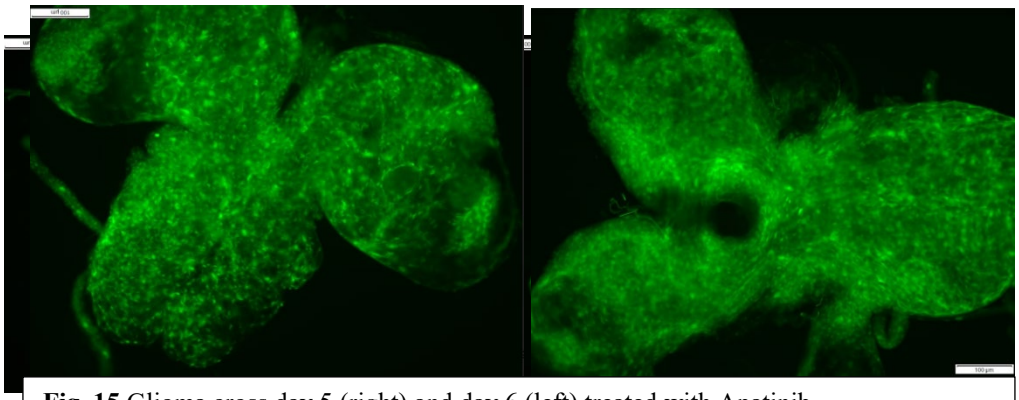


Fig. 15 Glioma cross day 5 (right) and day 6 (left) treated with Apatinib

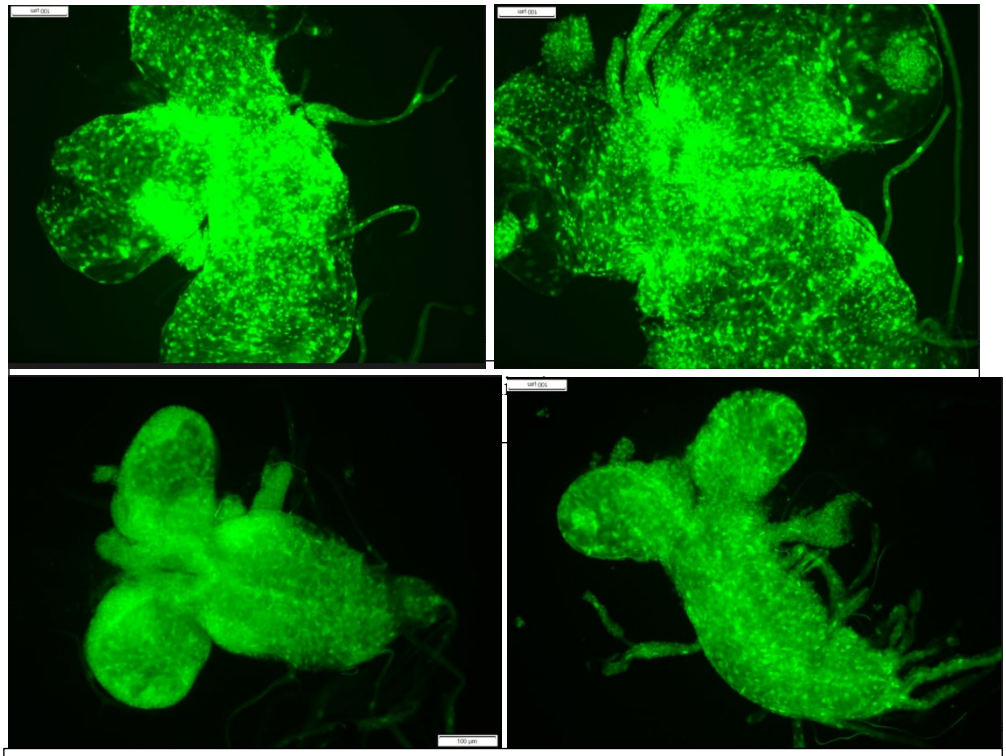


Fig. 14 Glioma cross day 6 (left) and day 5 (right) treated with Ibrutinib

4. Discussion

Overall, there was a range of effects seen in the images, indicating that the drugs had differing strengths of inhibition. The first set of images showing the *repo GFP* brain along with a glioma cross brain not treated with any drugs indicates the ways in which the tumor changes brain structure. The tumor mishaped the brain lobes and ventral nerve cord and also lead to an increase in glia density. This is because of the rapid division of cancer cells.

Some of the larvae that were fed the drugs did not survive, indicating that the drug given at that concentration was too lethal. Additionally, some larvae did not come up until day six or even the seventh day. This indicates that the drug may have slowed normal larvae growth and development. Further testing is needed to understand and quantify this finding.

The tumors treated with Saracatinib resulted in brains with decreased tumor sizes. These brains were also not deformed, but rather had wider ventral nerve cords as compared to the *repo GFP* brain. Additionally, there was still a high concentration of glia, specifically in the two lobes. Saracatinib shows promise in terms of reducing tumor size. This effect may be due to Saracatinib's ability to inhibit SRC as well as Fyn.

Tumors treated with Gefitinib had some of the most significant effects. These brains closely matched the shape of the unaffected brain, with clearly defined lobes and a ventral nerve cord. These structures did not seem to be overly enlarged or deformed due to tumor infiltration. However, there is still an increase in the glia density in both images. Since Gefitinib is able to bind to and inhibit EGFR, this drug could have played a role in slowing the division of cancerous glia or decreased cell proliferation.

Genistein showed promise as well, due to the decreased glia count in the outer portions of the brain lobes. The brains were also not as malformed as the untreated glioma brains. Genistein's ability to target MAPK and PI3K/Akt pathways may have caused this inhibition because these pathways are not well-regulated in glioma cells.

Ibrutinib also seemed to have a significant effect on the appearance of the brains, since they were near normal size and shape as compared to the unaffected brain image. Furthermore, the brain lobes and ventral nerve cord are clearly defined. Out of the the drugs studied, Ibrutinib seemed to have the strongest effect on glia cell count. Ibrutinib targets and inhibits Bruton's Tyrosine Kinase, which could be the reason behind the decreased tumor size and its lessened effects on the brain.

The brains treated with Apatinib were not as deformed as the untreated brains. However, the ventral nerve cord was not shaped like the control *repo GFP* brain. There was also a high density of glia throughout the brain, indicating that the cancer cells were dividing quicker than healthy glia. These effects may be due to the fact that Apatinib is a known VEGFR-2 inhibitor and the pathway associated with this receptor is dysregulated in GBM.

To summarize, these five drugs all had anti-cancer effects, as they shrunk the tumors and/or made the brain appear more like the *repoGFP* brain. However, the degree to which these drugs worked differed across the samples, indicating that some drugs were more effective than others. Further study and a deeper analysis of these drugs at varying concentrations is necessary in order to definitively conclude which ones may be useful in the treatment of human brain cancer.

5. Future Directions

First of all, the next step of this study involves testing the rest of the drugs. This will allow for more possible treatment options to study. Once all of the drugs have been tested, it is important to determine the EC50 of the ones found to be effective in decreasing tumor progression. This value will determine the best concentration to use the drug. Another possible next step includes using various drugs in combination with either each other or other drugs already used for the treatment of gliomas. This could more closely mimic treatment protocol for glioma patients, as chemotherapy regimens often entail a cocktail of powerful drugs. Down the road, the use of another model organism, such as mice may be useful to model tumors as well and test the anti-angiogenic effects of some of the drugs. This could also be a useful way to test a drug's ability to cross the blood-brain barrier.

The Role of p53 and E2F Mutations in Glioma Progression and Therapy Resistance

Gliomas are among the most deadly types of cancers, with the median survival after diagnosis being just 12-15 months. Although there are numerous types of gliomas that occur in adults and children, glioblastoma multiforme (GBM) is typically the most lethal. Patients diagnosed with this type of brain cancer often experience symptoms such as dizziness, headaches, nausea, seizures and a multitude of other neurological issues. Once diagnosed, the typical course of treatment includes a grueling regimen of surgery, chemotherapy and radiation therapy. The chances of a patient surviving even one year after diagnosis, in spite of these rigorous treatments is only 25%. The five year survival rates are even bleaker, as only 5% of patients will live past this point [31]. Even after a patient receives all of these therapies, the recurrence rate for GBM is extremely high. Oftentimes, the tumor recurs in the same location it originated in and is even more aggressive. When a patient relapses, there are even fewer therapeutic options, with most being experimental and eventually, the disease runs its course. Because of the devastating effects of this disease, it is critical to pursue research endeavors that will eventually benefit patients and improve survival odds.

Gliomas are solid primary brain tumors which arise from glia. Glia constitutes the connective tissue of the brain. These cells were originally thought to be the “glue” holding the brain together, although research has shown that glia are much more than just glue. Scientists are still working to grasp the roles of glia, which include modulating neurotransmission and even performing immune functions [33]. Like any other cancer, gliomas arise due to the rapid division of mutated cells and in this case, these cells are glia. The exact causes and mechanisms behind these mutations are unknown. Furthermore, the complete range of mutations found in gliomas are not fully understood.

However, the potential that certain mutations have to propel uncontrollable cell division is no secret. What were once a few unhealthy cells, can eventually form a large mass and kill a person within a matter of months.

The reason that these tumors are so deadly is the fact that they are often resistant to current therapy options. Therapy resistance refers to the ability of a cancer to no longer respond to treatments, either chemotherapy or radiation therapy, and continue to progress. Although the exact process of how cancer cells develop resistance is unknown, there are many studies which point to various mechanisms playing a role. Generally, there are changes happening at the genetic level in the cells as well as the tumor microenvironment. GBM cells are heterogeneous by nature, which points toward the cells having developed mechanisms to evade the body's way of destroying mutated cells. This heterogeneity results from the selection/adaptation processes happening in developing cancers [38].

Another contributor to therapy resistance in GBM is the different morphology of endothelial cells lining the blood vessels providing blood flow to the tumor. Interestingly, brain tumors have significantly higher blood vessel densities as compared to tumors in other parts of the body. Some types of brain tumors have blood vessel densities which are 50% higher than tumors in other locations. The mutated endothelial cells in GBM are able to migrate quicker and have a higher number of growth factors, which allow them to continue to support the growing tumor. The fact that these endothelial cells contain abnormal centromeres is believed to be a key player in preventing a proper response to therapy [37].

These same types of cells compose the blood brain barrier, which poses its own difficulty in delivering drugs to the brain. Although this mechanism works to protect the brain from infections and toxins, it also prevents the entry of many chemotherapeutic agents into the brain environment. New advances in nanotechnology aim to evade the blood brain barrier in order to deliver drugs to the brain, not only for the treatment of cancer, but many other neurological conditions [13]. The blood brain barrier is disturbed in GBM patients, which causes edema and increased pressure in the brain, which has the potential to damage remaining healthy tissue. Issues with the polarity of astrocytes are hypothesized to be reason that the blood brain barrier tends to be disturbed in cases of GBM [37].

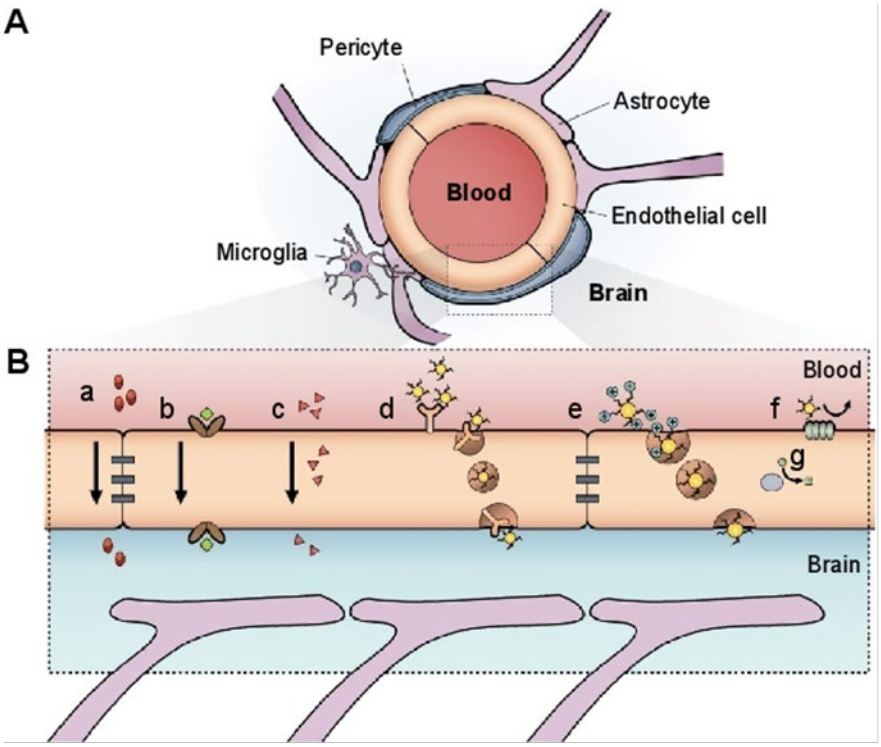


Fig 16 The blood brain barrier protects the brain, but can prevent the entry of chemotherapy into the brain.
Image retrieved from: Velasco, Carolina & Morales Zavala, Francisco & Gallardo-Toledo, Eduardo & Guerrero, Simon & Giralt, Ernest & Araya, Eyleen & Kogan, Marcelo. (2015). Peptides, and proteins used to enhance gold nanoparticle delivery to the brain: Preclinical approaches. International journal of nanomedicine. 10. 4919-36. 10.2147/IJN.S82310.

One major reason that these tumors are often resistant to therapies lies within the cancer cells themselves. There are a plethora of oncogenic mutations causing these cells to proliferate and divide uncontrollably. There are many known mutations, such as EGFR and IDH known to play a role in therapy resistance. EGFR, which has amplified activity in 40-60% of GBM cases, may respond positively to chemotherapies that target this mutation initially. However, due to unknown mechanisms, new mutations arise which make the cancer cells resistant to this therapy, causing the tumor to grow yet again. Patients with IDH mutations tend to fare slightly better than those with IDH wildtype. Although there are multiple types of IDH mutations, they eventually result in an increase in onco-metabolite d-2-hydroxy-glutarate, which in turn leads to oxidative stress and

eventually the damage of once healthy DNA [17]. Wildtype IDH is a marker for resistance to chemotherapy, as IDH mutants have shown reduced proliferation as compared to the wildtype [35]. In terms of radiation therapy, one mutation that contributes to this is ASAH1, which becomes upregulated after being exposed to radiation treatments, aiding in the proliferation of cancerous cells [17].

This project hinges on the fact that there are numerous mutations within GBM cells that make the cancer both difficult to treat and resistant to current therapies. This endeavor involved testing the effects of a *p53* mutation and an *E2F* mutation on glioma progression. Certain *p53* gain of function mutations result in a decreased response to Temozolamide (common GBM drug) and therefore, may be a marker for therapy resistance [20].

The reason for incorporating an *E2F* mutation into this experiment is because *E2F* operates in the same pathway as *p53* and helps to regulate its activity. The way in which this experiment addresses the issue of therapy resistance is by testing two mutations, *p53* and *E2F*, as possible contributors to the aggressive nature of the tumor. Subsequently, running models with these mutations through a slew of different chemicals may identify possible drugs which target them. The overarching goal of this project is to gain a better understanding of the cellular and biochemical defects occurring in these tumors in an effort to eventually improve outcomes for patients. Once targets are identified, treating a patient with drugs that combat certain mutations can more effectively treat their cancer.

When functioning normally, the TP53 gene (on chromosome 17) acts as a tumor suppressor and is nicknamed the "guardian of the genome." TP53 works by coding for *p53* proteins which bind to DNA, targeting the CDKN1A gene (on chromosome 6) to make the protein *p21*. This protein complexes with cdk2, which is a protein that stimulates cell division. Once this complex forms, the cell does not move forward in mitosis, essentially halting cell division [15]. This includes stopping division of mutated cells which may be cancerous. An issue with this pathway can lead to a disastrous hallmark of cancer: uncontrollable division of mutated cells.

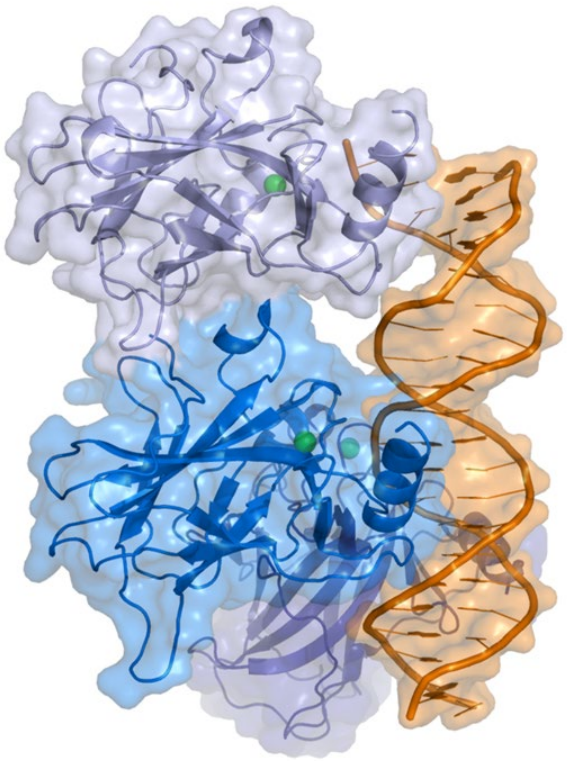


Fig. 17 *p53* protein bound to DNA.
Image retrieved from: Splettstoesser, T. (2006). Cartoon representation of a complex between DNA and the protein *p53*

A mutation within *p53* can have serious consequences for a patient, because it disrupts this important cellular process. A *p53* gene mutation leads to the translation of

mutated *p53* proteins. Not only do these proteins improperly bind to DNA and fail to control cell division, but they also develop oncogenic properties. High levels of these proteins are often found in cancer cells of various types. When functioning as an oncogene, *p53* helps sustain the survival of mutated cells. Therefore, mutations which inactivate *p53* contribute to tumorigenesis and eventually metastasis in many types of cancers. Interestingly, mutant *p53* can inactivate wildtype *p53*, although not always completely. The dominant negative mechanism of mutant *p53* renders the healthy allele unable to perform its usual functions [25]. The exact cause of these mutations in GBM patients remains unknown.

Evidence for the importance of *p53* is seen in people without two working copies of this gene. Individuals with Li Fraumeni syndrome have only one functional copy of *p53* inherited from one parent. This causes a predisposition to cancer because, if the only copy of *p53* is damaged, the affected cells lose their ability to properly regulate cell division. This is why nearly half of all people with Li Fraumeni syndrome will develop cancer before the age of 30 and 80% will be diagnosed before the age of 60. One organ in which people with Li Fraumeni syndrome commonly develop cancer is the brain, further implicating the way *p53* mutations cause progression of brain tumors [30].

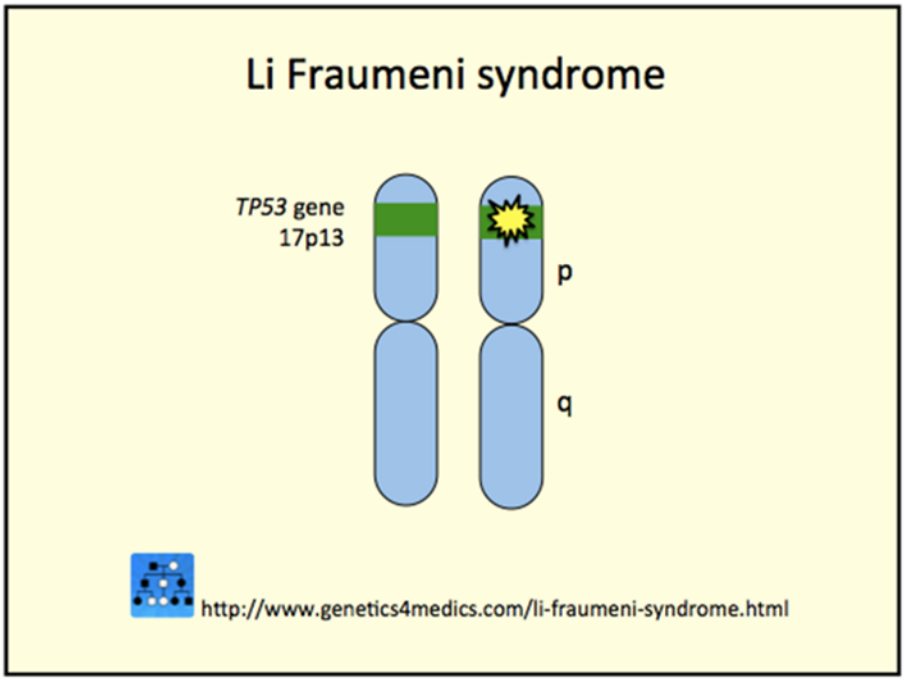


Fig. TP53 is located on the 17th chromosome and only one functional copy is inherited in Li Fraumeni syndrome.
Image retrieved from: Li-Fraumeni Syndrome: Inherited Cancer Disorder. *Emaze*. https://app.emaze.com/@ATRQWCWQ#1

Deregulation of the *p53* pathway is seen in 84% of GBM patients, indicating the vast impact of *p53* mutations in the development and progression of GBM. Evidence of *p53* mutations in astrocytomas and low-grade gliomas suggest that these mutations are early events in tumorigenesis. In addition to damaging a major apoptotic pathway, *p53* point mutations promote the gain of function of oncogenic variations of *p53* proteins. This is what promotes malignancy in gliomas, since they begin to work as transcription factors [39]. *p53* mutations are thought to be the reason behind certain gliomas' resistance to Temozolamide. This is because inactivation of *p53* promotes the activity of the DNA repair enzyme, MGMT [8]. Furthermore, *p53* promotes inflammation in gliomas, making patients' prognoses even more dismal [9].Because of these events, it is

necessary to identify treatments that can either destroy mutated *p53* or help restore its original tumor suppressor abilities.

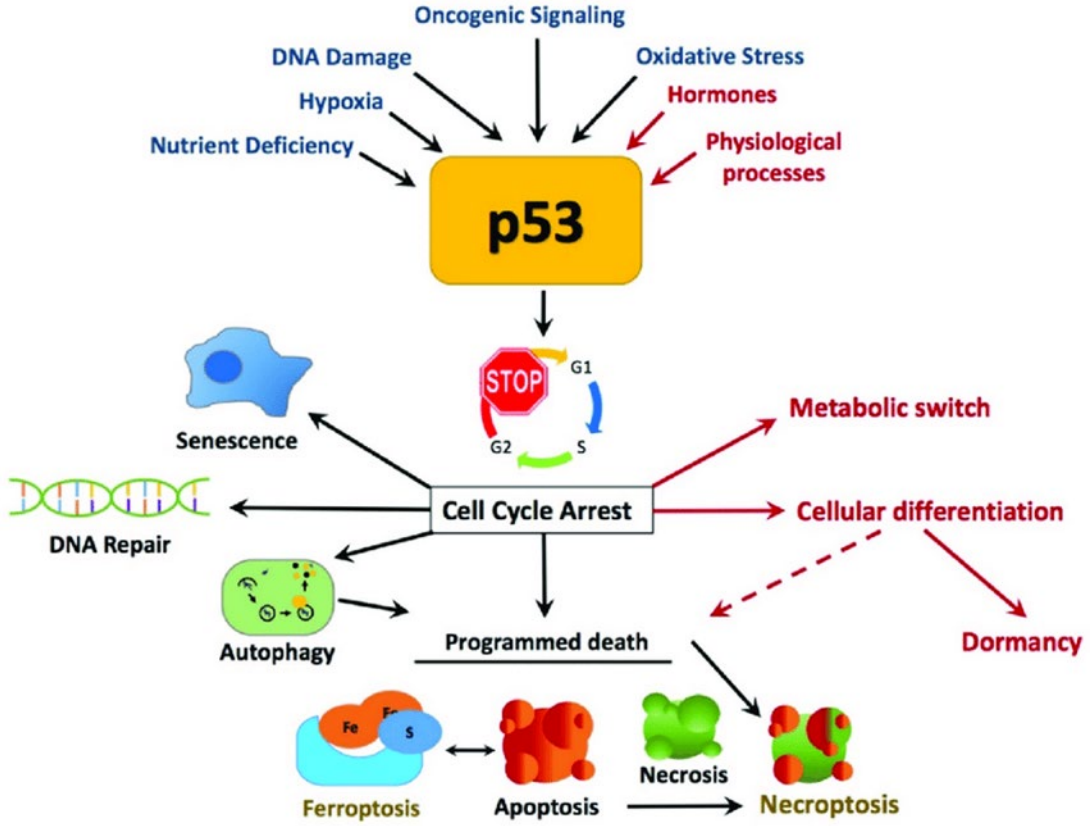


Fig. 17 *p53* regulates numerous cellular functions, which can become disturbed when *p53* is mutated.
Image retrieved from: Moulder, David & Hatoum, Diana & Tay, Enoch & Lin, Yiguang & Mcgowan, Eileen. (2018). The Roles of p53 in Mitochondrial Dynamics and Cancer Metabolism: The Pendulum between Survival and Death in Breast Cancer?. Cancers. 10. 189. 10.3390/cancers10060189.

It is important to recognize that *p53* does not operate independently, but rather is mediated by *E2F*. One author described the relationship between *p53* and *E2F* as "partners in life and death" [22]. The Rb–E2f and MDM2–p53 pathways are deregulated in a majority of tumors and operate independently. However, when *E2F* and *p53* function as transcription factors, they facilitate crosstalk between these two pathways [22]. When E2F1 is overexpressed, it activates *p53* in response, which in turn begins the signaling

cascade which leads to apoptosis [11]. Hence, *E2F* acts as a tumor suppressor and is an important factor to consider when studying *p53*.

E2F has been shown to induce apoptosis in *p53*-dependent and independent manners. Although the exact mechanisms for *p53*-dependent apoptosis are not fully understood, there are several theories. One such theory suggests that by targeting the p14(p19)/Arf tumor suppressor gene, *E2F* induces stability of *p53*. Further studies have shown that *E2F* still affects *p53* in the absence of Arf, suggesting there are other routes in which *E2F* functions. Another theory proposes that, in response to DNA damage, the cyclin A-binding domain present in *E2F* interacts with *p53.* This interaction stabilizes *p53*. There are also two hypotheses for the way in which *E2F* triggers apoptosis independent of activity with *p53*. *E2F* is thought to accomplish this by either interacting with *p73* (a transcription factor, part of the *p53* family) or by working with the tumor necrosis factor [5,26].

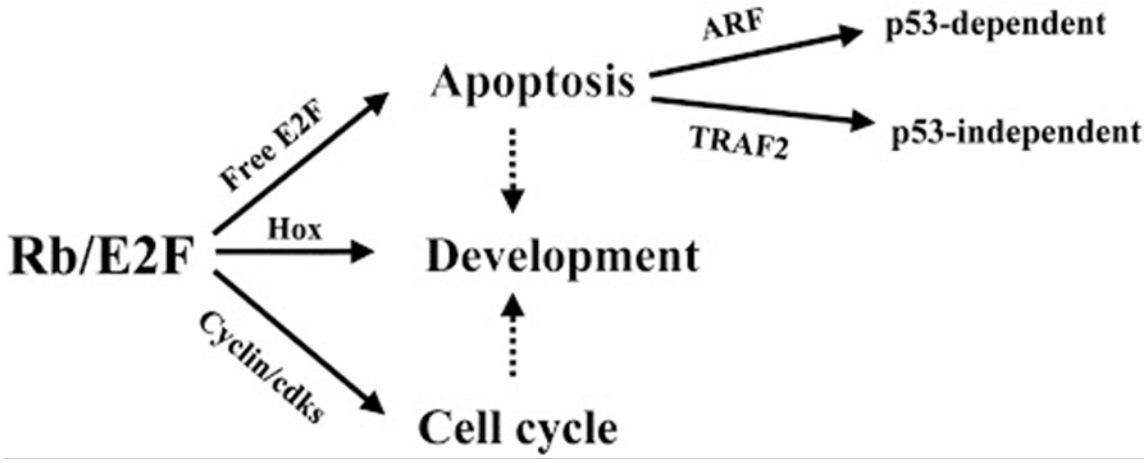


Fig. 18 The Rb/E2F pathway can regulate apoptosis, development and the cell cycle. It also regulates p53-dependent and p53-independent apoptosis.
Image retrieved from: The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes & Dev*. 2000. 14: 2393-2409. http://genesdev.cshlp.org/content/14/19/2393/F5.expansion.html

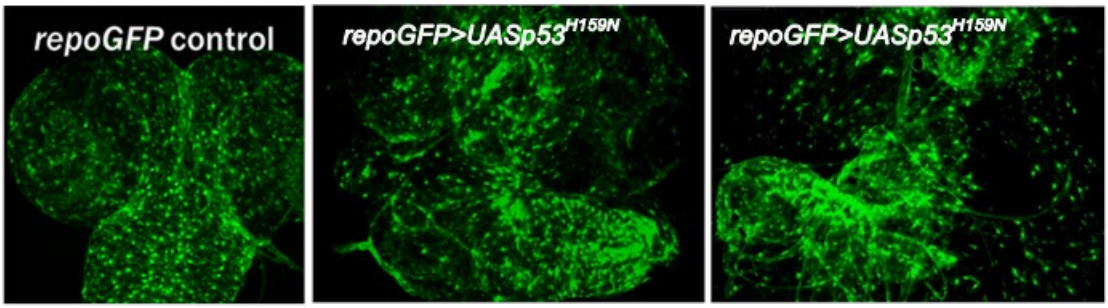
The transcription factors associated with *E2F* work to regulate genes important for various stages of the cell cycle, specifically, the G1 and S phases. This acts as a way to control cell division. When *E2F* genes are improperly expressed, cells continue to enter the S phase, unchecked and then begin to divide [10]. *E2F* has dueling abilities to both activate and repress transcription, so when mutated, it can function as an oncogene. When not mutated, E2F1 plays a role in responding to damaged DNA, as heightened levels of this protein have been detected in cells that had been given chemicals known to cause DNA damage [5]. Although the direct cause of mutations in the *E2F* family of genes in GBM is not known, *E2F* is regulated by the pRB family of proteins [6]. Therefore, dysregulation of this pathway may be partially to blame for the oncogenic effects of *E2F*.

Due to the well-established relationship between *E2F* and *p53* and their known role as oncogenes, this project seeks to understand how mutations in these genes contribute to gliomas. The plan for this project was to induce both of these mutations into *Drosophila* in addition to the tumor-driving $Pten^{RNAi}$; Ras^{V12} mutation used in the drug screen. In order to induce a *p53* mutation, a dominant negative approach was put into place. The *E2F* mutation was to be added by using an RNA interference approach to knock-down dE2F1. By creating a triple mutant, expressing these specific mutations, we would have a more complete model of human brain tumors. Testing how these tumors grow and progress would give insight into the way these mutations affect this process. Testing these mutants with various Tyrosine Kinase inhibitors would assess them for therapy resistance and hopefully identify agents which target these mutations.

Although the clear goal was established and a detailed plan of how to achieve it was devised, it was not fully realized. Due to contaminated fly food, many stocks died. At one point the project was restarted because the stocks did not survive. Once the project got back on its feet and started moving smoothly, the COVID-19 shutdown stopped all undergraduate research for the spring 2020 and fall semester. Therefore we have preliminary data to present, but no information regarding the tumor progression data and response to various drugs.

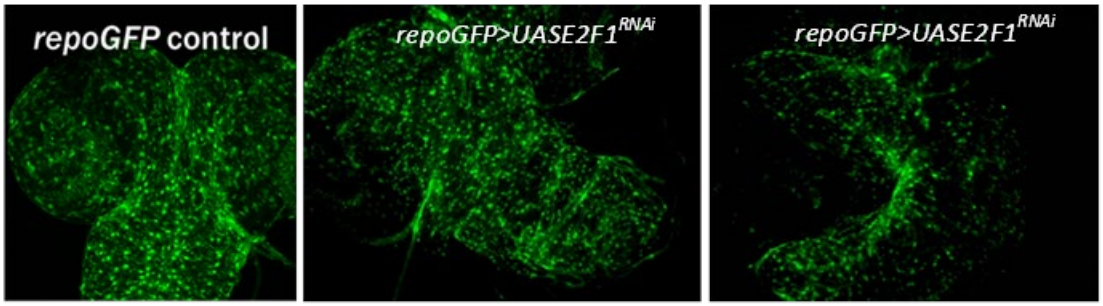
Results:

Fig.19



Results from *repoGFP>UAS-p53^{H159N}*: As compared to the *repoGFP* control, these mutants had a lower glia count, especially in the optic lobes. Additionally, these brains were deformed as the ventral nerve cord curved upward, instead of lying flat and straight in line with the lobes as seen in the control.

Fig. 20



Results from *repoGFP>UAS-E2f1RNAi:* Compared to the repoGFP brains, this cross had decreased glia in the optic lobes. The loss of E2F also resulted in deformed brains. In some cases, the ventral nerve cord curved upward and was attached to the optic lobes.

Even though this project remains unfinished, preliminary data points to this approach serving as a viable method for creating the triple mutants. The balancer stocks were healthy and viable. We are hopeful that in the future, other undergraduate lab members will be interested in understanding the role of *p53* and *E2F* in gliomas and pick up where we left off.

Citations

1. Aalipour, A., & Advani, R. H. (2014). Bruton's tyrosine kinase inhibitors and their clinical potential in the treatment of B-cell malignancies: focus on ibrutinib. *Therapeutic advances in hematology*, 5(4), 121–133. https://doi.org/10.1177/2040620714539906

2. Ahluwalia, M. S., de Groot, J., Liu, W. M., & Gladson, C. L. (2010). Targeting SRC inglioblastoma tumors and brain metastases: rationale and preclinical studies. *Cancer letters*, 298(2), 139–149. https://doi.org/10.1016/j.canlet.2010.08.014

3. Alhasan, S. A., Aranha, O., & Sarkar, F. H. (2001). Genistein elicits pleiotropic molecular effects on head and neck cancer cells. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 7(12), 4174-4181.

4. Arif, S. H., Pandith, A. A., Tabasum, R., Ramzan, A. U., Singh, S., Siddiqi, M. A., & Bhat, A. R. (2018). Significant Effect of Anti-tyrosine Kinase Inhibitor (Gefitinib) on Overall Survival of the Glioblastoma Multiforme Patients in the Backdrop of Mutational Status of Epidermal Growth Factor Receptor and PTEN Genes. *Asian journal of neurosurgery*, 13(1), 46–52. https://doi.org/10.4103/ajns.AJNS_95_17

5. Dimova, D., Dyson, N. The E2F transcriptional network: old acquaintances with new faces. *Oncogene* 24, 2810–2826 (2005). https://doi.org/10.1038/sj.onc.1208612

6. Dyson, N. The regulation of E2F by pRB-family proteins. *Genes & Dev*. 12, 2245-2262 (1998). http://genesdev.cshlp.org/content/12/15/2245.full.html

7. Elevar Therapeutics. (2020). *Rivoceranib Pipeline.* Retrieved from Apatinib: https://elevartherapeutics.com/rivoceranib-apatinib/

8. Forte, I.M., Indovina, P., Iannuzzi, C.A., Cirillo, D., Di Marzo, D., Barone, D. Giordano, A. (2019). Targeted therapy based on p53 reactivation reduces both glioblastoma cell growth and resistance to temozolomide. *International Journal of Oncology*, 54, 2189-2199. https://doi.org/10.3892/ijo.2019.4788

9. Ham, S.W., Jeon, HY., Jin, X. et al. TP53 gain-of-function mutation promotes inflammation in glioblastoma. *Cell Death Differ* 26, 409–425 (2019). https://doi.org/10.1038/s41418-018-0126-3

10. Johnson, D. G., & Schneider-Broussard, R. (1998). Role of E2F in cell cycle control and cancer. *Frontiers in bioscience : a journal and virtual library*, 3, d447–d448. https://doi.org/10.2741/a291

11. Kenta Kurayoshi, Eiko Ozono, Ritsuko Iwanaga, Andrew P. Bradford, Hideyuki Komori, Keigo Araki and Kiyoshi Ohtani (February 28th 2018). The Key Role of E2F in Tumor Suppression through Specific Regulation of Tumor Suppressor Genes in Response to Oncogenic Changes, Gene Expression and Regulation in Mammalian Cells - Transcription Toward the Establishment of Novel Therapeutics, Fumiaki Uchiumi, *IntechOpen*, https://www.intechopen.com/books/gene-expression-and-regulation-in

mammalian-cells-transcription-toward-the-establishment-of-novel therapeutics/the-key-role-of-e2f-in-tumor-suppression-through-specific regulation-of-tumor-suppressor-genes-in-re

12. Khaw, A. K., Yong, J. W., Kalthur, G., & Hande, M. P. (2012). Genistein induces growth arrest and suppresses telomerase activity in brain tumor cells. *Genes, chromosomes & cancer*, 51(10), 961–974. https://doi.org/10.1002/gcc.21979

13. Kon, K. (2020, November 12). *Glioblastoma Nanomedicine Breaks through Blood Brain Barrier in Mice*. Retrieved from Genetic Engineering and Biotech News: https://www.genengnews.com/news/glioblastoma nanomedicine-breaks-throughblood-brain-barrier-in-mice/

14. Lymphoma Action. (2020). *Ibrutinib*. Retrieved from Lymphoma Action: https://lymphoma-action.org.uk/about-lymphoma-treatment-lymphoma targeted drugs/ibrutinib

15. National Center for Biotechnology Information (US). Genes and Disease [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 1998-. *The p53 tumor suppressor protein*. Available from:https://www.ncbi.nlm.nih.gov/books/NBK22268/

16. National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 10302451, *Saracatinib.* Retrieved April 14, 2021 from https://pubchem.ncbi.nlm.nih.gov/compound/Saracatinib.

17. Nguyen, H. S., Shabani, S., Awad, A. J., Kaushal, M., & Doan, N. (2018). Molecular Markers of Therapy-Resistant Glioblastoma and Potential Strategy to Combat Resistance. *International journal of molecular sciences,* 19(6), 1765. https://doi.org/10.3390/ijms19061765

18. NIH. (2020). *Gefitinib*. Retrieved from National Cancer Institute: https://www.cancer.gov/publications/dictionaries/cancer terms/def/gefitinib

19. NIH. (2021, March 3). *Ibrutinib With Radiation and Temozolomide in Patients With Newly Diagnosed Glioblastoma.* Retrieved from Clinical Trials.gov: https://clinicaltrials.gov/ct2/show/NCT03535350

20. Oliver L, Lalier L, Salaud C, Heymann D, Cartron PF, Vallette FM. Drug resistance in glioblastoma: are persisters the key to therapy?. *Cancer Drug Resist* 2020, 3, 287-301. http://dx.doi.org/10.20517/cdr.2020.29

21. Paul, M. K., & Mukhopadhyay, A. K. (2004). Tyrosine kinase - Role and significance in Cancer. *International journal of medical sciences*, 1(2), 101–115. https://doi.org/10.7150/ijms.1.101

22. Polager, S., Ginsberg, D. p53 and E2f: partners in life and death. *Nat Rev Cancer* 9, 738 748 (2009). https://doi.org/10.1038/nrc2718

23. Reardon, D. A., Turner, S., Peters, K. B., Desjardins, A., Gururangan, S., Sampson, J. H., McLendon, R. E., Herndon, J. E., 2nd, Jones, L. W., Kirkpatrick, J. P., Friedman, A. H., Vredenburgh, J. J., Bigner, D. D., &

Friedman, H. S. (2011). A review of VEGF/VEGFR-targeted therapeutics for recurrent glioblastoma. *Journal of the National Comprehensive Cancer Network: JNCCN*, 9(4), 414–427. https://doi.org/10.6004/jnccn.2011.0038

24. Rich, J. et al. (2004). Phase II Trial of Gefitinib in Recurrent Glioblastoma. *Journal of Clinical Oncology*, 22(1), 133-142, https://ascopubs.org/doi/abs/10.1200/JCO.2004.08.110

25. Rivlin, N., Brosh, R., Oren, M., & Rotter, V. (2011). Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes & cancer*, 2(4), 466–474. https://doi.org/10.1177/1947601911408889

26. Rufini, A., Agostini, M., Grespi, F., Tomasini, R., Sayan, B. S., Niklison-Chirou, M. V., Conforti, F., Velletri, T., Mastino, A., Mak, T. W., Melino, G., & Knight, R. A. (2011). p73 in Cancer. *Genes & cancer*, 2(4), 491–502. https://doi.org/10.1177/1947601911408890

27. Shi, Y., Guryanova, O. A., Zhou, W., Liu, C., Huang, Z., Fang, X., Wang, X., Chen, C., Wu, Q., He, Z., Wang, W., Zhang, W., Jiang, T., Liu, Q., Chen, Y., Wang, W., Wu, J., Kim, L., Gimple, R. C., Feng, H., Bao, S. (2018). Ibrutinib inactivates BMX-STAT3 in glioma stem cells to impair malignant growth and radioresistance. *Science translational medicine*, 10(443), eaah6816. https://doi.org/10.1126/scitranslmed.aah6816

28. Sigismund, S., Avanzato, D., & Lanzetti, L. (2018). Emerging functions of the EGFR in cancer. *Molecular oncology*, 12(1), 3–20. https://doi.org/10.1002/1878 0261.12155

29. Spagnuolo, C. et al. Genistein and Cancer: Current Status, Challenges, and Future Directions, *Advances in Nutrition,* Volume 6, Issue 4, July 2015, 408–419, https://doi.org/10.3945/an.114.008052

30. St. Jude Children's Research Hospital. (2020, June). *Li-Fraumeni Syndrome*. Retrieved from Cancer Predisposition Clinic: https://www.stjude.org/disease/lifraumeni.html

31. The Brain Tumor Charity. (2021, June). Glioblastoma Fact Sheet. Retrieved from The Brain Tumor Charity: https://www.thebraintumourcharity.org/brain tumourdiagnosis-treatment/types-of-brain-tumour adult/glioblastoma/glioblastomaprognosis/#:~:text=Glioblastoma%20survival,survive%20more%20than%20fiv%20years

32. U.S. National Library of Medicine. (2017, December 1). EGFR Gene.Retrieved from Medline Plus: https://medlineplus.gov/genetics/gene/egfr/

33. Vallejo, R., et al. (2010). The role of glia and the immune system in the development and maintenance of neuropathic pain. Pain practice : the official journal of World Institute of Pain, 10(3), 167–184. https://doi.org/10.1111/j.15332500.2010.00367.x

34. Vlassov, V. (2019, August 26). Saracatinib. Retrieved from Alzheimer's News Today:https://alzheimersnewstoday.com/saracatinib/

35. Wang, J. B., Dong, D. F., Wang, M. D., & Gao, K. (2014). IDH1 overexpression induced chemotherapy resistance and IDH1 mutation enhanced chemotherapy sensitivity in Glioma cells in vitro and in vivo. Asian Pacific journal of cancer prevention:APJCP, 15(1), 427–432.https://doi.org/10.7314/apjcp.2014.15.1.427

36. Wang, Y., Meng, X., Zhou, S., Zhu, Y., Xu, J., & Tao, R. (2019). Apatinib Plus Temozolomide for Recurrent Glioblastoma: An Uncontrolled, Open-Label Study. OncoTargets and therapy, 12, 10579–10585.https://doi.org/10.2147/OTT.S226804

37. Wolburg, H., Noell, S., Fallier-Becker, P., Mack, A. F., & Wolburg-Buchholz, K. (2012).The disturbed blood-brain barrier in human glioblastoma. Molecular aspects of medicine, 33(5-6), 579–589. https://doi.org/10.1016/j.mam.2012.02.003

38. Zhang, X. et al. (2019). Chemoresistance caused by the microenvironment of glioblastoma and the corresponding solutions. Biomedicine and Pharmacotherapy, 109, 39-46. https://doi.org/10.1016/j.biopha.2018.10.063

39. Zhang, Y., Dube, C., Gibert, M., Jr, Cruickshanks, N., Wang, B., Coughlan, M., Yang, Y., Setiady, I., Deveau, C., Saoud, K., Grello, C., Oxford, M.,

Yuan, F., & Abounader, R. (2018). The p53 Pathway in Glioblastoma. Cancers, 10(9), 297.https://doi.org/10.3390/cancers10090297