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GSK-3 inhibitors in glioblastoma therapy: mechanisms of action

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Thesis

**GSK-3 INHIBITORS IN GLIOBLASTOMA THERAPY:
MECHANISMS OF ACTION**

by

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DEDICATION

I would like to dedicate this work to my parents, Edmund and Kathleen, for all their love, support, and sacrifice for all my endeavors, academic and otherwise. I could not have asked for better people to aspire to be.

I would also like to dedicate this work to my sister, Rachel, who is the most amazing and inspiring young woman. Baby sister, I can't wait to see what you become.

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GSK-3 INHIBITORS IN GLIOBLASTOMA THERAPY:

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MEGHAN VICTORIA HANDLEY

ABSTRACT

Glioblastoma multiforme (GBM) is the most malignant form of brain cancer. Therapies targeting glioblastoma have not consistently been able to give those diagnosed the best prognosis. Treatments that directly infiltrate into the tumor are highly sought after. Indirubins have been used to treat various types of cancers and are a promising avenue for future glioma research. In the current study, we further researched several key GSK-3 inhibitors, BIO (an indirubin) and CHIR99021, in addition to LiCl, to see their effects on the translocation of β -catenin to the nucleus, and the invasion and migration of cells in both a sphere assay and an aortic ring assay.

Here we studied anti-invasive therapies that may have a future role in GBM treatment. It is thought that combining conventional treatments with anti-invasive therapies will create cytotoxicity in and reduce migration of the tumor. Three types of cells were used throughout the experiments: HBMEC, HUVEC, and U251 glioma cells. We reported that GSK-3 inhibitors might have a valuable role in the treatment of GBM. The selected inhibitors (BIO, CHIR99021, and LiCl) all were shown to lessen cell migration and invasion *in vitro* in a range of assays and in all cell lines tested. All inhibitors tested cause a dose-dependent, reversible inhibition of glioma cell invasion in spheroid assays. BIO was shown to cause a rapid upregulation of total and nuclear β -

catenin. BIO, at higher concentrations, also created a toxic environment for cells, sometimes killing them. This shows that a more in-depth experiment involving different BIO concentrations is needed to test the optimal concentration for treatment.

Each of the experimented GSK-3 inhibitors also showed a change in the junctions between cells. NaCl as a control showed normal, spikey, junctions, while CHIR99021 and BIO caused the junctions to become more smooth. This suggests that GSK-3 inhibition has a role in either maintaining the ECM and/or in communication between cells. Also in this assay, there was a heterogeneity between cells treated with the same inhibitor and in the same dish, indicating that not all cells respond to each drug the same way. The reasons for this are not known and further investigation is required. A new construct was also made to report β -catenin transcriptional coactivation using luciferase expression as the reporter in response to these selected GSK-3 inhibitors. With the combined results of these experiments, we concluded that GSK-3 inhibitors may be a promising approach to the treatment of GBM. Further investigation is required before any treatments can be administered to those diagnosed.

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LIST OF ABBREVIATIONS

ABTA.....	American Brain Tumor Association
BBB	Blood-Brain Barrier
BIO.....	6-Bromoindirubin-oxime
CGH	Comparative Genomic Hybridization
CNA	Copy Number Alterations
CNS.....	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computed Tomography
DMSO.....	Dimethylsulfoxide
DPBS	Dubecco's Phosphate Buffered Saline
ECM.....	Extracellular Matrix
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
GBM	Glioblastoma Multiforme
GSK-3	Glycogen Synthase Kinase-3
HBMEC	Human Bone Marrow Endothelial Cell
HSV-1	Herpes Simplex Virus-1
HUVEC.....	Human Umbilical Vascular Endothelial Cell
IDH1	Isocitrate Dehydrogenase 1
IO	Indirubin 3'-oxime
LiCl.....	Lithium Chloride

MAPK	Mitogen-Activated Protein Kinase
MGMT	O6-methylguanine-DNA Methyltransferase
miR	microRNA
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
mTOR	Mammalian Target of Rapamycin
NaCl	Sodium Chloride
OSU	Ohio State University
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphoinositide 3 Kinase
PKA	Protein Kinase A
PTEN	Phosphatase and Tensin Homolog
Rb	Retinoblastoma
TCGA	The Cancer Genome Atlas
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

INTRODUCTION

1. GLIOBLASTOMA: THE MOST AGGRESSIVE OF PRIMARY BRAIN TUMORS

Glioblastoma Multiforme (GBM, World Health Organization Grade IV) is a neoplasm of the central nervous system (CNS) and is the most malignant form of brain cancer. GBM is a primary malignant brain tumor accounting for 80% of all high-grade primary CNS cancers, 12-16% of all intracranial neoplasms, and comprising more than half of all malignant gliomas (Ray, 2010). GBM is diagnosed in approximately 10,000 individuals in the United States each year. It has a higher incidence in males and in the Caucasian population, and typically affects those aged between 65 and 75, with the median age of diagnosis at 64 years. However, GBM is seen in all age groups including the pediatric population, although less frequently (Louis et al., 2007; Ray, 2010; Schwartzbaum, Fisher, Aldape, & Wrensch, 2006). After diagnosis, patients suffering with GBM typically have a median survival of 12 to 14 months with the current standard of care consisting of maximal surgical resection followed by concomitant radiation and temozolomide treatment (Stupp et al 2005). If diagnosed at an age younger than 44 years, however, patients typically have almost a 30% two-year survival rate (Ray, 2010). GBM is characterized by the presence of mitotic cells, necrosis and/or angiogenesis and by biological hallmark features: “uncontrolled cellular proliferation, diffuse infiltration, propensity for necrosis, robust angiogenesis, intense resistance to apoptosis, and rampant genomic instability” (Furnari et al., 2007).

1.1. Tumor Grading And Pathology

Although GBM is considered a malignant astrocytoma, its precise histogenesis remains unclear (Huse & Holland, 2010). Astrocytomas can be diffuse or localized and are graded by the World Health Organization (WHO) according to their histopathological features of the presence of mitotic cells, along with necrosis and/or angiogenesis. Malignant astrocytoma (grade III) has mitoses but necrotic regions and high levels of angiogenesis are not seen. Grade II astrocytoma is less aggressive with rare mitotic cells. The survival of patients with lower grade astrocytomas is correspondingly longer than patients with GBM, and can be over ten years. However, progression of these tumors is inevitable, and patients eventually succumb to GBM. This progressive GBM is called secondary GBM and accounts for approximately 5% of all cases. The remainder, which present as GBM at diagnosis, are known as primary GBM (Louis et al., 2007). Figure 1 (Preusser, et al., 2011) shows the relative frequencies of the different types of glioma.

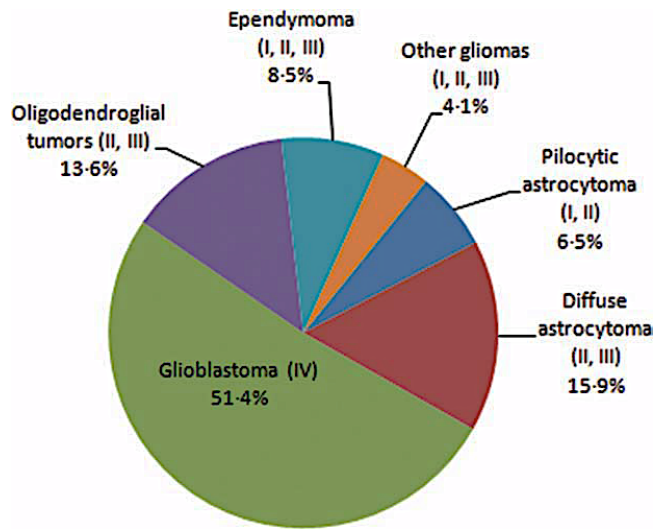


Figure 1. Relative Frequencies of Gliomas. WHO grades are given in parentheses. Grades III and IV represent high-grade gliomas (that have a worse prognosis), while grades II and I represent lower grade gliomas (better prognosis). Figure taken from Preusser et al., 2011.

The majority of GBM tumors are supratentorial (cerebral). A major problem in these tumors is that the tumor is not well delineated from the adjacent brain tissue. This is because the tumor cells infiltrate into the nearby brain parenchyma. This prevents complete surgical resection, and leads to inevitable recurrence.

The term “multiforme” is used to describe the various appearances on cross section: the tumor appears grayish white, the necrotic regions appear yellowish, and often there is a red-brown discoloration from a previous hemorrhage. Microscopically, the individual cells of GBM have a hyperchromatic nucleus and are of various size and shapes. The periphery of the tumor contains infiltrating tumor cells that are able to colonize healthy adjacent brain tissue. These infiltrating cells make complete surgical resection impossible because of the lack of delineation between the tumor and the normal

tissue (Ray, 2010). After surgical resection, approximately 90% of patients with GBM develop a recurrent tumor adjacent to the original. Satellite lesions have also been shown to occur nearby the location of the resected tumor (Lefranc, 2005). Figure 2 (Preusser et al., 2011) shows the neuropathology of GBM using slides that demonstrate its microscopic features.

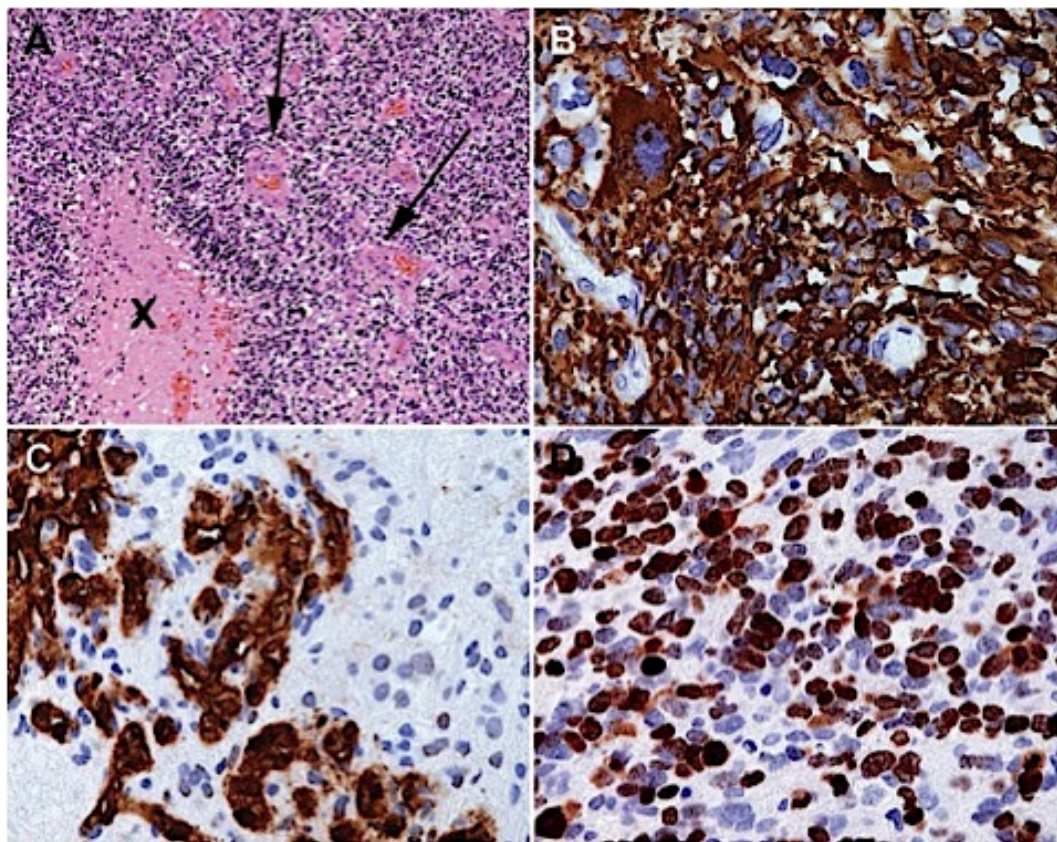


Figure 2. Neuropathology of Glioblastoma. (A) X marks cellular glial tumor tissue with central necrosis, arrows mark nuclear pseudopalisading and microvascular proliferates (H&E staining). (B) Immunostaining for glial fibrillary acidic protein (GFAP), tumor cells appear brown. (C) Immunostaining for CD34 showing glomeruloid microvascular proliferates. (D) Immunostaining for Ki67 (cell-cycle related antigen), shows tumor cells undergoing mitosis as brown. (Figure taken from Preusser, et al., 2011.)

1.2. Possible Causes

There are many hypotheses regarding the causes of the different types of glioma. A few rare hereditary syndromes, such as neurofibromatosis (neurofibromin mutations), tuberous sclerosis, and Li-Fraumeni syndrome (TP53 mutations) are associated with GBM accounting for a few percent of total cases. Other potential links include an inverse association with allergies (Schwartzbaum et al., 2006). The only known environmental cause of GBM is exposure to high doses of ionizing radiation (Preusser et al., 2011). There is a higher incidence in the United States and Europe than in Japan, suggesting that there may also be underlying environmental factors in the development of glioma tumors (Preusser et al., 2011; Schwartzbaum et al., 2006). One of the most controversial areas in brain tumor etiology is the effect of cell phone use on tumor incidence. Although some studies have been published supporting the idea that brain tumor incidence is linked to the use of cell phones, other larger studies did not establish a link. However, the National Brain Tumor Society recommends that cell phone use should be limited in children (Hardell & Carlberg, 2015).

1.3. Symptoms And Genetics

Symptoms of GBM include headache, nausea, vomiting, and drowsiness, which may be due to increased intracranial pressure as a result of tumor growth. The location of the tumor within the brain can also determine additional symptoms, for example, speech difficulties, vision changes, hemiparesis, seizures, and changes in personality and cognitive behavior (American Brain Tumor Association [ABTA], 2014).

Brain tumor cells arise from a transformation process that involves amplification or overexpression of oncogenes. This, combined with a loss or under expression of tumor suppression genes (p53, retinoblastoma [Rb], p16, p15, and PTEN [phosphatase and tensin homolog]), leads to the formation of tumor cells. Examples of oncogenes involved include platelet-derived growth factor (PDGF) and its receptor, epidermal growth factor (EGF) and its receptor, CDK4, mdm-2, Ras, Akt, and the mammalian target of rapamycin (mTOR). The combination of these two events leads to an imbalance between mitosis and apoptosis (Ray, 2010).

It is known that GBM tumors can be of two major subtypes: primary GBM (*de novo*) or a secondary GBM, which is a progression from a lower-grade astrocytoma (II or III) and only accounts for about 5% of glioblastomas (Yan et al., 2009). Primary GBMs are more common in older patients, while secondary GBMs occur more frequently in those patients under the age of 45 years (Furnari et al., 2007). While the isocitrate dehydrogenase 1 (IDH1) mutation is the defining feature of secondary GBM, TP53 mutation is theorized to be the initiating event, present in 60% of the tumors that eventually evolve to be GBM (Ohgaki & Kleihues, 2007). EGFR gene amplification occurs in about 40% of all GBM tumors, and the genes that are usually rearranged (Furnari et al., 2007; Louis et al., 2007). Recent investigations have concluded that primary and secondary GBMs have very different transcriptional patterns and different recurrent DNA copy number aberrations (Furnari et al., 2007). Patients with primary GBM have a shorter median survival than those with secondary GBM. With recurrence of these gliomas, “less than 50% of patients will survive more than a year” (Ray, 2010).

The genetics of GBM are now very well characterized due to the Cancer Genome Atlas (TCGA). In this effort, which started in 2006, approximately 500 patient tumor samples have been analyzed for chromosomal alterations, mutations, and gene expression. This showed that GBM is a heterogeneous disease, with no two tumors containing the same mutations. However, the majority of mutations occur in similar pathways: tyrosine kinase receptor signaling (often via EGFR amplification or mutation), cell cycle deregulation (through mutation of Rb signaling), and cell survival (through alterations in p53). Interestingly these efforts and others (Yan et al., 2009) revealed that the great majority secondary GBMs have a novel and distinct mutation in the gene encoding (IDH1). The most common mutation in IDH1 is R132H, which changes the biological activity of IDH1. This leads to the accumulation of 2-hydroxyglutarate within tumors, and this is thought to lead to epigenetic changes that drive tumor growth. IDH mutation tests could also become of use to distinguish between lower grade astrocytomas and those of a higher grade (Yan et al., 2009). A summary of the most common mutations is shown in Figure 3 (McLendon, et al., 2008).

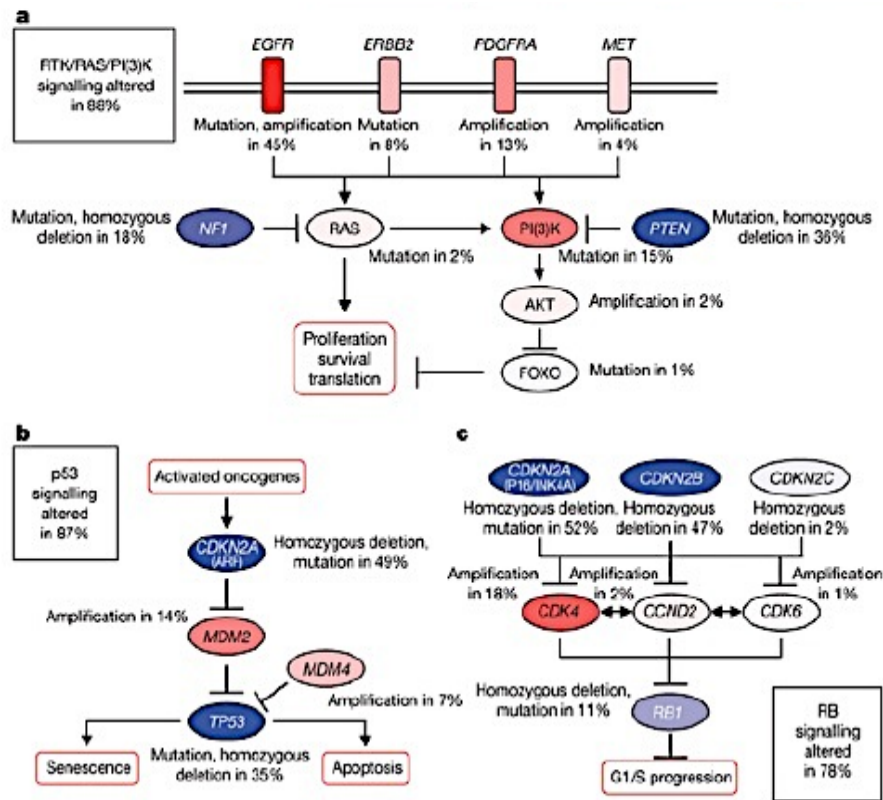


Figure 3. Core Pathway Alterations in GBM. Figure shows examples of pathway alterations in GBM. The top panel shows RTK/RAS/PI3K signaling, which is altered in 88% of GBM cases. The bottom left shows P53 signaling, which is altered in 87% of GBM cases, while the bottom right shows Rb signaling, which is altered in 78% of cases. The three panels together conclude that multiple alterations can happen in each GBM case. (Figure taken from McLendon et al., 2008.)

Comparative genomic hybridization (CGH) profiling of both primary and secondary glioblastomas has shown patterns of copy number alterations (CNA; alteration in a genome that results in an abnormal variation in the number of copies in a section of DNA). Coupling array CGH with additional genomic techniques has allowed more investigation into the identities and functions of oncogenes and tumor suppressor genes

that may have roles in the formation and progression of malignant gliomas (Furnari et al., 2007; Huse & Holland, 2010). These techniques also allow correlation CNA patterns with tumor subtypes and clinical outcome (Maher et al., 2006).

1.4. Tumor Diagnosis

An MRI (magnetic resonance imaging, shown in Figure 4) or CT (computed tomography) scan is needed after a thorough neurological exam is performed to obtain an accurate determination of the presence of a brain tumor. The initial clinical presentation is highly variable and depends on the size and location of the GBM tumor (Preusser et al., 2011). Sometimes, physicians will also perform a magnetic resonance spectroscopy (MRS) scan to measure chemical and mineral levels within the tumor. This scan can give clues to whether the tumor is malignant or benign. After these scans, an exact diagnosis to the type of tumor requires surgery or a biopsy to obtain a sample of the tumor and a thorough examination of the tumor histology using a microscope (Preusser et al., 2011; ABTA 2014).

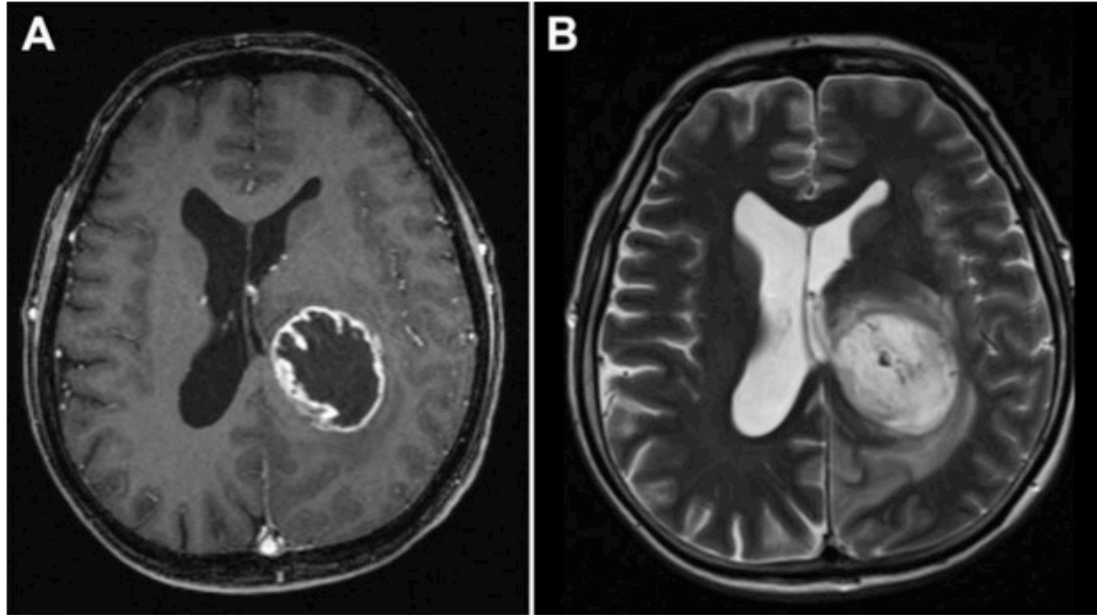


Figure 4. MRI of Glioblastoma. (A) Glioblastoma tumor showing irregular peripheral ring-like contrast enhancement in the left thalamic region. **(B)** MRI showing heterogeneous hyper-intense mass surrounded by hyper-intense signal extending along the adjacent white matter indicating tumor extension and edema. (Figure taken from Preusser et al., 2011.)

Macroscopically, the lesions usually involve more than one lobe of the brain. By crossing the corpus callosum, a white matter tract linking the right and left cerebral hemispheres, both sides of the brain could be affected by tumor growth, a phenomenon called a butterfly GBM.

1.5. Tumor Invasion: A Major Challenge

Cell migration is an important aspect of the development and evolution of GBM. It involves three biologic processes: adhesion, motility, and invasion. Adhesion consists

of both the adhesion of the cells with the extracellular matrix (ECM) and cell-cell adhesion. It has been shown that glioma cells can make and deposit modified ECM components (i.e. laminin) that help the cells to move within the brain. Integrins, cadherins, and lectins are also important adhesion molecules that help glioma cells interact with ECM components (Lefranc, 2005). A loss of cadherin expression is shown to promote metastasis in epithelial tumors, but little is known of the effect on GBM cells or astrocytes. N-cadherin can bind with β -catenin, which allows the cadherin to bind with the actin cytoskeleton. The expression of both N-cadherin and β -catenin may be involved in astrocytic tumors (Lefranc, 2005). Another study observed that in malignant gliomas, decreased levels of N-cadherin were associated with an increased probability of tumor invasion and transport via cerebrospinal fluid (CSF) (Asano et al., 1997). The motility of the cells is made possible by the reorganization of the actin cytoskeleton. This reorganization can happen because of the polarity of the cells and by actin polymerization. Invasion involves the degradation of matrix proteins by proteolytic enzymes secreted by the tumor (i.e. matrix metalloproteinases) (Lefranc, 2005).

1.6. Current And Future Prospects For GBM Treatment

One of the current strategies to treat GBM and other gliomas is to decrease the burden of the disease by detecting genetic, environmental, and developmental influences to the risk of having a glioma. Treatments used in the past and present include surgical removal, radiation, chemotherapy, and gene therapy, however while these have an effect on prognosis, they do not show a sufficient cure. Surgery followed by radiation therapy

with concomitant temozolomide is now the standard of care for patients diagnosed with GBM (Stupp et al 2005). The blood-brain barrier (BBB) and blood-tumor barrier create obstacles for drugs to even reach the tumor itself. Although the blood-brain barrier is somewhat disrupted in most malignant gliomas, it commonly remains intact in the borders where recurrent neoplasm tend to occur (de Vries, Beijnen, Boogerd, & van Tellingen, 2006). A few of the ideas currently being investigated to overcome the obstacle of the BBB are increasing the dosage of the drug (although this brings in the problem of toxicity), conjugating the drug with antibodies, peptides, or viruses, packaging drugs in liposomes or micelles, and direct infusion of the drug into the tumor via a catheter (Huse & Holland, 2010).

1.6.1. Radiation Therapy

Radiation therapy has been shown to improve the survival rate of those patients suffering from malignant gliomas (Ray, 2010) and is currently an important part of GBM treatment (Roger Stupp et al., 2005). A study including newly diagnosed GBM patients showed a significant improvement in survival when radiation was combined with temozolomide chemotherapy and followed by adjuvant temozolomide. Temozolomide is an oral drug that has shown antitumor activity when treating patients with primary gliomas. Giving temozolomide on a continuous schedule with radiotherapy allowed researchers to administer low, daily doses with an increase in dose intensity without the corresponding increase in toxicity. Temozolomide is an alkylating agent that depletes O6-methylguanine-DNA methyltransferase (MGMT), an enzyme that may be instigated by

radiotherapy and is needed to repair damage caused by alkylating agents like temozolomide. A low level of MGMT in tumor tissue seems to be associated with a longer survival (Hegi et al., 2005). By methylating the MGMT promoter, gene silencing is observed and is correlated with a survival benefit not seen with radiotherapy alone. In this study, the treatment group had an increased median survival of 14.6 months, an increase in survival of 2.5 months when compared with the control group (12.1 months), a relative reduction in the risk of death of 37 percent. After two years, the treatment group had an increase in survival from 10% (radiotherapy alone) to 26.6% (radiotherapy combined with temozolomide) (Roger Stupp et al., 2005).

1.6.2. *Viral Therapy*

Several viruses have shown oncolytic properties and will have a lasting effect in cancer therapy. Examples of these include, but are not limited to, adenovirus, herpes simplex virus-1 (HSV-1), and retrovirus. Oncolytic viruses can further be used to increase the effectiveness of tumor killing by incorporating therapeutic transgenes. Selection for tumors can be improved by using glioma-selective promoters to drive the expression of viral proteins or transgenes. Current investigations regarding transgenes include pro-drug activating (suicide) genes, intracellular signaling molecules, immune modulators, and inhibitors of angiogenesis and cell invasion (Lawler, Peruzzi, & Chiocca, 2006). Viral infection is designed so viruses will replicate in tumor cells and avoid normal cells. They should destroy the tumor by using a variety of mechanisms, for example, direct oncolysis, inductions of an anti-tumor immune response, destruction of

tumor vasculature, and cancer cell starvation. One of the most broadly studied viruses in glioma therapy is recombinant HSV with a deletion in the ICP34.5 gene, which makes the virus especially neuro-virulent (Kaufmann & Chiocca, 2014). Studies using oncolytic HSV-1 deleted in ICP34.5 showed a toleration, with two out of 21 patients living beyond four years after treatment (Rampling et al., 2000).

1.6.3. Signaling Pathways

Various cell-signaling pathways have been reviewed regarding targets for cancer therapy. Oncogenes, tumor suppressors, and apoptotic regulators all contribute to gliomagenesis, thereby giving multitudes of potential therapeutic targets (Lawler et al., 2006). One of these is the phosphoinositide 3 kinase (PI3K)/Akt pathway. The PI3K/Akt pathway is commonly upregulated in brain tumors, as a result of the excessive stimulation by growth factor receptors and Ras. GBM tumors have a mutation in the PTEN tumor suppressor gene in around 40% of primary glioblastomas (Lefranc, 2005; Miyashita et al., 2009). Normally, this gene will have an inhibitory effect on the activation of protein kinase B signaling, which is dependent on PI3K. PTEN has a role in angiogenesis inhibition. A mutation in PTEN will activate the PI3K pathway, a pathway correlated with an increased tumor grade, decreased apoptosis, and a worse clinical prognosis (Lefranc, 2005). It has also been suggested that GBMs with increased EGFR expression are more likely to have a mutation in the PI3K/Akt pathway (Choe et al., 2003; Narita et al., 2002). The multitude of data regarding this pathway implies that irregular PI3K/Akt signaling causes cell proliferation in glioma cells and causes atypical

cell migration due to the downregulation of the PTEN tumor suppressor (Lawler et al., 2006). With this irregularity, chemotherapy and other cytotoxic insults become ineffective as the tumor cells gain a resistance. Additionally, inhibition of the PI3K/Akt pathway (for example, with BKM120) returns and may strengthen the effectiveness of chemotherapy on the glioma cells (Shingu et al., 2003). Another study shows that migrating glioma cells have “increased amounts of phosphorylated Akt and its downstream substrate glycogen synthase kinase-3 as compared with migration-restricted cells” (Joy, 2003).

Another related pathway with promising potential for cancer therapy is the TOR pathway. Rapamycin is an inhibitor of mTOR, while mTOR is a direct target of the PI3K/Akt signaling pathway. Rapamycin has a role in the stoppage of cells from the G1 to S phase of mitosis. It inhibits the phosphorylation of the retinoblastoma protein, which commonly occurs in GBM cells as a result of p16 gene (tumor suppressor) inactivation (Sekulić et al., 2000). mTOR has also been shown to have a role in cell migration in GBM as well as a role in the induction of autophagy. These results show that “inhibition of mTOR activity represents a possible means to combat apoptotic-resistant migrating GBM cells” (Lefranc, 2005).

1.6.4. *Anti-angiogenic Therapy*

Glioblastoma multiforme is highly vascularized, making it a promising candidate for anti-angiogenic therapy. Recent experiments have shown that mutations involving the PTEN and EGFR genes may act as a switch for the initiation of angiogenesis. Tumor

microvessels tend to have poor architecture, meaning that the endothelial cells fail to make tight junctions and “have few pericytes and astrocytic foot processes, leaving the integrity of the BBB compromised” (Furnari et al., 2007). This can result in increased interstitial edema, decreasing local blood flow and leading to tumor hypoxia, microhemorrhages and, eventually, areas of necrosis (Furnari et al., 2007). Because angiogenesis is required by tumors growing larger than 2-3 millimeters in diameter, anti-angiogenic therapy has a place in current investigations. Vascular endothelial growth factor (VEGF), a highly pro-angiogenic molecule, is the most widely researched and has shown disruption of signaling when negative VEGF is virally delivered (Lawler et al., 2006). Currently, there are three approaches to target VEGF signaling pathways: monoclonal antibodies directed against VEGF or its receptor(s), small molecule inhibitors of VEGFR-2 tyrosine kinase activity, and soluble decoy receptors created from VEGFR1 receptor that selectively inhibit VEGF. Early clinical approaches to anti-angiogenic therapy have yielded modest results. Radiation and chemotherapy given in combination with an anti-angiogenic therapy has shown some benefits, although it is unknown if this is due to the effects of the anti-angiogenic therapies (i.e. improved drug delivery because of improved vascular flow, improved drug infiltration into the tumor because of reduced interstitial pressure, or improved radiation/chemotherapy response because of reduced tumor hypoxia) that increase the concentration of the drug within the tumor (Lawler et al., 2006).

Avastin (bevacizumab) is a monoclonal anti-VEGF antibody that has showed remarkable effects on imaging and promotion of survival in recurrent GBM. However,

two major trials in 2013 failed to show any survival benefit (Chinot et al., 2014). Thus, Avastin remains a useful tool, but it is not known how best to use it (Chi, Norden, & Wen, 2007).

1.6.5. *MicroRNAs*

An additional emerging connection has formed between gliomagenesis and microRNA (miR) biology. MiRNAs are single-stranded non-coding RNA molecules of around 20-22 nucleotides in length. Some microRNAs have appeared to act as oncogenes, called oncomirs. These may function by suppressing tumor suppressor genes, allowing the formation of a tumor (Lawler & Chiocca, 2009). It has also been suggested that microRNAs allow for continued tumor growth through the maintenance of the stem cell-like properties of tumor cells (Godlewski, Newton, Chiocca, & Lawler, 2010). MicroRNAs can regulate multiple targets; theorizing that microRNA therapy could offer an alternative to highly selective pathway inhibitors (examples mentioned previously) by targeting multiple gene networks (Lawler & Chiocca, 2009). For example, “it has recently been shown that amplification of miR-26a-2 leads to the overexpression of miR-25a in 12% of glioblastomas, promoting gliomagenesis through direct repression of PTEN, Rb, and MAP3K2” (Huse & Holland, 2010). Many microRNAs have already been identified that downregulate the levels of proteins in pathways related to tyrosine kinase signaling in GBM, for example, miR-21, miR-27, and miR-124 (Lawler & Chiocca, 2009). miR-7 has been shown to suppress EGFR expression and to inhibit the Akt pathway. In one study, miR-7 transfection decreased viability and invasiveness of

primary GBM cell lines (Kefas et al., 2008). miR-21, miR-124, and miR-128 (which each play a role in neural differentiation) have also been shown to be expressed more frequently in GBM compared with the normal brain, displaying a possible similarity between glioma cells and neural stem cells (Godlewski et al., 2010). In the near future, miRNA-based regulation of numerous pathways involved in gliomagenesis should allow for additional possible therapies. MicroRNAs could also be used as potential biomarkers for diagnosis of GBM and treatment monitoring (Lawler & Chiocca, 2009).

1.6.6. Future Prospects for Treatment and Therapy

Future glioma research should focus on the role of immune factors in the development of glioma tumors. Genetic and environmental factors should be taken further into consideration within future studies (Schwartzbaum et al., 2006). New treatments should also have more targeted mechanisms of action. Small molecule and antibody-based molecular therapeutics have a promising future in the treatment of GBM (Ray, 2010). Additionally, particular inhibitors should only be chosen if the target is present in the tumor tissue, requiring individualized treatment or molecular profiling based on the patient (O'Rourke, 2004). Unfortunately, targeting specific pathways may pose challenges of their own, as some are not fully understood and may have more functions than currently known. Transgene expression within these pathways may cause unforeseen results and effects. Using oncolytic vectors that can propagate throughout the tumor also poses a possible treatment (Lawler et al., 2006).

2. GSK-3 INHIBITORS AS POTENTIAL ANTI-INVASIVE CANDIDATES FOR GLIOBLASTOMA TREATMENT

Glycogen synthase kinase 3 (GSK-3), a serine-threonine protein kinase, has long been known as a key enzyme in glycogen metabolism, but is now known to have a role in various cellular functions. Examples of these functions include, but are not limited to, signaling by insulin, growth factors and nutrients, the fate of cells during embryonic development, cell division, apoptosis, and microtubule function (Cohen & Frame, 2001). There are two isoforms of GSK-3, GSK-3 α and GSK-3 β . Having so many functions, GSK-3 inhibitors have become a promising idea for the treatment of several types of cancers, as well as a therapy for diabetes and Alzheimer's disease (Cohen & Frame, 2001). Past research has concluded that GSK-3 has a role in cell proliferation and tumor promotion, with an overexpression in colon, pancreatic, liver, and ovarian tumors. GSK-3 has been shown to confer resistance to chemotherapy, radiation, and other targeted therapies, therefore targeting GSK-3 may reduce this resistance and improve patient prognosis (McCubrey et al., 2014).

GSK-3 is one of the enzymes that can phosphorylate glycogen synthase, but, unlike other kinases, GSK-3 inhibits the action of glycogen synthase rather than activating it. The three-dimensional structure of GSK-3 is very similar to that of mitogen-activated protein kinase (MAPK) (Cohen & Frame, 2001). GSK-3 can be inhibited in response to the phosphorylation of one serine residue in its structure (S9). Kinases that are known to phosphorylate GSK-3 β include protein kinase A (PKA) and Akt, among several growth factors, including EGF and PDGF (McCubrey et al., 2014).

GSK-3 has a role in migration-associated pathways, including Wnt (explained further below), Notch, growth factor, and G-protein coupled receptor signaling (Williams et al., 2011). GSK-3 is regulated by many upstream signals and can function independently in multiple pathways, suggesting that while GSK-3 inhibition may have effects on tumor invasion, it may also have oncogenic properties (Nowicki et al., 2008).

GSK-3 may function as a tumor suppressor in some tumor types by suppressing the Wnt/ β -catenin pathway. Wnts have a specific function in a pathway involved in the fate of cells during embryonic development, and their targets include a GSK-3 that can be complexed to β -catenin (a proto-oncoprotein). β -catenin is stabilized by GSK-3, giving GSK-3 β oncogenic properties (McCubrey et al., 2014). β -catenin is targeted for ubiquitination and proteosomal degradation after being phosphorylated by GSK-3. This complex is disturbed during Wnt signaling, which prevents GSK-3 from phosphorylating β -catenin. When β -catenin is not phosphorylated, it is no longer targeted for degradation and will increase its transcription, and will have a prominent role in tumor invasion and proliferation. This suggests that GSK-3 plays a role in glioma migration through the regulation of β -catenin, although other pathways may contribute. Unfortunately, since GSK-3 inhibitors mimic the Wnt signaling pathway, they could become oncogenic with prolonged use (Cohen & Goedert, 2004). An overview of the Wnt signaling pathway involving GSK-3 is shown in Figure 5 (McCubrey et al., 2014).

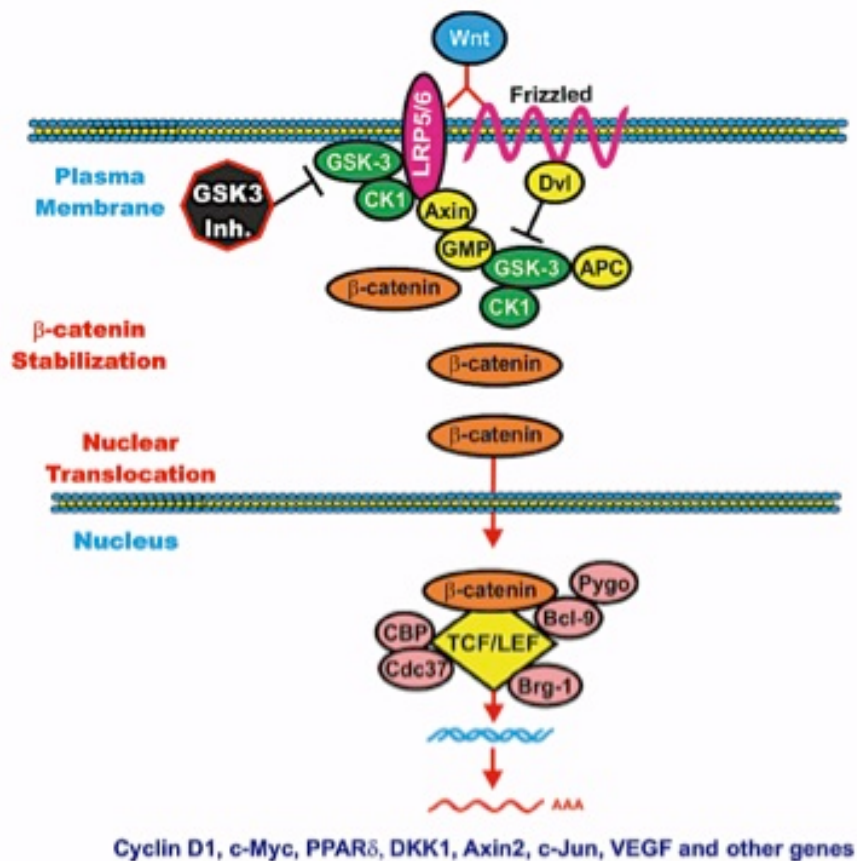


Figure 5. Wnt/β-catenin as Modulated by GSK-3. If Wnt is present, β-catenin is stabilized and can induce gene transcription. Wnt binds its co-receptors Frizzled and LRP5/6. Frizzled is shown as a squiggly line (as it is a transmembrane receptor) and LRP5/6 are shown as an oval. Yellow ovals represent various molecules that interact with the receptors. In the present of Wnt, β-catenin is stabilized and complexes with TCF/LEF (yellow diamond). Pink circles represent various proteins that can interact with the transcription factor complexes. (Figure taken from McCubrey et al., 2014.)

One study (Miyashita et al., 2009) showed that GSK-3β inhibition provides several benefits: reduced tumor proliferation and protection of brain tissue from degradation. Healthy brain tissue shows less expression of GSK-3β, making the neoplastic tissue more susceptible to inhibition. The same study found that GSK-3β

inhibition induced the expression of p53 and p21, two known tumor suppressor genes, in two different cell lines harboring wild-type p53. GSK-3 β also decreased the levels of Rb phosphorylation and activation. Thus, inhibition of GSK-3 β decreases proliferation and survival and increases apoptosis in GBM cells via an activating Rb-mediated pathway. CDK6 seems to have a role in regulation in the proliferation of these cells. Therefore, further investigation is necessary to determine the role of GSK-3 β in CDK6 activity. As a result of this study, it is thought that using GSK-3 β inhibitors will increase the radiosensitivity of GBM tumors to the combination of temozolomide and chemotherapy through decreases in Rb phosphorylation and CDK6 expression (Miyashita et al., 2009).

Another study showed that GSK-3 β phosphorylates PTEN (a tumor suppressor gene mentioned in section 1.3), which causes it to be destabilized. Inhibition of GSK-3 β allowed PTEN to regain stability in GBM cells, and helped to increase p53 activity and thereby cell cycle arrest (Miyashita et al., 2009). A third study by Williams et al. (2011) involving GSK-3 showed that β -catenin played a role in facilitating the effects of GSK-3 on glioma cell motility. This study showed that, among other things, β -catenin knockdown leads to less cell-cell adhesion and that β -catenin phosphorylation increases during a glioma cell migration assay.

One example of a GSK-3 inhibitor is lithium, which has already been used for some time in the treatment of bipolar disorder. Lithium also has effects on metabolism, neuronal communication, and cell proliferation and development, all of which are cell-type- and dose-dependent (Nowicki et al., 2008). A study by Nowicki et al. (2008)

showed that lithium chloride (LiCl) caused a near complete blockage of cellular invasion and slowed cell proliferation in glioma spheroids.

Indirubins, another example GSK-3 inhibitors, have been traditionally used by the Chinese Academy of Medicine for the treatment of certain types of leukemia (Kim, Shin, Kang, & Park, 2011). They have been shown to provoke cell cycle arrest at the G2/M phase by inhibiting GSK-3, among other things. Indirubins have a role in anti-angiogenic mechanisms that prevent tumor growth and invasion (Kim et al., 2011). Various indirubin derivatives have been examined, including 6-bromoindirubin-oxime (BIO), BIA, and indirubin 3'-oxime (IO). In one study (Williams et al., 2011), a rapid reduction of the phosphorylation of β -catenin was seen after treatment with BIO, but not with lithium chloride (LiCl), showing that BIO leads to a stronger GSK-3 inactivation than lithium and a decrease in cell migration. The same study concluded that indirubins are more effective at blocking migration than other inhibitors examined previously, improving survival. These data show that indirubins are viable candidates from which to base further anti-angiogenic and anti-invasive therapies for gliomas (Williams et al., 2011). BIO and other indirubins are very non-selective and inhibit many kinases, also making them valuable contenders for GBM therapy.

2.1. Research Question

Studies on the effects of these various GSK-3 inhibitors in tumors have shown diverse effects (e.g., anti-angiogenesis, promotion of cell death, and inhibition of cell motility). In this study, we aimed to further understand these effects by examining the

effects of a panel of GSK-3 inhibitors (BIO, CHIR99021, LiCl) on glioma cells and endothelial cells *in vivo*. As a second part of this study we also developed a glioma cell line stably expressing a luciferase gene downstream of the β -catenin promoter. This allowed a direct readout of GSK-3 inhibition in glioma cells, and will be useful for *in vivo* drug delivery studies in the Lawler Laboratory.

METHODS

1. BIOLOGICAL EFFECTS OF GSK-3 INHIBITORS

1.1. Cell Culture

Human glioblastoma cell lines U251 and G9 (#1062047 prepared on 10/12/06) were obtained from American Type Tissue Collection (Rockland, MD) and Ohio State University (OSU), respectively. Human tissue was procured using established procedures as defined in OSU tissue procurement facility protocols approved by the Institutional Research Board. HBMEC and HUVEC cells were obtained from Sciencell (Carlsbad, CA). U251 cells were maintained in DMEM supplemented with 10% fetal bovine serum. G9 cells were maintained in Neurobasal medium (Life Technologies, Grand Island, NY) supplemented with b27, EGF (epidermal growth factor), and FGF (fibroblast growth factor) and without Vitamin A. EGF and FGF were given at 10ng/mL and were obtained from Peprotech (Rocky Hill, NJ). HBMEC were cultured in Endothelial Cell Medium (Sciencell) supplemented with 10% fetal bovine serum and 5mL of endothelial cell growth supplement. HUVEC were cultured in Endothelial Cell Medium (Sciencell) supplemented with 10% fetal bovine serum and 5mL of endothelial cell growth supplement. All cells were kept at 37°C while in culture.

1.2. Immunohistochemistry

HUVEC, HBMEC, and U251 were all cultured on 8-chamber Lab-Tek glass slides. The cells were treated with selected GSK-3 inhibitors (LiCl, 20mM; BIO, 2uM;

CHIR99021, 2uM) and controls (NaCl, 20mM; and DMSO) and allowed to incubate overnight. Cells were washed once with cold DPBS (Dubecco's Phosphate Buffered Saline, Life Technologies). Cells were then fixed in 4% paraformaldehyde solution and washed with DPBS supplemented with 0.1% Triton X-100 (Sigma-Aldrich) solution. Blocking was carried out using with normal donkey serum (non-immunized, from Jackson ImmunoResearch, West Grove, PA). Slides were then incubated overnight in 4°C with the primary antibody, mouse anti-beta-catenin (1:500) (from Cell Signaling). Next, the cells were washed several times with the DPBS-Triton X-100 solution. Thereafter, a fluorescently labeled secondary antibody (488 nm Alexa Fluor anti-mouse, 1:1000; from Jackson ImmunoResearch) and blue channel DNA stain DAPI (Life Technologies) were applied and the cells were allowed to incubate for six hours. The cells were washed again several times with the DPBS-Triton X-100 solution. A coverslip was applied and images were recorded with a ZEISS LSM710 confocal microscope with appropriate filter settings.

1.3. Adherens Junctions in Live Cells

G9 cells were transfected with pEGFP-C1/beta-catenin using Lipofectamine 2000 (Life Technologies) using established transfection procedures. Individual clones were selected for under Geneticin conditions (Life Technologies). These cells were then seeded (20,000 cells per chamber) on a Nunc Lab-Tek chambered coverglass (Thermo Fisher Scientific Inc.) and imaged using a Nikon TE2000 with an on-stage incubator with a lens magnification of 60X. The cells were then exposed to selected GSK-3 inhibitors

(LiCl, 20mM; BIO, 2uM; CHIR99021, 2uM) and the control (NaCl, 20mM). Images were collected in a time-lapse fashion using appropriate settings.

1.4. Electric Cell-Substrate Impedance Sensing (ECIS)

HBMEC cells were cultured as described above. 25,000 cells were added to each chamber containing a single circular 250 μ m diameter active electrode (ECIS Cultureware Disposable Electrode Arrays, Applied Biophysics). The cells were grown to confluency (about four days). Drugs were added to the chambers immediately before running the ECIS machine (ECIS Z θ , Applied Biophysics, Inc., Troy, NY). Resistance was recorded for 24 hours for adherens junction data and for 20 days for tight junction data. The goal was to obtain a phase of plateau of resistance, which would indicate the maturation of tight junctions.

1.5. Rat Aortic Ring Assay

A Wistar rat was obtained from Charles River Laboratories International, Inc. The rat was killed using a ketamine overdose followed by a cervical dislocation as described in the protocol. The rat's abdominal aorta was extracted and was stripped from the connective tissue. Aortic rings were cut in 0.5mm portions and were embedded in a collagen I (neutralized and supplemented with 5% fetal bovine serum) matrigel mixture (90:10). The embedded rings were overlaid with full-grown medium supplemented with rat-FGF (10ng/mL). NaCl, LiCl, DMSO, CHIR99021, and BIO dilutions adjusted for

final concentration were applied to each well containing the aortic rings. The rings plus drugs were allowed to migrate for six days. Cells were stained by adding calcein AM (0.5mg/mL, Life Technologies). Images were collected in the green channel of the Nikon Ti fluorescent microscope. A 3D Z-stack was collected and collapsed with an increased depth of field.

1.6. HBMEC Sphere Assay

HBMEC were obtained as stated previously. Cells were put in a 96-well round bottom, low attachment plate (Corning, Inc.). They were incubated overnight to reach the desired cell aggregation. The medium was replaced with a neutralized collagen I solution. After polymerization, the gel was overlaid with medium containing the appropriate drugs dilutions (NaCl, 20mM; LiCl, 20mM; DMSO, 0.1%; CHIR99021, 10uM; BIO, 5uM). After 96 hours treatment, images were taken on the Nikon Ti microscope. Outgrowth areas and perimeters were measured.

2. CLONING AND TCF-LEF LUCIFERASE REPORTER ASSAY

The M50 and M51 (M50 - pTA/Super8XTOPflash – b-catenin binding; M51 - pTA/Super8XFOPflash – b-catenin NoBinding) plasmids were provided by Dr. Randall Moon, University of Washington, Seattle, WA. The plasmid DNA was then isolated from DH5 α bacterial cultures. The pTA plasmid provided a sequence of 8xTCF-LEF-TA (TCF-LEF – sequence allowing binding of TCF-LEF transcription factor, TA – minimal eukaryotic promoter) sequence that served as the insert to be cloned into pGL4.14

plasmid (Promega).

The plasmid DNA was isolated from 5mL cultures using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA). In more detail: Bacterial cultures were spun for 10 minutes at 6000 rpm to collect bacterial cells; Pellets were resuspended in resuspension buffer (50mM Tris-HCL, pH 8.0, 10mM EDTA) before being applied to the column; Cells were lysed using an alkaline/SDS procedure (200mM NaOH, 1% w/v SDS) and the lysate was applied to a silica membrane spin column that selectively binds plasmid DNA. Contaminants were removed using Wash Buffer and the plasmid DNA was eluted in preheated TE Buffer (10mM Tris-HCL, pH 8.0, 0.1mM EDTA). A microcentrifuge at room temperature was used for all procedural steps and was spun at >12,000 x g.

The purified pTA plasmids were cut using restriction enzymes HindIII-HF (sticky ends, high-fidelity) and SacI (sticky ends) (both New England BioLabs Inc.). pGL4.14 (obtained from Promega) served as the plasmid vector for new construct. The pGL4.14 plasmid was also cut with HindIII-HF and SacI restriction enzymes.

The digestion mixture consisted of 1 µg of the DNA plasmid obtained from the PureLink Quick Plasmid Miniprep Kit (Invitrogen), 1.0 uL of NEBuffer 2.1 (New England BioLabs Inc.), 0.5 µL of both HindIII and SacI, and 7 µL of deionized water. They were digested in a 37 degrees Celsius water bath for a period of one hour.

Nucleic acids digested were mixed with loading buffer (20 % Ficoll 400, 0.1 M Na₂EDTA, pH 8, 1.0% sodium dodecyl sulfate, 0.25 % bromphenol blue, 0.25 % xylene cyanol) and run on a 1% agarose/ethyl bromide/1xTAE gel. 1ug Ready load 1kb DNA

ladder (Invitrogen) was run as a standard. The gel was run at 5 volts per cm of tank length (150V). The gel was visualized using the BioRad ChemiDoc XRS. The desired vector and desired insert was cut out of the gel and purified using the protocols established in the PureLink Quick Gel Extraction Kit (Invitrogen).

The purified insert vector were ligated using protocols established in the Quick Ligation Kit (New England BioLabs Inc.). Bacteria (DH5-alpha competent E. Coli) were transformed with 1.0 uL of the ligation mixture and plated overnight. Colonies were expanded to 5ml liquid culture and DNA was isolated as established in the PureLink Quick Plasmid Miniprep Kit (Invitrogen). This DNA was cut with the same restriction enzymes (HindIII and SacI as mentioned previously) to determine if our desired DNA fragment had been inserted. The cloning procedure yielded two new DNA constructs named: pGL4.14/Super8XTOPflash or pGL4.14/8xTCF-LEF-TA-b-catenin-Binding and pGL4.14/Super8XFOPflash or pGL4.14/8xTCF-LEF-TA-b-catenin-NO-Binding.

The amount of plasmids DNA was scaled up as established in the PureLink HiPure Plasmid Filter Maxiprep Kit (Life Technologies). Using Lipofectamine 3000 reagent, the plasmids were introduced into U251 glioma cells. The mixture of stable clones was selected using Hygromycin B (150 ug/mL, Life Technologies). The U251/pGL4.14/8xTCF-LEF-TA-b-catenin-Binding cells were plated in 96-well white wall plates, 10,000cells/well, in 84 wells.

The chosen GSK-3 inhibitors, BIO and CHIR99021 were used in seven dilutions (serial two times dilution from 10uM). Each dilution was done in triplicate, with a 6-hour incubation period to allow TCF-LEF-TA-dependent accumulation of Luciferase. The

U251/pGL4.14/8xTCF-LEF-TA-b-catenin-No-Binding cells were used as a negative control, reflecting basal (very low) level of luciferase translation from the TA minimal eukaryotic promoter. Medium was removed, and cells were lysed using the Steady-Glo Luciferase Assay System (Promega), which also provides a luciferin substrate. Light output from luciferase directly reflects promoter activity; signal was determined by measuring luciferase levels in a Fluostar Optima plate reader (BMG Labtech, Durham, NC).

RESULTS

As described in the introduction, small molecule GSK-3 inhibitors have been previously shown to have potentially beneficial effects *in vitro* and in animal models of GBM. In order to further understand the potential roles of GSK-3 inhibition in GBM we performed two sets of experiments. First we studied cultured GBM cells and endothelial cells in order to gain insight into the effects of these inhibitors on the behavior of these cells, with particular emphasis on the GSK-3 substrate β -catenin. Second, we created a GBM cell line carrying a genetic reporter to enable the visualization of GSK-3 inhibition *in vivo* by luciferase expression.

1. BIOLOGICAL EFFECTS OF GSK-3 INHIBITORS

1.1. Immunohistochemistry

β -catenin is a one of the best studied substrates of GSK-3, and is involved in the regulation of cell junctions, and also in regulating gene transcription programs. However, its response to GSK-3 inhibitors has not been well studied in GBM or endothelial cells. Therefore, in this experiment we used immunostaining techniques to investigate the upregulation of β -catenin after employing three GSK-3 inhibitors (BIO, CHIR99021, and LiCl) in three cell lines (HBMEC, HUVEC, and U251 glioma cells) (Figures 6-8). Controls used were NaCl and DMSO. Cells were fixed and stained for β -catenin 24 hours after drug treatment. There was an up-regulation of total and nuclear β -catenin in cells treated with all three GSK-3 inhibitors. The endothelial cell samples had a greater total β -catenin upregulation than the glioma cells. LiCl is previously known as an effective

GSK-3 inhibitor, and this experiment shows that BIO is even more potent while CHIR99021 has the greatest ability to induce β -catenin accumulation.

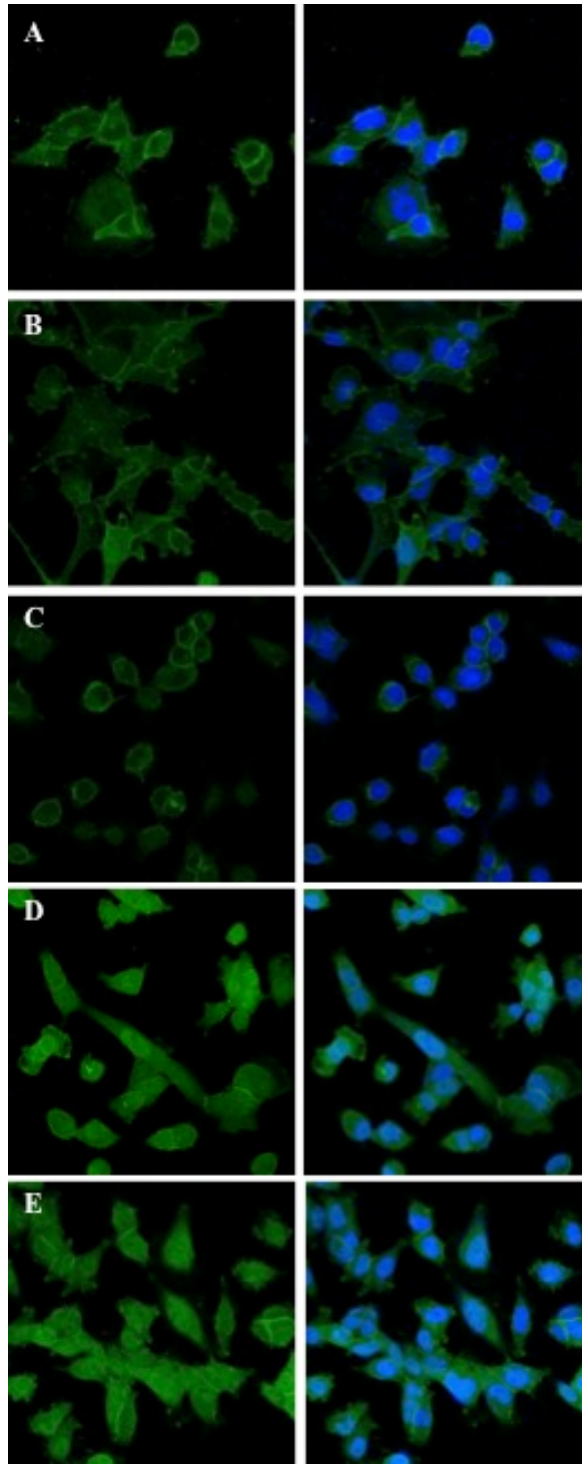


Figure 6. U251 Glioma Cell Immunohistochemistry. Figure shows more up-regulation of total and nuclear β -catenin as compared with endothelial cells (Figures 7 and 8). **(A)** DMSO, 0.1% **(B)** NaCl, 20mM **(C)** LiCl, 20mM **(D)** BIO, 2 μ M **(E)** CHIR99021, 2 μ M.

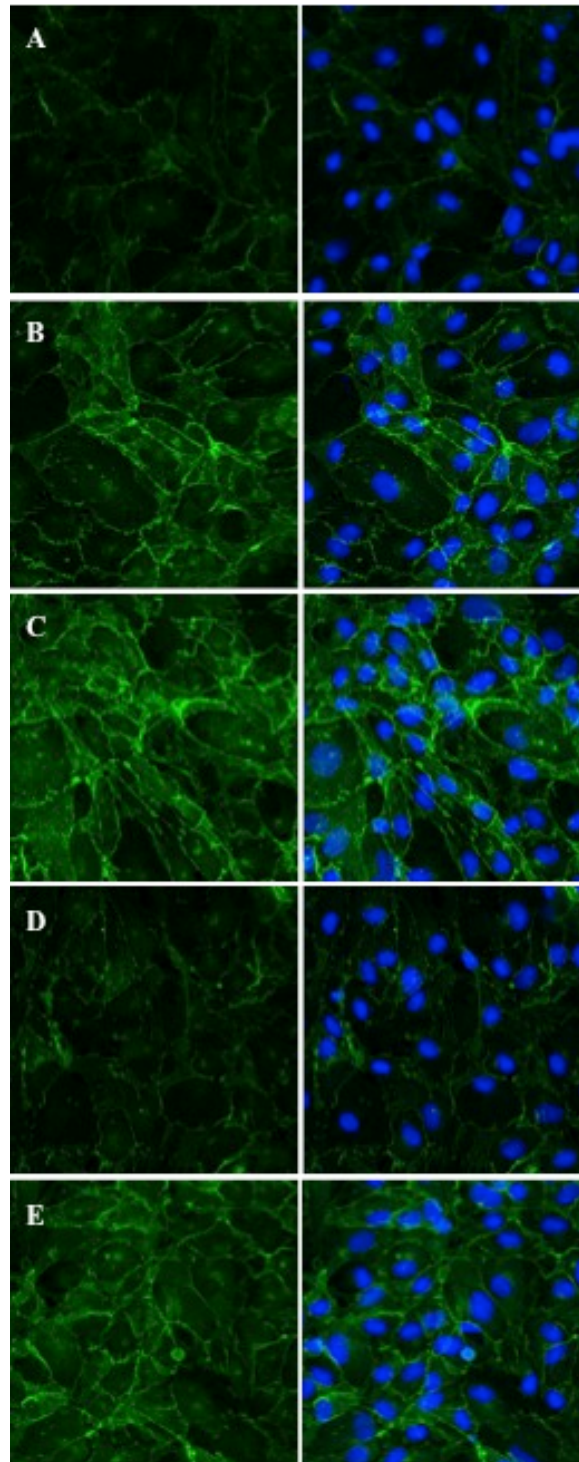


Figure 7. HUVEC Immunohistochemistry. Figure shows less up-regulation of total and nuclear β -catenin as compared with glioma cells (A) DMSO, 0.1% (B) NaCl, 20mM (C) LiCl, 20mM (D) BIO, 2 μ M (E) CHIR99021, 2 μ M.

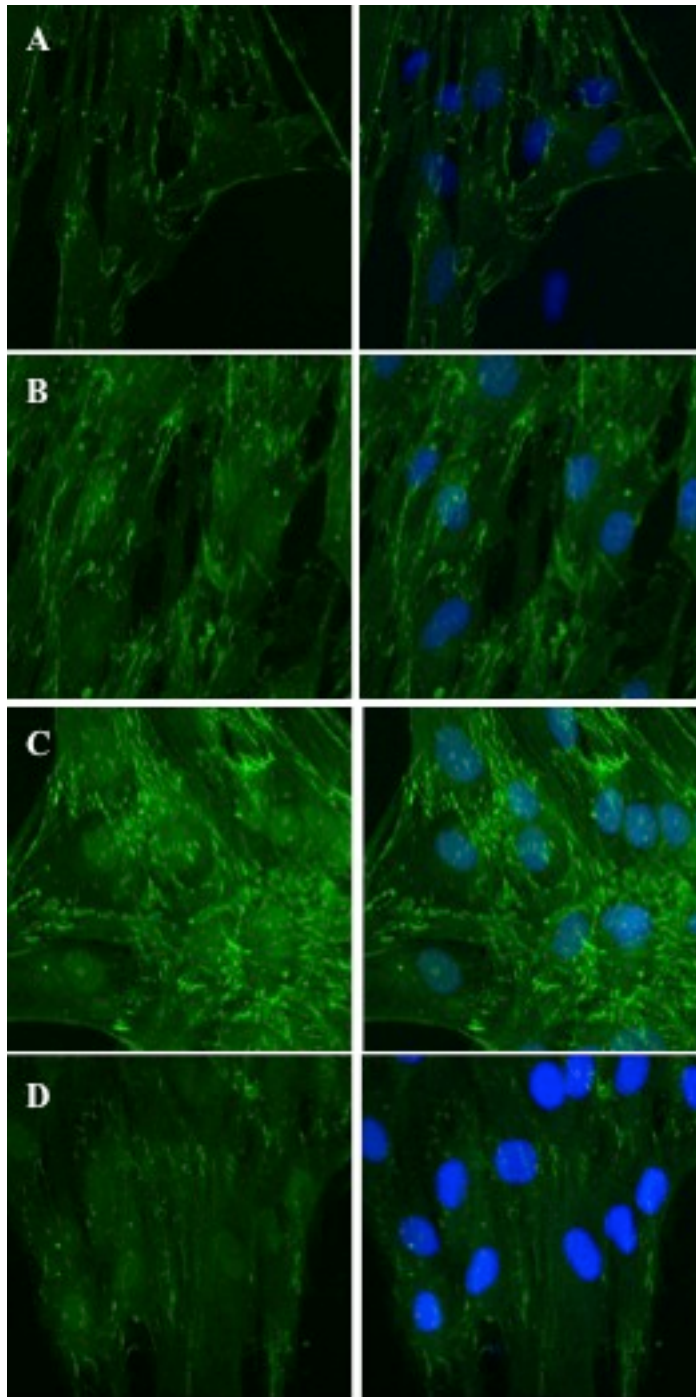


Figure 8. HBMEC Immunohistochemistry. Figure shows less up-regulation of total and nuclear β -catenin as compared with glioma cells (A) DMSO, 0.1% (B) NaCl, 20mM (C) LiCl, 20mM (D) BIO, 2 μ M.

1.2. Adherens Junctions in Live Cells

An adherens junction is a cell junction whose cytoplasmic face is linked to the actin cytoskeleton. Because we wanted to observe the changes in the cell-to-cell junction appearance in glioma cells after being treated with GSK-3 inhibitors, junctions were visualized using EGFP- β -catenin fusion protein in G9 glioma cells. This result was not observed in immunostaining of other cell lines. As shown in Figures 9 and 10, NaCl as a control showed fine, spikey cell-to-cell junctions in the cell membranes. LiCl led to an expected increase in the translocation of β -catenin to the nucleus as seen in other cells. Both CHIR99021 and BIO showed a total upregulation and nuclear translocation of β -catenin, in addition to the cell-to-cell junctions becoming more smooth as compared to the spikey control NaCl. There was a heterogeneous response to both drugs between cells in the same dish, indicating that not all cells in the sample responded to the drugs in the same way.

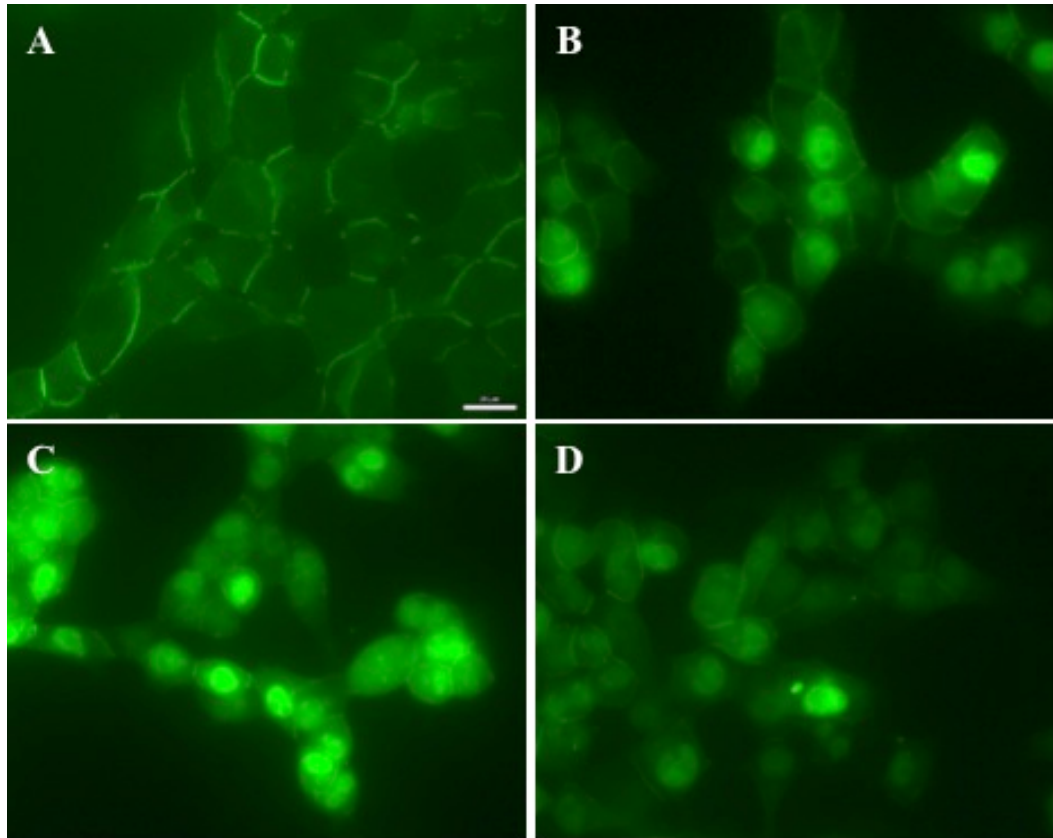


Figure 9. Selected G9 Clones After 24-Hour Treatment With Drugs. Smoother junctions are seen with the BIO and CHIR99021 samples, while LiCl showed a translocation of β -catenin to the nucleus. **(A)** NaCl, 20mM **(B)** LiCl, 20mM **(C)** BIO, 2 μ M **(D)** CHIR99021, 2 μ M.

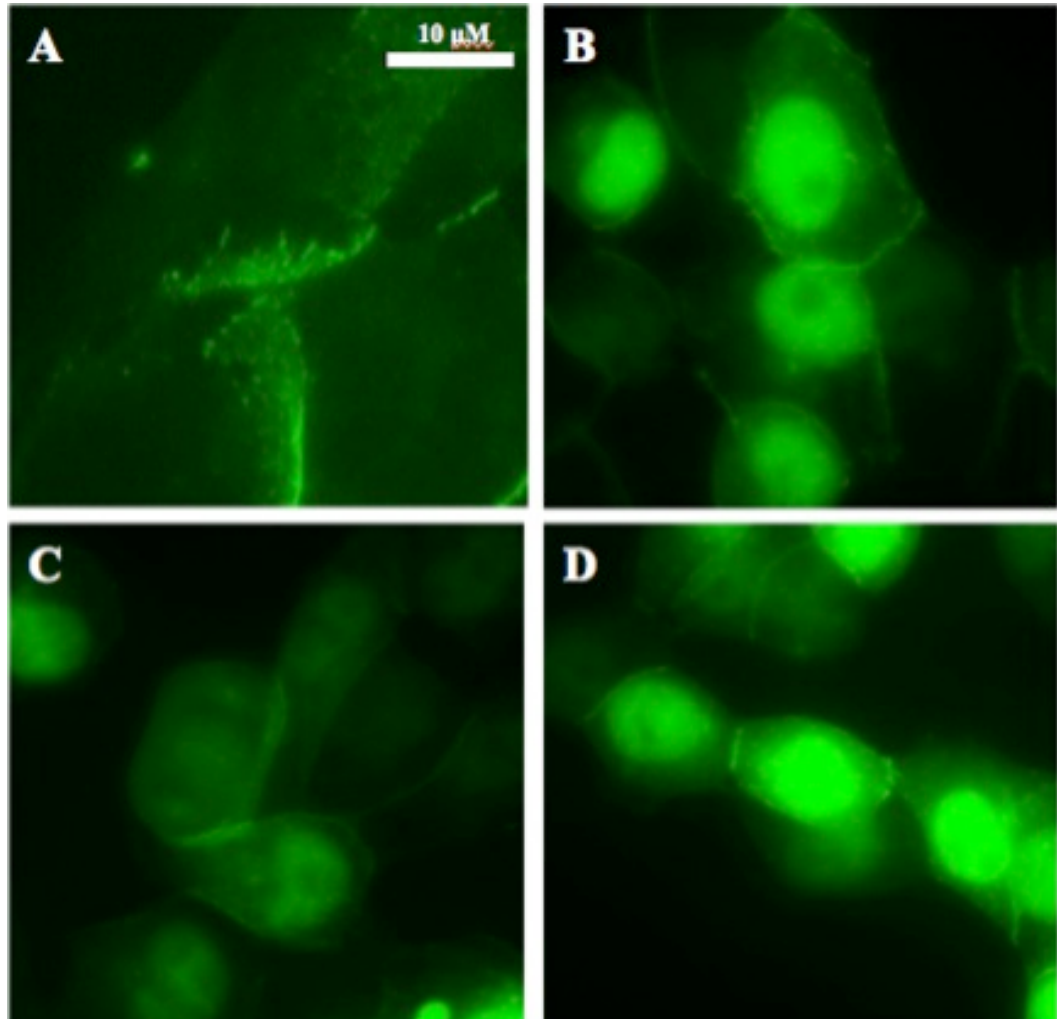


Fig 10. Tight Junctions in Selected G9 Clones After 24-Hour Treatment With Drugs. Smoother junctions are seen with the BIO and CHIR99021 samples, while LiCl showed a translocation of β -catenin to the nucleus. (A) NaCl, 20mM (B) LiCl, 20mM (C) BIO, 5 μ M (D) CHIR99021, 10 μ M.

1.3. Electric Cell-Substrate Impedance Sensing (ECIS)

Another method which allows the assessment of cell-cell interaction is electrical cell-substrate impedance sensing (ECIS). In this technique the resistance to electrical flow created by a cell monolayer is measured using a slide with electrodes on the underside. Because we wanted to see the change in membrane permeability in HBMEC after treatment with GSK-3 inhibitors, we performed an experiment using ECIS, measuring cell layer resistance to current at 4,000 Hz. Both BIO and CHIR99021 were found to decrease the integrity of the cell monolayer and reduce resistance (Figure 11A). The top graph shows the data for monolayer of cells that was formed over 24h period, we expected that the cells did not have enough time to form mature tight junctions; therefore these results show adherens junction permeability and resistance. The bottom graph (Figure 11B) shows the data after a longer period of time (20 days) when the cells were able to form a mature monolayer (maturation of tight junctions was represented by stable and non-increasing resistance of the monolayer over period of at least 24 hours); therefore these show tight junction permeability and resistance. Both BIO and CHIR99021 show a significant decrease in resistance over time, while NaCl, LiCl, and DMSO each only have a slight reduction associated with changing condition of culture medium.

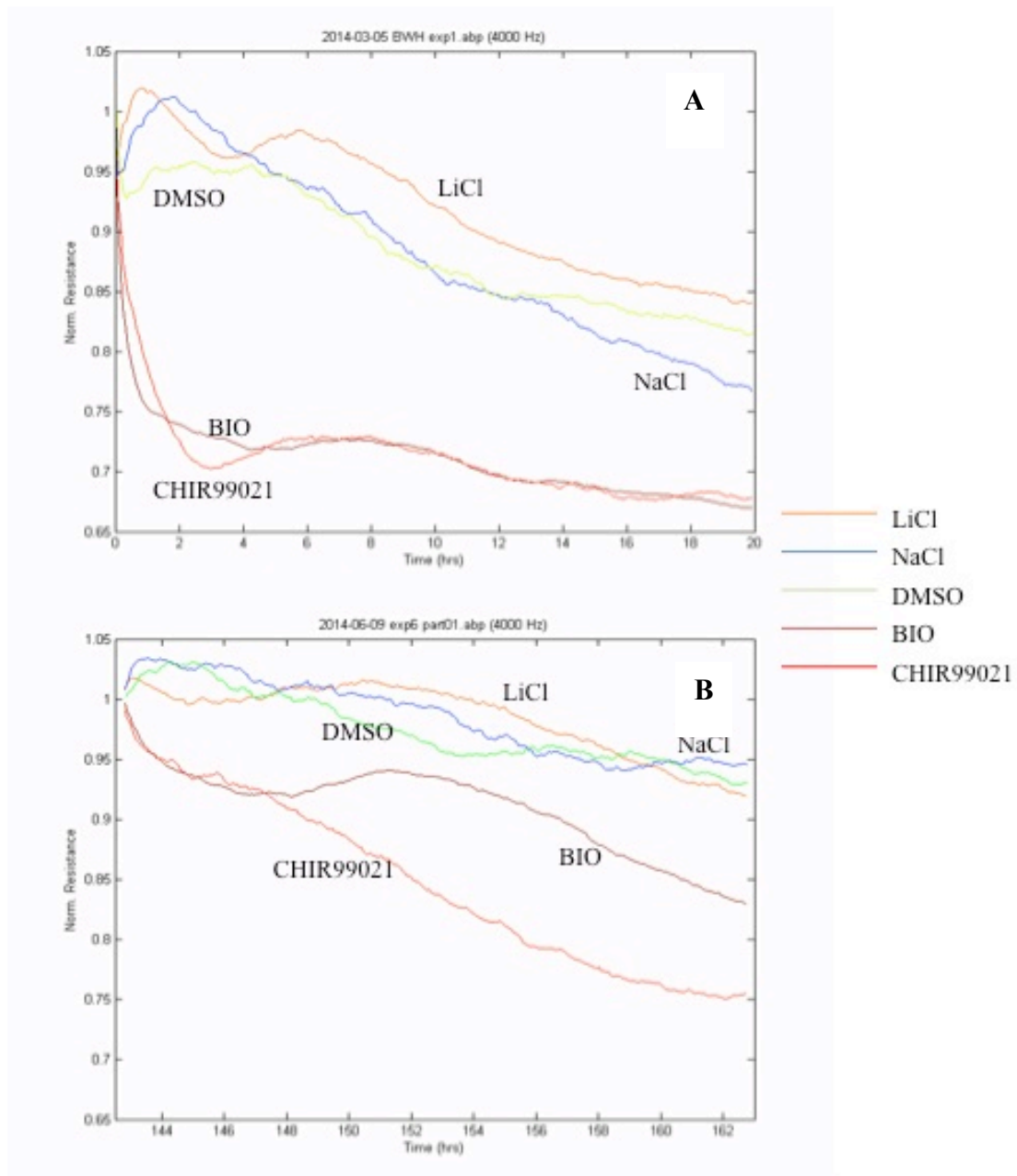


Figure 11. ECIS Graphs Measuring Normalized Resistance of HBMECs. (A) Cells did not form a mature monolayer, data show adherens junctions. (B) Mature monolayer was formed, data show tight junctions. (NaCl, 20mM; LiCl, 20mM; 0.1% DMSO; CHIR99021, 10 μ M; BIO, 5 μ M).

1.4. Rat Aortic Ring Assay

In this experiment, we wanted to see the effects of the GSK-3 inhibitors on the migration of endothelial cells from rat aortic rings (Figures 12 and 13). The controls NaCl and DMSO gave an expected result that showed the most migration as compared with the drugs used. LiCl showed the least amount of migration (approximately 2 million μm^2), while CHIR99021 and BIO showed slightly more (approximately 7 million μm^2 and 4 million μm^2 , respectively).

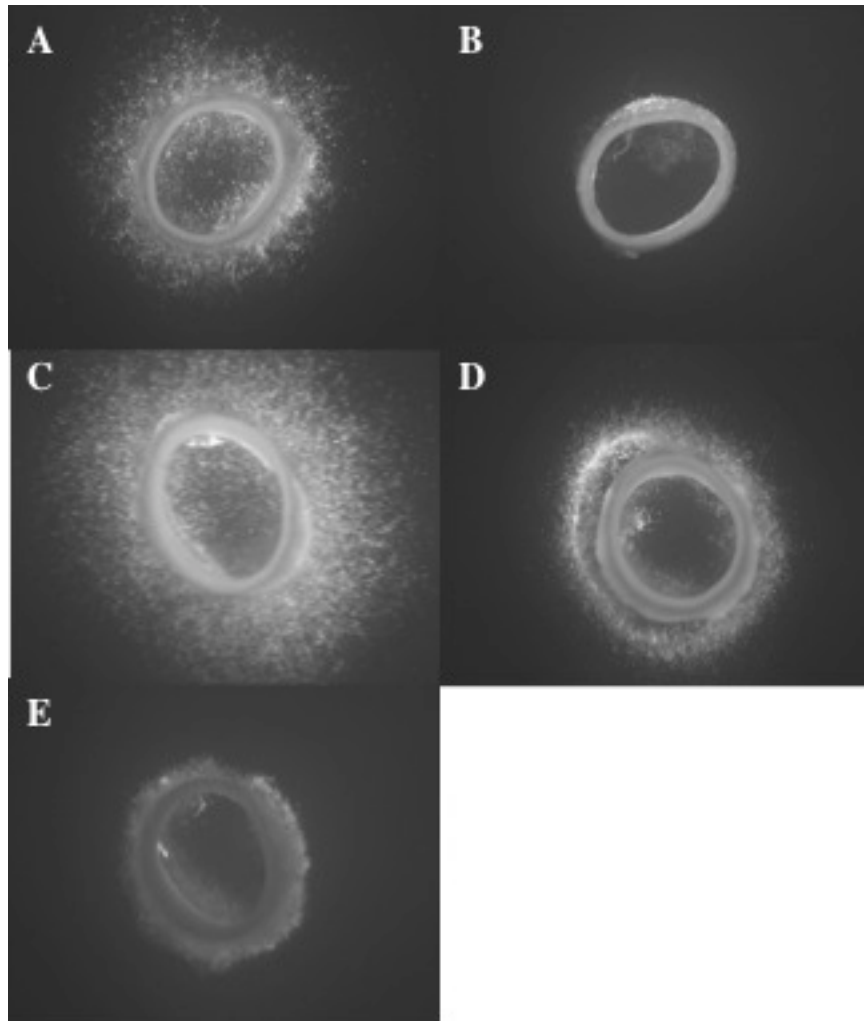


Figure 12. Rat Aortic Ring Assay. LiCl stopped all migration almost completely, while CHIR99021 and BIO slowed it. DMSO and NaCl showed no stoppage of migration. **(A)** NaCl, 20mM. **(B)** LiCl, 20mM. **(C)** DMSO. **(D)** CHIR99021, 10µM. **(E)** BIO, 5µM.

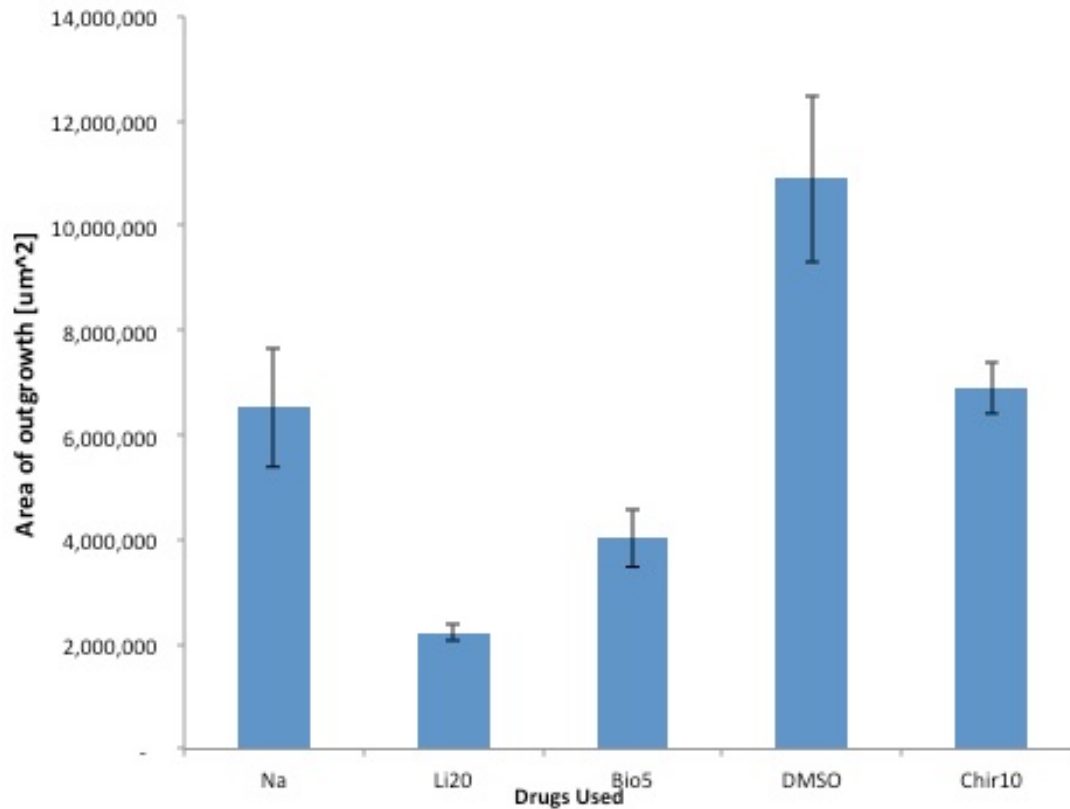


Figure 13. Outgrowth Area in GSK-3 Inhibitor Treated Rat Endothelial Aortic Rings.

1.5. HBMEC Sphere Assay

Because we wanted to see the extent of the invasion of cells after treatment with GSK-3 inhibitors, we performed a sphere assay. In this experiment, HBMEC cells were cultured and treated with the drugs and their invasion was measured using area and perimeter of the sphere and invasive zone. As expected, NaCl and DMSO showed the most invasion of cells, while the drugs LiCl, BIO, and CHIR99021 showed reduction.

The perimeter of the NaCl and DMSO treated spheres were the most, also suggesting a high rate of invasion. CHIR99021 did not limit invasion as much as LiCl. The spheres treated with BIO showed a mild toxicity, making these results somewhat inconclusive.

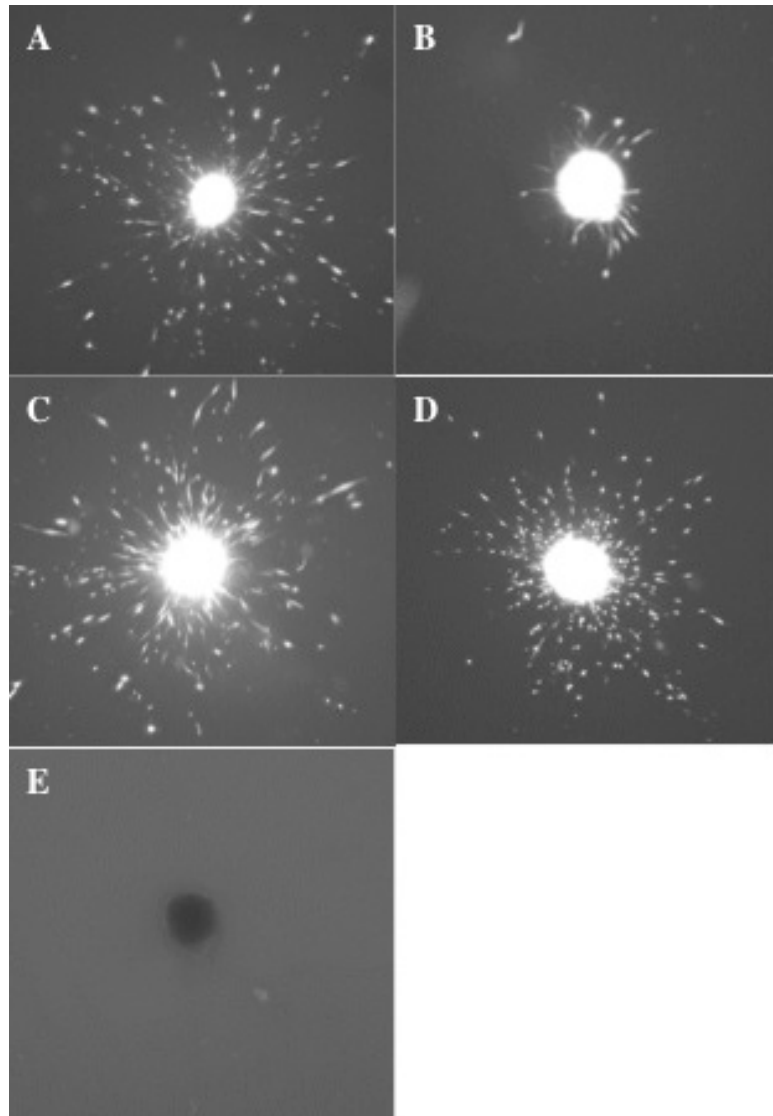


Fig 14. Sphere Outgrowth in GSK-3 Inhibitor Treated HBMECs. BIO showed a toxicity, killing most all cells, while CHIR99021 showed a reduction in invasion. NaCl and DMSO showed no decrease in invasion. **(A)** NaCl, 20mM. **(B)** LiCl, 20mM. **(C)** 0.1% DMSO. **(D)** CHIR99021, 10µM. **(E)** BIO, 5µM.

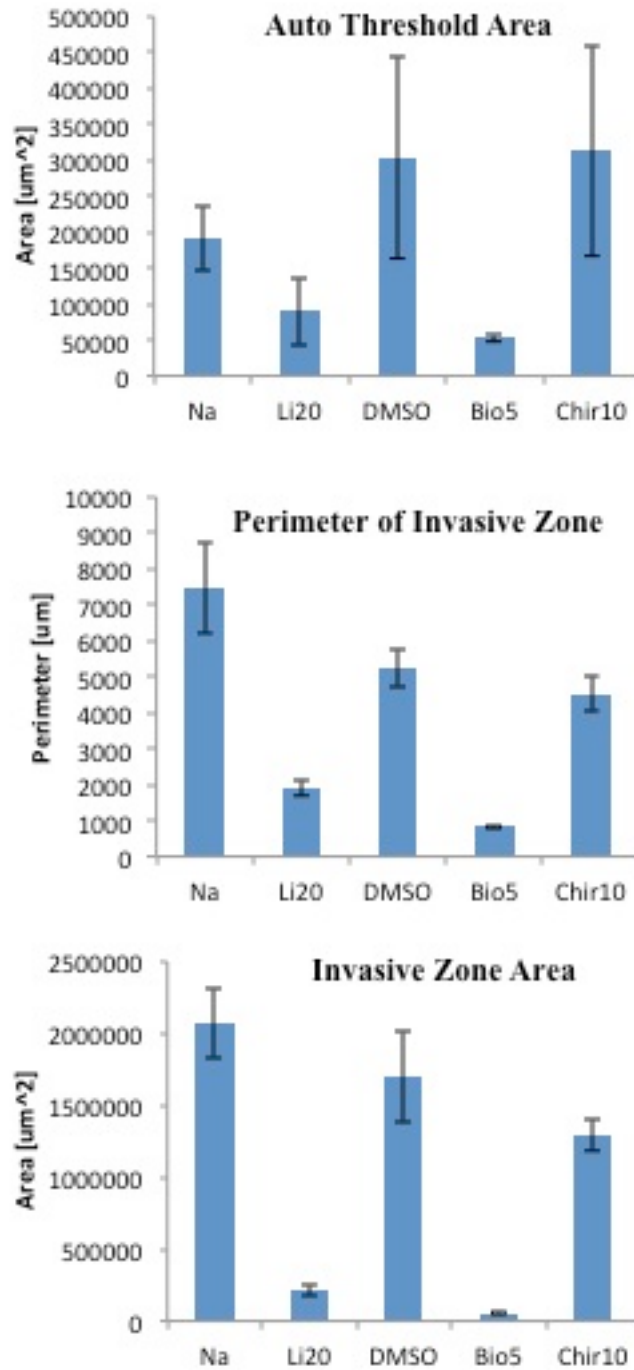


Figure 15. Quantitative Data for HBMEC Sphere Outgrowth. (Top) Auto-Threshold Area of HBMEC Spheres After GSK-3 Inhibitor Treatment. **(Middle)** Perimeter of HBMEC Spheres After GSK-3 Inhibitor Treatment. **(Bottom)** Area of HBMEC Spheres After GSK-3 Inhibitor Treatment.

2. DEVELOPMENT OF A GSK-3 INHIBITOR REPORTER CELL LINE

2.1. Cloning and TCF-LEF Luciferase Reporter Assay

For cloning, we used a plasmid vector and a desired insert (see Methods 2: Cloning and TCF-LEF Luciferase Reporter Assay) to create a new construct containing luciferase, which was used as a reporter of β -catenin accumulation. We used the TCF/LEF reporter plasmid (pGL4.14/8xTCF-LEF-TA- β -catenin-binding) as a reporter of β -catenin transcriptional coactivation in response to GSK-3 inhibition. In order to determine the level of GSK-3 inhibition in drug-treated glioma cells, we assayed the activity of a β -catenin responsive luciferase reporter plasmid in GSK-3 inhibitor treated U251 cells. We were unable to transfect HUVEC and HBMEC cells with reporter plasmid in levels sufficient enough to observe β -catenin up-regulation. The luciferase signal was measured 6 hours after treatment with drugs. GSK-3 inhibition at 5 μ M BIO showed the highest level of luciferase signal. At concentration 10 μ M, the signal was less, suggesting BIO toxicity. CHIR99021 has the highest luciferase signal at 10 μ M, with a steady decrease at lower concentrations. The β -catenin transcriptional co-activation was observed at concentration as low as 0.66 μ M. Background signals are shown in gray for error/comparison purposes.

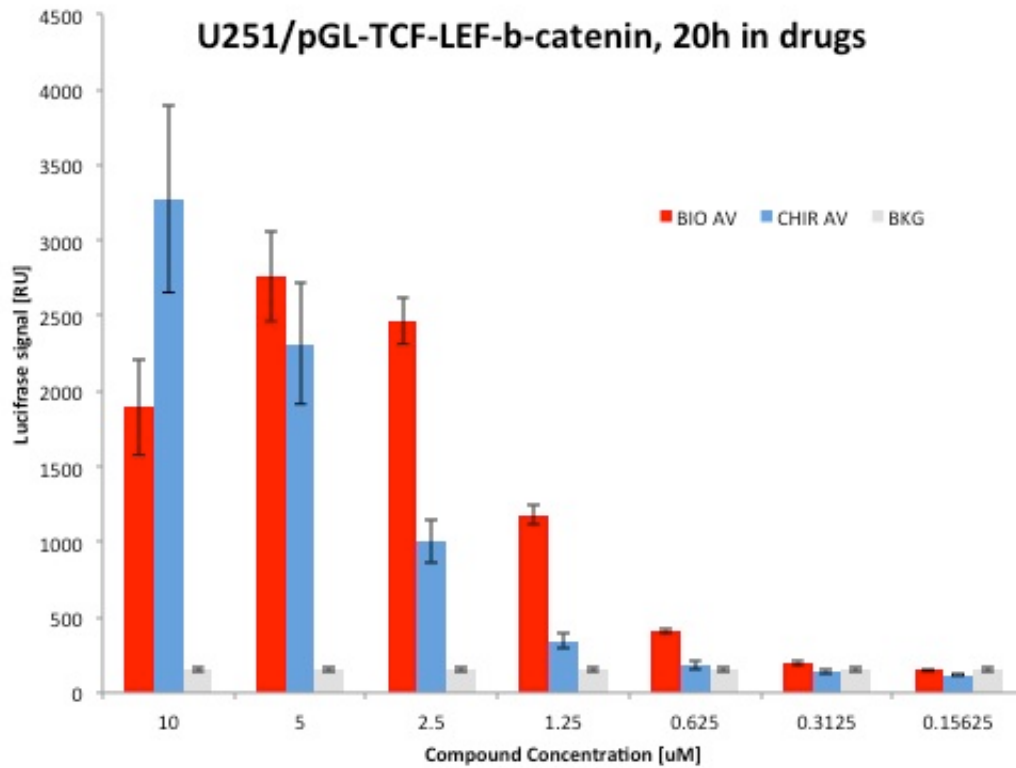


Fig 16. Luciferase Signal of U251 Cells Transfected with pGL-TCF-LEF- β -catenin After 20 Hours in Drugs. (Red: BIO; Blue: CHIR99021; Gray: Background).

DISCUSSION

As the most malignant form of primary adult brain tumors, glioblastoma treatments and cures are highly sought after. Patients with this cancer usually succumb to death within one year (Ray, 2010). GBM is a devastating diagnosis. GBM cells have a high rate of invasion and angiogenesis into nearby normal brain tissue, making completely surgical resection almost impossible, and creating a devastating prognosis for those afflicted with GBM. Conventional treatments like radiotherapy and cytotoxic chemotherapy definitely have an effect on prognosis, but are not an adequate cure as they are unable to overcome the malignant biology of the tumor cells and usually result in a recurrence. It is obvious that new treatment modalities must be developed to have a more targeted and adequate mechanism of action and to have the ability to give a much better prognosis for patients. New treatments also need to focus on the invasion and migration of GBM tumor cells.

GSK-3 is a multifunctional serine-threonine protein kinase that regulates many processes, including metabolism, cell fate specification, cell division, and cell death. Examples of pathways where GSK-3 is involved are Wnt, notch, tyrosine kinase, G-protein couple receptor signaling, and PI3K/Akt/mTOR. Because of these roles and its role in the regulation of cell motility, GSK-3 has become a very important target for multitudes of treatments for cancers, Alzheimer's disease, diabetes, and bipolar disorder (Williams et al., 2011). There have also been concerns about possible consequences of inhibiting GSK-3, as the inhibition upregulates the phosphorylation of β -catenin, which has been shown to have oncogenic effects in some cell types, however, patients who have

been treated with GSK-3 inhibitors have not been shown to have an increase in tumor number. Past investigations have concluded that tumor growth can be controlled with the use of GSK-3 inhibitors: an increase in cell death and a decrease in tumorigenicity (Kotliarova et al., 2008).

Previous work has shown that lithium, at high concentrations, will block glioma cell migration in part through GSK-3 inhibition. Unfortunately the concentration for lithium must be 20mM to have a significant effect, and this concentration is quite harmful to humans, who have a tolerance of 2mM. Based on previous observations, at lower concentrations lithium may not create enough of a blockade of glioma cell migration. Therefore more studies need to be done to identify suitable and more effective candidates for anti-invasive therapies of GBM.

Here we studied anti-invasive therapies that may have a future role in GBM treatment. It is thought that combining conventional treatments with anti-invasive therapies will create cytotoxicity in and reduce migration of the tumor. We reported that GSK-3 inhibitors might have a valuable role in the treatment of GBM. The selected inhibitors (BIO, CHIR99021, and LiCl) all were shown to lessen cell migration and invasion *in vitro* in a range of assays and in all cell lines tested. All inhibitors tested cause a dose-dependent, reversible inhibition of glioma cell invasion in spheroid assays. BIO was shown to cause a rapid upregulation of total and nuclear β -catenin. BIO, at higher concentrations, also created a toxic environment for cells, sometimes killing them. This shows that a more in-depth experiment involving different BIO concentrations is needed to test the optimal concentration for treatment.

Each of the experimented GSK-3 inhibitors also showed a change in the junctions between cells. NaCl as a control showed normal, spikey, junctions, while CHIR99021 and BIO caused the junctions to become more smooth. This suggests that GSK-3 inhibition has a role in either maintaining the ECM and/or in communication between cells. Also in this assay, there was a heterogeneity between cells treated with the same inhibitor and in the same dish, indicating that not all cells respond to each drug the same way. The reasons for this are not known and further investigation is required.

As is previously known, a tight monolayer gives greater electrical resistance. The ECIS technique is often used as an *in vitro* method to assess barrier functions in endothelial cells. Tight junctions require a longer period of time to form within a monolayer, while adherens junctions take a shorter period. BIO and CHIR99021 show a reduction in resistance and a decrease in the integrity of the cell monolayer over time, suggesting a role of GSK-3 inhibitors in the increase in permeability of endothelial cells. This is a possible avenue to increase the effectiveness of the drug in reaching the tumor.

The degree of GSK-3 inhibition, which we measured by a luciferase reporter assay of β -catenin transcriptional activation, showed an inverse correlation with the degree of invasion, suggesting a direct link between GSK-3 activity and the rate of glioma invasion. β -catenin is a key mediator of glioma migration. In the β -catenin luciferase assay both BIO and CHIR99021 led to a greater activation of β -catenin than LiCl.

The further development and research of GSK-3 inhibitors is a promising path for the future of the treatment of GBM and the future of medicine.

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CURRICULUM VITAE

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EDUCATION

Boston University School of Medicine

Candidate for Master of Science in Medical Sciences

Boston, MA

Expected Graduation: May 2015

University of Virginia

Bachelor of Science in Kinesiology: Sports Medicine Concentration

Charlottesville, VA

May 2013

- *Curry School of Education*

- *GPA – 3.22*

- *Dean's List – Spring 2012, Spring 2013*

Tidewater Community College

- *GPA – 3.84*

- *President's Honor Roll – Fall 2009, Spring 2010*

Chesapeake, VA

August 2009 – May 2010

PROFESSIONAL EXPERIENCE

Brigham and Women's Hospital, Department of Neurosurgery

Research Trainee (30 hours/week)

Boston, MA

September 2014 – Present

- Interact with the principal investigator and numerous post-doctoral fellows to design an experiment and complete the thesis requirement for the Master of Science in Medical Sciences degree at Boston University School of Medicine
- Study various drugs and inhibitors in an experiment based on obstructing angiogenesis and cell migration in glioblastoma tumors
- Compile literature relating to my project to compose an in-depth analysis of the inhibitors being studied
- Gain knowledge in molecular biology techniques in a laboratory setting, including, but not limited to western blots, cell culturing, gel electrophoresis, and bacterial DNA isolation

U.Va. University Career Services

Health Professions/Law Advising Intern (10 hours/week)

Charlottesville, VA

August 2012 – May 2013

- Coordinated related panels, programs, workshops (logistics, publicity, speakers), and library resources
 - Managed communication vehicles for students interested in health professions (listservs, newsletter)
 - Interacted with professors and employees to ensure timely execution of programs and opportunities for students
 - Developed interactive media including Twitter, Blogpost, and Pinterest using established content
 - Participated in applicant search, interviewing, and hiring process for new Health and Law Professions Advisor
-

SHADOWING AND CLINICAL EXPERIENCE

U.Va. Health System, Department of Orthopaedic Surgery and Sports Medicine

Practicum Student (10-12 hours/week)

Charlottesville, VA

August 2012 – December 2012

- Observed practiced physician as he performs surgeries relating to orthopaedic conditions, such as ACL reconstructions, MPFL reconstructions, meniscus repairs, and arthroscopies
- Interacted with surgical staff by maintaining active dialogue concerning patient history and surgical process during procedures
- Communicated with physician assistants and doctors in a clinical setting regarding medical history and treatment options of patients

Research Assistant

January 2013 — May 2013

- Found research from peer-reviewed journals relating to Sports Hernias and Athletic Pubalgia
- Interacted with physicians and editors to compile resources, organize data, and compose the medical chapter
- Intention was to contribute chapter entitled “Sports Hernia and Athletic Pubalgia: Diagnosis and Treatment” to a Springer textbook

Sentara Norfolk General Hospital, Department of Pathology

Norfolk, VA

Student Observer (10 hours total)

July 2012

- Observed the daily activities of a non-patient seeing clinician as he diagnosed disease using histopathology, cytology, and autopsy
- Witnessed the interactions of pathologists with physicians regarding the diagnoses of their patients
- Became aware of a more research-based side of medicine as it related to the treating of patients

U.Va. Health System, Department of Cardiovascular and Thoracic Surgery

Charlottesville, VA

Student Observer (5 hours/week)

August 2011—December 2011

- Observed practiced physician as he performed surgeries relating to cardiothoracic conditions, such as aortic valve replacement, coronary artery bypass grafts, and left ventricular assisting device installation
- Interacted with perfusionists to learn about the physiological conditions associated with cardiovascular bypass
- Communicated with anesthesiology team to gain knowledge of the physiological and metabolic needs of the patients

VOLUNTEER EXPERIENCE

SquashBusters

Boston, MA

Academic Tutor/Volunteer (3 hours/week)

January 2014—Present

- Tutor and advise underprivileged middle and high school aged students/squash players in all subjects
- Provide a warm and caring environment for the students to learn, succeed, and squash train
- Aid in the writing of resumes, in applying for jobs, and in applying for college

Volunteers for International Students and Scholars, and Staff

Charlottesville, VA

Student Language Consultant (2 hours/week)

January 2011—May 2011

- Met with international student once per week to discuss American culture and traditions
- Answered questions and alleviated concerns than student had regarding life in the United States
- Reinforced English language and grammar skills while maintaining friendly relationship

Youth Exalting the Savior Mission Team

Portsmouth, VA

Team Member (1 week/summer)

Summer 2006, 2008

College Adult (1 week/summer)

Summer 2009, 2010

- Participated in mission trips each summer to rural areas of Virginia, North Carolina, and Georgia

- Collaborated with other members of the team to determine best course of action for various endeavors
- Gained ability to partake in home-improvement projects, then demonstrated skills to other members
- Assisted with organization of fundraisers throughout Spring season

WORK EXPERIENCE

Camp Laurel

Mount Vernon, ME

Swimming Instructor, Camp Counselor, Lifeguard (8 weeks/s

Summer 2013

Head of Swimming (9 weeks/summer)

Summer 2014

- Collaborated with camp directors and other counselors to ensure the safety and happiness of campers
- Participated in the planning and implementing of day and evening programs
- Taught and ensured water safety while maintaining an active swim instruction program
- Responsible for campers aged 13-15 while living in shared cabins and common spaces

Strikezone Bowling Center

Portsmouth, VA

Recreation Assistant

March 2010 – August 2010

- Managed front desk including cash register and equipment rental while handling customer suggestions and complaints
- Organized and supervised league play while maintaining and ensuring a clean, safe center
- Communicated with other members of the staff to assist in maintenance and lane repairs

Deep Creek United Methodist Child Care Center

Chesapeake, VA

Child Care Worker

June 2008—September 2009

- Supervised children aged 3-10 years while ensuring safety and aiding children in solving interpersonal conflicts
- Organized and chaperoned field trips
- Responsible for preparing play and learning materials to set up children's environment

PUBLICATIONS

Gilmore, CJ, DR Diduch, MV Handley, and JB Hanks. "Sports Hernia-History and Physical Examination: Making the Diagnosis with Confidence." In *Sports Hernia and Athletic Pubalgia: Diagnosis and Treatment*, edited by DR Diduch and LM Brunt, 75–85. New York, NY: Springer, 2014.

ACTIVITIES

- Boston University Aquatics Swim Instructor 2014-Present
- U.Va. Student Council Curry School of Education Representative 2012-2013
- Tidewater Community College Student Ambassador 2009-2010