2016

Investigation of the anti-migratory properties of GSK-3 inhibitors in glioblastoma

Rolfs, Hillary

http://hdl.handle.net/2144/19472

Boston University
INVESTIGATION OF THE ANTI-MIGRATORY PROPERTIES OF GSK-3 INHIBITORS IN GLIOBLASTOMA

by

HILLARY ROLFS

B.S., University of Notre Dame, 2014

Submitted in partial fulfillment of the requirements for the degree of Master of Science

2016
Approved by

First Reader
Vickery Trinkaus-Randall, Ph.D.
Director, Cell and Molecular Biology Graduate Program
Professor of Ophthalmology

Second Reader
Sean Lawler, Ph.D.
Assistant Professor
Department of Neurosurgery
Brigham and Women’s Hospital
Harvard Medical School
DEDICATION

I would like to dedicate this page to my family, who continue to support and inspire me as I chase my dreams.
ACKNOWLEDGMENTS

I would like to thank Dr. Michal Nowicki for being an incredible mentor, for challenging me to learn, and for teaching me a great deal, and I want to extend thanks to Dr. Maria Speranza for her role in training and mentoring me. I would like to thank Dr. Sean Lawler for allowing me this opportunity, and helping and supporting me throughout. I would also like to thank Sylwia Wojcik for ensuring that my time in lab was enjoyable, and for helping me organize training. Lastly, I would like to acknowledge my patient advisor, Dr. Vickery Trinkaus-Randall for giving her time and commitment to my success.
INVESTIGATION OF THE ANTI-MIGRATORY PROPERTIES OF GSK-3 INHIBITORS IN GLIOBLASTOMA

HILLARY ROLFS

ABSTRACT

Glioblastoma is the most malignant form of brain cancer. Due to its aggressive nature, extensive research has been performed, but little progress has been made in identifying effective treatment options. Glycogen synthase kinase-3 (GSK-3) is a ubiquitous, multifaceted protein kinase. Previous studies have shown that small molecule inhibitors of GSK-3 block the migration of glioblastoma cells and may prevent spread of tumor in the brain. However, these studies were performed using non-selective GSK-3 inhibitors (LiCl and an indirubin derivative, BIO), thus it was unclear whether GSK-3 was the most important target. In this study, we used recently generated highly selective GSK-3 inhibitors (CHIR99021, AZD1080, and AZD2858, as well as BIO) to investigate these questions. These were applied to four glioblastoma cell lines: G30, G9, U251, and U1242, in three migration assays: transwell, spheroid, and wound healing (scratch) assay to further assess the suitability of GSK-3 as a target in glioblastoma. We also utilized the ATP Luciferase reporter assay for cell viability to assess the influence of our panel of drugs on cell migration versus viability. In addition, the TOPFlash Luciferase reporter assay was performed as an indicator of the level of GSK-3 inhibition.

The TOPFlash assay showed that all GSK-3 inhibitors were able to increase luciferase levels. This indicates that GSK-3 was inhibited in our cells after drug treatment. The transwell assays showed us that the GSK-3 inhibitors were able to block
migration significantly in all cell lines tested in a dose-dependent manner. The effectiveness of GSK-3 inhibition in the three-dimensional collagen spheroid assays was cell line-dependent, with the non-selective GSK-3 inhibitor BIO showing the most potent effects. Cell migration was not blocked by any of the three selective GSK-3 inhibitors in the wound healing scratch assay. Thus we have found that the three distinct highly selective inhibitors of GSK-3 block glioblastoma cell migration, but only work consistently in the transwell assay. Therefore, we conclude that GSK-3 might be important in the contraction and morphological changes necessary for glioblastoma cells to migrate through the 8 micron pores in the transwell. Further investigation into this observation is necessary. Though results were variable between assays, we conclude that the inhibition of GSK-3 is a promising potential therapeutic strategy for glioblastoma treatment.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT PAGE</td>
<td>ii</td>
</tr>
<tr>
<td>READER APPROVAL PAGE</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1. GLIOBLASTOMA: THE MOST COMMON AND THERAPEUTICAL CHALLENGING MALIGNANT BRAIN TUMOR</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Epidemiology</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Potential Causes</td>
<td>2</td>
</tr>
<tr>
<td>1.2.1 Rare Hereditary Syndromes</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2 Susceptibility Variants from Genome-wide Studies</td>
<td>3</td>
</tr>
<tr>
<td>1.2.3 Growth Factor Downstream Signaling</td>
<td>3</td>
</tr>
<tr>
<td>1.2.4 Ionizing Radiation</td>
<td>4</td>
</tr>
<tr>
<td>1.2.5 Allergies and Atopic Disease</td>
<td>4</td>
</tr>
</tbody>
</table>

viii
1.1 A luciferase reporter to show the upregulation of beta-catenin, a hallmark of GSK-3 inhibition ................................................................. 36

1.2 Immunohistochemistry ............................................................................. 37

2. CELL INVASION ASSAY ............................................................................. 40

3. WOUND HEALING ASSAY (SCRATCH) ..................................................... 44

4. CELL VIABILITY (ATP) .............................................................................. 46

5. PROLIFERATION AND INVASION (SPHERES) ....................................... 46

DISCUSSION .................................................................................................. 52

REFERENCES ............................................................................................... 54

CURRICULUM VITAE .................................................................................... 62
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glioma 2-year Survival by Age and Histological Subtype, Based on Individuals Diagnosed Between 1973 and 2002</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Horizontal Section of Glioblastoma Pathology</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Histology of Glioblastoma</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Divergent Signaling in Glioblastoma</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Histology of Differential Brain Tumors</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>Imaging Examples of Glioblastoma</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>Control of GSK-3</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>Insulin Signaling Utilizing GSK-3</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>Wnt Signaling Pathway</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>U251pGL-B, TCF/LEF-luc GSK-3 Inhibitors</td>
<td>37</td>
</tr>
<tr>
<td>11</td>
<td>Immunostaining of β-catenin on Glioblastoma G9 Cells</td>
<td>39</td>
</tr>
<tr>
<td>12</td>
<td>Area of GSK-3 Inhibitor CHIR99021 Treated U251pCDH, G9pCDH, &amp; U1242 Glioblastoma Cell Lines as Seen in an 8μM Transwell Assay</td>
<td>41</td>
</tr>
<tr>
<td>13</td>
<td>Area of GSK-3 Inhibitor BIO Treated U251pCDH, G9pCDH, &amp; U1242 Glioblastoma Cell Lines as Seen in an 8μM Transwell Assay</td>
<td>42</td>
</tr>
<tr>
<td>14</td>
<td>Area of GSK-3 Inhibitor AZD1080 Treated U251pCDH, G9pCDH, &amp; U1242 Glioblastoma Cell Lines as Seen in an 8μM Transwell Assay</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15</td>
<td>Area of GSK-3 Inhibitor AZD2858 Treated U251pCDH, G9pCDH, &amp; U1242 Glioblastoma Cell Lines as Seen in an 8µM Transwell Assay</td>
<td>44</td>
</tr>
<tr>
<td>16</td>
<td>Area of Scratch and Consequentially ATP Levels in G30pCDH, G9pCDH, and U1242 Cell Lines respectively, Treated with GSK-3 Inhibitors BIO, CHIR90221, AZD1080, AZD2858, and control DMSO.</td>
<td>45-46</td>
</tr>
<tr>
<td>17</td>
<td>Mask of Sphere Outgrowth in GSK-3 Inhibitor Treated U251pCDH Collagen Spheroids</td>
<td>47</td>
</tr>
<tr>
<td>18</td>
<td>Quantitative Data for CHIR99021 Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth</td>
<td>48</td>
</tr>
<tr>
<td>19</td>
<td>Quantitative Data for AZD1080 Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth</td>
<td>49</td>
</tr>
<tr>
<td>20</td>
<td>Quantitative Data for AZD2858 Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth</td>
<td>50</td>
</tr>
<tr>
<td>21</td>
<td>Quantitative Data for BIO Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth</td>
<td>51</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2HG</td>
<td>2-hydroxyglutarate</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli Protein</td>
</tr>
<tr>
<td>Antigen-Presenting Cell</td>
<td></td>
</tr>
<tr>
<td>Arg180</td>
<td>Arginine180</td>
</tr>
<tr>
<td>Arg96</td>
<td>Arginine96</td>
</tr>
<tr>
<td>Asn95</td>
<td>Asparagine95</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>Coiled-coil Domain Containing 26</td>
<td></td>
</tr>
<tr>
<td>CDK5</td>
<td>Cyclin-dependent Kinase 5</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytolytic CD8+ T cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>eIF2B</td>
<td>Eukaryotic Initiation Factor 2B</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>18F-FET</td>
<td>18F-fluoroethyl-l-thyrosine</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>Gln89</td>
<td>Glutamine89</td>
</tr>
</tbody>
</table>
GS .............................................................. Glycogen Synthase
HDAC ........................................................... Histone Deacetylase
HSP ............................................................. Heat Shock Proteins
IARC ............................................................ International Agency for Research on Cancer
IDH1 or 2 ....................................................... Isocitrate Dehydrogenase-1 or -2
IGFBP-2 ........................................................ Insulin-like Growth Factor Binding Protein 2
IL-2 ...................................................................... Interleukin-2
IO ......................................................................... Indirubin 3'-oxime
KLH ................................................................. Keyhole Limpet Hemocyanin
KPS ................................................................. Karnofsky Performance Status
LEF ..................................................................... Lymphoid Enhancer Factor
Lys205 ................................................................ Lysine205
MAPK .................................................................. p38 Mitogen-activated Protein Kinase
MGMT ............................................................. O6-Methylguanine-DNA-methyltransferase
MHC .................................................................. Major Histocompatibility Complex
NF1 ...................................................................... Neurofibromatosis 1 gene
OSU ................................................................. Ohio State University
PARP .................................................................. poly(ADP-ribose) Polymerase 1
PBS ...................................................................... Phosphate-Buffered Saline
PCNSL ................................................................ Primary CNS Lymphoma
PD-L1 .................................................................. Programmed Death Ligand I
PDGFR ................................................................ Platelet Derived Growth Factor Receptor
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe67</td>
<td>Phenylalanine67</td>
</tr>
<tr>
<td>PHLDB1</td>
<td>Pleckstrin Homology-like domain family B member 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase &amp; Tensin Homolog</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat Sarcoma</td>
</tr>
<tr>
<td>RTEL1</td>
<td>Regulator of Telomere Elongation Helicase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>S6K1</td>
<td>p70 Ribosomal S6 Kinase</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>Serine/Threonine</td>
</tr>
<tr>
<td>TCF</td>
<td>T Cell Factor</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptors</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolide</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms Tumor Protein</td>
</tr>
<tr>
<td>YKL-40</td>
<td>Chitinase-3-like Protein</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. Glioblastoma: The Most Common and Therapeutical Challenging Malignant Brain Tumor

Glioblastoma is the most aggressive malignant primary brain tumor; its destructive nature combined with minimal success in advancing treatment options has led to a prognosis of 14-16 months median survival, with approximately 5% of patients surviving more than 5 years past diagnosis (Knights, 2015; Neagu & Reardon, 2015). The tumor’s sensitive location, and the presence of infiltrating cells prevent complete surgical resection, and cause inevitable recurrence (Knights, 2015). Though glioblastoma cells are highly invasive, they typically do not metastasize outside of the central nervous system (Omuro A & DeAngelis LM, 2013). Glioblastoma is also highly resistant to treatment because of its heterogeneity, and the challenge of drug delivery across the blood brain barrier (BBB) (Knights, 2015).

1.1 Epidemiology

Glioblastoma has an incidence of 2-3 cases per 100,000 people in Europe and North America. Though this may appear to be a low number, glioblastoma is the fourth greatest cause of cancer death (Weingart, McGirt, & Brem, 2010). Glioblastoma is more common with increasing age, with the highest incidence in 75-84 year olds (Ostrom et al., 2014); however, cases are also reported in infants (Urbańska, Sokołowska, Szmidt, & Sysa, 2014). The ratio of cases in men as compared to women is 1.26:1 (Ostrom et al., 2014).
Figure 1. Glioma 2-year survival by age and histologic subtype, based on individuals diagnosed between 1973 and 2002. Seen here is the decreased survival rates of the elderly population with glioblastoma. Figure taken from (Schwartzbaum, Fisher, Aldape, & Wrensch, 2006).

1.2 Potential Causes

1.2.1 Rare Hereditary Syndromes

Around 5% of glioblastomas arise from hereditary syndromes (Malmer et al., 2001). Ostrom et al. have recognized mutations in the tumor suppressor gene p53 in approximately 30% of gliomas (Ostrom et al., 2014). Li-Fraumeni Syndrome is a rare hereditary disorder commonly associated with germline mutations in p53, and is consequently a risk factor for glioblastoma (Omuro A & DeAngelis LM, 2013). In studies by Therkildsen et al., 14% of patients with Lynch Syndrome developed brain tumors, with 56% of these tumors being glioblastomas (Omuro A & DeAngelis LM, 2013). Cowden syndrome, Turcot syndrome, Neurofibromatosis type 1 and 2, tuberous sclerosis, melanoma-neural system tumor syndrome, and familial schwannomatosis are other hereditary diseases associated with glioblastoma development (Barani & Larson,
2015; Omuro A & DeAngelis LM, 2013). More recent additions to this list include Ollier disease/Maffucci syndrome (Barani & Larson, 2015).

1.2.2 Susceptibility Variants from Genome-wide Studies

The Cancer Genome Atlas (TCGA) has characterized approximately 500 patient glioblastoma tumor samples by comprehensive genomic and transcriptomic profiling. From this data they found no two tumors have the same mutations, i.e. glioblastoma is a heterogenous disease (Omuro A & DeAngelis LM, 2013). However, 7 genomic variants that increased glioma risk were identified: telomerase reverse transcriptase (TERT), epidermal growth factor receptor (EGFR), coiled-coil domain containing 26 (CCDC26), pleckstrin homology-like domain family B member 1 (PHLDB1), p53, and regulator of telomere elongation helicase (RTEL1) (Barani & Larson, 2015).

1.2.3 Growth Factor Downstream Signaling

Growth factor downstream signaling also plays a large role in glioblastoma development; signaling pathways are altered in 88% of glioblastoma cases (Knights, 2015). Amplification of the EGFR gene and overexpression of protein was indicated in 40% of glioblastoma cases. This promotes cell proliferation via the Phosphoinositide 3-kinase (PI3K)/Protein Kinase B (Akt) pathway, enhancing Shc and Grb2 coordination, and Ras activity, as well as promoting cell survival by increasing expression of Bcl-\(X_L\) and other antiapoptotic proteins, and dysregulating cell cycle inhibitors such as \(p27^KiP1\) (Knights, 2015). Mutations in EGFR also enhance angiogenesis and cell migration via vascular endothelial growth factor, interleukin-8, and matrix metalloproteinase 13 (Knights, 2015). Also commonly mutated is the upstream inhibitor of PI3K, phosphatase
and tensin homolog (PTEN), in addition to the neurofibromatosis 1 gene (NF1) (Barani & Larson, 2015).

1.2.4 Ionizing Radiation

The sole firmly established environmental risk factor for developing glioblastoma is ionizing radiation (Knights, 2015). The propensity for persons exposed to ionizing radiation to develop glioblastoma is also correlated with a genetic predisposition for cancer (Knights, 2015). Ionizing radiation damages DNA by inducing single- and double-strand DNA breaks and introducing genetic changes that can lead to cancer (Knights, 2015).

1.2.5 Allergies and Atopic Disease

There has been some inconclusive evidence that indicates allergies may have a protective effect against glioblastoma via a heightened immune system. Studies by Ostrom et al. have shown elevated IgE (>100 kU/L) significantly reduced glioma risk; the data for glioblastoma was not significant.

1.2.6 Cellular Phones

The International Agency for Research on Cancer (IARC) published a study in 2011 showing no correlation between cell phone usage and glioma risk (Ostrom et al., 2014). In more recent years two cohort studies and three incidence rate over time studies have shown no significant risk of developing glioblastoma with cell phone use (Ostrom et al., 2014) However, one small case-control study did show significant data supporting a link between cell phone use and glioblastoma (Ostrom et al., 2014). Results remain inconclusive and further data is being generated on this topic.
1.3 Tumor Grading and Pathology
1.3.1 Classical Presentation

Glioblastoma multiforme is an outmoded term that hints at the heterogeneity of glioblastoma. Glioblastoma is thought to be initiated by stem cell-like populations of cells that also promote its rapid migration (Knights, 2015). Though glioblastoma migrates swiftly, the majority are supratentorial and do not metastasize outside of the central nervous system. Typically glioblastoma is considered a Grade 4 malignant astrocytoma, but histogenesis is unclear, and there can be differentiated cells within the same tumor (A. L. Cohen & Colman, 2015). Uncontrolled proliferation, diffuse infiltration, propensity for necrosis, robust angiogenesis, intense resistance to apoptosis, and genomic instability are all hallmark traits of glioblastoma. On histological cross sections one can observe the heterogeneity of glioblastoma through the greyish-white tumor, the necrotic yellow regions, and red-brown discoloration from hemorrhage (Urbańska et al., 2014). Also notable is the dense hyperchromatic nucleus that is indicative of rapid cell proliferation. However, glioblastoma can be difficult to distinguish from normal tissue because of its ability to assimilate and infiltrate into healthy tissue (Urbańska et al., 2014).
Figure 2. Horizontal Section of Glioblastoma Pathology. This image demonstrates the necrotic and hemorrhagic nature of glioblastoma. (Ray-Chaudhury, 2010)

Figure 3. Histology of Glioblastoma. Glioblastoma diagnosis is dependent on identification of cellular pleomorphism with presence of mitotic figures, and glomerular structures as seen in this figure. Typically present is tumor cell necrosis, usually at the center of the tumor. (Ray-Chaudhury, 2010)

1.3.2 Tumor Grading

Glioblastoma is currently classified as either the more prevalent primary glioblastoma or as secondary glioblastoma. Secondary glioblastomas progress from a lower grade tumor, commonly a low-grade astrocytoma (II) or an anaplastic astrocytoma (III); these make up only 5% of glioblastomas (A. L. Cohen & Colman, 2015). Secondary glioblastomas are more likely to have Isocitrate Dehydrogenase -1 or -2 (IDH1 or 2), or p53 mutations (A. L. Cohen & Colman, 2015). Primary glioblastomas, on the other hand, present as grade IV tumors at diagnosis (A. L. Cohen & Colman, 2015). They are typically found in older patients and present with increased EGFR gene expression (A. L. Cohen & Colman, 2015).
Figure 4. Divergent Signaling in Glioblastoma. An example of the mutated pathways that contribute to the development of primary or secondary glioblastoma. (Carrabba, Mukhopadhyay, & Guha, 2010)

Figure 5. Histology of Differential Brain Tumors. Figure highlights the histological differences during the progression to secondary glioblastoma. (Al-Hussaini, 2013).
1.3.3 Classification of Adult High Grade Gliomas via Gene Expression

The Cancer Genome Atlas (TCGA) has identified four subgroups based on transcriptional profiling. The first of the subgroups is proneural, which is the classic presentation in secondary glioblastoma (A. L. Cohen & Colman, 2015). Classical glioblastomas present with enhanced EGFR expression, most commonly EGFRvIII (A. L. Cohen & Colman, 2015). The third subgroup is mesenchymal; these tumors are likely to present with amplified MET and NF1 mutation, increased angiogenesis, hypoxia, and inflammatory response, and mesenchymal tumors are generally associated with poorer prognosis (A. L. Cohen & Colman, 2015; Kahlert et al., 2012). The fourth subgroup is neural, and is not well studied (A. L. Cohen & Colman, 2015).

1.4 Current Methods of Diagnosis
1.4.1 Clinical Presentation

The clinical presentation of glioblastoma is vastly dependent on the location and size of the tumor, but normal manifestations include aphasia (language problems), paresthesia (tingling), hemiparesis (weakness of one side of the body), and visual disturbances (Preusser et al., 2011). Also typical of glioblastoma patients are mood/personality changes, increased intracranial pressure, nausea, vomiting, headaches, and seizures (Preusser et al., 2011). Headaches are the typical indication, present in about 50% of patients (Omuro A & DeAngelis LM, 2013). Not to be confused with benign headaches, the presence of a brain tumor can be differentiated by rapid onset in patients older than 50, nonspecific pain pattern, unilateral localization, and increasing intensity (Omuro A & DeAngelis LM, 2013). It is also necessary to rule out other syndromes that
imitate glioma symptoms; these include brain abscess, subacute stroke, multiple sclerosis, and other inflammatory diseases of the brain (Omuro A & DeAngelis LM, 2013).

1.4.2 Magnetic Resonance Imaging (MRI)

MRI is the primary method of diagnosis; the images are usually enhanced with Gadolinium contrast (Omuro A & DeAngelis LM, 2013). Diffuse Tractor MRI-Based Tractography is a useful tool that utilizes the diminished amount of water present in myelinated white matter tracts to locate these areas, making it easier to avoid them in surgery (Ohue, Kohno, Kumon, & Ohnishi, 2014).

1.4.3 CT Scan

CT scans are a less popular option for imaging glioblastoma, but are necessary for patients who cannot undergo MRI for reasons such as having a pacemaker (Omuro A & DeAngelis LM, 2013).

1.4.4 Positron Emission Tomography (PET) Scan

PET scans use radioactive tracers to examine tissues and organs. Radioiodinated poly(ADP-ribose) polymerase 1 (PARP1) is a biomarker overexpressed in glioblastoma.
tissue and is expressed at low levels in the healthy brain (Salinas et al., 2015). Salinas et al. developed a novel selective tracer 12-PARPi to aid in the imaging of tumors. 18F-fluoroethyl-l-thyrosine (FET) PET is a radiopharmaceutical used clinically to image gliomas (Buchmann et al., 2016). FET is extremely useful as a predictive metabolic agent, and was found to be even more sensitive than MRI at detecting residual tumor post-resection (Bénard, Romsa, & Hustinx, 2003; Buchmann et al., 2016).

On the horizon of glioblastoma imaging is a chlorotoxin purified from scorpion venom. $^{[131]}$I-radiolabeled versions are in clinical trial, and it is thought that this compound is particularly applicable to gliomas because the mechanism potentially involves binding of matrix metalloproteases secreted by gliomas (Le Roux & Schellingerhout, 2014). Optical labeling techniques like those mentioned are exciting developments for delineating tumors from healthy brain tissue.

### 1.4.5 Blood Biomarkers

Blood biomarkers allow physicians to detect certain cancers solely through circulating proteins in the blood. Biomarkers have been established for many cancers; for example, the prostate specific antigen CA15-3 which is indicative of prostate cancer (Preusser, 2014). Thus far, no circulating biomarkers have been discovered for glioblastoma, but Perez-Larraya et al. have recently suggested a combination of proteins that might be a useful diagnostic (Gállego Pérez-Larraya et al., 2014). Glial fibrillary acidic protein (GFAP) is an intermediate filament of astrocytes; high levels of expression were found in 20% of glioma patients. Chitinase-3-like protein 1 (YKL-40) is a glycoprotein secreted by many cell types. YKL-40 is secreted at high levels in
glioblastoma patients, but is also amplified in a multitude of other diseases, and is therefore potentially useful as a diagnostic in coordination with elevated GFAP levels. Insulin-like growth factor binding protein 2 (IGFBP-2) has also been identified as a prognostic marker in cancer. The profile of all three of these circulating proteins could potentially give a reliable glioblastoma diagnosis.

1.5 Prognosis Indicators & Treatment Options
1.5.1 Karnofsky Performance Status

The Karnofsky Performance Status (KPS) is a scale assessing the mental status of a patient, and is used in glioblastoma as a principal indicator of the severity of the tumor and symptoms. This scale includes 11 positions, and ranges from no evidence of disease (100 points) to complaints of forgetting location of objects, to death (0 points). It is currently the most powerful clinical prognostic factor representing the mental health of a patient (Swagerty & Johnson, n.d.; Urbańska et al., 2014).

1.5.2 Histological Subtyping with Genomic Analysis

Histological subtyping aids physicians in determining the heterogeneity of glioblastoma tumors and is beneficial in predicting overall survival and deciding the best avenue of patient-specific treatment. One common prognostic marker is O6-Methylguanine-DNA-methyltransferase (MGMT) methylation. MGMT is a DNA repair protein, which when expressed can repair DNA damage caused by temozolomide. MGMT is methylated in 30-35% of glioblastoma patients and its methylation is characteristic of longer overall survival (Rudà et al., 2015). MGMT methylation is also of predictive value, i.e. it is useful in choosing how to treat a patient; in the elderly, MGMT
promoter unmethylated tumors have shown to have no survival benefit in undergoing chemotherapy in coordination with radiation (Rudà et al., 2015). Isocitrate dehydrogenase 1 or 2 (IDH1 or 2) gene mutations are present in 5% of primary glioblastomas and >50% of secondary glioblastomas (Rudà et al., 2015). IDH1 mutated tumors are thought to represent a related but histologically distinct class of glioma. The mutated enzyme has the ability to produce 2-hydroxyglutarate (2HG), and is prognostic of increased survival compared with wild-type IDH1 expressing gliomas (Amelot et al., 2015). 2HG is thought to promote oncogenic behavior through targeting specific enzymes involved in DNA methylation (Amelot et al., 2015).

1.5.3 Symptomatic Treatment

Peritumoral edema may be the root of many symptomatic presentations; moderate relief may be provided by administering corticosteroids (Omuro A & DeAngelis, 2013). The most popular choice of corticosteroid is Dexamethasone because of its low mineralocorticoid activity, but side effects can still be substantial (Omuro A & DeAngelis LM, 2013). It is pertinent to monitor the patient for primary CNS lymphoma (PCNSL) before administering corticosteroids as these drugs are lympholytic (Omuro A & DeAngelis LM, 2013). In cases where the patient presents with seizures, the use of antiepileptic drugs is necessary; Levetiracetam is preferable because it can work synergistically with chemotherapeutic agents (Omuro A & DeAngelis LM, 2013).

1.5.4 Surgical Resection

Surgery is typically the initial line of treatment, but because of the delicate location of tumors it is often impossible to completely resect (Preusser et al., 2011). Even
when complete macroscopic resection is an option, infiltrative cells make for extraordinarily high recurrence rates (Preusser et al., 2011). Surgery is commonly indicated even for patients with inoperable tumors to obtain stereotactic biopsies for histological analysis (Omuro A & DeAngelis LM, 2013).

1.5.5 First-Line Adjuvant Treatment

Carmustine Polymer (Gliadel) wafers are often placed at the site of resection during surgery. These wafers are a biodegradable matrix implanted with bis-chloroethylnitrosourea (carmustine) as an extended release carrier system (Venur, Peereboom, & Ahluwalia, 2015). There is no statistically significant difference in survival, but the results are still positive with a median of 13.5 months with wafers in comparison to 11.4 in placebo (Venur et al., 2015). However, use of carmustine wafers comes with increased risk of cerebrospinal fluid (CSF) leakage and intra-cranial hypertension (Venur et al., 2015). Additional radiotherapy in combination with chemotherapy is the usual course of action. Radiotherapy is commonly given in 60 Gy doses divided in 30 fractions (Venur et al., 2015). Temozolomide (TMZ) is a relatively new inclusion to the protocol for glioblastoma. TMZ is a DNA alkylating agent commonly used to treat other cancers. TMZ given concomitantly with radiotherapy has increased median survival to 15 months from 12 months with radiotherapy alone, and the use of this agent is particular beneficial to patients with MGMT promoter methylation (Venur et al., 2015).

1.5.6 Novel Therapeutic Targets
1.5.6.1 Vascular Endothelial Growth Factor
Glioblastoma is one of the most vascularized tumors, and is thus a great target for anti-angiogenic therapies. Angiogenesis promotes upregulation of VEGF and tumors with high levels of VEGF are associated with rapid development and poor survival (Venur et al., 2015). Bevacizumab is a recombinant humanized monoclonal antibody against VEGF-A that demonstrated significant improvement in progression free survival (PFS) of 10.6 versus 6.2 months in combination with radiotherapy and TMZ, but didn’t change overall survival time. In a second Phase III trial, it didn’t have significant PFS or OS. Further research of Bevacizumab and other anti-angiogenic therapies such as Aflibercept are underway, but none have shown significant impact on patient survival (Venur et al., 2015).

1.5.6.2 Integrins

Integrins play a key role in cell substrate adhesion, and are therefore an ideal drug target to inhibit migratory tumor cells. Multiple trials have been performed using Cilengitide, an integrin inhibitor targeting $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins. Cilengitide was adequately delivered to tumors, which can be an issue for glioblastoma, but didn’t elicit any results worth noting. For this reason Cilengitide is no longer being researched as treatment for high grade gliomas (Jordan & Wen, 2015).

1.5.6.3 Tyrosine Kinase Inhibitors

Tyrosine kinase inhibitors target receptor tyrosine kinases (RTKs) and are one of the most prominent anti-neoplastic therapeutic agents as these enzymes regulate signaling cascades controlling cell growth and survival (Jordan & Wen, 2015). The most commonly amplified RTKs in glioblastoma are epidermal growth factor receptor
(EGFR), vascular endothelial growth factor receptor (VEGFR), and platelet derived growth factor receptor (PDGFR). EGFR is overexpressed in 40-90% of glioblastomas, and is thought to be a promising target, but results have been disappointing because of poor delivery across the BBB (Jordan & Wen, 2015; Venur et al., 2015). PDGFR is amplified or mutated in 10% of glioblastomas, but drug trials of the PDGFR inhibitor Imatinib showed a lack of efficacy, and trials of Dasatinib showed no significant benefit and adverse hematologic effects (Jordan & Wen, 2015). All tyrosine kinase inhibitors targeting VEGFR had no clinical benefits (Jordan & Wen, 2015).

Signaling downstream of RTKs is often mediated through the PI3K/Akt/mTOR pathway. PI3K is an enzyme regulating cell survival and growth, and is commonly overactivated in glioblastoma usually as the result of mutated PI3K genes, overactivation of RTKs, or loss of PTEN inhibition. Buparlisib and Px-866 (oncothyreon) were both well tolerated in phase I trials, but buparlisib was not efficacious in phase II trials and Px-866 is currently undergoing trials. Everolimus and Temsirolimus (mTOR inhibitors) both showed toxicity when combined with TMZ and radiation (Jordan & Wen, 2015).

Another downstream signaling cascade of RTKs is mediated through the GTPase Ras, and its pathway involving RAF/MEK/ERK protein kinases. Drugs targeting this pathway have difficulty penetrating the BBB and showed no significant improvement in patient outcome (Jordan & Wen, 2015).

1.5.6.4 Isocitrate Dehydrogenase-1 and 2

Isocitrate dehydrogenase (IDH) is a metabolic enzyme that when mutated produces 2-hydroxyglutarate (2HG) as a byproduct; as discussed above this enzyme is
strongly implicated in secondary glioblastoma (Jordan & Wen, 2015). Drugs, such as AGI-5198 selectively inhibit this enzyme and deplete 2HG levels, which has been shown to slow tumor growth in vitro. This drug is currently in a Phase I clinical trial (Jordan & Wen, 2015).

1.5.6.5 Histone Deacetylase (HDAC)

Vorinostat (SAHA) is an anti-neoplastic agent commonly used to treat other forms of cancer such as cutaneous T-cell lymphoma. It is now undergoing research in glioblastoma, and is in Phase II studies. Vorinostat inhibits histone deacetylases, which prevents histones from being removed to enable gene transcription (Jordan & Wen, 2015). One of the benefits of this drug is that it crosses the BBB, but there have been issues of thrombocytopenia in about 1/5 of patients (Venur et al., 2015).

1.5.6.6 Proteasome inhibitors

Proteasomes breakdown unwanted proteins in healthy cells. When the proteasome is inhibited, the cell-cycle becomes dysregulated and apoptosis ensues, therefore drugs targeting the proteasome are an interesting target for cancer research (Jordan & Wen, 2015). However, studies utilizing the proteasome inhibitor Bortezomib have not had promising results, partially because of limited BBB penetration (Jordan & Wen, 2015).

1.5.6.7 Microtubules

Microtubules are dynamic components of the cell cytoskeleton, and are important in cell movement and as transport structures, as well as organization of cytoskeletal elements during mitosis. Inhibition of microtubule dynamics prevents cells from dividing. Paclitaxel has been tested with 35% of patients having improved disease, but because of
the generalized role of microtubules in cells, this drug is highly toxic (Jordan & Wen, 2015). To limit the toxic side effects researchers conjugated Paclitaxel with L-glutamic acid to improve water solubility and target delivery, but in this study there was hematologic toxicity. To improve targeted delivery to the tumor and lower necessary dosage, Paclitaxel was then conjugated with Angiopep-2 and renamed ANG1005. ANG1005 employs LDL receptor-related protein 1 to cross the BBB adequately. Studies using ANG1005 showed good delivery, but similar toxicity profile to Paclitaxel in phase I trials, and phase II studies are ongoing (Jordan & Wen, 2015).

1.5.7 Immunotherapy

Immunotherapy is defined as any process that targets malignancy by harnessing the patient’s immune system. Typically, immunotherapy utilizes the patients’ cytolytic CD8+ T cells (CTLs) to bind class I major histocompatibility complexes (MHCs) displayed on antigen-presenting cells (APCs) and destroy the malignant tumor cells (Neagu & Reardon, 2015). Because tumors are often immunosuppressive, especially in the case of gliomas, certain non-specific agents are also administered to boost immune processes. For example, interleukin-2 (IL-2) facilitates the clonal expansion of T cells (Neagu & Reardon, 2015). Interferons and toll-like receptor (TLR) agonists stimulate T cell activity also, through pro-inflammatory pathways (Neagu & Reardon, 2015). There are currently two categories of immunotherapy that elicit more specific responses. Passive immunotherapy has been successful in some cancer types because it is extraordinarily specific, and it relies on administering antibodies or immune cells not made by the patient to target an antigen expressed by the tumor (Neagu & Reardon,
Passive immunotherapy is limited in that the tumor must express that specific marker, and therapy only lasts as long as treatment is continued (Neagu & Reardon, 2015). Active immunotherapy utilizes the patient’s immune system to elicit a response, either by boosting insufficient responses or training the immune system to observe new targets (Neagu & Reardon, 2015). Active immunotherapy can have one or more targets, and cells will be trained to re-initiate an immunological response if prompted.

Applying immunotherapy techniques to gliomas is particularly challenging because the brain is fairly immunopriveleged being protected by the BBB (Bloch, 2015). The BBB under normal conditions is mostly impermeable to peripheral immune cells, and antigen recognition is monitored by microglia and astrocytes (Neagu & Reardon, 2015). Gliomas develop extremely immunosuppressive environments; even when immune cells have invaded only 5-10% of them are T cells and the tumor is able to escape recognition by the peripheral immune system until it is very advanced (Neagu & Reardon, 2015). Vaccines are thought to be the best avenue to combat gliomas because they educate the immune system to target tumor-specific peptides instead of just stimulating nonspecific responses.

**1.5.7.1 Peptide Vaccines**

Peptide vaccines deliver specific tumor peptides or tumor cell lysates to dermal APCs for uptake and display; this activates the CTLs to expand and elicit a response (Neagu & Reardon, 2015). Often this process is enhanced via immune stimulants such as keyhole limpet hemocyanin (KLH) or leukocyte growth factors such as GM-CSF or IL-2.
30-40% of glioblastoma patients express a mutated EGFR, EGFRvIII, which is not expressed in other cells (Bloch, 2015). An antigenic vaccine for EGFRvIII conjugated to KHL has undergone and continues to undergo trials; the primary outcome measure of these trials was median PFS which increased for all trials. A phase III trial of EGFRvIII vaccine with adjuvant TMZ is underway (Neagu & Reardon, 2015). Other potential antigenic vaccines target Wilms Tumor (WT1) protein, surviving peptide, CMV antigens, and telomerase; this research is ongoing.

One of the challenges of specific peptide vaccines is their use is limited to patients that express the target; in the case of EGFRvIII that is only 30-40% of patients. The heterogeneity of glioblastoma tumors poses another complication because when cells that express the target have been eradicated, other malignant cells may remain that facilitate tumor recurrence. To account for this researchers developed a multivalent vaccine by screening a large number of tumor samples and selecting 11 HLA-A2 synthetic tumor-associated peptides (Neagu & Reardon, 2015). This vaccine demonstrated a positive immunologic response in 81% of HLA-A2 positive patients in a phase I trial and is undergoing further investigation (Neagu & Reardon, 2015).

1.5.7.2 Dendritic Cell Vaccines

Dendritic cell (DC) vaccines are far more personalized for the patient. In this approach the patient’s dendritic cells are extracted through plasmapheresis and pulsed ex vivo with a predetermined immune-stimulatory peptide (Neagu & Reardon, 2015). The activated DCs are now ensured to display the peptide on their cell surface and can stimulate an antigen specific T cell response on reintroduction into the patient. Early
phase clinical trials using this method have been reported with mixed results. For example the ICT-107 vaccine assimilates six HLA-A1/2 restricted peptides with DCs. This vaccine had an exceptional median PFS of 16.9 months in a small phase I study, but the placebo-controlled phase IIb trial didn’t show difference between placebo and treatment (Neagu & Reardon, 2015). Numerous studies developing patient-specific multivalent vaccines are in development and are highly awaited.

1.5.7.3 Heat Shock Protein Vaccines

Heat shock proteins (HSPs) are ubiquitously expressed, and are another route to deliver antigenic peptides to endogenous APCs. HSP complex 96 can be extracted from tumor cell lysates and has been utilized to express specific antigenic peptides on APCs (Neagu & Reardon, 2015). The single arm trial results using this method have been successful in increasing PFS, but more research is necessary.

1.5.7.4 Immune Modulators

As discussed above cancer, in particular glioblastoma, can be extremely immunosuppressive. The results of immunotherapy vaccines of all types in glioblastoma have procured results that extend PFS or OS by months, not years, and the resilience of glioblastoma and its ability to resist contribute greatly to this. Researchers are now exploring avenues to combat the ability of glioblastoma to escape the full immune response. CD4+ T cells are a subclass of regulatory T cells that produce cytokines the halt the effects of APCs and effector T cells (Neagu & Reardon, 2015). In the healthy individual these cells act as a checkpoint to ensure that the appropriate immune response is initiated. CD4+ T cells are enriched in the blood of glioblastoma patients, and have
therefore been suggested to contribute to the immunosuppressive environment (Neagu & Reardon, 2015). Patients with low levels of regulatory T cells have a greater humoral response to vaccination, and studies are ongoing to evaluate the results of these findings.

Activation of effector T cells requires binding of its cofactor B7 with its receptor, also on the T cell. B7 binding to CD28 will activate the T cell and stimulate an immune response, but binding to CTLA-4 receptors will inactivate the T cell (Neagu & Reardon, 2015). Inhibition of CTLA-4 receptors will prevent T cell inactivation and generate a robust immune response (Neagu & Reardon, 2015). Use of CTLA-4 inhibitors, such as ipilimumab, has been shown to improve the survival of melanoma patients, and trials have been initiated in glioblastoma patients (Neagu & Reardon, 2015).

Programmed death ligand I (PD-L1) is a modulator of T cell activity that is expressed on a variety of immune cells. When bound to its receptor, programmed death I (PD-1), T cell apoptosis occurs (Neagu & Reardon, 2015). The expression of PD-L1 on cancer cells is a large factor of the immunosuppressive environment of tumors. Not only does PD-L1 expression contribute to local immunoresistance, but increased systemic PDL-1 contributes to a muted immune response that is tumor-independent. High expression of PD-1 on circulating monocytes in newly diagnosed glioblastoma patients has been linked to worsened survival. Trials of the PD-1 inhibitor nivolumab are ongoing in patients with recurrent glioblastoma (Neagu & Reardon, 2015). Currently there are no successful anti-invasive therapeutics, but as aforementioned there are some in development, and glycogen synthase kinase-3 (GSK-3) is a candidate for this research.
2. GLYCOGEN SYNTHASE KINASE-3

2.1 Introduction and Structure

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine (Ser/Thr) protein kinase that regulates a wide variety of substrates via phosphorylation at specific amino acids. GSK-3 has a wide range of functions, including regulation of glycogen metabolism, protein synthesis, cell proliferation, cell differentiation, microtubule dynamics, cell motility, cell-division cycle, transcription factors, and apoptosis. Such a large distribution of control has attributed GSK-3 dysfunction to the development of diseases such as diabetes, cancer, bipolar disorder, Huntington’s disease, and Alzheimer’s (Frame & Cohen, 2001; Rayasam, Tulasi, Sodhi, Davis, & Ray, 2009). GSK-3 is unusual in being one of the few kinases that is constitutively active in cells, and is inhibited when phosphorylated. GSK-3 has two well-studied isoforms, alpha and beta, and they share a 97% sequence homology within their kinase catalytic domains. GSK-3α has an extended N-terminal glycine-rich tail, and thus a higher molecular weight (Frame & Cohen, 2001). Though the two isoforms are not functionally redundant, there is overlap in certain pathways; for example both GSK-3α and GSK-3β contribute to axon development in hippocampal neurons (Rayasam et al., 2009).

2.2 Regulation

As mentioned above, GSK-3 is inhibited via phosphorylation, but can also be regulated by cellular localization and protein-protein interactions. Unlike most enzymes, phosphorylation of GSK-3’s substrates usually leads to their inactivation (Frame & Cohen, 2001). GSK-3 contains a docking site for substrates that have been previously
phosphorylated, or “primed”. These primed substrates have a phosphate group added to the consensus sequence Ser/Thr-X-X-X-pSer/pThr with X being any amino acid and pSer/pThr being phosphorylated serine/threonine (Frame & Cohen, 2001). The priming phosphate then specifically recognizes and interacts with arginine96 (Arg96), Arg180, and Lysine205 (Lys205) in a binding pocket on GSK-3 (Rayasam et al., 2009). Aside from this binding pocket, substrate specificity is also seen through the requirement of Phenylalanine67 (Phe67), Glutamine89 (Gln89), and Asparagine95 (Asn95) to position the substrate for phosphorylation by GSK-3β (Rayasam et al., 2009). Some identified primed substrates of GSK-3 are glycogen synthase (GS), eukaryotic initiation factor 2B (eIF2B), and beta catenin. GSK-3 can also phosphorylate unprimed substrates such as cyclin D3, axin, adenomatous polyposis coli (APC), but this occurs less frequently (Rayasam et al., 2009).

2.2.1 Regulation by insulin and Growth Factors

As previously mentioned, GSK-3 is inhibited by phosphorylation; this occurs at Ser21 for GSK-3α and Ser9 for GSK-3β. Once phosphorylated, it can act as a pseudosubstrate and bind in the pocket that would ordinarily interact with a phosphate group of a primed substrate (Rayasam et al., 2009).

Insulin signaling will activate Akt/PKB, which inactivates GSK-3 via phosphorylation at the amino terminus. This will release GS of inhibition and induce glycogen synthesis.
Figure 7. Control of GSK-3. Regulation of GSK-3 highlighting the ability of the N-terminus phosphate to act as a pseudosubstrate and inhibit GSK-3. (P. Cohen & Frame, 2001)

Figure 8. Insulin Signaling Utilizing GSK-3. A classic representation of the insulin pathway and control of glycogen synthesis. (P. Cohen & Frame, 2001)
GSK-3 is also phosphorylated at Ser21/Ser9 by AGC kinase, p70 ribosomal S6 kinase (S6K1), p90 ribosomal S6 kinase. S6K1 is a potential mechanism inhibition of GSK-3 by amino acid availability (Frame & Cohen, 2001). Inhibition of GSK-3 also occurs via cAMP kinase mediated phosphorylation of Ser21/Ser9 (Frame & Cohen, 2001).

Another method of inhibition is phosphorylation at the carboxyterminus by p38 mitogen-activated protein kinase (MAPK). This manner of inactivation occurs only for GSK-3β on Thr390, which is not present in GSK-3α, and occurs primarily in the brain and thymocytes (Rayasam et al., 2009; Thornton et al., 2008). Phosphorylation can also lead to stimulation of GSK-3. The kinases that perform this phosphorylation are not well characterized but the phosphorylation occurs at Tyr279 for GSK-3α and 216 for GSK-3β (Rayasam et al., 2009).

2.2.2 Wnt Pathway Regulation

The Wnt pathway was discovered using Wingless, the Drosophilia homolog of Wnt (P. Cohen & Frame, 2001). Implicated in embryonic development, researchers discovered that knockout or overexpression of this gene changed patterns of segment polarity in Drosophilia (Cohen & Frame, 2001; Rayasam et al., 2009). Wnt is also of high importance in cancer development and dysregulated gene expression through the regulation of stem cell biology. The Wnt pathway utilizes a different method of regulation, via sequestration of GSK-3 in a multiprotein complex including axin, beta-catenin, and (APC) protein (Rayasam et al., 2009). Without a Wnt signal, GSK-3 actively
phosphorylates beta-catenin, and targets it via ubiquitylation for proteasome-mediated degradation (Rayasam et al., 2009). Inhibition occurs via the FRAT and DVL protein complex, which disrupt the interaction of GSK-3 and axin; and promote beta-catenin stabilization and nuclear translocation where it interacts with transcription factors lymphoid enhancer factor (LEF) and T cell factor (TCF). This promotion of transcription factors activates target genes like c-myc and cyclin D1 and increases cell proliferation, explaining the role of this pathway in cancer development (Rayasam et al., 2009).

Figure 9. Wnt Signaling Pathway. A clear image showing the canonical Wnt pathway and the role of GSK-3. (P. Cohen & Frame, 2001).
2.3 Implication in Glioblastoma

The role of GSK-3 in cancer cell migration is a subject with conflicting sides. Some research has suggested GSK-3 inhibition promotes cancer cell migration via epithelial to mesenchymal transition (Nowicki et al., 2008). However, GSK-3 inhibition has also been shown to inhibit tumor invasion, and several of its well-known substrates (paxillin, FAK, NFκB, β-catenin, and SNAIL) have roles in cell migration (Nowicki et al., 2008). It is thought that GSK-3 is regulated via localization in distinct subcellular pools, which may explain the conflicting data. In studies performed by Nowicki et al., GSK-3 inhibition via lithium halts glioma invasion. Research has also shown that healthy brain tissue expresses lower levels of GSK-3β, and that inhibition of GSK-3β reduces glioma proliferation, and has protective benefits for brain tissue. GSK-3β inhibition also activated tumor suppressor genes p53 and p21, as well as decreased levels of Rb phosphorylation leading to apoptosis (Miyashita et al., 2009). Studies also show that GSK-3β inhibition stabilizes PTEN, which will arrest the cell cycle and promote glioblastoma cell proliferation (Miyashita et al., 2009). Research by Williams et al. demonstrates that GSK-3 inhibitors of the indirubin family successfully inhibit glioblastoma invasion at a 4,000 fold lower concentration than LiCl, and the use of these indirubins does not exhibit high toxicity. Indirubins were discovered through a Chinese medicine Dang Gui Long Hui Wan, and were primarily investigated as anti-leukemia agents that function to inhibit angiogenesis (Kim, Shin, Kang, & Park, 2011). The work of Williams et al. examined three indirubins, 6-bromoindirubin-oxime (BIO), BIA (6-bromoindirubin-acetoxime), and indirubin 3’-
oxime (IO). These three indirubins showed a more robust inhibition of GSK-3 than previously observed using LiCl, as indicated by a reduced phosphorylation of β-catenin. This research shows that GSK-3 inhibitors, and more specifically indirubins, are viable candidates for anti-invasive therapies for glioblastoma.

2.4 Research Question

Studies utilizing LiCl and indirubins to inhibit GSK-3 and prevent tumor cell migration have demonstrated varying levels of success, but have shown promise in application to glioblastoma. Recently, a number of more selective GSK-3 inhibitors have been developed, and we investigated their anti-invasive properties to examine whether GSK-3 is a good target. BIO was included in our panel of GSK-3 inhibitors; this compound inhibits GSK-3 with an IC50 of 5 nM, and also has some effects on cyclin dependent kinases (>16-fold selectivity over cyclin-dependent kinase 5 (CDK5)), as well as targeting the pan-JAK pathway (“BIO | GSK-3 inhibitor | Read Reviews & Product Use Citation”). BIO interacts with the ATP-binding pocket of these kinases (“BIO | GSK-3 inhibitor | Read Reviews & Product Use Citation”). CHIR-99021 (CT99021) is another GSK-3 inhibitor in common use, but is more selective than BIO and does not exhibit cross-reactivity against CDKs (“CHIR-99021 (CT99021) | GSK-3 inhibitor | Read Reviews & Product Use Citation”). The newest developed GSK-3 inhibitor in our panel, AZD1080 is highly selective for GSKα/β with no other known targets (“AZD1080 | GSK-3 inhibitor | Read Reviews & Product Use Citation”). The fourth GSK-3 inhibitor utilized is AZD2858; a selective inhibitor which activates Wnt signaling (“AZD2858 | GSK-3 inhibitor | Read Reviews & Product Use Citation”). This study aims to
characterize the anti-invasive properties of these four GSK-3 inhibitors in four glioma cell lines using three assays in hopes of providing evidence to guide their future clinical application. Our hypothesis is that highly selective inhibitors of GSK-3 will allow us to understand better the suitability of GSK-3 as a target in the treatment of glioblastoma.
METHODS

1. Cell Lines & Chemicals

Human glioblastoma cell lines U251 and U1242 from ATCC, and G9, and G30 obtained from Ohio State University (OSU), were utilized throughout this study. Established protocols, approved by the Institutional Research Board, were used by the OSU tissue procurement facility to acquire human tissue.

Marker vector copGFP (System Biosciences, empty pCDH lentiviral vector) was transfected into U251, G9, and G30 cell lines to be utilized for imaging. RFP- lpp-hluc-lv-206-025-c (Genecopeia) was utilized for U1242 imaging.

U251 and U1242 were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with the addition of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. G9 and G30 cell lines were cultured in Neurobasal medium (Life Technologies, Grand Island, NY, USA) and supplemented with b27, epidermal growth factor (EGF), fibroblast growth factor (FGF), and without Vitamin A. EGF and FGF were given at 10ng/mL and obtained from Peprotech (Rocky Hill, NJ, USA). All cells were sustained in culture at 37°C with 5% CO₂.

Dimethyl Sulfoxide (DMSO) was used as a solvent for all compounds and a negative control for all assays. CHIR99021, BIO, AZD1080, and AZD2858, known GSK-3 inhibitors, were obtained from Tocris and tested in all assays.

2. Transwell (Boyden Chamber) Assay
24-well plates with 8µm or 3µm pore size inserts (ISC Bioexpress, Kaysville, UT, USA) were coated with 50µl of 10µg/ml Fibronectin in phosphate-buffered saline (PBS). U251, U1242, G9, or G30 cells grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin were counted and allotted at 50,000 cells/well to be incubated with either 10µm or 1µm of the selected treatment (DMSO, CHIR99021, AZD1080, AZD2858, BIO) for three hours. The bottom chamber of the plate was washed and medium with drugs was added. Cells were transferred to the insert and permitted to migrate for a minimum of twelve hours while being monitored using a Nikon TE2000 microscope system with on-stage incubation for live-cell imaging.

3. Collagen Spheroid Assay

For three-dimensional spheroid cultures, 1,000 cells were cultured in 96-well ultra low attachment plates in either DMEM with 10% FBS and 1% penicillin-streptomycin (U251 and U1242 cell lines) or neurobasal medium with growth factors and b27 (G9 and G30 cell lines). After spheroid/aggregate formation, the medium was partially removed (10µl leftover) and supplemented with liquid 40µl neutralized collagen I solution (PureCol, Inamed, Fremont, CA, USA). The medium was removed and replaced with 300µl neutralized collagen I solution (PureCol, Inamed, Fremont, CA, USA). Collagen was neutralized to pH 7.5 using 1N NaOH and fortified with .5X DMEM for U251 and U1242 cell lines and .5X neurobasal medium for G9 and G30 cell lines. The collagen I solution was allowed to polymerize into a gel at 38°C. Medium (50µl) with drugs (2x concentration) was added on top of the collagen I. Spheroid expansion was monitored up to 96 hours using a Nikon TE2000 microscope system with on-stage incubation.
4. Wound Healing (Scratch) Assay

Cells were seeded at 20,000/well on a 48-well plate and allowed to grow in DMEM media with 10% FBS and 1% penicillin-streptomycin until a monolayer was formed. Drugs were added and wells were scratched using a 200µl pipet tip (100-300µm gap). Microscopic visualization was done over a period of 24 hours using a Nikon TE2000 or Nikon Eclipse Ti, both with on-stage incubation.

4.1 ATP Luciferase Reporter Assay for Cell Death

After the scratch assay, medium was removed and cells were lysed using the Steady-Glo Luciferase Assay System (Promega), which provides Luciferase and substrate D-luciferin. A limiting factor for signal strength is ATP from analyzed cells. Light output of luciferase was detected using Fluostar Optima plate reader (BMG Labtech, Durham, NC).

5. TCF-LEF Luciferase Reporter Assay

M50 and M51 (M50-pTA/Super8XTOPflash-β-catenin binding; M51-pTA/Super8XFOPflash-β-catenin NoBinding) plasmids were provided by Dr. Randall Moon, University of Washington, Seattle, WA. Plasmid DNA was isolated from DH5α bacterial cultures. The sequence 8xTCF-LEF-TA (TCF-LEF sequence allowing binding of TCF-LEF transcription factor, TA-minimal eukaryotic promoter) was chosen from the pTA plasmid to be inserted and cloned into pGL4.14 plasmid (Promega).
The plasmid DNA was isolated from 5mL cultures using the PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA, USA). Bacterial cultures were spun at 6000 rpm for 10 minutes; pellets were then re-suspended in buffer (50mM Tris-HCL, pH 8.0, 10mM EDTA) before being applied to the column. Lysis was performed 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. Wash buffer was applied to remove contaminants, and the plasmid DNA was eluted in preheated TE Buffer (10mM Tris-HCL, pH 8.0, .1mM EDTA). A microcentrifuge at room temperature was used for all procedural steps and spun at >12,000 x g.

Restriction enzymes HindIII-HF (sticky ends, high-fidelity) and SacI (sticky ends) (New England BioLabs Inc.) were employed to cut purified pTA plasmids. pGL4.14 (Promega) provided the plasmid vector for new construct, also having been cut with HindIII-HF and SacI restriction enzymes.

Next a mixture of 1µg of DNA plasmid obtained from the PureLink Quick Plasmid Miniprep Kit (Invitrogen), 1.0µL of NEBuffer 2.1 (New England BioLabs Inc.), 0.5µL of HindIII and SacI, and 7µL of deionized water were digested in a 37°C water bath for 1 hour.

The digested nucleic acids were mixed with loading buffer (20% Ficoll 400, 0.1M Na2EDTA, pH 8, 1.0% sodium dodecyl sulfate, 0.25% bromphenol blue, 0.25% xylene cyanol) and run on 1% agarose/ethyl bromide/1xTAE gel. 1µg Ready load 1kb DNA ladder (Invitrogen) was run as a standard. The gel was run at 5 volts per cm of tank length (150V), and visualized using a BioRad ChemiDoc XRS. The chosen vector and
insert were cut out of the gel and purified using the protocols of the PureLink Quick Gel Extraction Kit (Invitrogen).

Protocols established in the Quick Ligation Kit (New England BioLabs Inc.) were used to ligate purified insert vector. Bacteria (DH5α competent E.Coli) were plated overnight with 1.0µL of the ligation mixture. Colonies were then expanded to 5ml liquid culture and DNA was isolated using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). The same restriction enzymes, HindIII and SacI, were used to cut DNA to determine if our fragment had been inserted. The cloning procedure indicated two new DNA constructs, named: pGL4.14/Super8XTOPflash or pGL4.14/8xTCF-LEF-TA- β-catenin-Binding and pGL4.14/Super8XTOPflash or pGL4.14/9xTCF-LEF-TA- β-catenin-NO-Binding.

The established procedure of the PureLink HiPure Plasmid Filter Maxiprep Kit (Life Technologies) was used, included the suggested scaling up of plasmid DNA purification. The plasmids were introduced to G9 glioma cells using the Lipofectamine 3000 reagent. The stable clones were selected using Hygromycin B (150µg/mL, Life Technologies). The G9/pGL4.14/8xTCF-LEF-TA- β-catenin-Binding cells were plated in 96-well white wall plates, 10,000 cells/well, in 84 wells.

CHIR99021, AZD1080, AZD2858, and non-treated cells were incubated with cells for 6 hours to allow TCF-LEF-TA-dependent accumulation of Luciferase. Medium was removed and cells were lysed using the Steady-Glo Luciferase Assay System (Promega), which also provides the luciferin substrate. Light output reflects promoter
activity, and was determined using Fluostar Optima plate reader (BMG Labtech, Durham, NC).

6. Immunohistochemistry

G9 cells were cultured on 8-chamber Lab-Tek glass slides, and treated with CHIR99021, BIO, AZD1080, AZD2858 and control DMSO all at 10µm for 6 hours. Cells were washed with ice-cold DPBS (Dubecco’s Phosphate Buffered Saline, Life Technologies) and fixed using 4% paraformaldehyde solution and washed with DPBS supplemented with .1% Triton X-100 (Sigma-Aldrich). Normal donkey serum was used to block (non-immunized, Jackson ImmunoResearch, West Grove, PA). Primary antibody, mouse anti-beta-catenin (1:500) (Cell Signaling) was added and slides were incubated overnight at 4ºC. Cells were washed multiple times using a DPBS-Triton X-100 solution, and fluorescent labeled secondary antibody (488nm Alexa Fluor anti-mouse, 1:1000; Jackson ImmunoResearch) was incubated with cells for 6 hours to be utilized for visualization. Cells were repeatedly washed with the DPBS-Triton X-100 solution, and images were produced with a ZEISS LSM710 confocal microscope.

7. Statistical Analyses

All image processing was performed using ImageJ (Schindelin, 2015), and all statistical analysis was done in Microsoft Excel 2010.
RESULTS

As formerly stated, GSK-3 inhibitors have proven to be potential therapeutic agents for glioblastoma. However, the GSK-3 inhibitors used in these previous studies were not selective, and inhibit other kinases in addition to GSK-3. Therefore, we have investigated newer highly selective GSK-3 inhibitors, which allows a more precise view of how they function in a variety of assays, and provides valuable information for future use. We utilized four gliomas cell lines in three differential assays to garner information on the suitability of GSK-3 as a drug target, and to hypothesize about how its inhibition may prevent glioblastoma cell invasion.

1. BIOLOGICAL EFFECTS OF GSK-3 INHIBITION

1.1 A luciferase reporter to show the upregulation of beta-catenin, a hallmark of GSK-3 inhibition

Firstly it was important to confirm that GSK-3 was indeed being inhibited by our specific inhibitors: CHIR99021, BIO, AZD1080, and AZD2858 (utilizing DMSO as control). This was accomplished with the U251 cell line, using the TCF/LEF-luc assay as discussed in methods. The inhibition of GSK-3 promotes nuclear localization of β-catenin, which acts as a transcription factor and promotes formation of luciferin (P. Cohen & Frame, 2001; “TCF/LEF Reporter”). Here it was shown that GSK-3 inhibitors CHIR99021, AZD1080, and AZD2858 demonstrated a dose-dependent increase in β-catenin expression. The largest increase in expression was found in cells treated with AZD1080, but notably AZD2858 maintained a high signal at lower drug
concentrations. CHIR99021 and AZD1080 maintained a signal until a concentration of about 1.25µM, whereas AZD2858 inhibited GSK-3 signaling at about 0.3µM.

Figure 10. U251pGL-B, TCF/LEF-luc activity in the presence of GSK-3 Inhibitors. Strong luminescent signals were obtained at high concentrations of CHIR99021, AZD1080, & AZD2858 indicating a high level of GSK-3 inhibition. AZD2858 continued to maintain luminescence at lower concentrations than CHIR99021 & AZD1080.

1.2 Immunohistochemistry

β-catenin is a well-studied substrate of GSK-3 involved in regulation of cell junctions, and is also of particular importance as a transcription factor (P. Cohen & Frame, 2001). β-catenin therefore has many implications and a wide range of effects; its localization plays an important role in its functions. Typically the inhibition of GSK-3
prevents the ubiquitin-mediated proteolysis of β-catenin, allowing its translocation into the nucleus where it regulates gene transcription. However, this is a complex pathway, and the sequestration of β-catenin into differential pools can produce a variety of results (P. Cohen & Frame, 2001). Nonetheless, the upregulation of β-catenin transcriptional activity can be used as an indication of inhibition of GSK-3, and the effect of our GSK-3 inhibitor panel over a range of concentrations produced a varying level of β-catenin increase in glioblastoma cells.

We utilized immunostaining techniques to show the β-catenin upregulation in G9 cells. Cells were fixed and stained for β-catenin 6 hours after drug treatment, and the experiment was also performed 24 hours after drug treatment. Our results indicated that CHIR99021 and AZD1080 produced the highest levels of β-catenin upregulation, but all drugs demonstrated a significant increase compared with DMSO controls.
Figure 11. Immunostaining of β-catenin in Glioblastoma G9 cells. Immunostaining techniques were used to indicate the upregulation of β-catenin seen via the prevention of ubiquitin-mediated proteolysis of initiated by inhibition of GSK-3.
2. CELL INVASION ASSAY

The transwell assay, also known as the Boyden chamber assay, is used to quantify the migration of cells through a porous membrane. In this technique, cells are seeded in an upper chamber and must intercalate through pores to a bottom compartment. This assay is useful because the size of the pores can be controlled to mimic the environment in the brain through which the cells must migrate. We first tested 3µM and 8µM pores; which are comparable to the in vivo environment. After success in both 3µM and 8µM, it was decided to continue with 8µM membranes; it was assumed if migration could be prevented in a model that is easier to move through than its application then results could be reproduced in the brain. Quantification involved counting the cells that have invaded the bottom compartment.

CHIR99021 inhibited glioma cell migration in a dose-dependent manner in 3/3 cell lines. Results were replicated three times in U251pCDH with both 3µM and 8µM pores, and twice in G9. AZD1080 was efficacious in 3/3 cell lines also, with only 1µM not showing significant data in G9. AZD2858 also had concentration-dependent significant data in 3/3 cell lines, with the exception of 10µM in U1242 cells. BIO also proved to be a potent inhibitor of migration in 3/3 cell lines. In three of the four cell lines treated cells migrated through the pores significantly less, with the fourth cell line, G30, unable to migrate under any conditions.
Figure 12. Area of GSK-3 Inhibitor CHIR99021 Treated U251pCDH, G9pCDH, & U1242 Glioblastoma Cell Lines as Seen in an 8µM Transwell Assay.
Figure 13. Area of GSK-3 Inhibitor BIO Treated U251pCDH, G9pCDH, & U1242 Glioblastoma Cell Lines as seen in an 8µM Transwell Assay.
Figure 14. Area of GSK-3 Inhibitor AZD1080 Treated U251pCDH, G9pCDH, & U1242 Glioblastoma Cell Lines as Seen in an 8µM Transwell Assay.
3. WOUND HEALING ASSAY (SCRATCH)

The transwell assay measures chemotactic behavior; to test haptotactic behavior we used another migration assay. A wound-healing scratch assay was performed by scratching a monolayer of cells and analyzing the gap area after treatment and 24 hours in comparison with the initial area. The results of this assay were less promising; the drugs
did very little to prevent the closure of the scratch. Most successful of the treatments in majority of the cell lines was BIO, but no data was significantly different from DMSO.
4. CELL VIABILITY (ATP)

After performing the wound-healing assay, treated cells were then utilized for a cell viability assay. It was necessary to determine whether the effects of CHIR99021, AZD1080, AZD2858, and BIO influenced glioma cell migration and not only cell viability. The ATP assay is a relatively simple method that images via luminescence the primary energy currency of cells, ATP, and in our case to thus determine the viability of the cells. Our results show that GSK3 inhibitors were not killing the cells, even at high drug concentrations of 10µM. The amount of luminescence produced by cells treated with drugs was comparable to the amount produced by the positive control and cells that received no treatment at all, indicating cells are alive, and their viability was not affected under the experimental conditions.

5. PROLIFERATION AND INVASION (SPHERES)

For the final migration assay we employed a 3-D assay where glioma spheroids were implanted in a type I collagen matrix. This is a profitable assay, in particular for studying cancer invasion, because the spheroid mimics a cell-dense core of a tumor, and cells invade outwards mimicking brain tumor invasion while employing process extension and matrix remodeling. The most successful GSK-3 inhibitor in this assay was
BIO, which showed concentration-dependent inhibition of invasion in 4/4 cell lines tested. For the remaining drugs tested, results were cell line dependent. U1242 appeared to be the most sensitive cell line tested; AZD2858 prevented invasion at all concentrations, but CHIR99021 and AZD1080 were only successful at high drug concentrations. AZD2858 inhibited collagen invasion at 10µM in U251, but AZD1080 worked only at 20µM. CHIR99021 had very poor results in U251, and was tested multiple times with no success at any concentration. CHIR99021, AZD1080, and AZD2858 demonstrated a complete lack of inhibition of migration in G9 and G30 cell lines.

Figure 17. Mask of Sphere Outgrowth in GSK-3 Inhibitor Treated U251pCDH Collagen Spheroids. Cells were allowed to migrate for 96 hours.
Figure 18. Quantitative Data for CHIR99021 Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth. Cells were allowed to migrate for 96 hours, the X-axis indicates time, and the Y-axis shows area migrated.
Figure 19. Quantitative Data for AZD1080 Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth. Cells were allowed to migrate for 96 hours, the X-axis indicates time, and the Y-axis shows area migrated.
Figure 20. Quantitative Data for AZD2858 Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth. Cells were allowed to migrate for 96 hours, the X-axis indicates time, and the Y-axis shows area migrated.
Figure 21. Quantitative Data for BIO Treated U251pCDH, G9pCDH, G30pCDH, and U1242 Collagen Spheroid Outgrowth. Cells were allowed to migrate for 96 hours, the X-axis indicates time, and the Y-axis shows area migrated.
DISCUSSION

Our results indicate that the panel of GSK3 inhibitors tested, CHIR99021, AZD1080, AZD2858, and BIO are viable candidates for anti-invasive glioblastoma treatment. Replicable, dose-dependent, significant success was had in 3/3 cell lines in the transwell assay testing all four drugs. This led us to hypothesize that GSK3 is potentially tied to the ability of glioblastoma cells to contract and intercalate through the pores of the membrane, whereas this motion was not procured by the other assays.

Indeed, it was discovered that the application of this panel of GSK3 inhibitors in another assay performed by Nowicki et al. inhibited the contractile capability of HUVEC and HBMEM epithelial cells. Attempts were made to reproduce this assay utilizing the glioblastoma cell lines, but the setup was not technically feasible.

Mixed results were produced in the collagen spheroid assay. It was clear that BIO was able to inhibit cellular invasion in the collagen matrix. The remaining drugs tested had some success at high concentrations in U1242 cells, and with the exception of CHIR99021, in U251. As stated above, no significant inhibition occurred in G9 or G30 cells for CHIR99021, AZD2858, or AZD1080.

Both GSK-3α and GSK-3β are implicated in a wide variety of pathways including Wnt, notch, tyrosine kinase, and G-protein coupled receptor signaling, and it is no surprise inhibition has been shown to have differential results. For example, lithium, a potent GSK3 inhibitor, has stimulated cell proliferation in mammary tumor cells, but inhibits proliferation in melanoma and hepatocellular carcinoma (Nowicki et al., 2008).

Our results support the work of Williams et al. in that GSK3 inhibition blocks invasion in
the transwell assay, and that BIO is also capable of inhibiting cell invasion in a collagen spheroid assay. Our novel panel of drugs also demonstrated some success in the collagen spheroid assay, but no significant success in the wound-healing assay.

Application of our panel of specific small molecule GSK3 inhibitors, BIO, CHIR99021, AZD1080, AZD2858, to the variety of assays tested shows that they should not be ruled out as potential therapies for glioblastoma. Given the extremely rapid onset and lethality of glioblastoma, with high recurrence and little progression in treatment options, there is a clear need for alternatives. We propose CHIR99021, AZD1080, AZD2858, and BIO have inherent potential to be anti-invasive treatment options for glioblastoma, and they importantly don’t exhibit cytotoxicity; supplemental research is implicated to further asses their suitability, with a particular focus on examining a potential relationship between GSK3 and contractility of glioblastoma cells.
REFERENCES


http://doi.org/10.1371/journal.pone.0130596


http://doi.org/10.1016/j.wneu.2016.02.032

CHIR-99021 (CT99021) | GSK-3 inhibitor | Read Reviews & Product Use Citation.

(n.d.). Retrieved February 23, 2016, from
http://www.selleckchem.com/products/CHIR-99021.html

http://link.springer.com.ezproxy.bu.edu/chapter/10.1007/978-3-319-12048-5_2


http://doi.org/10.1002/cncr.28949


Retrieved from


http://doi.org/10.1038/nrneurol.2014.208

Miyashita, K., Kawakami, K., Nakada, M., Mai, W., Shakoori, A., Fujisawa, H., ...


Nowicki, M. O., Dmitrieva, N., Stein, A. M., Cutter, J. L., Godlewska, J., Saeki, Y., ...


http://link.springer.com.ezproxy.bu.edu/chapter/10.1007/978-94-007-7037-9_4
http://doi.org/10.1001/jama.2013.280319

http://doi.org/10.1093/neuonc/nou087


CURRICULUM VITAE

Hillary B. Rolfs
hill.rolfs@gmail.com  •  (262) 309-2472  •  Year of Birth: 1992

Current Address: 178 Marlborough St, Apt #5, Boston, MA 02116
Permanent Address: 4978 N Maple Lane, Nashotah, WI 53058

EDUCATION

Boston University, Division of Graduate Medical Sciences, Boston, MA
•  M.S. in Medical Sciences, Expected September 2016

The University of Notre Dame, South Bend, IN
•  B.S. in Pre-Professional Science, 2014
•  Studied at London Campus, Fall 2012

RESEARCH EXPERIENCE

Harvard Institutes of Medicine in association with Brigham and Women’s Hospital, Boston, MA
Research Assistant with Dr. Sean Lawler, July 2015- Present
Investigation of the anti-migratory properties of GSK-3 inhibitors in glioblastoma
•  Cultured various glioblastoma cell lines and ran proliferation, migration, and contractility assays to determine the effectiveness of assorted GSK-3 inhibitors
•  Practiced immunohistochemistry on glioblastoma cells and rodent brain tissue sections to detect proteins of interest and observe tumor migration extent
•  Utilized the Western Blot method to monitor changes in protein levels and degree of phosphorylation of relevant proteins between drugged and control cells

The University of Notre Dame, South Bend, IN
Research Assistant with Dr. Benjamin Ridenhour, January- May 2014
- Extracted DNA from ticks and hosts, and processed it using PCR and gel electrophoresis to look for carriers of the parasites
- Executed DNA sequencing upon identification of a potential parasite to confirm host infection
- Conducted survey of which ticks and hosts could act as carriers

**SKILLS and TECHNIQUES**

- DNA extraction, DNA sequencing PCR, gel electrophoresis
- Statistical analysis
- Histological identification of tissues, cells, pathologies, etc.

**EXPERIENCE**

**Children’s Hospital of Philadelphia, PA**

*Medical Intern* with multiple physicians at the Voorhees, Paoli, and Camden hospitals, May-July 2011
- Assisted orthopedic doctors with paperwork and phone calls
- Shadowed doctors to gain knowledge about injuries, diagnostics, and reading scans
- Learned about the medical field and doctor-patient relationships

**Hospital Pedro Vicente Maldonado, Ecuador**

*Medical Intern* with Dr. David Gaus of Andean Health & Development, May-June 2010
- Shadowed doctors in Emergency care, Outpatient, and a Cesarean section
- Gained valuable insight into the difference that access to healthcare can make in rural settings

**ADDITIONAL ACTIVITIES & INTERESTS**

**ZGiRLS, 2015-2016**
- Volunteered as a mentor for adolescent girls involved in athletics
- ZGiRLS is an organization dedicated to using athletics as a foundation to provide young girls with an environment to discuss relevant issues such as body image, teamwork, and trust to create the next generation of healthy, powerhouse individuals

**GlobeMed Club of Notre Dame, 2012, 2014**
- Participated in presentations and debates on issues in global healthcare
• Aided in fundraising for Promotion for Education and Development Association (PEDA) in Vientiane, Laos

**ND8 Service Club**, 2011, 2012
• Worked to bring awareness and fundraise for the 8 United Nations Millenium Development Goals
  • Focused particularly on awareness of sex trafficking

**Outdoor Leadership and Backpacking Trips**
• Summitted Mt. Kilimanjaro
• Backpacked the Rocky Mountain Range
• Multiple canoe trips to the Boundary Waters, Minnesota

**Club & Intramural Sports**
• Club Ski Team
• Interhall Lacrosse Team
• Intramural Volleyball Team