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Discovering Glioma Inhibitors via Chemical-Genetic Screens in Drosophila Cancer Models

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Abstract

Today, there are over 250 drugs being used to cure over 100 different types of cancer. We hypothesize that one of the drugs already being used in cancer treatment will either show positive or negative growth when applied specifically to glioblastoma, a type of brain tumor with poor prognosis. Using the fruit fly, *Drosophila melanogaster*, and 150 tyrosine kinase inhibitors, we can induce tumors and track their growth in response to the drugs. This would open the doors to researching similarities between drugs present in hundreds of chemical libraries. Additionally, the drugs that prove successful through the screenings could start being used in mammalian and clinical trials in the future.

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Chapter 1

History of *Drosophila*

Drosophila has been proven to be a useful model organism in the scientific field since it was first used in 1901 by Thomas Hunt Morgan (Jennings, 2011). Jennings states in her research that Morgan, along with many other scientists, found *Drosophila* more beneficial than vertebrae models for a number of reasons, including they are easy and inexpensive to culture in laboratory conditions, have a much shorter life cycle of about 12 days, represented in Figure 1, and they produce large numbers of externally laid embryos that can be genetically modified. Morgan used the defining of genes and ability to establish that they were on the chromosomes to further define the theory of inheritance that was proposed by Gregor Mendel (Jennings, 2011). It was this redefining that led Morgan to receive The Nobel Prize of Physiology or Medicine in 1933 for "*his discoveries concerning the role played by the chromosome in heredity*" ("Nobel Prize and Literature", 2018). This Nobel Peace Prize opened the doors to different research that could be conducted using *Drosophila*, and it was this continuation of research that went on to win many other awards of their own. This is demonstrated through the findings of Jeffery C. Hall, Michael Rosbash and Michael W. Young, who in the 1980s discovered the molecular mechanism that controls circadian rhythms using this model organism (Huang, 2018). Areas of their research concerned the rapid reproduction of recombinant DNA, which allowed them to both characterize and clone the *Drosophila* clock gene named *period* independently. It is this rich legacy of past research that allows this model organism to be used for comparisons to continually be made between different biological diseases as well as other physiological differences.

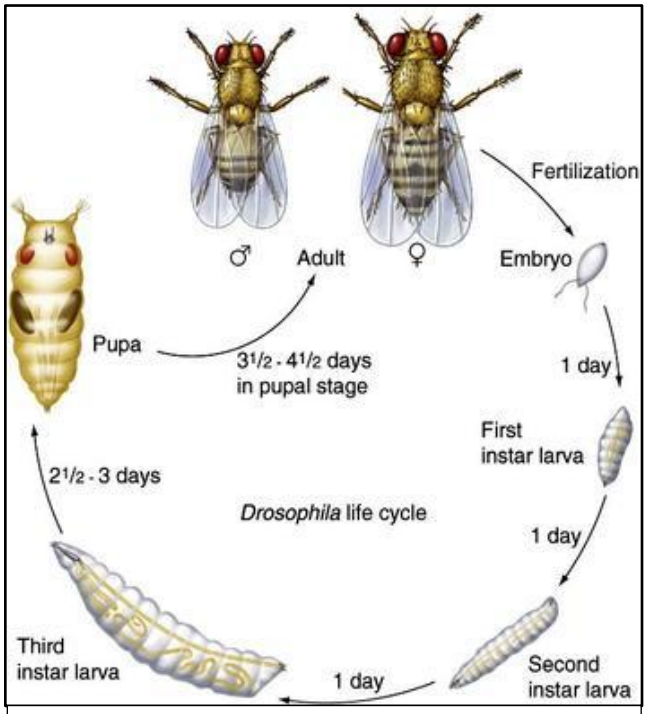


Fig. 1. Lifecycle of *Drosophila Melanogaster*: For both male and female flies, they go through 5 different growth stages, starting with the embryo and ending with pupa. Image from: Creative Diagnostics, 2009-2018.

Comparisons Between *Drosophila* and Humans

Besides the ability to easily induce desired genetic pathways, *Drosophila* also makes a good model organism due to it sharing 70% of disease genes with humans (Read et al. 2009). *Drosophila* has approximately 13,600 genes in its genome, which was sequenced for the first time in 2000 (Mark et al. 2000). Of these 13,600 genes, there was a separate study completed that looked at the 929 human disease genes that were associated with at least one mutant allele discovered in *Drosophila's* own genome (Reiter et al. 2001). The OMIM, or Online Mendelian Inheritance in Man, was used as the human comparison group. In Reiter et. al. research they found that of the 929 OMIM genes, 714 contain highly similar ($E \leq 10^{-10}$) cognates in *Drosophila* (77%). The researchers created a database, appropriately named Homophila, to build the connection between *Drosophila* and humans, allowing for the variety of many successful experiments to be conducted in the future. A subset of shared genes are illustrated in Figure 2, which represent the different cancer types that can be a modelled using *Drosophila*.

In the past, other research has been conducted that focused on the genetic comparisons between *Drosophila* and human disease (Fortini et al. 2000; Rubin et al. 2000). Although these studies all proved successful, they were a more restrictive study than that performed by Reiter et. al., only looking at a subset of 289 linked genes. Fortini and Rubin's research does not take into account the remaining 425 genes (when compared to the linked 714 in Reiter's study), nor do they examine the vast possible genetic effects of the found 289 genes. Fortini and Rubin in their studies further solidified the genetic connection between *Drosophila* and humans, but a much wider gene pool was necessary for the most accurate data.

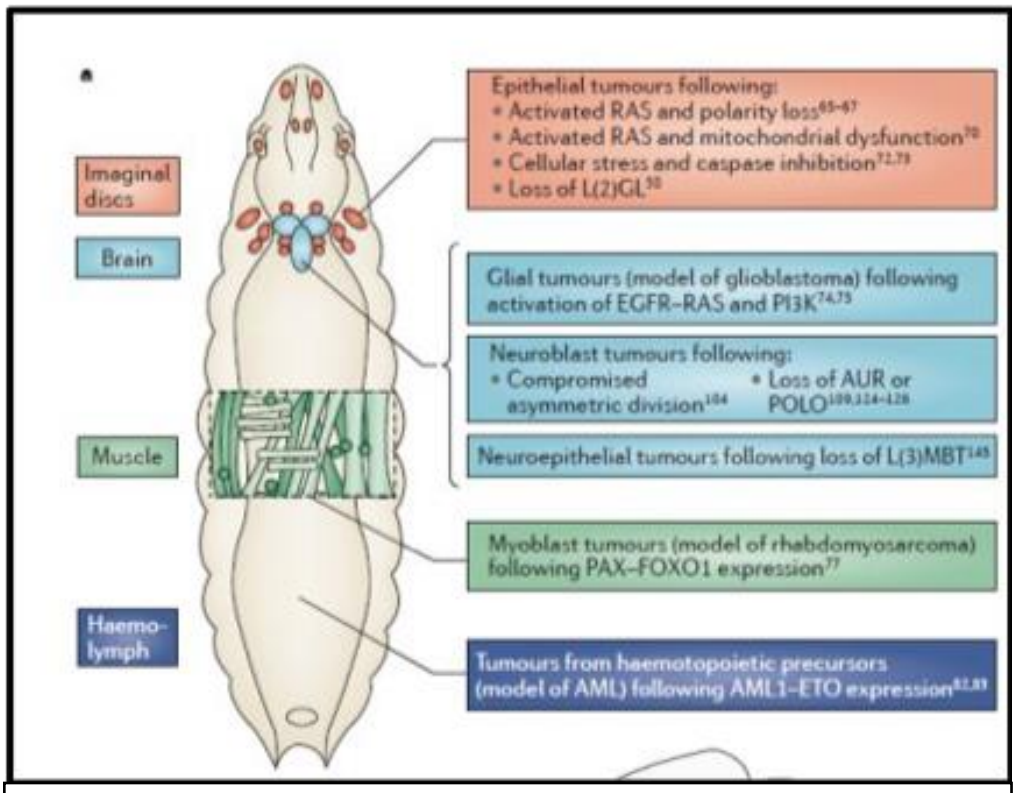


Figure 2. Illustration of tumor expression in *Drosophila*. Tumors ranging from Imaginal Disks, Brain, Muscle, and Haemo-lymph tumors can be expressed. These can be further divided into sub-tumors as listed on the right along with the type of pathways used for each. Showcases how *Drosophila* is a top-tier model organism for expressing the wanted tumors. Figure from Villegas, 2017.

Inducing Glioma in *Drosophila*

The model of glioma that can be replicated repeatedly in *Drosophila* is useful in studying the possible causes, as well as changes that can act as inhibitors to the tumor growth. The fly brain is composed of two dorsal hemispheres and the ventral nerve cord, that forms the central nervous system of the fly. The CNS is mainly comprised of neuron and glia that make up ~10% of cells in the fly CNS (Witte et al., 2011). This substantial percentage gives good insight into what makes glioblastoma so malignant, given the fact it is a glial driven tumor. Other areas that Witte, Jeibmann, etc. cover in their research are the pathways that can be induced in *Drosophila* to effectively produce glioma. They found promising molecular targets for therapeutic intervention included the tyrosine kinase receptors epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR) and their downstream signaling cascades, the phosphatidylinositol 3-phosphate kinase (PI3K)/AKT and Ras/mitogen-activated protein kinase pathway (Witte et al. 2009). Additionally, it was found that using the GAL4-UAS system proved the most effective when testing possible pathways to inhibit glioma via different measures, and this is shown in Figure 3. Although this research does exhibit the GAL4-UAS system as an effective pathway, it does not fully explain what aspects of this pathway led them to choosing it for their research.

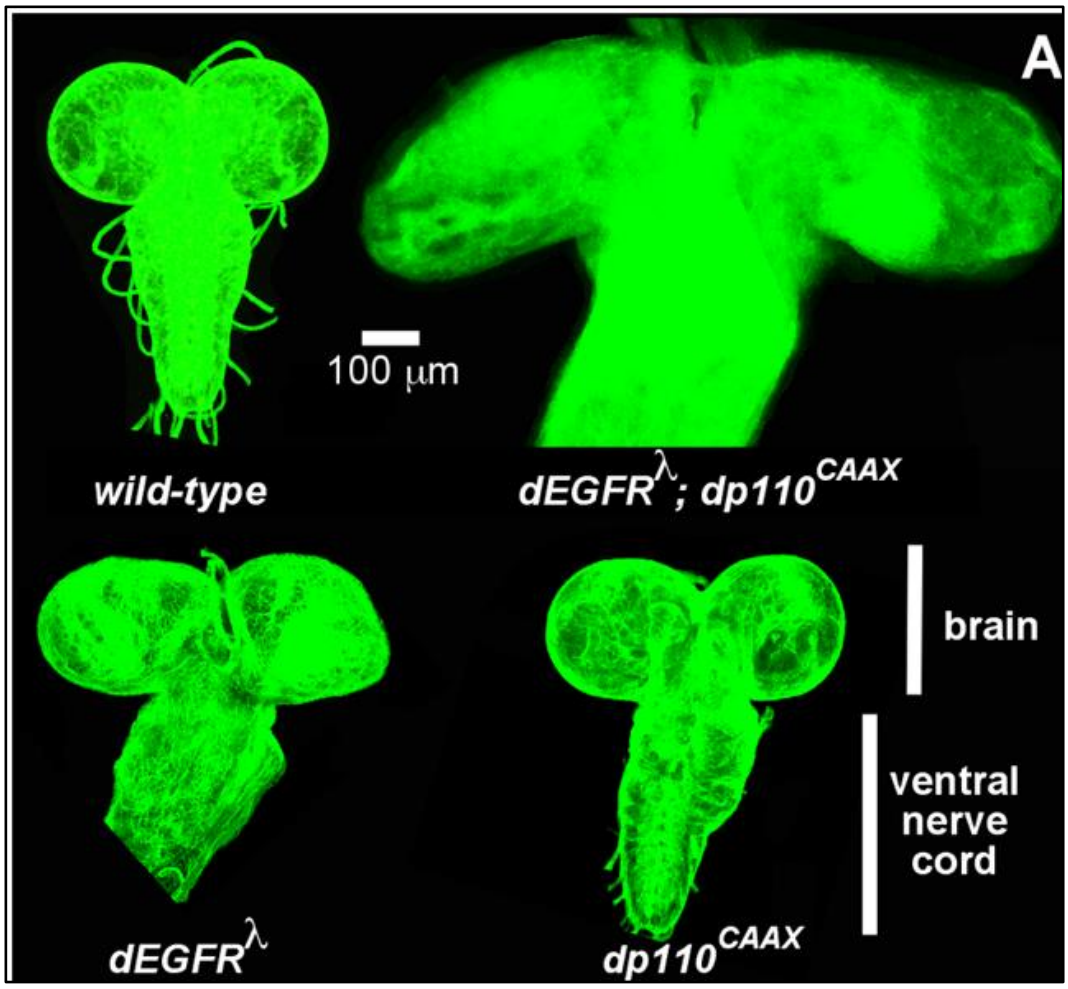


Figure 3. Optical projections of whole brain-ventral nerve cord complexes from late 3rd instar larvae approximately 130 hr AED, displayed at the same scale. Dorsal view; anterior up. Each brain is composed of 2 symmetrical hemispheres attached to the ventral nerve cord (VNC). In *repo>dEGFR$^{\lambda}$;dp110^{CAAX}* larvae, both brain hemispheres and the VNC are enlarged and elongated relative to other genotypes. Figure by Read et al., 2009.

The P13K Ras Pathway

The three core signaling pathways that are commonly activated in glioma patients are the tumor protein p53 [p53] pathway, the receptor tyrosine kinase/Ras/phosphoinositide 3-kinase signaling pathway, and the retinoblastoma pathway (Davis, 2016). Despite these separate pathways, the EGFR-Ras and PI3K pathway continues to be one of the most effective models, as shown in Figure 4. The mutation or amplification of the Epidermal Growth Factor Receptor (EGFR) tyrosine kinase continually shows the most frequent genetic damage in gliomas, as stated in Read et al. research concerning this specific pathway. The network EGFR-Ras/ PI3K pathway coordinately stimulates oncogenic behaviors, such as, cell cycle entry and progression, protein translation, and inappropriate cellular growth and migration. The article states that it is these behaviors exhibited in the coactivation of these pathways that creates tumor-like growths that mimic human glioma, which is what makes it so useful in a laboratory setting. When PI3K Ras pathway was activated using the *repo-Gal4* driver, it induced an accumulation of ~50-fold excess glia (Read et al. 2009). In spite of their being different pathways initiated to produce glioma, in Read et al. study they found glial-specific coactivation of EGFR-Ras and PI3K stimulated glial neoplasia, giving rise to CNS enlargement and malformation, neurologic defects, and late larval lethality.

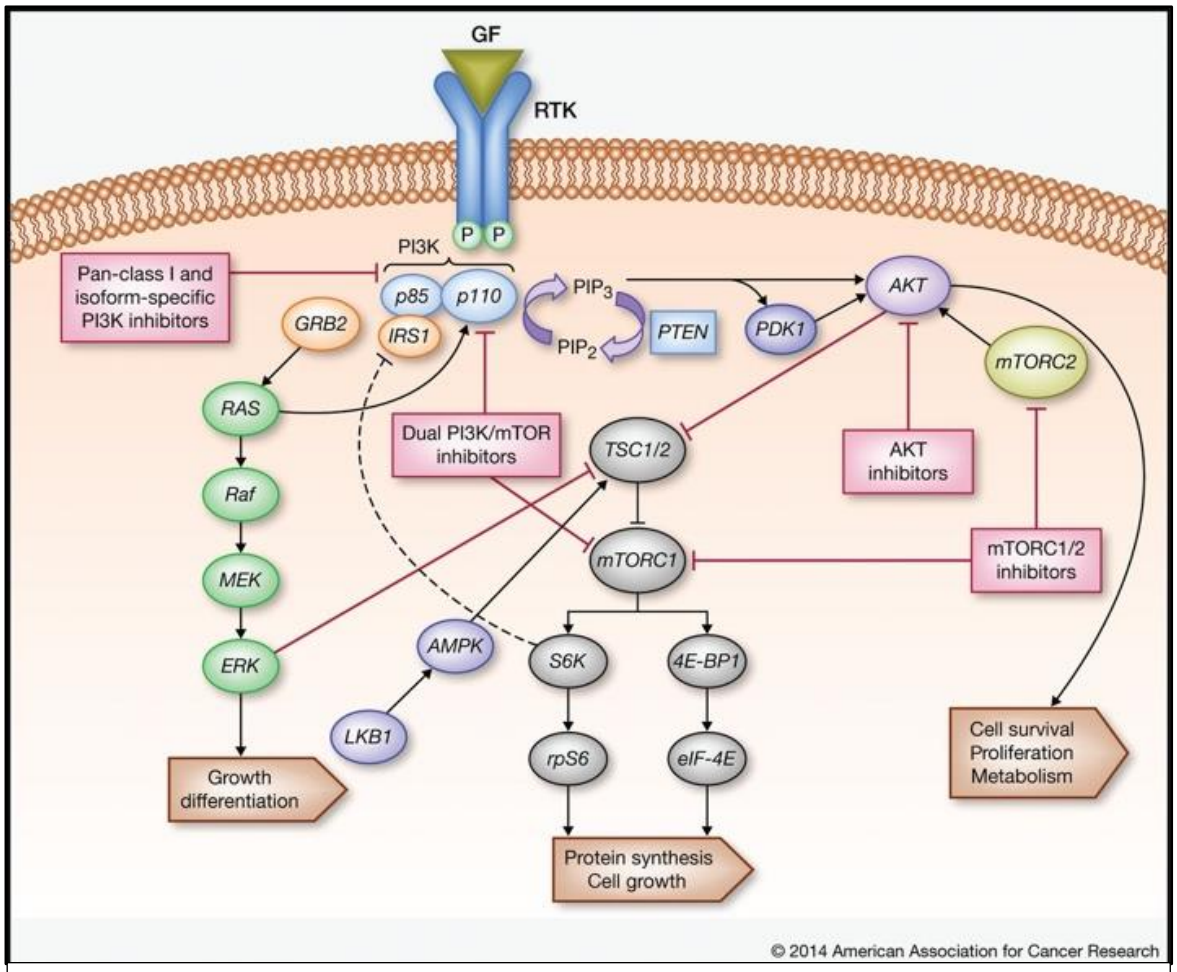


Figure 4. Overview of the PI3K/AKT/mTOR pathway and drug targets. Activating nodes (PI3K, AKT, PDK1, mTORC1 and mTORC2) and negative regulators (PTEN, TSC complex) are highlighted. Interaction with RAS and LKB1/AMPK pathways is also displayed.

Tyrosine Kinase Function

Tyrosine kinases are a family of enzymes, which use ATP to catalyze phosphorylation of select tyrosine residues in target proteins. It is these enzymes, through covalent post-translational modification, that act as a key component of normal cellular communication and maintenance of homeostasis (Paul & Mukhopadhyay, 2004). Some of these cellular functions include: cell proliferation, differentiation, migration, metabolism and programmed cell death. Paul and Mukhopadhyay go on in their research of tyrosine kinases to state that it is the ligand binding to the kinase's extracellular domain that triggers a response, as seen in Figure 5. These ligands are extracellular signal molecules (e.g. EGF, PDGF etc.) that induce receptor dimerization, which is a chemical reaction that joins two molecular subunits, forming a dimer (Chemistry LibreTexts, 2017)

There are two separate classifications of Tyrosine Kinases, either Receptor Tyrosine Kinase (RTK) or non-receptor tyrosine kinase (NRTK). The RTK are cell surface transmembrane receptors that also possess kinase activity. Paul and Mukhopadhyay found in their research that the RTK are ligand specific, contain a single pass transmembrane hydrophobic helix and a cytoplasmic portion with a tyrosine kinase domain. NRTK are cytoplasmic proteins, that contain a kinase domain and possess several additional signaling or protein-protein interacting domains (Paul & Mukhopadhyay, 2004). It was found that the activation mechanism of NRTK is more complex than that of RTK, which they hypothesized made the RTK more frequently activated.

Tyrosine kinases activity are tightly regulated in normal cells, but they have the ability to acquire transforming functions due to mutation(s), overexpression and autocrine paracrine stimulation, leading to malignancy (Paul & Mukhopadhyay, 2004). It is these different functions that the kinase takes on that allow it to represent a major portion of the oncoproteins that play a powerful role in the plethora of cancers, which includes glioma.

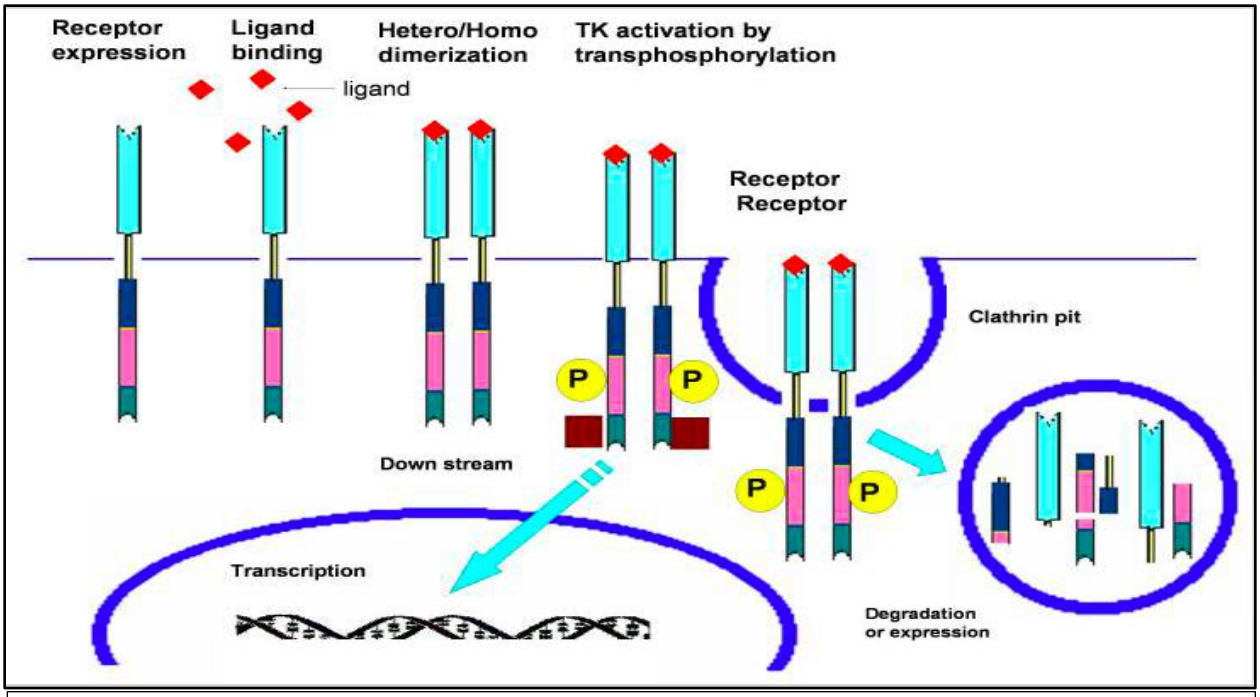


Figure 5. Mechanism of action of tyrosine kinase. 1. Receptor expression at membrane claveola 2. Ligand binding occurs 3. Hetero/homodimerization leads to tyrosine kinase activation and tyrosine transphosphorylation 4. Signal transduction throughout the cell 5. Receptor internalization 6. Receptor activates response of either degradation or re-expression. Figure by Paul & Mukhopadhyay, 2004.

Tyrosine Kinase Role in Cancer

To ensure normal tissue patterning in an organism, there must be tight control of cell proliferation and morphogenesis in conjunction with programmed apoptosis. It is when imbalances occur within these cell signals that oncogenesis can occur (Sangwan & Park, 2006). As stated in earlier research (Mukhopadhyay & Paul, 2004), it is the more frequently activated RTKs that have the potential to cause dimerization to the kinases, and this change in conformation leads to activation of the kinase and transphosphorylation of the receptor on specific tyrosine residues. The phosphorylated tyrosine residues are a critical aspect because they provide docking sites on the receptor for signaling proteins, and it is these signaling proteins that act to relay the signal from the receptor into the cell. When these signaling pathways become altered through mutations or chromosomal translocation, the RTKs can deliver a continuous or enhanced signal, forming it into a potent oncogene (Sangwan & Park, 2006). As of 2001, 58 genes encoding RTKs have been identified in the human genome, 30 of which have been found to be dysregulated in human cancers (Blume-Jensen & Hunter, 2001). Different discovered tyrosine kinases inhibitors are shown in Figure 6.

Together with these behaviors, Sangwan and Park (2006) found that growth-factor stimulation within a cell also aids in producing oncogenic behaviors. It is the non-scheduled expression of these growth factors that may result in a constant stimulation of cell growth in addition to a block in apoptosis. The combination of the mis-regulated RTKs and growth factors plays a critical role in regulating the tumor microenvironment, by enhancing both the proliferation and invasion by tumor cells. This invasion of tumor cells can form lung, breast, and brain cancer, and Sangwan & Park in their research go to explain how the pathways are initiated, and also possible routes that can be taken to halt the tumor progression in its track.

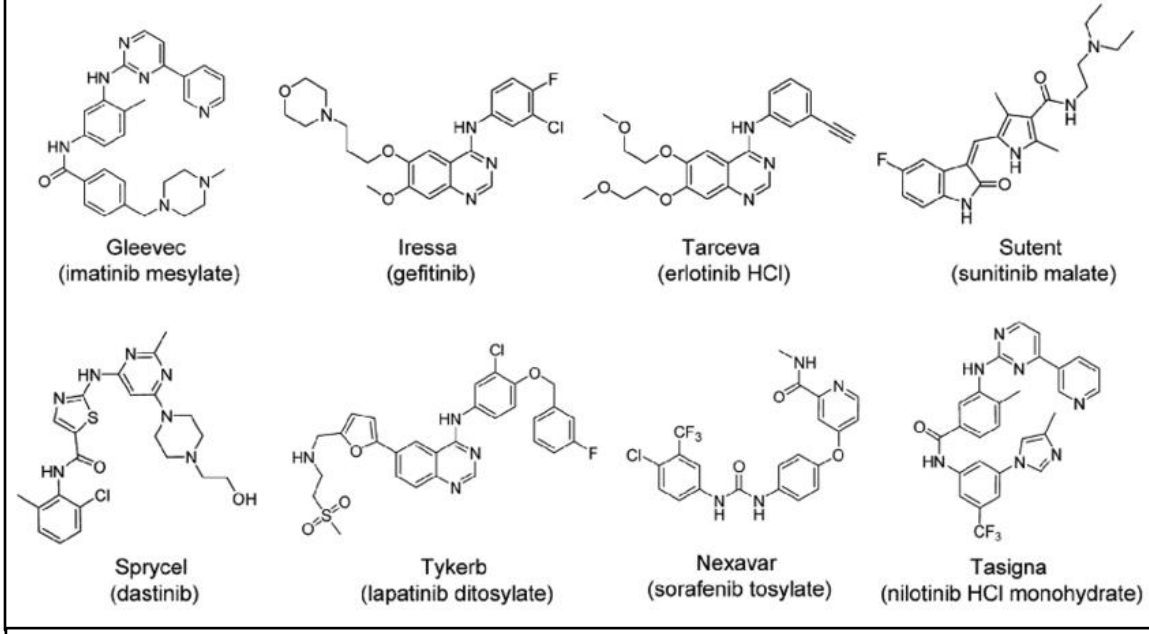


Figure 6. These are structures of some of the important kinase inhibitors discovered so far. Figure by Grant, 2008.

Tyrosine Kinase Expression in *Drosophila*

Through the sequencing completed by the Human Genome Project, it was found that the human genome contains 90 tyrosine kinases (Robinson et al., 2000). Of these 90 tyrosine kinases, 58 are the receptor types (RTKs). Of this large amount of RTKs present in the human genome, only 20 RTKs have been identified in the *Drosophila* genome. Just like what has been found in human kinases, these different receptors share many of the same effectors and their hierarchical organization is retained in biological contexts (Sopko & Perrimon, 2013). While in their research they may have found this to be true for the identified *Drosophila* tyrosine kinase receptors, very little is still known for approximately half of them.

Of the many pathways that RTKs can signal in the *Drosophila* genome, the one most commonly linked to glioma is the Epidermal Growth Factor Receptor (EGFR). EGFR plays a multitude of roles inside the genome, such as dorsal/ventral patterning of the embryonic ectoderm, establishment of neuroectoderm, wing development, photoreceptor differentiation, and the specification of muscle precursors (Sopko & Perrimon, 2013). It is able to signal these specific pathways by predominantly mediating short-range signaling that is restricted to cells producing EGF or to cells positioned 1-2 cells away. A pathway that falls into this category is the Ras/Raf/MEK/ERK pathway, showcased in Figure 7. There are four EGFR ligands in *Drosophila*: Spitz, Keren, Gurken, and Vein, and Sopko & Perrimon found in their study that all four play a critical role in the signaling of the pathway. By monitoring these different ligands and sub-pathways in EGFR, Sopko and Perrimon opened up the research field to possibly triggers or onsets of diseases, such as ones like glioma.

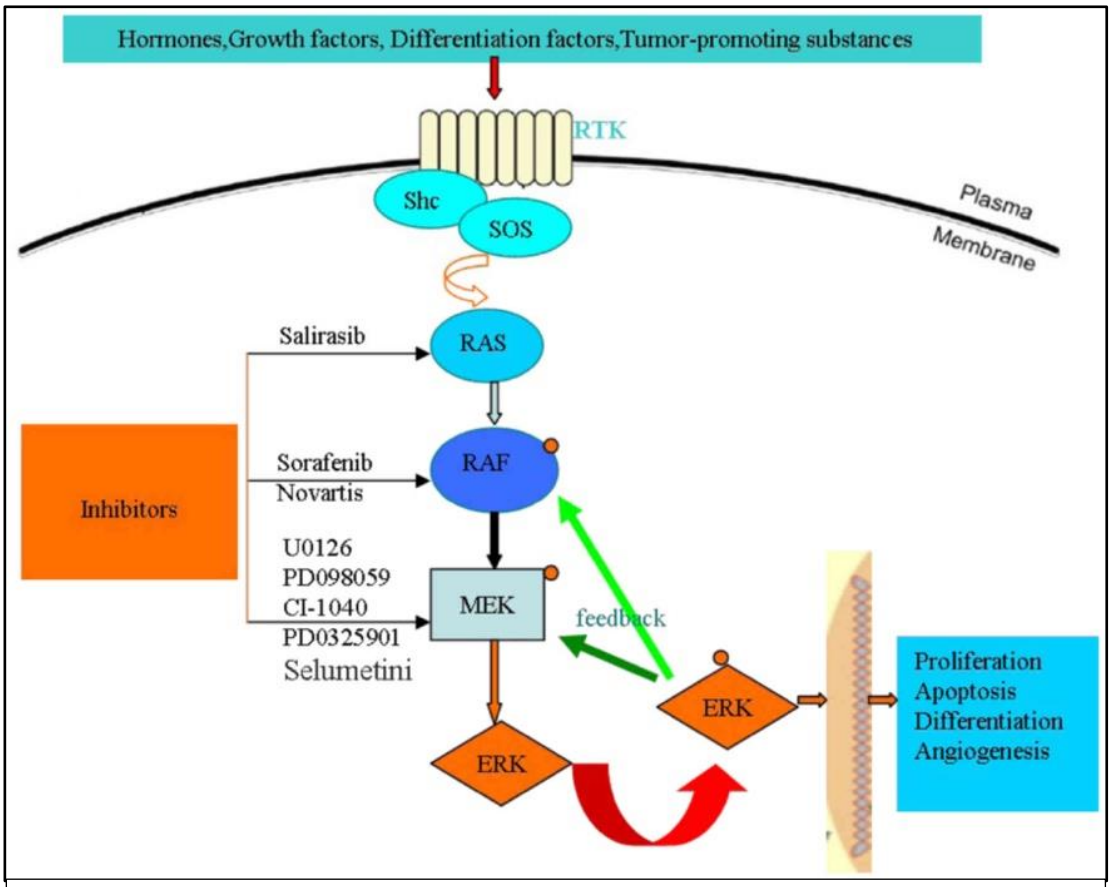


Figure 7. Activation of Ras/Raf/MEK/ERK signaling pathway. RTK, receptor tyrosine kinase; SOS, mammalian son-of-sevenless; Shc, homology 2 domain-containing protein; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase. Figure by Yang & Yang, 2017.

Success of Tyrosine Kinase Inhibitors with Glioma

The modes of oncogenic activation can be targeted using different approaches for tyrosine kinase inhibition, such as, small molecule inhibitors, monoclonal antibodies, heat shock proteins, immunoconjugates, antisense and peptide drugs (Mukhopadhyay, Paul 2004). The small-molecule compounds that inhibit the kinase domain have recently changed clinical practice for several cancers. Lapatinib has shown positive effects in HER2-positive metastatic breast cancer (Geyer et al., 2006); sunitinib positively influences metastatic renal-cell carcinoma (Motzer et al., 2007); and sorafenib is beneficial in carcinoma treatment due to it inhibiting the targeted kinase domain (Joseph et al., 2008). Taking this knowledge on the kinase inhibitors that work, De Witt Hammer (2010) systematically reviewed the efficacy, toxicity, and tissue analysis of small-molecule kinase inhibitors in adult patients with glioblastoma as reported in published clinical studies. De Witt Hammer also determined which kinases have been targeted by the inhibitors used in these studies, by using publications from a MEDLINE search. From the search, 60 studies qualified for inclusion, and 2385 glioblastoma patients receiving kinase inhibitors could be evaluated. The extracted data included radiological response, progression-free survival, overall survival, toxicity, and biomarker analysis. This data could be analyzed to determine the overall effects of kinase inhibition from past studies, by looking at the effects it showed on the patients during and after treatment.

De Witte Hammer found through analysis that (i) efficacy of small-molecule kinase inhibitors in clinical studies with glioblastoma patients does not yet warrant a change in standard clinical practice and (ii) 6 main kinase targets for inhibitors have been evaluated in these studies: EGFR, mTOR, KDR, FLT1, PKCβ, and PDGFR. Although in this study the promise of kinase inhibitors being effective in cancer treatment was not strong, De Witte Hammer overlooks the fact that there are many limitations to his study. Some of these limitations include, not having a control group, small sample sizes, and many of the 60 studies were not designed to determine the efficacy of therapy. The efficacy of the therapy is especially important, because by not having that the pathobiology of the drug may not be accurately studied in glioblastoma patients, along with the inhibitor may have

failed to inactivate the target in glioblastoma cells. Knowing the efficacy would aid to ruling out these errors and producing more accurate studies in the future.

Different from De Witte Hammer's study (2010), Mellinghoff et al. (2012) looked more specifically at how kinase inhibitors perform as glioblastoma drugs, specifically when targeting the PI3K pathway. Throughout their study they look at results from clinical trial, the structure of the human genome, and techniques that can be applied to glioblastoma to halt tumor growth. The clinical study looked specifically at mTOR, which was the first member of the PI3K pathway for which a clinical grade inhibitor became available, and its effect on patients with PTEN-deficient, recurrent glioblastoma (Podsypanina et al., 2001). After 1-2 weeks, the effect that inhibition of mTOR had on glioblastoma was analyzed, and it was found that although there was reduction of neoplasia, it was not enough to cause effective change on the tumor. Although these results do not seem promising, it must be taken into account that the information was preliminary because tumors with the most informative genotype(s) and strong basal pathway activation were generally underrepresented in the studies and because of difficulties to assemble a sufficient drug-naive "control" tumor sample (Mellinghoff et al., 2012). In their study, Mellinghoff et al. (2012) also talk about factors that would increase the therapeutic window of individual kinase inhibitors. It is stated that these are different dosing schedules (e.g., intermittent or "pulsatile" dosing) (Shah et al. 2008) and isoform-specific (e.g., PI3K) or mutant-specific (e.g., BRAF) compounds. The different dosage levels effectiveness can be represented through cytotoxicity, and the results are shown in Figure 8. The different dosage levels are the strongest candidate for positive results, given it was this fact that was also highlighted in De Witte Hammer's (2010) study of kinase inhibitors with glioblastoma.

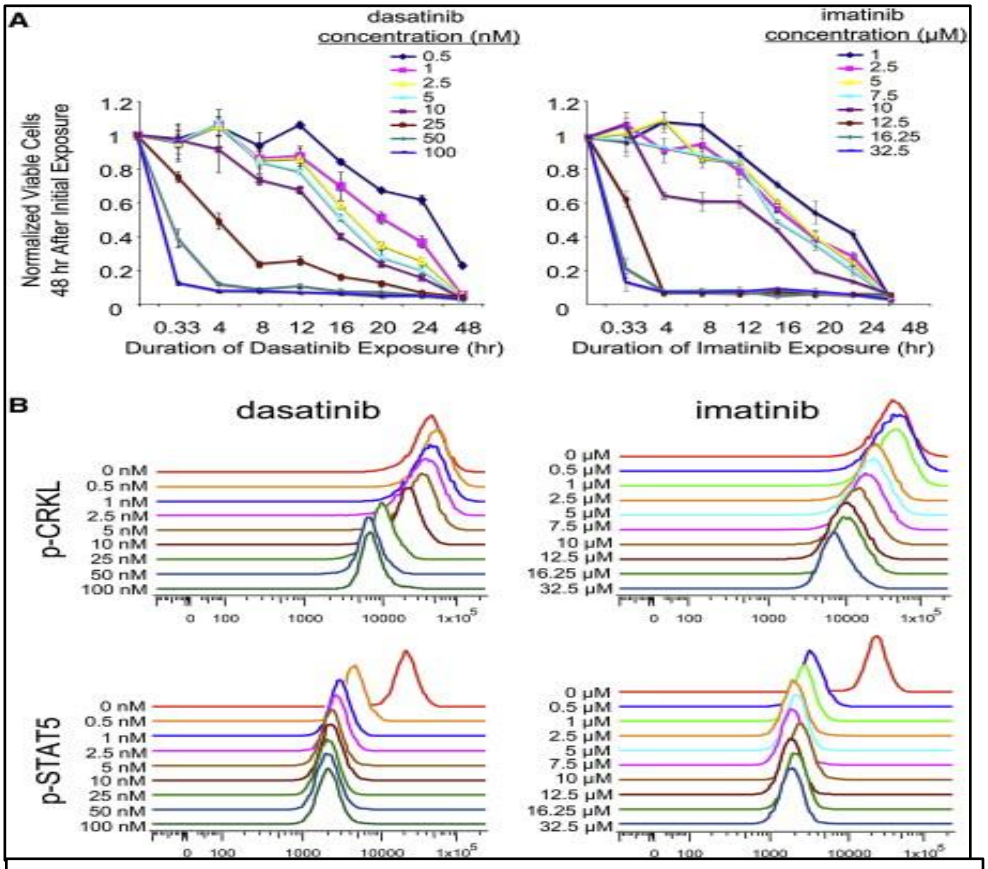


Figure 8. (A) The results establish a relationship between concentration and treatment duration with different kinase inhibitors. Cytotoxicity approached 100 percent in cells exposed continuously to 0.5 or 1 nM dasatinib (left) but was substantially diminished with shorter exposure times. Similar results were observed using concentrations of imatinib (1 μM) (right). (B) Assessment of BCR-ABL kinase activity in K562 cells through analysis of phospho-CRKL and phospho-STAT5 following treatment for 20 min with varying concentrations of dasatinib (left) and imatinib (right). Figure from Shah et al. 2008.

Glioma Treatment

Surgery

Standard treatment of glioblastoma includes maximal safe surgical resection, followed by concurrent radiation with temozolomide (TMZ) (Temodar®), an oral alkylating chemotherapy agent, and then adjuvant chemotherapy with TMZ (National Comprehensive Cancer Network [NCCN], 2015). Although surgically removing the tumor would seem like a promising therapy, extensive and complete surgical resection of glioblastoma is difficult because these tumors are frequently invasive and are often in eloquent areas of the brain, including areas that control speech, motor function, and the senses (Davis, 2016). In the study conducted by Kuhnt et al. (2011), they focus on how the more total resection for the patients possible, the more beneficial surgery will be in glioblastoma therapy. They came to this conclusion by having 135 glioma patients undergo tumor resection aided by 1.5T intraoperative MRI (iMRI) and integrated multimodal navigation. The media survival was 14 months for patients who underwent an extent of resection ≥98%, which is a significant improvement in patient survival. Kuhnt et al. (2011) in their study came to the conclusion that results like this can be achieved with iMRI and an intraoperative update of navigation data, along when performed on patients <65 years of age. Although, Kuhnt et al. (2011) study showed promising results, the prognosis for patients with GBM remains poor, with a median survival of 15 months (Thakkar et al., 2014). In conclusion, both Thakkar et al. (2014) and Kuhnt et al. (2011) found that patients with a lower age and higher performance status experience longer survival.

Chemoradiation

Chemoradiation as means of glioblastoma treatment has been seen to produce more promising results compared to chemotherapy and radiation alone. Although it is one of the most popular choices of glioblastoma therapy, usually occurring around 4 weeks after surgery, it holds the potential to cause more consequences than advantages. Shih and Batchelor's study (2017) analyzed the different techniques of chemoradiation practiced (Adjuvant radiotherapy, treatment target, Intensity-modulated RT, etc.) and stated the similar limitations found in each of them. It was found that the similar consequences of

chemoradiation on glioblastoma include radiation-induced gliomas, neurocognitive toxicity, RT-induced leukoencephalopathy, and endocrinopathies (Shih & Batchelor, 2016). Other side effects do occur because of chemoradiation, but they are more specific to the route of chemoradiation chosen. Together with all these side effects, it was found that the survival rate of patients was still low, with at 17 months after treatment 72% of patients developed recurrent glioblastoma (Milano et al, 2010). Comparing Shih and Batchelor's (2016) findings to present chemoradiation practice, the adequate dose of chemoradiation necessary to cause a high survival rate with low cytotoxicity levels is required to maximize the survival benefit. Furthermore, these studies done in the past on glioblastoma chemoradiation treatment, which included the benefits and limitations of it, showed that further therapy options must be taken either before or after this to ensure an increase in overall patient survival.

Temozolomide

Chemoradiation started showing much higher patient survival rates and less lethal side effects when it contained Temozolomide (TMZ), rather than simple radiation alone, shown in Figure 9. A separate study was conducted to test and analyze the results of TMZ used in clinical practice, and the results from it were promising (Stupp et al., 2005). This study was conducted with 573 patients who randomly received radiotherapy alone or radiotherapy plus continuous daily temozolomide, followed by six cycles of adjuvant temozolomide. At the median follow-up of 28 months, the median survival was 14.6 months with radiotherapy plus temozolomide and 12.1 months with radiotherapy alone (Stupp et al., 2005). The two-year survival rate was 26.5 percent with radiotherapy plus temozolomide and 10.4 percent with radiotherapy alone. These results were clinically beneficial and showed statistically significant survival benefit with minimal additional toxicity.

Another study looked at the combinatorial effect of high-linear transfer radiation (high-LET) combined with Temozolomide, versus with conventional radiation like the study above. Glioblastoma in treatment is known as a radioresistant tumor, meaning that even when treated with conventional radiation the survival rates are still low. Barazzuol et al.

(2012), believe that by using the new technology of high-LET combined with Temozolomide on glioblastoma tumors, then this will show a much greater success rate than what conventional radiation has shown in the past. To accomplish this, they tested these combinations on four different human glioblastoma strains and analyzed their cell survival, DNA damage and repair, and cell growth (Barazzuol et al., 2012). The results from this study did not find any additive effects between high-LET and TMZ, but did present data that supports the notion that the cytotoxic effects of TMZ and high-LET are not likely to be correlated. This recognizes and supports past data that TMZ cytotoxicity needs one or two cell divisions before DNA damage can be recognized. The cytotoxicity of TMZ must be considered in both experimental or clinical procedures, and this data further supports the positive effects of TMZ when in combination with either radiotherapy or other chemotherapy drugs.

Temozolomide acts through DNA alkylation, and this analysis led to another strong predictor of patient-related outcomes: the methylation of the MGMT gene (Stupp et al., 2009). Methylated (not activated) MGMT exhibit compromised DNA repair, so when MGMT becomes activated it can interfere with the effects of treatment. Radiotherapy and alkylating chemotherapy exert their therapeutic effects by causing DNA damage, cytotoxicity, and triggering apoptosis. Therefore, the expression of methylated *MGMT* is beneficial for patients undergoing temozolomide chemotherapy and radiation. In this study conducted by Stupp et al. (2009), the methylation of *MGMT* was a strong predictor of better outcomes for temozolomide treatment.

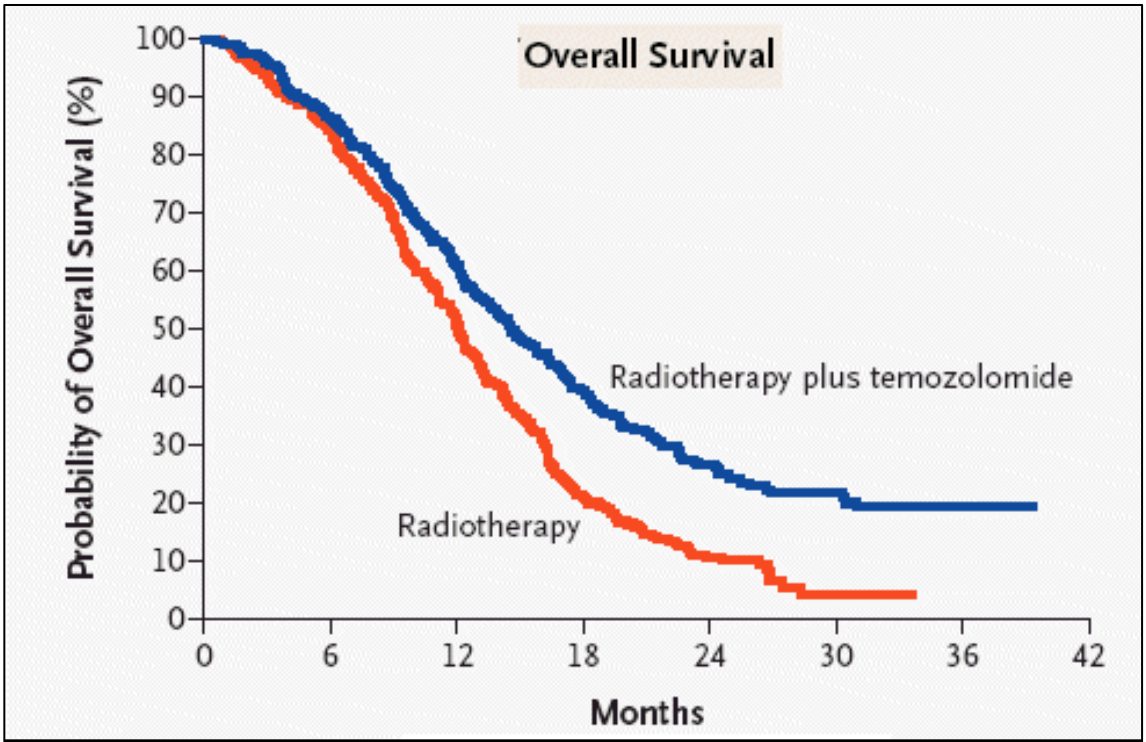


Figure 9. Results from clinical trial of patient treated with Radiotherapy plus Temozolomide vs. those treated with Radiotherapy alone. The results showed a much higher probability of survival over a 42 month time span for those treated with Temozolomide vs. without. Figure from Stupp et al., 2005.

Chapter 2

Materials and Methods

Blind Study

The first step in completing this combinatorial drug screen was determining which Tyrosine Kinase Inhibitors proved to be the most successful. This was done by completing a blind study over the course of the semesters. Blind studies are ones that can be utilized in both the laboratory setting and also in clinical trials of medicine. Blind studies by definition are studies done in which the subjects involved in the study do not know which experimental condition they are receiving (Clinical Trials and Screening, 2020). There are both double-blind and single-blind studies that can be conducted, but in the case of the drug screen it is only a single-blind study. In dealing with the Tyrosine Kinase Inhibitors, the researchers are unaware of which Tyrosine Kinase Inhibitor they are testing. It is done so by having the drugs named and labeled as a letter and numerical value (i.e. A11). This blinding is especially important in experimental usage due to its ability to prevent bias from influencing the results (Clinical Trials and Screening).

Many of the blind studies that are in the field of treating cancer, specifically with glioblastoma, are experimented on the clinical trial side of medicine. These experiments are done by recruiting a group of people that will allow for external validity to be applied and who's consent is properly given. An example of one of these blind studies took place in 2017 with the combined use of TMZ with Rindopepimut (Weller et al., 2017). Rindopepimut is a vaccine that targets the *EFGR* deletion mutation EGFRvIII. It does so due to it being composed of a an EGFRvIII-specific peptide conjugated to keyhole limpet haemocyanin (NCI Drug Dictionary). In this study patients with newly diagnose EGFRvIII-expressing glioblastoma were treated with either Rindopepimut plus TMZ or Rindopepimut plus control over the course of 6-12 cycles of treatment (Weller et al., 2017). The results were collected using a randomized, double-blind trial, meaning that neither the subject nor the researcher knew what treatment they were receiving. At the end of the trial when compared to the control group, it was discovered that Rindopepimut did not increase the survival rate in patients diagnosed with glioblastoma.

A successful blind study which was done at the laboratory level looked at drug resistance in Mycobacterium tuberculosis with the drugs isoniazid (INH) and rifampin (RMP). In this study consecutive isolates of Mycobacterium tuberculosis were coded and sent to two external laboratories for genotypic analysis of INH and RMP resistance by PCR-single-strand conformation polymorphism (SSCP) analysis (Enriquez et al. 1997). The study was considered blind given that the external laboratories were not aware of which of the two drugs they would be testing, which allowed for no bias to negatively impact the results. Through this study it was found that resistance can be accurately detected for both INH and RMP when the study is limited to analyzing four main genetic regions (Enriquez et al. 1997).

Dissection and Mounting

The phenotypes present in the fruit fly will be assessed from the larval to the pupae stage to monitor growth regulation and cell proliferation in the adult brain from the third instar larval stage following standard protocol. Dissection and mounting of the adult brain will be necessary to track and get clear images of the progression of the tumor. *Drosophila* larval brains are useful in modeling human brain degenerative diseases, mapping neuronal circuitries in adult brains, and studying the molecular and cellular basis of higher brain functions (Tito et al. 2016). In this drug screen dissection of the brain was done on the third day of the larvae's exposure to the specific drug. These drugs can either be (a) a Tyrosine Kinase Inhibitor, (b) TMZ, or (c) Tyrosine Kinase Inhibitor plus TMZ. The protocol calls for use of a Petri Dish and .55 forceps to ensure that a majority of the eye and tracheal tissues normally associated with the brain are removed so no interference is encountered in the later imaging steps (Tito et al. 2016).

The method used for proper dissection was to gently hold the larval body with one pair of forceps, and with a second pair of forceps, hold the larval mouth hook. Pull the two pairs of forceps apart gently to cause the mouth hook to detach from the body, allowing to the brain to be isolated (Wu & Wu, 2006). Once brains are properly dissected, place them in a 150µL phosphate-buffered saline (PBS) and 50µL Para-formaldehyde (PFA) solution to fix for 20 minutes. Next add 1mL of cold 1xPBST (3.2 mM Na_2HPO_4, 0.5 mM NaH_2PO_4,

1.3 mM KCl, 135 mM NaCl, 0.05% Triton X-100) and place on a rotator for 10 minutes. After ten minutes, vacuum out PBST and repeat the wash two more times.

For mounting there were no antibodies used, so a primary and secondary stain were not completed for the samples. Mounting was completed on a glass slide using .55 forceps to isolate the unwanted tissues from around the brains. Once properly isolated, the brains were coated in Vectashield to inhibit the rapid photobleaching of fluorescent proteins and fluorescent dyes (VECTASHIELD®, 2020). The brains were then organized in a line, a cover slip was placed properly on top, and nail polish was used to inhibit the cover slip from sliding. Mounted slides were then labeled and placed in a -20˚C freezer until imaging could be completed.

Imaging

The samples taken from the *Drosophila* models, once properly dissected and mounted, will be scanned in the Laser Confocal Scanning microscope. The adult flies will be photographed by using Olympus Bx51 Florescence microscope or Zeiss Apotome. The images that are generated from these microscopes will be used to more clearly track the growth of the tumor as well as be analyzed for statistical significance using Image J programming. Images will be analyzed based on both their glial cell presence and size and shape of the brain itself.

Layout of Drug Screen

Tyrosine Kinase Inhibitors

To analyze the effects of Tyrosine Kinase Inhibitors in treating glioblastoma, dissection, mounting and imaging of the brain were completed. The stocks of *Pten^{i}; Ras^{v12}*, and Repo Gal4 UAS-GFP served as controls, and the F1 larvae from the *Pten^{i};Ras^{v12}*x Repo GFP cross constituted the experimental samples in which glioma was induced in the larval CNS. All inhibitors were first tested at the 300μL dose. By following the protocol that is demonstrated in Figure 1, the brains were analyzed for two characteristics: (a) the number of glial cells left after treatment, and (b) the change in overall size of the brains. A successful treatment of Tyrosine Kinase Inhibitors would show the glioma brains shrinking back to a moderate normal size, and also have the glial cell population decrease (Figure 2). A negative effect of the drug would either show no change from the original or a sharp increase in glial cells and a larger abnormal brain lobe shape (Figure 2). The inhibitors that proved to show success will be tested at different concentrations and also be used in the combinatorial drug screen with TMZ.

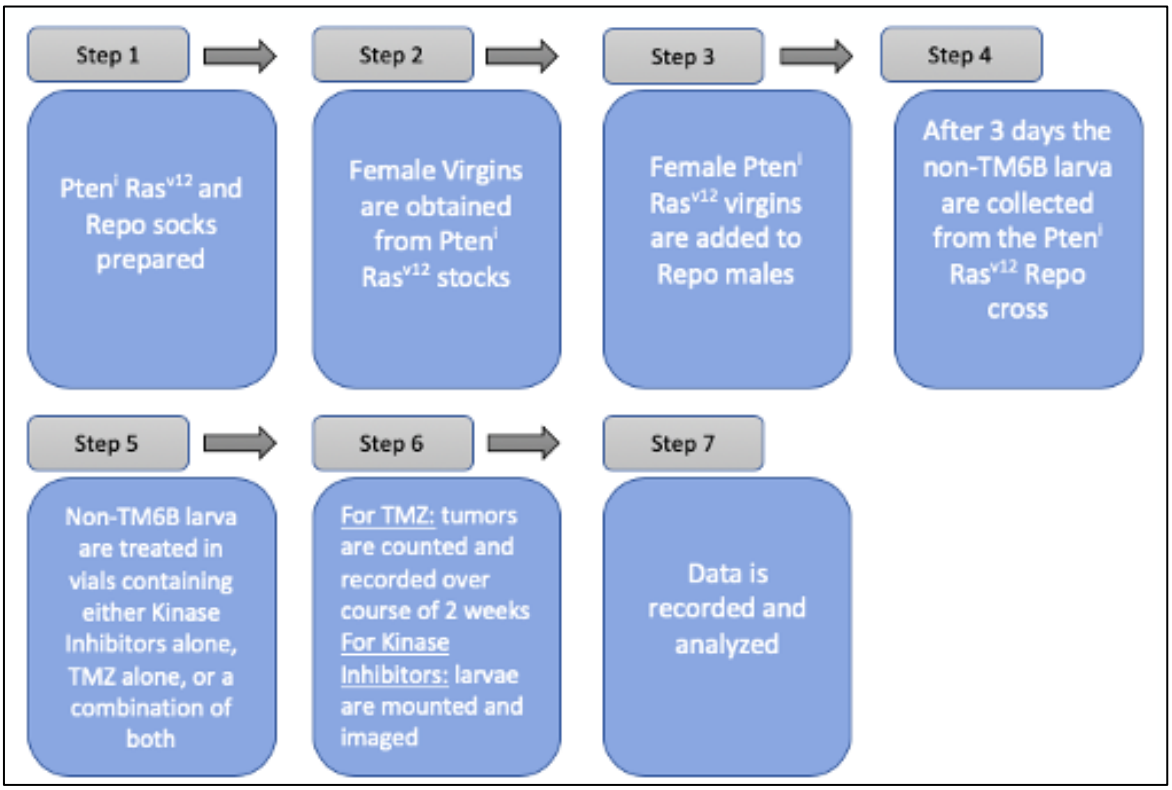


Figure 10. The seven-step protocol followed when testing both the Tyrosine Kinase Inhibitors and TMZ on the Drosophila glioma model.

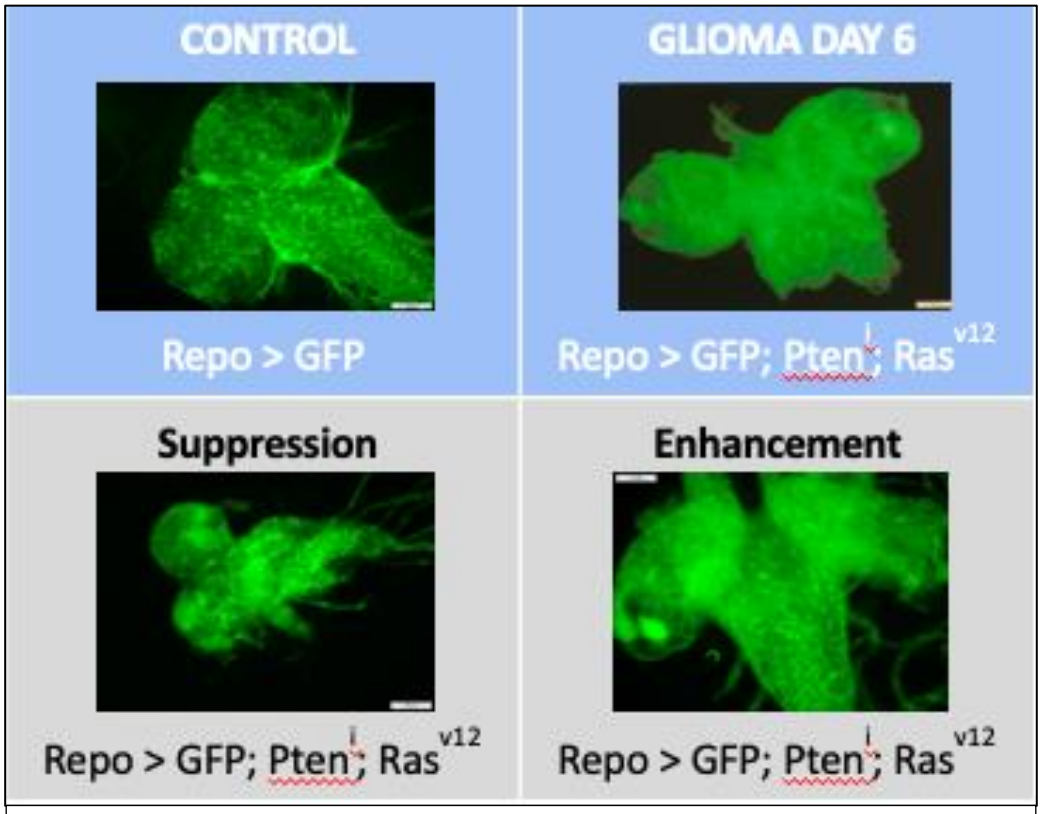


Figure 11. These are results used to help determine if a specific tyrosine kinase inhibitor showcases suppression, enhancement, or no change in tumor size and shape when compared to the control groups. The control group is a normal, healthy fly brain.

Combinatorial Drug Screen

Given that both TMZ and the tested Tyrosine Kinase Inhibitors showed positive results when used on the *Drosophila* glioma model separately, they went on to be further tested when used in combination. The five successful tyrosine kinase inhibitors were tested with the control value of TMZ (3mM) at a set value of 300μM on Repo GFP and $Pten^{i}$; Ras^{v12}x Repo GFP. The five tyrosine kinase inhibitors were A4, A9, B4, B6, and B9. The 300μM was chosen to test for the initial success of TMZ combined with the kinase inhibitors. These set values of TMZ and kinase inhibitors were added to the larvae's food by protocol, and the success of the screen was analyzed the same way the initial TMZ screen was. On day-three 50 larvae were added into the drug concentrated food, and it was starting on day-five that the larvae could begin to get analyzed and counted. By counting the amount of small, medium, large tumors and possible alive flies present, the combination was determined to be successful at this concentration range.

The most successful of these combinations was A9 (300μM) + TMZ (3mM). To determine if A9 would be more successful at a different concentration range, it was tested by changing the concentration using a log scale. These log scale concentrations tested were 10μM, 30μM, 100μM, and 300μM. Over the course of several weeks these combinations were tested and analyzed by counting the number of different tumors present in the larvae and the vitality of the larvae was also analyzed.

Chapter 3

Results

Tyrosine Kinase Results

The primary drug screen that has looked solely at the effectiveness of Tyrosine Kinase Inhibitors has been an entire group effort between students in Dr. Kango-Singh's lab. This is still an ongoing project as well to continue to determine tyrosine kinase inhibitors that show suppression in our *Drosophila* glioma model. Like stated earlier, the results of tyrosine kinase inhibitors by themselves are tested through dissection and mounting of the larval brains. From this analysis and comparing images of the brains, it was determined there were six inhibitors that showed strong suppression effects on our tumor model. These drugs were: P1A4, P1A9, P1B4, P1B6, P1B9 and P1G10. The results from one of these successful drugs, P1G10, is pictured in Figure 12. P1G10 is determined to show suppression due to that both brain lobes and the ventral nerve cord (VNC) decreased relatively close to the control, and the glia cell population has not increased. All other tyrosine kinase inhibitors listed above also followed a similar suppression trend as seen in P1G10.

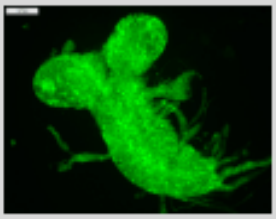
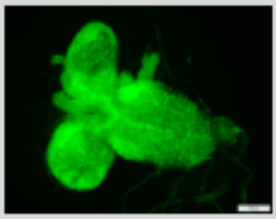
	Day 5	Day 6	Day 7
P1G10			No larva able to image on this day

Figure 12. Results from our glioma tumor being tested with Tyrosine Kinase Inhibitor P1G10. Results were looked at over the course of three days to determine effectiveness. Over the course of the analysis period P1G10 continued to show strong suppression results of the tumor.

Initial Temozolomide Screen

The initial screen of TMZ was done in order to test the concentration that it works best at in our *Drosophila* glioma model. TMZ was tested using a log scale from values 10-1000μM, and then an additional 3mM and 5mM. To analyze the effects that TMZ has on the *Drosophila* glioma model, mounting and dissection will not take place. Given that TMZ is a DNA alkylating agent, most of the larvae will not progress into the alive fly stage but will be halted in their growth at the pupal stage. The growth arrest phenotypes were categorized based on the tumor size which was tracked by the expression of GFP. The pupae were categorized as small, medium, and large tumors and the effect of particular concentration of TMZ was tested.

The different tumor sizes along with the control group can be seen in Figure 13. A small tumor is observed when the larvae progresses all the way to producing red eyes and wings but does not progress all the way to hatching. A medium tumor takes up more a presence within the larvae but has not completely taken over, as what would be seen in a large tumor. A large tumor completely takes over the larvae and produces necrotic spots along the pupae. With a large tumor it looks as if a larva never even formed within the pupal casing, but instead always remained empty. The TMZ concentration with the largest amount of small tumor larvae, along with the larvae that show the greatest vitality, will be seen as the most successful concentration of TMZ.

Another aspect that was analyzed when determining the successful TMZ concentration was the vitality of the larvae after treatment to the drug. TMZ effectiveness was tested by adding fifty larvae from each stock to every concentration tested. The larvae that would showcase the most vitality after being treated would lend to it being determined a successful concentration of treatment. High vitality was determined by the greatest number of larvae that came up from food concentrated with TMZ. If only ten out of fifty larvae come up from the food after treatment, this would showcase low vitality given a majority of larvae died due to the drug concentration. This death could either occur from the concentration being too low to have an effect on the glioma, or that it was too high

and was cytotoxic. The concentration of TMZ that shows the most success will go on to be used as the control value for the combinatorial drug screens between TMZ and the experimentally determined effective Tyrosine Kinase Inhibitors.

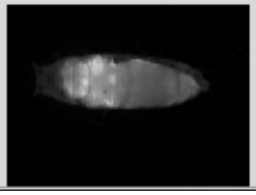
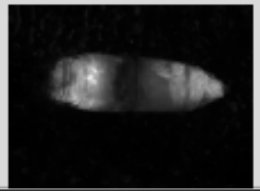
	Small Tumor	Medium Tumor	Large Tumor
Pten^{i};*Ras*^{vt2}; Repo GFP treated with 3mM			

Figure 13. Images of a small, medium, and large tumor after treatment with the effective 3mM concentration of TMZ. The small tumor is identified by its presence of both wings and eye disks within the pupal casing. Large and medium tumors are distinguished based on the large tumors having necrotic spots presence and also less larvae present in the case.

Testing the effects of Temozolomide on glioma growth

The effective control concentration of Temozolomide when used on the Drosophila glioma model (*Pten^{i}; Ras^{v12}*x Repo GFP) was determined to be 3mM. This was determined due to the 3mM concentration having the largest number of alive flies (9) and small tumors (7) present after treating with the drug. Given that other concentrations tested also had a large number of small tumors present, 3mM was selected due to the large number of alive flies that hatched over the time of observation. Alive flies are highly uncommon when in treatment with TMZ due to TMZ acting as an alkylating agent, so the presence of them gains immense interest. The least of effective concentration of TMZ was 1.5mM, given that this had the largest number of large tumors present (17) after treatment and no live flies hatching from their pupal casing. The results from the different concentrations can be viewed in Table 1. Images of an example of the small, medium, and large tumors from the effective 3mM concentration can be seen in Figure 13.

Pteni; Rasv12 x Repo				
Concentrations	small	medium	large	alive
1mM	16	5	11	1
1.5mM	3	3	17	0
2.0mM	8	11	13	2
2.5mM	5	7	18	2
3mM	7	4	10	9
5mM	4	5	23	3

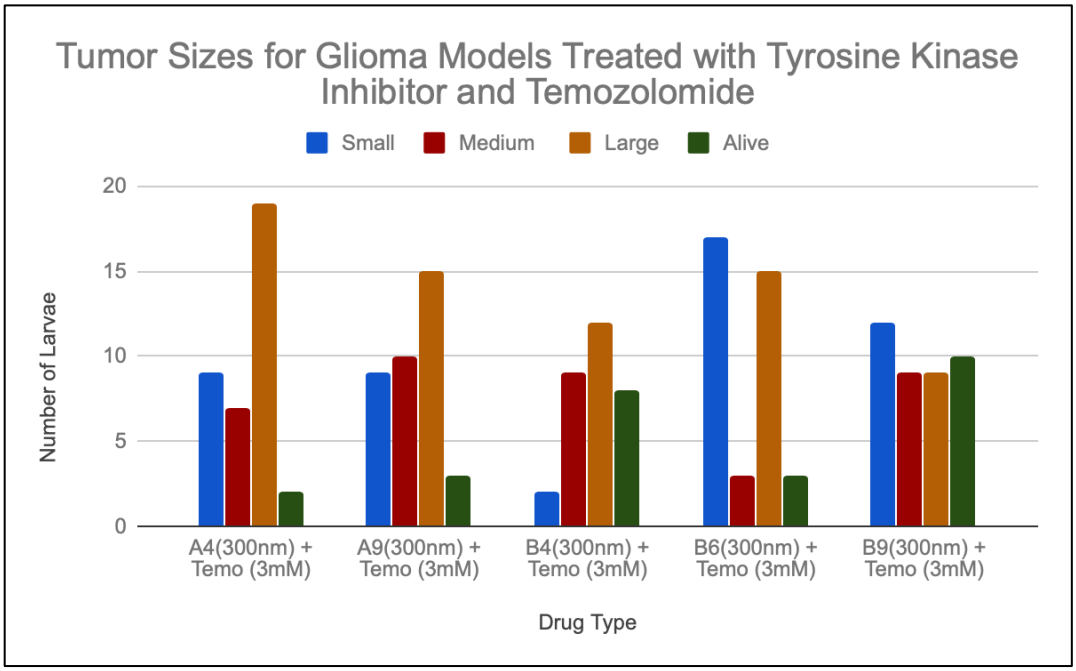
Table 1: The different tumor counts in the Drosophila after treatment with different concentrations of TMZ. It was through these results and the large amount of both small tumor and alive flies seen at the 3mM range that made it be chosen as the control value for future experiments containing TMZ

Tyrosine Kinase and Temozolomide Combinatorial Results

In the combinatorial research there was a total of five different tyrosine kinase inhibitors tested along with the concentration value of 3mM TMZ. The five tyrosine kinase inhibitors that went on to be further tested at the 300μM concentration level were: A4, A9, B4, B6, and B9. Out of these combinations A4(300μM) + TMZ(3mM) was one combination that showed significant suppression of the tumor. This suppression was determined based off the significant number of small tumors and alive flies present after addition to the drug. As seen in Table 2, there was a total of 9 small tumors and 3 alive flies present. Although there was still a sizable number of large tumors present (15 total), it was the fact that there were alive flies that hatched that kept promise of success of this specific combination. Another combination that stood out as being a potential successful suppressor was B9(300μM) + TMZ(3mM). Looking at both Table 2 and Graph 1, the most substantial result from this combination is the large number of alive flies that hatched out of the treated fifty. There was a total of ten alive flies that hatched, which is the most out of any other combination tested. Given that both of these showed substantial results, it was the A4(300μM) + TMZ(3mM) combination that was selected first to go through a more thorough testing of different concentrations that might be more effective than the 300μM value.

	***Pteni; Rasv12 x Repo* Combinatorial Drug Results of Number of Tumors Present with Various Tyrosine Kinase Inhibitors**			
Drug Type	Small Tumor	Medium Tumor	Large Tumor	Alive Flies
A4 (300µM) + TMZ (3mM)	9	7	19	2
A9 (300µM) + TMZ (3mM)	9	10	15	3
B4 (300µM) + TMZ (3mM)	2	9	12	8
B6(300µM) + TMZ (3mM)	17	3	15	3
B9 (300µM) + TMZ (3mM)	12	9	9	10

Table 2. This table is showing the different number of small, medium, and large tumors present in the larvae after each individual concentration of Tyrosine Kinase Inhibitor (300nm) + TMZ (3mM) was added. The total number of larvae added to each combination was 50 and the type of tumor was counted and recorded in the table above to help track both the success of the combination and



Graph 1. This graph shows the results from the different tyrosine kinase inhibitors when combined with 3mM TMZ. This graph was creating using the values in Table 2. Looking at the difference in size of tumor it was both A9 and B9 that showcased the most success as a tumor suppressor when aligned on a side by side graph like above.

Various concentrations of A9 + Temozolomide (300mM) Results

To further test the success that the Tyrosine Kinase Inhibitor A9 + TMZ could have, it was selected and observed separately using different concentration values. The concentrations selected were based on a log scale and the same amount of fifty larvae were added to each. Looking at the table and graph below there were significant results from the data collection. One of these results was the high success seen in the concentration value of 100μM versus the originally tested 300μM. In the 100μM concentration value there is a large number of small tumors present (18) and a significant number of alive flies (8) when compared to the medium and large tumor presence at this concentration (6 and 5, respectively) as seen in Table 3.

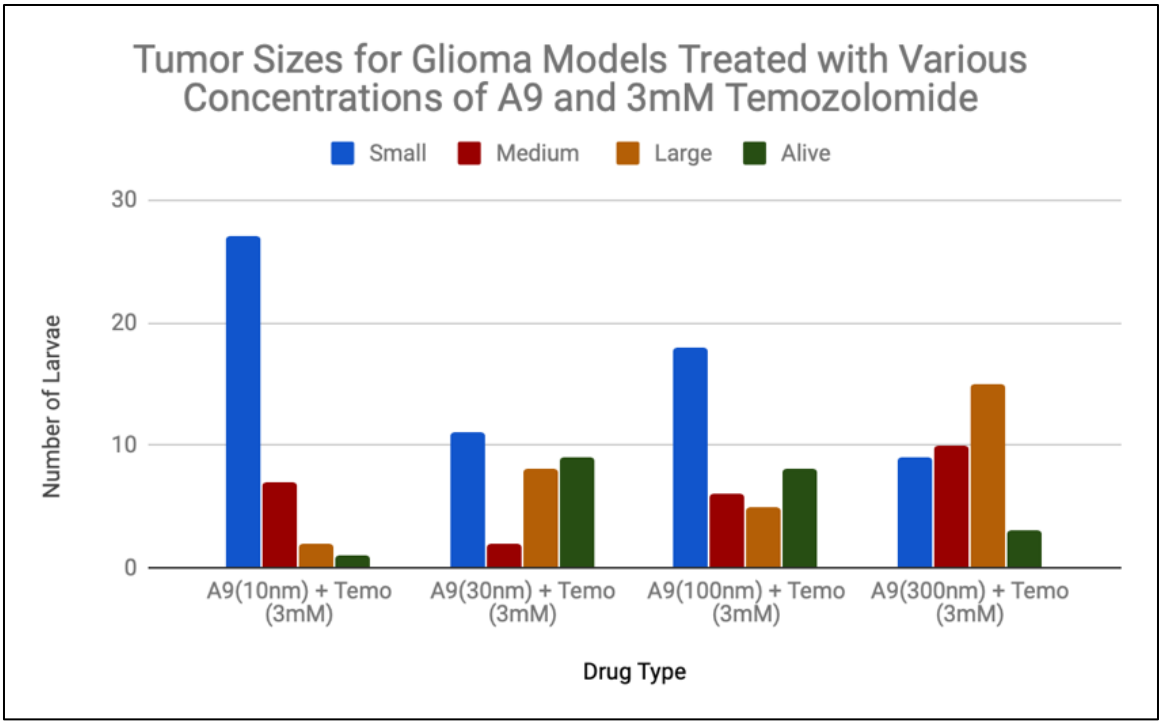
Another concentration that showed promise due to the substantial amount of suppression present was the A9 (10μM) + TMZ (3mM). Looking at Graph 2, it is this combination that shows the largest number of small tumors present when compared to the other three concentrations of A9 tested. Given this high number of large tumors present, it shows potential to be looked into through further testing. When compared to the results of A9 (100μM) + TMZ (3mM) that were described earlier, the 10μM concentration combination still did not succeed the effects of it due to the very low number of alive flies present and not significant halting of the tumor growth. The presence of alive flies at these concentrations hold much more importance in showing the effects it has on the tumor than the number of small tumors present. Taking this into consideration, it is the large number of alive flies that hatched (as seen in the 100μM combination) that proves more success than a large number of small tumors (as seen in the 10μM).

The concentration that showed the least amount of suppression was at the 300μM level, which was a significant result given it was this concentration value that showed the most promise in the original combinatorial study. This combination value of A9 (300μM) + TMZ (3mM) was determined to have the least amount of suppression compared to the others based off of it having the highest number of large tumors present when compared to both the small, medium, and alive flies at its same concentration value. With this leading amount of 15 large tumors present as seen in Graph 2, it was deemed that the

combination was not as successful as originally thought it was. This decrease in success is hypothesized to be due to the amount of 300μM being too high of a concentration for it to show any significant results. The concentration of drug starts to either cause cytotoxicity to the larvae or the concentration is too large for it to produce a successful suppression effect.

	***Pteni; Rasv12 x Repo* Results of Number of Tumors from A9 at various combinations + 3mM TMZ**			
Drug Type	Small Tumor	Medium Tumor	Large Tumor	Alive Flies
A9 (10μM) + TMZ (3mM)	27	7	2	1
A9 (30μM) + TMZ (3mM)	11	2	8	9
A9 (100μM) + TMZ (3mM)	18	6	5	8
A9 (300μM) + TMZ (3mM)	9	10	15	3

Table 2. This table is showing the different number of small, medium, and large tumors present in the larvae after each individual concentration of Tyrosine Kinase Inhibitor A9 + TMZ (3mM) was added. The total number of larvae added to each combination was 50 and the type of tumor was counted and recorded in the table above to help track both the success of the combination and vitality of the drug.



Graph 1. This graph shows the results from the tyrosine kinase inhibitor A9 at different concentrations on a log scale when it is combined with the control value of TMZ at 3mM. This graph was creating using the values in Table 3. Looking at the difference in size of tumor it was the A9 (100μM) that showcased the most success.

Conclusion

In conclusion we found that there were significant combinatorial effects when both the Tyrosine Kinase Inhibitor and Temozolomide were added to treat glioblastoma. With the poor prognosis that Glioblastoma has, these results are significant in the possibility of helping reduce these deathly diagnoses. One of the initial takeaways from these results is the promise that TMZ by itself showed in our *Drosophila* model. Given that TMZ is not supposed to have any alive flies hatch due to it acting as a DNA alkylating agent, it was surprising to see so many appear at the 3mM range. This result could be due to the amount of TMZ treating the glioma was not enough for it to become lethal to larvae, while at the same time not allowing all of the DNA to become alkylated. The presence of alive flies was something that carried on into the other experiments containing TMZ, which helped support the validity that the alive flies at 3mM were not an outlier. It was the effectiveness of TMZ alone that was seen in our *Drosophila* glioma model that helped further support its effectiveness as a chemotherapy drug, which is how it is currently being used in treatment of glioblastoma.

The effective concentration of TMZ (3mM) was combined with the Tyrosine Kinase Inhibitors that proved successful throughout past experiments in order to determine if tumor suppression could be seen in combination of drugs. The five successful tyrosine inhibitors were initially tested at a control value of 300μM when in combination with the 3mM TMZ. The findings from these results showed that there were significant suppression effects in some of the combination of Tyrosine Kinase Inhibitors and TMZ. It was in the combinations of A9 (10μM) + TMZ (3mM) and B9 (10μM) + TMZ (3mM) that the most impressive results were found. Both of these concentrations not only had a significant number of small tumors present when in comparison to the medium and small tumors, but they also had a noticeable number of alive flies' present. Although the high presence of small tumors does lead to the combination being a tumor suppressor, the presence of alive flies holds more weight when it comes to analyzing results. This is due to it being relatively uncommon in the treatment of TMZ, so the presence of them leads to the drug treating the glioma enough to allow the larvae to fully hatch. When looking at the combinations, the high frequency of alive flies could be due to the positive effect the

Tyrosine Kinase Inhibitor has on suppressing the glioma, and this effect could override the alkylating effect of the TMZ present. Also, TMZ alone at 3mM showed a high percentage of alive flies, so this concentration from past experiments is not known to fully alkylate all the DNA present in the larvae. It was the success that was found throughout this combinatorial experiment that led to further testing with these two chemotherapy drugs.

Isolating A9 at different combinations with TMZ (3mM) also showed significant results in the suppression of the glioma tumor. A9 was selected for isolation due to the strong tumor suppression results it showed in the earlier experiment discussed above. Looking at the results, it was clear to see that the initially tested 300μM of Tyrosine Kinase Inhibitor was not the best concentration value when combined with the control TMZ. Looking at Graph 2 it is clear to see the varying results across the four tested concentration ranges. Given that it was tested on a log scale there is some variability between all of the values, but the most effective concentration tested came to be the A9 (100μM) + TMZ (3mM). This was a lesser concentration than the 300μM initially tested, which leads to the conclusion that A9 at a lesser concentration is more effective than at larger ones (300μM<x). By determining the concentration range that this specific drug works best, future testing could be done to help narrow the specific range down even further.

When studying the Tyrosine Kinase Inhibitors in the primary drug screen there were over seventy drugs beings tested on the *Drosophila* glioma model. The need to conduct this blind study on the variety of different tyrosine kinase inhibitors was due to each activating a different protein pathway in order to exert their effect on the tumor. Looking at the results it was clear to see that each inhibitor tested did not show the same results, but instead showed a range of suppression and enhancement on the glioma. These results allowed for it to be further confirmed that although a category of treatment drugs have the same end result (i.e. adding a phosphate group), they do not activate the same pathways to get there. This activation of different pathways allows for different effects to be placed on the tumors which creates a wide variety of choices to choose in treatment of an individual suffering glioblastoma. To narrow down which protein pathways are

specifically activated in the successful suppression tyrosine kinase inhibitors tested, Western Blotting must be run. Western Blotting will be able to show the exact proteins that are activated in the pathway, and these results can be compared to other drugs that also use the same pathway. This comparison will allow for a quicker selection of drugs to be tested and a connection between a wider range of chemotherapy drugs.

Finally, given that this was a primary drug screen to test the initial effects of both tyrosine kinase inhibitors and TMZ on glioblastoma, there is further testing needing to be done to determine the overall effectiveness on other model organisms. The results of these combinatorial results showed success in our *Drosophila* glioma model, which leaves the question open if these results can be replicated in other systems. These combinations found to be successful in reducing the tumor in the fruit fly are worth testing in other organisms (i.e. mice). Mice are a helpful model in determining the success a drug would have in humans due to them sharing 85% of its genomes with humans and their genes being able to be added or removed easily for further testing (Why Mouse Matters, 2010). If the results are replicated in a different organism's system, then more credibility is added to the ability of the drug to act as a suppressor and therefore would show more promise to continue testing into clinical trials. Clinical trials are the last in drug testing to determine the overall success. If our combinatorial chemotherapy treatment makes it to clinical trials and continues to show suppression in human models, this shows great promise and confirms the success of this treatment in suppression glioblastoma tumors.

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