I, Robert F Koncar, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Systems Biology and Physiology.

It is entitled:
Exploiting genetic vulnerabilities to overcome treatment resistance in adult gliomas

Student’s name: Robert F Koncar

This work and its defense approved by:

Committee chair: El Mustapha Bahassi, Ph.D.
Committee member: Zalma Abdelmalek, Ph.D.
Committee member: Nelson Horseman, Ph.D.
Committee member: Carolyn Price, Ph.D.
Committee member: Peter Stambrook, Ph.D.
Committee member: Yana Zavros, Ph.D.
Exploiting genetic vulnerabilities to overcome treatment resistance in adult gliomas

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of Doctor of Philosophy

In the department of Molecular and Cellular Physiology of the College of Medicine by

Robert F. Koncar

B.S. University of St. Francis 2011

Committee Chair: El Mustapha Bahassi, Ph.D.
Abstract

**Background:** Malignant gliomas currently have a poor prognosis as recurrence remains nearly inevitable despite aggressive treatment. Gliomas are histologically graded as I-IV and the grade IV glioblastoma (GBM) is the most common and lethal malignant glioma with a median survival time of just over one year. Primary GBM arises spontaneously while secondary GBM develops from a recurrent, progressive, lower grade tumor. While primary and secondary GBM are histologically indistinguishable, their genetic profiles and molecular characteristics differ substantially. Primary GBM usually displays amplification or mutation of at least one receptor tyrosine kinase, most often the epidermal growth factor receptor (EGFR). Secondary GBM is typified by the presence of a mutant isocitrate dehydrogenase 1 (IDH1) allele and rarely exhibits EGFR amplification. Despite the striking differences in the molecular development of primary and secondary GBM, they are currently treated with the same general strategy which usually involves surgical resection, radiation therapy, and treatment with temozolomide, a DNA alkylating agent. While this is the most effective treatment plan currently available, it almost universally results in fatal disease recurrence.

**Objective:** The objective of this dissertation was to further our understanding of treatment resistance in adult gliomas and identify targetable vulnerabilities conferred by the genetic characteristics of the tumor to improve treatment efficacy.
Results: To evaluate GBM-specific mechanisms of resistance to the EGFR tyrosine kinase inhibitor, gefitinib, EGFR-overexpressing U87 cells were treated with increasing doses of gefitinib. RNA sequencing of gefitinib resistant cells revealed overexpression of the receptor tyrosine kinase ROS1, which was confirmed in multiple resistant clones by western blot. A cell viability assay showed gefitinib resistant cells to be sensitive to treatment with a combination of gefitinib and a small molecule inhibitor of ROS1, which caused cell cycle arrest in S phase, followed by apoptosis. To verify that our findings were not isolated to the U87-EGFR cells, we generated gefitinib resistant 1048 cells, which were early passage primary GBM cells with EGFR amplification and confirmed ROS1 overexpression and sensitivity to a ROS1 inhibitor.

Immortalized astrocytes transduced with wild type or R132H mutant IDH1 were compared to test the effect of IDH1 mutation on response to temozolomide. Following temozolomide exposure, IDH1 mutant cells exhibited significantly better survival and an accelerated G2 checkpoint exit despite the presence of unrepaired DNA damage. IDH1 mutant astrocytes had increased Polo-like kinase 1 (PLK1) activation and decreased Checkpoint kinase 1 activation. Sensitization of the IDH1 mutant cells to temozolomide was achieved in vitro and in a mouse xenograft with a combination of temozolomide and the PLK1 inhibitor BI2536. We confirmed our findings in cells from an IDH1 mutant patient-derived xenograft.
Conclusions: We identified combination therapy strategies tailored to specific genetic alterations prevalent in primary and secondary GBM. We are the first to report ROS1-mediated gefitinib resistance in GBM cells and that these cells are remarkably sensitive to ROS1 inhibition in combination with gefitinib. Additionally, mutant IDH1 promotes checkpoint adaptation which can be exploited therapeutically with the combination of TMZ and a PLK1 inhibitor.
Acknowledgements

The work presented here would not have been possible without a great deal of help and support. I would like to especially thank the following individuals:

My mentor, Dr. El Mustapha Bahassi, for guiding and supporting me throughout this long process. You were always accessible, providing essential input and direction while also allowing me the freedom to work independently. I have enjoyed the time in your lab which was critical in developing my ability and confidence as a researcher.

My committee members, Drs. Zalfa Abdel-Malek, Nelson Horseman, Carolyn Price, Peter Stambrock, and Yana Zavros for taking time to provide me with critical feedback.

Current and former coworkers and lab members, especially Mike Furgason, Hashim Aljohani, Mohamed Aittaleb, and Ola Gaber for your valuable discussion and friendship and Zhengtao Chu for teaching me the orthotopic brain tumor model.

Dusten and Magaly Unruh, for their friendship and countless scientific discussions. When everyone else was sick of talking science, I could always count on you.
My parents, Ray and Annette, for their constant support and for instilling in me the value of hard work. My siblings for their encouragement and visits to Cincinnati.

Most importantly, I would like to thank my wife, Jamie, to whom I owe this accomplishment. You first recommended applying to PhD programs and have made innumerable sacrifices throughout this process. You critiqued my presentations, edited my manuscripts, and lovingly picked up my slack whenever needed while also working on your own PhD. I will be forever grateful for your support and sacrifices over the past 5+ years.
Table of contents

Abstract .................................................................................................................................................. ii
Acknowledgements .................................................................................................................................. vi
Table of contents ..................................................................................................................................... viii
List of Figures .......................................................................................................................................... xiii
Abbreviations .......................................................................................................................................... xv
Chapter 1: Introduction ............................................................................................................................. 1

1.1 Glioma epidemiology and classification ............................................................................................ 2

1.2 Treatment of malignant gliomas ......................................................................................................... 4
1.2.1 Temozolomide .................................................................................................................................... 4
1.2.2 Targeted Therapies to treat gliomas ................................................................................................. 12

1.3 Molecular development and biology of gliomas ................................................................................. 13
1.3.1 Primary glioblastoma ....................................................................................................................... 13
1.3.2 Secondary glioblastoma and lower grade gliomas ......................................................................... 16

1.4 IDH1 and EGFR as drivers of gliomagenesis ...................................................................................... 20
1.4.1 IDH1 ................................................................................................................................................ 20
1.4.2 EGFR .............................................................................................................................................. 27
Chapter 2: ROS1 amplification mediates resistance to gefitinib in glioblastoma cells

2.1 Abstract: .................................................................38
2.2 Introduction ................................................................39
2.3 Results ........................................................................42
  2.3.1 Identification of ROS1 and DDR1 as mediators of gefitinib resistance in U87 cells overexpressing EGFR protein........................................42
  2.3.2 The increase in ROS1 and DDR1 transcripts in the resistant cells correlates with an increase in protein expression .........................43
  2.3.3 Gefitinib-resistant cells that overexpress ROS1 protein are highly sensitive to ROS1 inhibitors ...............................................................45
  2.3.4 ROS1 inhibition leads to S phase cell cycle arrest followed by cell death in gefitinib-resistant cells.....................................................46
2.4 Discussion ......................................................................48
2.5 Materials and Methods.................................................50
  2.5.1 Isolation of gefitinib-resistant clones..........................50
  2.5.2 RNA Isolation and RNA sequencing ............................51
  2.5.3 RNA Sequencing: ...................................................51
  2.5.4 MTS assay: ..........................................................52
Chapter 3: PLK1 inhibition enhances temozolomide efficacy in IDH1 mutant gliomas ........................................................................................................................................61

3.1 Abstract: ........................................................................................................................................................................63

3.2 Introduction.......................................................................................................................................................................64

3.3 Results .............................................................................................................................................................................66

3.3.1 IDH1 mutant-associated D2HG promotes TMZ resistance..........................................................66

3.3.2 IDH1 mutation promotes premature G2 checkpoint exit following TMZ treatment. .................................................................69

3.3.3 Inhibition of Polo-like kinase 1 sensitizes IDH1 mutant astrocytes to TMZ .................................................................70

3.3.4 A new in vivo model of IDH1 mutant glioma .................................................................71

3.3.5 BI2536 enhances TMZ anti-tumor efficacy in vivo.................................................................72

3.3.6 PLK1 inhibitor plus TMZ combination is effective in IDH1 mutant patient-derived cells .................................................................73

3.4 Discussion .......................................................................................................................................................................74

3.5 Materials and Methods..................................................................................................................................................78
3.5.1 Cell lines ................................................................. 78
3.5.2 Western blotting ....................................................... 78
3.5.3 Nuclear magnetic resonance spectroscopy ..................... 79
3.5.4 Clonogenic survival assay ......................................... 79
3.5.5 Cell Viability assay .................................................. 80
3.5.6 Propidium Iodide staining/Cell cycle analysis ................ 80
3.5.7 Comet Assay ........................................................... 81
3.5.8 Testing drug efficacy in vivo ......................................... 81
3.5.9 Intracranial xenograft ............................................... 82
3.5.10 Statistical Analysis .................................................. 82

3.6 Acknowledgments and conflict of interest: ........................ 83

3.7 Funding support: .......................................................... 83

Chapter 4: Discussion .......................................................... 93

4.1 ROS1 mediates gefitinib resistance in GBM cells ................ 94
  4.1.1 Summary .............................................................. 94
  4.1.2 Future directions ..................................................... 95

4.2 PLK1 inhibition enhances TMZ efficacy in IDH1 mutant gliomas .... 100
  4.2.1 Summary .............................................................. 100
4.2.2 Future Directions.................................................................102

References ......................................................................................106
List of Figures

1.1. Diagram of how TMZ generates cytotoxic double strand DNA damage. ........ 8

1.2. Schematic of key components of TMZ-induced DNA damage G2 checkpoint arrest and recovery .................................................................................................................. 9

1.3. Common genetic alterations in primary glioblastoma .................................. 15

1.4. Genetic alterations in grade II/III glioma ...................................................... 18

1.5. Molecular development of primary and secondary glioblastoma ................. 19

1.6. Reactions catalyzed by wild type and mutant IDH1 ................................. 26

2.1. ROS1 and DDR1 mRNA is upregulated in gefitinib-resistant GBM cells ....... 54

2.2. ROS1 and DDR1 protein expression in gefitinib-resistant cells .................. 55

2.3. ROS1 inhibitor sensitizes gefitinib-resistant cells to the gefitinib ............... 56

2.4. Gefitinib plus ROS1 inhibitor inhibits RTK signaling pathways and induces apoptosis in gefitinib-resistant cells .................................................................................................................. 57

2.5. Upregulation of ROS1 and DDR1 in gefitinib resistant low passage tumor-derived GBM cell line .................................................................................................................. 58

2.6. Overall survival and progression free survival in GBM patients with upregulated ROS1 and DDR1 expression ................................................................. 59
2.7. Treatment of gefitinib-resistant cells with crizotinib ............................... 60

3.1. IDH1 mutation promotes resistance to TMZ by D2HG production .............. 84

3.2. IDH1 mutation does not affect levels of DNA damage following TMZ treatment .................................................................................................................. 85

3.3. Inhibition of PLK1 sensitizes IDH1 mutant cells to TMZ ............................. 86

3.4. Combination of TMZ and BI2536 leads to a marked tumor regression in IDH1 mutant tumors .......................................................................................................................................................................................... 87

3.5. Confirmation of IDH1 WT and R132H mutant cell lines ............................ 88

3.6. Effect of TMZ and BI2536 combination treatment on IDH1 WT NHA ......... 89

3.7. In vivo model of IDH1-mutant glioma .......................................................... 90

3.8. TMZ+BI2536 combination therapy does not cause significant weight loss in mice .................................................................................................................................................................................. 91

3.9. BI2536 sensitizes IDH1 mutant patient-derived xenograft cells to TMZ ....... 92
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>53BP1</td>
<td>tumor protein p53 binding protein 1</td>
</tr>
<tr>
<td>5hmC</td>
<td>5 hydroxymethyl cytosine</td>
</tr>
<tr>
<td>5mC</td>
<td>5 methyl cytosine</td>
</tr>
<tr>
<td>αKG</td>
<td>alpha ketoglutarate</td>
</tr>
<tr>
<td>AKT</td>
<td>AKT serine/threonine kinase</td>
</tr>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>ALKBH</td>
<td>Alkylation repair homolog</td>
</tr>
<tr>
<td>ALT</td>
<td>alternative lengthening of telomeres</td>
</tr>
<tr>
<td>ATM</td>
<td>ATM serine/threonine kinase (formerly ataxia telangiectasia mutated)</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATR serine/threonine kinase (formerly ataxia telangiectasia and Rad3 related)</td>
</tr>
<tr>
<td>ATRX</td>
<td>ATRX, chromatin remodeler (formerly alpha thalassemia/mental retardation syndrome X-linked)</td>
</tr>
<tr>
<td>BIM</td>
<td>BCL2 like 11</td>
</tr>
<tr>
<td>Bora</td>
<td>aurora borealis</td>
</tr>
<tr>
<td>CCDC26</td>
<td>CCDC26 long non-coding RNA</td>
</tr>
<tr>
<td>CD74</td>
<td>CD74 molecule</td>
</tr>
<tr>
<td>CDC25</td>
<td>cell division cycle 25</td>
</tr>
<tr>
<td>CDK1</td>
<td>cyclin dependent kinase 1</td>
</tr>
<tr>
<td>CDK4</td>
<td>cyclin dependent kinase 4</td>
</tr>
<tr>
<td>CDK6</td>
<td>cyclin dependent kinase 6</td>
</tr>
<tr>
<td>CDKN1C</td>
<td>cyclin dependent kinase inhibitor 1C</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>cyclin dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>cyclin dependent kinase inhibitor 2B</td>
</tr>
<tr>
<td>CHK1</td>
<td>checkpoint kinase 1</td>
</tr>
<tr>
<td>CHK2</td>
<td>checkpoint kinase 2</td>
</tr>
<tr>
<td>CIC</td>
<td>capicua transcriptional repressor</td>
</tr>
<tr>
<td>D2HG</td>
<td>D-2-hydroxyglutarate</td>
</tr>
<tr>
<td>DDR1</td>
<td>discoidin domain receptor tyrosine kinase 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>E2F1</td>
<td>E2F transcription factor 1</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGLN</td>
<td>egl-9 family hypoxia inducible factor</td>
</tr>
<tr>
<td>ERBB2</td>
<td>erb-b2 receptor tyrosine kinase 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ERBB3</td>
<td>erb-b2 receptor tyrosine kinase 3</td>
</tr>
<tr>
<td>ERBB4</td>
<td>erb-b2 receptor tyrosine kinase 4</td>
</tr>
<tr>
<td>ERK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>EZR</td>
<td>ezrin</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FIGF</td>
<td>vascular endothelial growth factor D</td>
</tr>
<tr>
<td>FIG</td>
<td>fused in glioblastoma</td>
</tr>
<tr>
<td>FUBP1</td>
<td>far upstream element binding protein 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GBM</td>
<td>glioblastoma</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GRB2</td>
<td>growth factor receptor bound protein 2</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>H3K27</td>
<td>histone 3, lysine 27</td>
</tr>
<tr>
<td>H3K79</td>
<td>histone 3, lysine 79</td>
</tr>
<tr>
<td>H3K9</td>
<td>histone 3, lysine 9</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HIF-1a</td>
<td>hypoxia inducible factor 1 alpha subunit</td>
</tr>
<tr>
<td>IDH1</td>
<td>isocitrate dehydrogenase 1</td>
</tr>
<tr>
<td>IDH2</td>
<td>isocitrate dehydrogenase 2</td>
</tr>
<tr>
<td>JmjC</td>
<td>Jumonji C domain</td>
</tr>
<tr>
<td>LAMTOR</td>
<td>late endosomal/lysosomal adaptor, MAPK and MTOR activator</td>
</tr>
<tr>
<td>LRIG3</td>
<td>leucine rich repeats and immunoglobulin like domains 3</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinases</td>
</tr>
<tr>
<td>MDM2</td>
<td>MDM2 proto-oncogene</td>
</tr>
<tr>
<td>MDM4</td>
<td>MDM4, p53 regulator</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MET</td>
<td>MET Proto-Oncogene, Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>MGMT</td>
<td>O-6-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MLH1</td>
<td>MutL Homolog 1</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MSH2</td>
<td>MutS Homolog 2</td>
</tr>
<tr>
<td>MSH6</td>
<td>MutS Homolog 6</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target Of Rapamycin</td>
</tr>
<tr>
<td>MYC</td>
<td>V-Myc Avian Myelocytomatosis Viral Oncogene Homolog</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromin 1</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NHA</td>
<td>normal human astrocytes</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOD-scid</td>
<td>nonobese diabetic-severe combined immunodeficient</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>O6meG</td>
<td>O-6-methylguanine</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-Ribose) Polymerase</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PDX</td>
<td>patient-derived xenograft</td>
</tr>
<tr>
<td>PFS</td>
<td>progression free survival</td>
</tr>
<tr>
<td>PHLDB1</td>
<td>Pleckstrin Homology Like Domain Family B Member 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-4,5-Bisphosphate 3-Kinase</td>
</tr>
<tr>
<td>PLK1</td>
<td>Polo Like Kinase 1</td>
</tr>
<tr>
<td>PMS2</td>
<td>PMS1 Homolog 2, Mismatch Repair System Component</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase And Tensin Homolog</td>
</tr>
<tr>
<td>RAF</td>
<td>Raf Proto-Oncogene, Serine/Threonine Kinase</td>
</tr>
<tr>
<td>RAS</td>
<td>RAS Proto-Oncogene, GTPase</td>
</tr>
<tr>
<td>RB1</td>
<td>RB Transcriptional Corepressor 1</td>
</tr>
<tr>
<td>RC</td>
<td>resistant clone</td>
</tr>
<tr>
<td>RHO</td>
<td>Rhodopsin</td>
</tr>
<tr>
<td>ROS1</td>
<td>ROS Proto-Oncogene 1, Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>RT</td>
<td>radiation therapy</td>
</tr>
<tr>
<td>RTEL1</td>
<td>Regulator Of Telomere Elongation Helicase 1</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SC</td>
<td>sensitive clone</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDC4</td>
<td>Syndecan 4</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SLC34A2</td>
<td>Solute Carrier Family 34 Member 2</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOS</td>
<td>SOS Ras/Rac Guanine Nucleotide Exchange Factor</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer And Activator Of Transcription 3</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>TET</td>
<td>Tet Methylcytosine Dioxygenase (formerly Ten-Eleven Translocation)</td>
</tr>
<tr>
<td>TKI</td>
<td>tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TMZ</td>
<td>temozolomide</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor Protein P53</td>
</tr>
<tr>
<td>TPM3</td>
<td>Tropomyosin 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>VAV3</td>
<td>Vav Guanine Nucleotide Exchange Factor 3</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>WEE1</td>
<td>WEE1 G2 Checkpoint Kinase</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1.1 Glioma epidemiology and classification

Gliomas are tumors arising from glial cells and account for over 80% of malignant brain tumors. Between 2008 and 2012, nearly 110,000 gliomas were diagnosed in the United States (1). The World Health Organization designates four histological grades based on how malignant the tumor appears. Gliomas most frequently display astrocytic origin, oligodendrocytic origin or both. Astrocytomas may present as grades I-IV while oligodendrogliomas and oligoastrocytomas may present as grades II-III. Grade IV astrocytoma, also referred to as glioblastoma (GBM), is the most common form of glioma as well as the most lethal, with a 5 year survival rate of about 5% and a median survival time of 12-15 months (1,2). Over 12,000 GBM cases were expected in the US in 2016 (1).

Risk for development of a grade III anaplastic astrocytoma and grade IV GBM increases with age and peaks in the range of 75-84 years old while risk of oligodendroglial tumors and oligoastrocytomas is highest in adults 35-44 years old. With the exception of grade I pilocytic astrocytoma, gliomas are more common in males than females. In the United States, gliomas occur in non-Hispanic whites at a higher rate than in blacks, American Indians/Alaskan natives, and Asian/Pacific Islanders (1,2).
A very small percentage of gliomas are associated with a Mendelian cancer syndrome. Excluding these rare cancer syndromes, a family history of glioma is associated with 5%-10% of glioma patients (3). Genome wide association studies have identified heritable single nucleotide polymorphisms (SNP) in seven genes which increase the risk of glioma development (4). SNPs in TERT, EGFR, CCDC26, CDKN2B, PHLDB1, TP53, and RTEL1 are associated with increased incidence of glioma, however not in a monogenic fashion (4–9). Rather, glioma risk and development is best explained by a multifactorial polygenic model (10). While a minority of gliomas result from a genetic susceptibility, over 90% of cases are not linked to a heritable predisposition.

In addition to genetic risk factors, allergic conditions and exposure to ionizing radiation affect glioma risk. Individuals who suffer from asthma, hay fever, eczema, or food allergies are 20-40% less likely to develop a glioma (11–15). The reason for this decrease in risk is unclear, but it does not seem to be related to antihistamine use (13–16). Conversely, studies of atomic bomb survivors and individuals treated for tinea capitis with ionizing radiation determined that exposure to ionizing radiation increases the risk of glioma development in a dose-dependent manner (17,18). Additionally, children who receive cranial radiation therapy as part of treatment for brain tumors or leukemia have an elevated risk of developing a primary glioma as adults (19–21).
1.2 Treatment of malignant gliomas

1.2.1 Temozolomide

The current treatment for malignant gliomas includes surgical resection of the tumor, radiation therapy (RT), and chemotherapy with the DNA alkylating agent temozolomide (TMZ) (22). TMZ offers the greatest benefit to GBM patients when administered concurrently with RT followed by several additional cycles of TMZ. Following surgical resection, concurrent TMZ and RT extends the median survival time from 12.1 months with RT alone, to 14.6 months (23).

TMZ is taken orally and has a half-life of 1.8 hours (24). It is able to cross the blood brain barrier and cerebrospinal fluid concentrations can reach as high as 40% of plasma concentrations (25). TMZ is stable in acidic conditions, however, upon reaching the slightly alkaline bloodstream, it spontaneously hydrolyzes to 5-(3-methyl triazen-1-yl)imidazole-4-carboxamide breaks down into the inactive 5-aminoimidazole-4-carboxamide and the reactive methyldiazonium cation. Methyldiazonium is the reactive species responsible for the DNA methylating activity of TMZ and preferentially targets the N7 position of guanine (~70%), the N3 of adenine (~10%), and the O6 of guanine (~6-8%) (26,27). However, N-7-methylguanine and N-3-methyladenine are efficiently repaired via base excision
repair (28,29). Despite accounting for less than 10% of TMZ-induced lesions, O-6-methylguanine (O6meG) is responsible for its cytotoxic effect (26,30).

O-6-methylguanine methyltransferase (MGMT) is an enzyme which repairs O6meG, converting it back to guanine by transferring the methyl group to itself, after which it is ubiquitinated and degraded via the proteasome (26). If O6meG is not repaired by MGMT, thymine is mispaired with O6meG during DNA replication, triggering DNA mismatch repair (MMR). In an effort to correct the mispair, the thymine on the daughter strand is excised, but a thymine is then reincorporated opposite O6meG, leading to repeated cycles of DNA resection and resynthesis. During the subsequent cell cycle, continuous resection of DNA at sites of O6meG via MMR results in single-stranded DNA gaps that lead to replication fork collapse and formation of DNA double strand breaks which, if unrepaired, lead to apoptotic cell death (29,31–33) (Figure 1.1).

**Effect of temozolomide on cell cycle**

In cells with functional MMR, TMZ elicits DNA damage checkpoint activation and prolonged G2 arrest after two rounds of DNA replication (34–37). G2 checkpoint activation usually involves activation of CHK1 and CHK2, by ATR and ATM, respectively. CHK1 and CHK2 then phosphorylate targets including P53, WEE1, and CDC25 in an effort to inactivate CDK1/Cyclin B and prevent progression
to mitosis (38,39) While ATR and CHK1 are indispensable for DNA damage-induced G2 arrest, ATM and P53 are not essential, especially in the context of TMZ treatment (34,40–43). CDK1 requires removal of inhibitory phosphates by CDC25 for activation, which is necessary for mitotic entry. Therefore, inhibitory phosphorylation of CDC25 by CHK1 promotes G2 arrest. CHK1 also inhibits CDK1 by activating WEE1, an anti-mitotic kinase that phosphorylates CDK1 at tyrosine 15 (44) (Figure 1.2).

Following DNA damage and G2 arrest, there are three possible outcomes: checkpoint recovery, checkpoint adaptation, or cell death. Checkpoint recovery involves progression into mitosis following repair of DNA damage while checkpoint adaptation allows cells to progress through the cell cycle and divide with damaged DNA. Both of these processes require Polo-like kinase 1 (PLK1) in order to inactivate the G2 checkpoint and progress into mitosis (38,45). Checkpoint recovery is the better understood of the two processes and is potentially less deleterious than adaptation. PLK1 activation is inhibited in response to DNA damage (46). Once DNA damage is repaired, Aurora kinase A, with its cofactor Bora, activates PLK1 by phosphorylating threonine 210. PLK1 then phosphorylates WEE1 and Claspin, a required mediator of CHK1 activation by ATR, targeting them for degradation and consequently silencing the CHK1 pathway and preventing CDC25 and CDK1 inactivation (47). PLK1 also phosphorylates CDC25 and cyclin B, promoting their nuclear localization and mitotic entry (48,49). The ATR-CHK1 and
Aurora A-PLK1 signaling axes act antagonistically. While PLK1 prevents CHK1 activation by targeting Claspin, ATR inhibits PLK1 activation by phosphorylating Bora at threonine 501, which targets it for ubiquitination and degradation (50).

The alternative mechanism for progressing into mitosis following DNA damage and G2 arrest, checkpoint adaptation, involves silencing of checkpoint signaling without repair of DNA damage. Checkpoint adaptation is a poorly understood process that is heavily reliant upon PLK1 activation and CHK1 inactivation (51). In unicellular organisms such as yeast, evading cell death and proceeding with replication despite irreparable levels of DNA damage offer clear survival and proliferative advantages. However, because checkpoint adaptation has the potential to promote genomic instability and cancer development, the benefits of checkpoint adaptation in higher eukaryotic organisms is unknown (38,43). It has been hypothesized that checkpoint adaptation may allow cells a chance to repair DNA damage in G1 when conditions favor different repair mechanisms or provide an opportunity to segregate the damaged DNA into one daughter cell and leave one cell relatively healthy. It has also been proposed that checkpoint adaptation may prevent cells from all dying simultaneously upon tissue exposure to a DNA damaging agent, which would lessen the immune response and limit the amount of potentially cancer-causing inflammation (43,52). Further investigation is required to determine if any of these hypotheses are correct.
Figure 1.1. Diagram of how TMZ generates cytotoxic double strand DNA damage.

TMZ methylates guanine, generating O6meG. If O6meG is not directly repaired by MGMT, it mispairs with thymine during DNA replication. In mismatch repair deficient cells the paired thymine is tolerated and results in mutation. In mismatch repair proficient cells, the mispaired thymine is excised as part of the mismatch repair process but is continually replaced with another thymine which is also excised. The futile cycles of mismatch repair result in sites of single strand DNA which turn into cytotoxic double strand breaks upon encountering the replication forks during the second round of DNA replication. Figure adapted from (289)
Figure 1.2. Schematic of key components of TMZ-induced DNA damage G2 checkpoint arrest and inactivation. TMZ treatment elicits checkpoint activation and sustained G2 arrest that is dependent upon ATR and CHK1 activation. If DNA damage can be repaired, checkpoint inactivation and progression into mitosis requires PLK1 activation.
Mechanisms of temozolomide resistance

MGMT expression

As previously mentioned, MGMT is an enzyme responsible for removing methyl groups from O6meG lesions. MGMT activity prevents the cytotoxic action of TMZ and MGMT expression is an established predictor of TMZ efficacy. The promoter of MGMT is methylated in 45-70% of primary malignant gliomas, silencing MGMT expression (26). Patients whose tumors have methylated MGMT promoters and lack of MGMT expression derive the greatest benefit from TMZ while those that express MGMT receive little or no benefit (53–55). When primary gliomas lacking MGMT expression are treated with TMZ, recurrent tumors often have increased MGMT expression and are nonresponsive to TMZ. However, in the absence of TMZ treatment, recurrent tumors do not exhibit elevated MGMT (56). It is not clear whether TMZ exerts a selective pressure for a preexisting population of MGMT-expressing cells, or if TMZ induces MGMT expression.

Mismatch repair defects

The genotoxic effect of TMZ requires MMR processing of O6meG lesions and loss of MMR ability is another mechanism of TMZ resistance common in recurrent tumors. Thymine-O6meG pairs are recognized by the MUTSα heterodimer of MSH2 and MSH6. MUTSα recruits the MUTLα heterodimer (MLH1 and PMS2), facilitating
recruitment of Exonuclease 1 which removes a segment of the daughter strand that includes the mispaired thymine (29). The polymerase then reinserts a thymine opposite O6meG, the process is continually repeated, and replication fork collapse ensues when the replication fork meets the MMR-dependent single stranded DNA, generating the lethal double strand DNA breaks. Without MMR, the pairs of O6meG-thymine are tolerated by the cell and lead to point mutations rather than double strand breaks (32,37,57,58). TMZ resistance-associated MMR deficiency most often occurs through mutation of a MUTSα or MUTLα component. Although MSH6 mutation has been reported in treatment naïve gliomas, it is much more common for MMR deficiency to emerge in a recurrent tumor following TMZ treatment (59–61). More active MMR is likely to enhance TMZ efficacy and a comparison of matched primary and recurrent tumors revealed decreased MSH2, MSH6, and PMS2 expression in secondary tumors as compared to treatment naïve primary tumors (62,63).

*Increased DNA repair by homologous recombination*

TMZ-induced double strand breaks are preferentially repaired via homologous recombination (HR) and cells with impaired HR capability are especially susceptible to TMZ (64). Work with orthotopic mouse models indicates that after TMZ treatment, recurrent tumors may acquire TMZ resistance through augmented homologous recombination and rapid double strand break repair (65).
However there is also evidence that acquired TMZ resistance may be mediated by a defective DNA damage response and failure to activate the G2 checkpoint (66).

### 1.2.2 Targeted Therapies to treat gliomas

The anti-VEGF antibody, bevacizumab, is the only targeted therapy FDA approved to treat GBM and its approval is restricted to use as a second line therapy in tumors that recur following treatment with TMZ and radiation therapy. While bevacizumab may delay disease progression, it does little to extend overall survival times and was rejected by the European Medicines Agency for use in GBM (67,68). Inhibition of targets other than VEGF, including PDGFR, mTOR, and EGFR has been tested in GBMs, however clinical trials have not proven these inhibitors to be beneficial (69). Inhibitors of mutant IDH1 and IDH2 have been developed and are in phase III trials for acute myeloid leukemia and cholangiocarcinoma. However they are still in phase I trials for glioma so there is no clinical data regarding efficacy (70).
1.3 Molecular development and biology of gliomas

1.3.1 Primary glioblastoma

GBMs can occur as either primary or secondary tumors. Primary GBMs rapidly develop de novo while secondary GBMs progress over time from lower grade, usually recurrent, astrocytomas. Primary GBMs account for 80-85% of all GBMs and while histologically identical, primary and secondary GBMs are molecularly distinct pathologies (3,71,72). Primary GBMs are heterogeneous tumors which are typically driven by activation of one or more of three pathways: the PI3-kinase (PI3K) pathway, P53 pathway, and RB pathway (Figure 1.3 A,B).

The PI3K pathway is affected in 90% of GBMs and 39% of the tumors contain multiple alterations to the pathway (73). PI3K activation is most often a result of receptor tyrosine kinase (RTK) mutation or amplification (73,74). Nearly 70% of primary GBMs have at least one altered RTK gene. About 25% of GBMs harbor a mutated PI3K gene. In another 34-40% of tumors, the antagonist to PI3K signaling, PTEN, is mutated or deleted (73,75). Ras-mediated PI3K activation occurs through mutation or deletion of NF1 in 10-18% of GBMs (73,75–77). NF1 inactivates Ras by stimulating Ras GTPase activity (78).
Genomic alterations affecting the P53 pathway are present in over 85% of GBMs and the TP53 gene is mutated or deleted in 28-35% of GBMs (73,75). Amplification of MDM2 or MDM4, which results in P53 inactivation, occurs in about 20% of GBMs and these amplifications are mutually exclusive with TP53 alteration (73,75,79). CDKN2A, the gene for P14ARF, is deleted in 50-60% of GBMs (73,75,76). P14ARF prevents the MDM2-mediated degradation of P53 and CDKN2A deletion results in an effective loss of P53 (80).

Approximately 80% of GBMs have a dysfunctional RB pathway, however only 8-11% exhibit RB1 deletion or mutation (73,75). The RB pathway is most commonly affected by loss of CDKN2A and CDKN2B (50-60%) which are found in the same region of chromosome 9. In addition to coding for P14ARF, CDKN2A also codes for P16INK4A through a different reading frame and CDKN2B encodes P15INK4B. Both P16INK4A and P15INK4B prevent CDK4 and 6 from phosphorylating RB1 (73,75,76,81). Additionally, amplification of the CDK4 or CDK6 gene occurs in about 15% of GBMs (73).

In addition to activation of the three pathways previously described, primary GBMs rely on telomerase activation for telomere maintenance. There is a point mutation in the TERT promoter of 86% of primary GBMs, which results in increased expression of telomerase and guards against telomere shortening (82).
Figure 1.3. Common genetic alterations in primary glioblastoma. A. Alteration rates for commonly altered genes of the RTK/PI3K/MAPK, P53, and RB pathways. Data is presented for 273 primary GBMs from TCGA provisional dataset. Each column represents a single tumor specimen. Plot generated with cBio portal (290). B. The most commonly altered genes and their rate of alteration in GBM mapped by pathway. Adapted from (73). Rates are based on TCGA 2013 GBM dataset.
1.3.2 Secondary glioblastoma and lower grade gliomas

Secondary GBMs develop progressively from lower grade gliomas, usually astrocytomas, and are characterized by a mutation in the isocitrate dehydrogenase 1 (IDH1) gene. While very rare in primary GBMs, about 80% of grades II-III gliomas and secondary GBMs have a single base substitution on a single IDH1 allele at codon 132, or less frequently at codon 140 or 172 of IDH2 (71,83,84). The most common IDH1 mutation, 395G>A results in an R132H mutant protein and accounts for over 90% of all IDH1 mutations in gliomas (85).

IDH1 mutation occurs early in gliomagenesis and is likely responsible for tumor initiation (84,86). Only a few mutations co-occur with the IDH1 mutation at a high rate (Figure 1.4) and they tend to associate with specific cell lineages (Figure 1.5). Astrocytic tumors are characterized by the presence of TP53 and ATRX mutations. ATRX is a histone loader responsible for depositing histone 3.3 at heterochromatin regions. Oligodendrocytic tumors typically present with telomerase expression resulting from a mutation in the TERT promoter, a loss of 1p/19q, and mutation of CIC, FUBP1, or both. (87–94). CIC is a transcriptional repressor whose inactivation by MAPK facilitates changes in gene expression in response to RTK signaling (95). FUBP1 may act as either a transcriptional activator or repressor of MYC (96). Mutation of the TERT promoter and ATRX gene are associated with different mechanisms of telomere maintenance. While TERT promoter mutation is indicative of telomere maintenance by telomerase, loss of
ATRX protein is associated with the alternative lengthening of telomeres (ALT) which is a telomerase independent, DNA recombination-mediated mechanism of telomere elongation (91,97,98).

Patients with IDH1 mutant gliomas tend to be younger than patients with IDH1 wild type gliomas. Comparisons of IDH1 mutant and wild type GBM patients report mean ages ranging from 33 to 41 years for IDH1 mutant patient and 53 to 56 years for wild type patients (76,99). Independent of age, IDH1 mutation is a positive prognostic factor associated with an increased overall survival time compared to tumors with wild type IDH1 regardless of tumor grade. Patients with IDH1 wild type grade II-III gliomas have a median survival time of 1.7 years and patients with an IDH1 wild type GBM have a median survival time of only 1.1 years. If a GBM harbors an IDH1 mutation, median survival time increases to 2.1 years. Loss of 1p19q is also associated with longer survival time. Patients with a Grade II-III glioma containing both an IDH1 mutation and loss of 1p19q have a median survival time of 8.0 years while IDH1 mutant tumors of the same grade but lacking 1p19q codeletion are associated with a 6.3 year median survival time (88).
Figure 1.4. Genetic alterations in grade II/III glioma. Alteration rates for genes of the RTK/PI3K/MAPK, P53, and RB pathways. Data is presented for 283 grade II or III oligodendrogliomas and astrocytomas from the TCGA Low Grade Glioma provisional dataset. Each column represents a single tumor specimen. Plot generated with cBio portal (290).
**Figure 1.5. Molecular development of primary and secondary glioblastoma.** Alterations common to each grade and cell lineage are listed for lower grade gliomas which usually have an IDH1 mutation and progress to secondary GBM in a stepwise fashion. Primary GBMs rapidly develop de novo and there is no identified order in which specific mutations or alterations occur.

LOH, loss of heterozygosity
1.4 IDH1 and EGFR as drivers of gliomagenesis

The development and molecular characteristics of primary and secondary GBMs are clearly independent processes leading to different pathologies, similar only in their histology (Figure 1.5). Therefore, primary and secondary GBMs may benefit from different treatment strategies that target the specific characteristics of each disease. IDH1 and EGFR are the most frequently altered genes in their respective pathologies. Identifying and targeting vulnerabilities conferred by mutant IDH1 and EGFR is an appealing strategy that first requires an understanding of their basic function.

1.4.1 IDH1

IDH1 forms a homodimer and catalyzes the reversible oxidative decarboxylation of isocitrate in the cytoplasm and peroxisomes of cells, generating alpha-ketoglutarate (αKG) and NADPH (100) (Figure 1.6). IDH1 is the main producer of NADPH in the cytoplasm of cells and the largest producer of NADPH in the human brain (101). NADPH is essential for redox homeostasis, most importantly through the reduction of oxidized glutathione. Because IDH1 is a key
producer of NADPH, it’s activity is important in protecting cells from oxidative damage (102–104).

A mutation at arginine 132 alters the conformational state of the IDH1 dimer and prevents isocitrate binding. The mutant subunit instead binds αKG, which it converts to structurally similar D-2-hydroxyglutarate (D2HG) while consuming, rather than producing NADPH. (105,106) (Figure 1.6). Surprisingly, while D2HG concentrations in IDH1 mutant gliomas can range from 5mM to over 30mM, which is about 100 times higher than IDH1 wild type gliomas, mutant IDH1 has little effect on αKG and NADPH levels in cells (106–110). However due to its structural similarity to αKG, D2HG has been shown to competitively inhibit several αKG-dependent enzymes (111–114).

There are approximately 80 known αKG dependent hydroxylases including the Jumonji-C domain-containing histone demethylase (JmjC), Ten-eleven translocation methylcytosine hydroxylase (TET), prolyl hydroxylase (EGLN), and DNA alkylation repair homolog (AlkBH) families of enzymes. All of these hydroxylases require Fe(II), O₂, and αKG to hydroxylate their respective targets while generating CO₂ and succinate (115). Though all αKG-dependent hydroxylases facilitate essentially the same reaction on different substrates, they vary in their sensitivity to D2HG (112).

The EGLN family of hydroxylases are the oxygen sensors of cells and regulate HIF-1a by hydroxylation, which targets it for degradation via the Von
Hippel Lindau pathway. It has been proposed that mutant IDH1 promotes oncogenesis by D2HG mediated inhibition of the EGLN enzymes, which results in HIF-1a stabilization and increased HIF signaling (111). However, other hydroxylases such as the JmjC demethylases are up to 100 times more sensitive to D2HG than the EGLN proteins (112). Alternatively, it has been suggested that EGLNs are activated by D2HG and excessive HIF degradation is responsible for oncogenesis (116). It is also possible that aberrant HIF-1a regulation is not an important consequence of D2HG production since no difference in HIF-1a has been detected between IDH1 mutant and wild type gliomas and no association was found between IDH status and HIF target gene expression in acute myeloid leukemia (117–119).

The JmjC and TET families of αKG dependent hydroxylases are particularly sensitive to D2HG, and their inhibition results in histone and DNA hypermethylation which seems to be one of the oncogenic effects of mutant IDH1 activity. JmjC histone lysine demethylases are among the most sensitive hydroxylases to D2HG. Mutant IDH1 expression or D2HG administration increases H3K9 trimethylation across a variety of cell lines and increases H3K27 and H3K79 methylation in a context-dependent manner (111–113). Most importantly, elevated histone methylation is characteristic of gliomas with an IDH1 mutation (111,113).

TET enzymes are 5-methylcytosine (5mC) hydroxylases that convert 5mC to 5-hydroxymethylcytosine (5hmC) as part of a DNA demethylation process (120).
In human glioma tissue, 5hmC levels are higher in \textit{IDH1} wild type tumors than mutant tumors, which is consistent with D2HG inhibiting TET activity (111). Additionally, expression of IDH1 R132H decreases 5hmC levels in cell lines and knock-in mice expressing IDH1 R132H in neural stem cells also display decreased 5hmC (111,121). Gliomas with an \textit{IDH1} mutation typically display a specific glioma CpG island methylation phenotype, which can be recapitulated in human astrocytes by expression of IDH1 R132H (114). Notably, mutation of \textit{IDH1} or \textit{IDH2} also results in DNA hypermethylation in acute myeloid leukemia, where \textit{IDH} mutant is mutually exclusive with \textit{TET2} mutation, as well as in cholangiocarcinoma and chondrosarcoma, which also have CpG island hypermethylation profiles similar to those of \textit{IDH} mutant gliomas (122–124). Expression of a mutant \textit{IDH} gene in HCT116, a colorectal cancer cell line, HEK293T cells, or murine mesenchymal progenitor cells also results in DNA hypermethylation, suggesting that dysregulation of histone and DNA methylation may be characteristic of all \textit{IDH} mutant malignancies (123–125).

An important potential consequence of altered histone and DNA demethylation dynamics is that the epigenetic modifications may affect a cell’s differentiation state. Mutant \textit{IDH}-induced epigenetic changes have been shown to inhibit differentiation in a variety of cell lines including mouse neural stem cells and hematopoietic cells, and to promote the expression of stem cell markers in immortalized human astrocytes (113,114,124). In mice, mutant \textit{IDH} impairs
hepatocyte differentiation and causes an increase in hematopoietic progenitors (124,126). The development of mutant-specific IDH1 and IDH2 inhibitors revealed that pharmacological inhibition of the mutant IDH enzymes promotes cellular differentiation of glioma and leukemia in mice (127,128). Differentiation of \textit{IDH} mutant cells can also be achieved with methyl transferase inhibitors (129,130). However, the relationship between \textit{IDH} mutation, DNA and histone methylation, cell differentiation, and tumorigenesis is not fully understood. Methyl transferase inhibition lowers DNA methylation and promotes differentiation but does not lower DHG levels. The mutant IDH1 inhibitor is able to decrease D2HG levels and inhibit tumor growth in mice at a dose that does not affect DNA methylation, histone methylation, or differentiation. A higher dose of the inhibitor further decreases D2HG levels and can induce differentiation and reverse H3\textsuperscript{K9} trimethylation, though it does not affect DNA methylation or overall anti-tumor efficacy (127). This discrepancy between the concentrations needed for tumor growth inhibition and for differentiation indicates that the IDH1 mutation also promotes tumor growth via pathways independent of DNA or histone demethylation. These other pathways seem to be more acutely dependent on mutant IDH1 activity, though their activation may only be oncogenic in the context of hypermethylation-induced differentiation impairment, since hypomethylating agents also inhibit tumor growth in mice (129,130).
It would be expected that the neomorphic activity of IDH mutant enzymes would greatly affect the levels of TCA cycle metabolite in addition to producing high concentrations of D2HG. However, the tumor cells seem to be able to compensate for the mutant enzyme as αKG, malate, fumarate, succinate, and isocitrate levels remain relatively unaffected in IDH1 mutant gliomas (106). One compensatory mechanism is the generation of αKG by glutaminolysis, which leaves the cells with decreased glutamine and glutamate pools (131). The dependence upon glutamate to generate αKG and maintain normal levels of TCA cycle metabolites has been shown to leave cells expressing mutant IDH1 vulnerable to inhibition of glutaminase, the enzyme that converts glutamine to glutamate (132). Additionally, IDH1 mutant gliomas overexpress the glutamate dehydrogenases GLUD1 and GLUD2, which generate αKG from glutamate (133).
Figure 1.6 Reactions catalyzed by wild type and mutant IDH1. Wild type IDH1 and IDH2 (gray reaction) convert isocitrate to \( \alpha \)-KG and generate NADPH. Mutant IDH1 and IDH2 (red reaction) convert \( \alpha \)-KG to D2HG while consuming NADPH. \( \alpha \)-KG and D2HG are structurally very similar and differ only by a single hydrogen atom (hydroxyl group in red). Modified from (291)
1.4.2 EGFR

*EGFR*, which codes for the epidermal growth factor receptor, is the most commonly altered gene in GBM, in which it affects nearly 60% of all tumors and accounts for about 80% of all RTK gene alterations (73). The *EGFR* gene is frequently amplified, mutated, or both and *EGFR* copy number and mutation status can vary substantially within different cells of a single GBM (73,134). Canonical EGFR activation requires binding of the ligand, epidermal growth factor (EGF), to the extracellular portion of a membrane-associated EGFR protein. Upon ligand binding, EGFR monomers dimerize and autophosphorylate intracellular tyrosine residues (135,136). Additional EGFR ligands including amphiregulin, betacellulin, and transforming growth factor $\alpha$ are also able to induce EGFR dimerization. In addition to forming homodimers, EGFR may also dimerize with other EGFR family members (ERBB2, ERBB3, ERBB4) (137). Whether EGFR forms homodimers or heterodimers is likely determined in part by what ligand is bound (138).

Upon EGFR dimerization and autophosphorylation, intracellular GRB2 and SOS bind to EGFR and recruit RAS. SOS activates Ras by promoting the exchange of Ras-associated GDP for GTP (139). RAS then activates the MAP kinase pathway through RAF, as well as the PI3K pathway through direct activation of the p110 catalytic subunit. EGFR also recruits the p85 regulatory PI3K subunit which initiates p110 activation and conversion of PIP2 to PIP3 at the cell membrane, to which the pro-survival kinase, AKT, is recruited (140,141).
In addition to gene amplification several other genomic alterations involving EGFR have been reported in GBM including gene fusions, large deletions affecting the extracellular, transmembrane, or C terminal domains, and point mutations in the extracellular and kinase domains (73). Approximately one-third of GBMs have a deletion of exons 2-7 in the EGFR gene which is usually also amplified (73). This variant, referred to as EGFRvIII, results in a transcript with a junction of exons 1 and 8, separated by a new glycine residue. Exons 2-7 correspond to the extracellular domain of the protein, rendering ligands unable to bind EGFRvIII which is then constitutively active at low levels (142). While sufficiently active to stimulate downstream signaling cascades including the PI3K/AKT and RAS/MAPK pathways, its low level of activity prevents it from interacting with endocytic proteins and results in high retention at the cell membrane (143–149). In addition to forming homodimers, EGFRvIII is also able to dimerize with and activate wild type EGFR and the RTK MET in a ligand independent manner (150–152).

**EGFR inhibitors**

Due to frequent alteration in GBM and the variety of inhibitors already approved to treat other malignancies, EGFR has long been a target of interest (69). To date, FDA approved therapies targeting EGFR fall into two categories. The first class of drugs are tyrosine kinase inhibitors (TKI) which are small molecules that block ATP binding at the intracellular tyrosine kinase domain of EGFR and inhibit its
kinase activity. The first clinically available EGFR TKI is gefitinib which was approved in 2003 for use in non-small cell lung cancer (NSCLC) (153). Erlotinib was then approved in 2004 with several more being approved since and even more in clinical development (140). The second class of EGFR-targeted therapies consists of monoclonal antibody-based treatments. Cetuximab, a human-mouse chimeric antibody, was FDA approved for colorectal cancer in 2004 and panitumumab, a fully human monoclonal antibody, received approval in 2006 (154). Monoclonal antibody therapies bind the extracellular portion of EGFR and inhibit activation by preventing ligand binding and RTK dimerization (155–157).

**Resistance to EGFR inhibitors**

While many EGFR inhibitors are available and GBMs are known to be dependent on EGFR signaling pathways, no EGFR-targeted therapies are FDA approved for GBM treatment despite the numerous clinical trials for both antibody and TKI therapies (158–165). Their lack of effectiveness against GBMs can be attributed to both intrinsic and rapidly acquired resistance to EGFR inhibitors (166–169). Resistance is an obstacle for all targeted therapies and specific resistance mechanisms have been identified in various types of cancers treated with EGFR inhibitors. One such mechanism is the presence or development of a secondary EGFR alteration, either innate or acquired, to overcome the mechanism of inhibition. Following treatment of NSCLC with an EGFR TKI, about 60% of tumors
present with a T790M mutation in the tyrosine kinase domain (170). TKIs such as gefitinib and erlotinib are reversible ATP competitive inhibitors and the T790M mutation increases affinity for ATP which then outcompetes the TKIs (171,172). In colorectal cancer, an S492R mutation in EGFR’s extracellular domain has been reported following cetuximab treatment and is associated with cetuximab resistance as the mutation affects the antibody epitope (173).

Another mechanism of EGFR inhibitor resistance is an intrinsic defect in apoptosis induction, eliminating dependence on EGFR and its pro-survival signaling pathways (154). Apoptosis in response to growth factor withdrawal is mediated by the upregulation of the pro-apoptotic protein BIM (174). BIM is also essential for EGFR TKIs to induce apoptosis, however, some individuals have a deletion polymorphism in one BIM allele. Tumors in these individuals are inherently resistant to EGFR TKIs since the tumor cells will not undergo apoptosis in the absence of growth factor signaling (175).

A third mechanism of resistance to EGFR-targeted therapies, and the one most commonly associated with GBMs, is EGFR-independent activation of the EGFR pathway. This pathway activation can occur through an alternative RTK or alteration of a downstream effector (168,170,176–178). While a secondary EGFR mutation is the most common means of resistance in lung cancer, it is rare in GBMs which tend to develop resistance to EGFR inhibitors by signaling around EGFR rather than through it (179). A 2011 study, in which 22 recurrent GBM patients
received at least five days of gefitinib treatment preoperatively, examined EGFR and downstream signaling activation in the surgically resected tumors (168).(168) Gefitinib accumulated to high levels in the tumors and EGFR was efficiently inhibited. However, examination of downstream EGFR pathway effectors showed no difference in activation when compared to untreated GBMs, indicating EGFR was not necessary for activation of the EGFR pathway in these tumors. GBMs are heterogeneous tumors and the RTK status of different cells within a single tumor can be highly variable (74,134,180). Different RTKs such as PDGFR, MET, FGFR, ERBB2, and ERBB3, which are also capable of activating PI3K and RAS, may be amplified or overexpressed in different cell populations of the tumor or altered concurrently with EGFR in the same cells (73,74,176,177,180–184).

The intratumoral heterogeneity of GBMs was clearly illustrated by Francis et al. and Patel et al. through single cell DNA sequencing and RNA sequencing, respectively (74,134). Single cell DNA sequencing revealed a diverse array of subclonal populations with different EGFR alterations, occurring in a mutually exclusive fashion (134). Single cell RNA sequencing revealed that different cells within the tumor rely on EGFR to varying extents. While some cells appear to rely exclusively on EGFR for activation of survival and proliferation pathways, subpopulations of cells highly express other RTKs in addition to, or instead of, EGFR (74). The heterogeneity of GBM tumors makes it difficult to predict which
patients will respond to EGFR inhibitors since the potentially resistant subpopulations of cells cannot yet be routinely identified.

While intratumoral heterogeneity and variable RTK expression result in an intrinsic resistance to EGFR inhibitors, another mechanism of intrinsic resistance involves RTK-independent activation of downstream signaling pathways. Active EGFR dimers recruit and activate PI3K which then activates additional proliferative kinases such as AKT. Activating mutations in PI3K genes or deletion of PTEN, both of which are common in GBM, result in constitutive activation of PI3K signaling and eliminate the need for EGFR activation. Loss of PTEN has specifically been implicated in resistance to both EGFR TKIs and antibody therapies, however not all studies have found PTEN status to be predictive of patient response to therapy (165,169,185).

In addition to possessing innate resistance, GBMs may also acquire resistance to EGFR inhibitors. EGFR activation has been reported to repress expression of PDGFRβ, another RTK, through activation of both MAPK and AKT/mTOR. Inhibition of EGFR can remove the repression of PDGFRβ, the expression of which can compensate for EGFR inhibition (186). Interestingly, GBM cells also seem to be able to actively adjust their dependence on EGFR through changes in extrachromosomal DNA copy number. Treatment of GBM cells with EGFR TKIs can cause a decrease in copy number of extrachromosomal EGFRvIII which is reversible upon removal of the TKI (187). It is possible that asymmetric
segregation of extrachromosomal DNA to daughter cells may account for this phenomenon as mice injected with GBM cells containing either a high or low copy number of EGFR form tumors consisting of cells with both high and low EGFR copy number (187,188).

**Strategies to overcome resistance to EGFR inhibitors**

Several strategies have been employed in an effort to overcome resistance to EGFR inhibitors. Secondary EGFR mutations may be largely addressed with an alternative EGFR inhibitor. The S492R mutation, which confers resistance to cetuximab, does not affect response to panitumumab, an alternative anti-EGFR antibody therapy (173). Additionally, the development of MM-151, an antibody that bind multiple sites on the EGFR extracellular domain, may avoid or delay the development of resistance via secondary mutation (189). T790M mutation, which facilitates gefitinib and erlotinib resistance, is being addressed with mutant-specific inhibitors such as osimertinib, which irreversibly binds specifically to EGFR T790M mutants (190,191).

Use of combination therapy is another strategy of overcoming EGFR TKI resistance. Use of a histone deacetylase (HDAC) inhibitor may be able to overcome resistance mediated by a defective BIM allele. Nakagawa et al and Tanimoto et al showed that gefitinib and osimertinib sensitivity can be restored in
NSCLC cells with a mutant BIM allele by co-treating the cells with the HDAC inhibitor, vorinostat (192,193). Vorinostat promotes the expression of the functional BIM isoform. Combination therapy may also be beneficial for treating GBMs which tend to compensate for EGFR inhibition with other RTKs or by constitutive activation of downstream signaling components. Because EGFR inhibitors may upregulate PDGFRβ expression, a treatment strategy including EGFR and PDGFR inhibitors has been proposed (186). Inhibition of effectors downstream of EGFR is also a potentially promising strategy, especially in tumors with mutant PI3K or PTEN deletion. A combination of anti-EGFRvIII antibody and rapamycin, an inhibitor of the EGFR pathway downstream effector mTOR, was shown to kill PTEN deficient, EGFRvIII-expressing glioma cells much more effectively than antibody therapy alone (185).

While combination therapy is a promising treatment strategy, efforts so far have failed to significantly extend the overall survival time of malignant glioma patients beyond that achieved with the standard treatment plan of surgery, radiation, and temozolomide. Most research concerning resistance to EGFR inhibitors was performed in the context of lung or colon cancer. However, it is now apparent that the most common TKI resistance mechanisms of lung and colon cancers are uncommon in GBMs and additional work is needed to understand GBM-specific resistance. It is also apparent that primary and secondary GBMs develop through different mechanisms and while histologically the same, they are
molecularly separate diseases that may benefit from different treatment strategies. We hypothesized that the specific molecular characteristics of primary and secondary GBMs confer unique vulnerabilities to the tumors that can be exploited with combination therapy. In chapter 2 we identify a unique mechanism of resistance to gefitinib in GBM cells, upregulation of the RTK ROS1, and demonstrate that gefitinib resistant cells are highly susceptible to the combination of gefitinib and a ROS1 inhibitor. In chapter 3 we describe how mutant IDH1 promotes temozolomide resistance through attenuation of the G2 checkpoint, which can be reversed by PLK1 inhibition.
Chapter 2: ROS1 amplification mediates resistance to gefitinib in glioblastoma cells
ROS1 Amplification Mediates Resistance to Gefitinib in Glioblastoma Cells

Hashim Aljohani¹#, Robert F. Koncar¹#, Ahmad Zarzour², Byung Sun Park³, So Ha Lee³ and El Mustapha Bahassi¹

¹Department of Internal Medicine; Division of Hematology/Oncology. 231, Albert Sabin Way, Cincinnati, OH. 45267-0508.

²Department of Internal Medicine, Cleveland Clinic Foundation. 9500 Euclid Ave, Cleveland, OH 44195.

³Chemical Kinomics Research Center, Korea Institute of Science and Technology, Hwarangno 14-gil 5, Seongbuk-gu, Seoul 136-791, Republic of Korea

# Equal contribution

Presented with slight modification as published in (194): 

2.1 Abstract:

Glioblastoma (GBM) is the most aggressive brain tumor in adults and remains incurable despite multimodal intensive treatment regimens. The majority of GBM tumors show a mutated or overexpressed EGFR, however, tumors treated with tyrosine kinase inhibitors (TKIs) will inevitably recur highlighting the need to identify signalling pathways involved in GBM resistance. To this end, we treated GBM cells that overexpress EGFR with increasing concentrations of gefitinib and isolated resistant clones. These clones were subject to RNAseq and the expression of several genes was found to be upregulated. These genes are mainly tyrosine kinase receptors and include ROS1, DDR1 and PDGFRA known to control several downstream targets of EGFR. The upregulation of ROS1 and DDR1 was confirmed at the protein level by western blot. Treatment with a potent and highly specific pyrazole ROS1 inhibitor in ROS1 overexpressing clones led to a sensitization of these cells to low concentrations of gefitinib. Combined treatment with gefitinib and ROS1 inhibitor induces apoptosis following a prolonged S phase cell cycle arrest. Our current study led to the discovery of alternative pathways used by GBM cells to evade cell death following gefitinib treatment and identifies new therapeutic targets to prevent GBM cell resistance to the drug.
2.2 Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults (195,196). Despite multimodal therapy with radiation and the alkylating agent temozolomide, median survival is a dismal 15 months (23). The most common genetic aberration associated with GBM is amplification of the epidermal growth factor receptor gene (EGFR, also referred to as ERBB1 or HER1), with a frequency of about 50% (195). EGFR is a member of the HER superfamily of receptor tyrosine kinases, together with ERBB2, ERBB3, and ERBB4 (197). The structure of each of the members comprises a ligand-binding ectodomain with two cysteine-rich regions, a single transmembrane region, and a cytoplasmic tyrosine kinase (TK) domain (198). Binding of a cognate ligand to the ligand-binding site results in the autophosphorylation of the receptor and induction of downstream signaling through the PI3K/Akt and the MAPK pathways, among others leading to cell differentiation, proliferation, and survival (197,199).

*EGFR* amplification and mutations are also found in breast, lung, and prostate cancers (142). In spite of this, therapies that have been effective for these solid tumors have shown limited efficacy against GBM. EGFR-specific inhibitors have been approved for use in patients with non-small cell lung carcinoma (NSCLC), and are currently in clinical trials for GBM (161,200,201). However, the
clinical experience has been that many GBM patients do not respond to these therapies and those that do eventually show progression (202). Successful treatment of GBM continues to be a major therapeutic challenge due to both inherent and acquired resistance (166,203). Mechanisms causing resistance to EGFR inhibitors have been studied in a number of solid tumors. Some of the documented mechanisms include the acquisition of secondary EGFR point mutations, co-activation and/or amplification of other receptor tyrosine kinases (RTKs), and up-regulation of drug efflux pumps, however, mechanisms of resistance that are unique to glioma are not clearly defined (166,203).

Specific drugs that target EGFR signaling include erlotinib and gefitinib, which reversibly inhibit the EGFR tyrosine kinase domain by competitively binding with ATP, and the monoclonal antibodies (mAbs) cetuximab (a chimeric mouse-human IgG1 antibody) and panitumumab (a fully humanized IgG2 antibody). Cetuximab and panitumumab block ligand binding to the extracellular domain of EGFR, promote receptor internalization and mediate antibody- and complement-mediated cytotoxicity (204). The common EGFR-activating mutations, exon 19 deletions and L858R, which account for 85% of all EGFR mutations, predict sensitivity to the EGFR TKIs (gefitinib, erlotinib and afatinib) in preclinical models and in patients with lung cancer. However, these mutations are largely absent in brain tumors.
To determine the mechanism by which glioblastoma cells acquire resistance to RTK inhibitors, U87 cells overexpressing EGFR were treated with increasing concentrations of gefitinib and resistant clones were isolated, expanded and subject to RNA sequencing (RNAseq). Data analysis revealed that the resistant clones show overexpression of the orphan RTK c-ros oncogene 1 (ROS1), discoidin domain receptor tyrosine kinase 1 (DDR1) or the platelet-derived growth factor receptor, alpha (PDGFRA). Other proteins from the AKT/mTOR pathway were also mildly amplified. Overexpression of ROS1 and DDR1 proteins was confirmed by western blotting. Using a pyrazole ROS1 inhibitor in four of the resistant clones, we were able to sensitize them to gefitinib confirming that the resistance was mediated by ROS1 in these cells. We also showed that both gefitinib and ROS1 inhibitors induce cell death by apoptosis following an S phase cell cycle arrest.
2.3 Results

2.3.1 Identification of ROS1 and DDR1 as mediators of gefitinib resistance in U87 cells overexpressing EGFR protein

To identify genes and pathways that mediate resistance to the EGFR inhibitor gefitinib, U87 glioma cells expressing high levels of EGFR (U87-EGFR) were treated with increasing concentrations of the drug. Kill curve assay showed that the gefitinib IC$_{50}$ concentration for U87-EGFR is 0.75 $\mu$M. We therefore started the screen at 0.75 $\mu$M and gradually escalated the dose up to 3.25 $\mu$M over a period of eight weeks. Cells that survived at this concentration were expanded, pooled together, and subject to RNA-seq. Non treated U87-EGFR gefitinib-sensitive cells were used as controls. The study design is described in Figure 2.1A. Three plates from either non treated or treated cells were used for RNA extraction and RNA sequencing. RNA-seq results showed that besides a statistically significant increase in AKT1, AKT2, AKT3, PDGFB, LAMTOR1, LAMTOR2, LAMTOR3 and FIGF (Figure 2.1B), three tyrosine kinase receptor genes ROS1, DDR1 and PRGFRA showed the most significant increase in the gefitinib resistant cells. Figure 2.1B-D shows a 12 times increase in ROS1 transcript in gefitinib-resistant cells compared to non-
treated cells. Similarly, DDR1 transcript levels were much higher in the gefitinib-resistant cells compared to the sensitive ones (Figure 2.1D). This increase was not specific to U87-EGFR cells and was also observed in a low passage tumor-derived GBM cell line (Figure 2.5). While PDGFRA overexpression has been shown to mediate resistance to EGFR inhibitors, to our knowledge, this is the first report of the involvement of ROS1 and DDR1 in TKI resistance in GBM. Interestingly, a survey of The Cancer Genome Atlas (TCGA) data indicates that ROS1 and DDR1 upregulation correlates with shorter overall survival (OS) and progression free survival (PFS) (Figure 2.6).

2.3.2 The increase in ROS1 and DDR1 transcripts in the resistant cells correlates with an increase in protein expression

Oncogenic activation of ROS1 is observed in a subset of patients with glioblastoma, non–small-cell lung cancer (NSCLC), and cholangiocarcinoma (205–207). In most cases, ROS1 signaling is activated by interchromosomal translocation or intrachromosomal deletion that results in N-terminal ROS1 fusion genes. Several ROS1 kinase fusion proteins have been identified, including the Fused in Glioblastoma–ROS1 (FIG–ROS) that was first discovered in a human glioblastoma cell line and more recently in patients with NSCLC, cholangiocarcinoma, and serous ovarian carcinoma (207–209). The SLC34A2–ROS1 (SLC–ROS) fusion is present in a subset of patients with NSCLC and gastric cancer.
(203,209,210). Other ROS1 fusions include CD74–ROS1, EZR–ROS1, LRIG3–ROS1, SDC4–ROS1, and TPM3–ROS1 (207). To test whether the observed increase in transcript levels of ROS1 and DDR1 translates to an increase in protein level expression, lysates from gefitinib-resistant and gefitinib-sensitive cells were tested for ROS1 protein expression by western blotting. To this end, single clones were derived from the original pool of resistant cells. Single clones were expanded and tested by western blotting for the expression of ROS1 protein. Four clones showed overexpression of different ROS1 fusion proteins (Figure 2.2A). Interestingly, one of the four clones (RC1) shows both overexpression of ROS1 and its ligand VAV3 (Figure 2.2B), a Rho GTPase guanine nucleotide exchange factor, associated with tumor growth, apoptosis, invasion and metastasis, and angiogenesis, which has also been shown to be phosphorylated and activated by ROS1 (211). The pooled resistant cells were also tested for DDR1 protein expression. DDR1 is a receptor tyrosine kinase that is identified during the search for tyrosine kinase proteins expressed in human malignancies (212). DDR1 kinase contains a homology domain to discoidin, which is distinct from other members of the large receptor tyrosine kinase and could be activated by various types of collagens and is found to be involved in cell attachment, migration and invasion (213). Accumulated evidence indicates that DDR1 is overexpressed in invasive tumors including breast, prostate, and lung and cancer cells overexpressing DDR1 display increased migration and invasion (214,215). For instance, upregulated
DDR1 expression promotes cancer development by enhancing cancer cell survival and invasion, and high DDR1 expression is associated with short hormone resistance interval in prostate carcinoma (216). Figure 2.2C shows that while the sensitive cells showed an almost undetectable level of endogenous DDR1 protein, DDR1 protein is highly expressed in lysates from the resistant cells. These data show that ROS1 and DDR1 are upregulated both at the RNA and protein levels and may be responsible for resistance to gefitinib. Since ROS1 inhibitors are readily available, we decided to follow up on the ROS1 overexpressing clones and test the possibility of rendering them sensitive to low concentrations of gefitinib using a pyrazole ROS1 inhibitor that we have previously shown to specifically target ROS1 protein but not a large panel of kinases including tyrosine kinase receptor proteins.

2.3.3 Gefitinib-resistant cells that overexpress ROS1 protein are highly sensitive to ROS1 inhibitors

We have previously described a number of pyrazole compounds that have a potent and selective activity with IC$_{50}$ value of 199 nM for ROS1 (217). Recently, we synthesized a new pyrazole compound that shows excellent inhibition of ROS1 enzymatic activity with an IC$_{50}$ value of 23.2 nM and it also showed high selectivity for ROS1 kinase (Figure 2.3A) (218). To test whether gefitinib-resistant cells that overexpress ROS1 fusions can be sensitized with the ROS1 inhibitor to low
concentrations of gefitinib, the four clones that show over-expression of the ROS1 fusions as well as gefitinib-sensitive cells were subject to increasing concentrations of the ROS1 inhibitor either singly or in combination with 1 μM gefitinib. Data in Figure 2.3B show that gefitinib-resistant cells are sensitive to ROS1 inhibitor and a 2X the IC$_{50}$ of the drug (23.2 nM) combined with 1 μM of gefitinib achieved an almost complete growth inhibition of the resistant cells in 36 hours while ROS1 inhibitor only did not show a dramatic effect on gefitinib-sensitive cells. Crizotinib, an FDA approved drug for treatment of Non-Small Cell Lung Carcinoma (NSCLC) patients with rearranged ROS1 was used to treat these resistant cells and showed a moderate effect compared to the pyrazole ROS1 inhibitor (Figure 2.7). These data show that ROS1 inhibition provide a therapeutic alternative in gefitinib-resistant cells that overexpress ROS1 fusions.

2.3.4 ROS1 inhibition leads to S phase cell cycle arrest followed by cell death in gefitinib-resistant cells.

To investigate the mechanism by which ROS1 inhibitor induces cell death in gefitinib-resistant cells, we analyzed the cell cycle profile of both gefitinib-sensitive and gefitinib-resistant cells after treatment with either gefitinib, ROS1 inhibitor or a combination of both inhibitors (Figure 2.4A). While the non-treated cells show a normal cell cycle distribution in both gefitinib-sensitive and gefitinib-resistant cells,
despite small differences mainly in G2 and S phase populations, the cell cycle profile of gefitinib-resistant cells did not show any noticeable changes after treatment with 3 μM gefitinib. However, the gefitinib-sensitive cells showed an increase in S phase population, a slight increase in G2 population and the accumulation of a sub-G1 population, suggestive of active cell death likely by apoptosis. Treatment of gefitinib-resistant cells with gefitinib only led to a slight increase in S phase population. Treatment with pyrazole ROS1 inhibitor led to a slight increase in S phase cells and the start of the appearance of a small sub-G1 population. Such a sub-G1 population was completely absent in the sensitive cells suggesting that sensitive cells do not rely on ROS1 for their growth. Combination of gefitinib and pyrazole ROS1 inhibitors led to a sharp S arrest, an almost complete absence of mitotic cells, a sharp decrease in G1 population and accumulation of cells in sub-G1 in both resistant and sensitive cells. These data indicate that ROS1 inhibition of cell growth in gefitinib-resistant cells is mediated through a prolonged S phase checkpoint arrest followed by accumulation of a sub-G1 population indicative of cell death by apoptosis. The apoptotic phenotype is supported by an increase in PARP cleavage in cells treated by the ROS1 inhibitor and ROS1 inhibitor combined with gefitinib (Figure 2.4B). Since ROS1 activates some of the same pathways that are activated by EGFR, we investigated whether the ROS1 inhibitor compound was able to inhibit the downstream EGFR effectors, mainly AKT1 and p42 MAPK proteins. Indeed, while gefitinib alone was unable to
inhibit the active forms of these proteins in the resistant clones, ROS1 inhibitor alone or in combination with gefitinib efficiently inhibited the expression of both p-AKT1 and p-p42 MAPK (Figure 2.4C).

2.4 Discussion

Resistance to EGFR therapy is an endemic problem faced in the clinic every day. While the majority of brain tumors show an overexpressed or mutant EGFR, TKI remain inefficient and even patients that show some response eventually recur as a result of acquired resistance. While in lung cancer, mutations that are associated with TKI sensitivity are well established and secondary mutations that lead to acquired resistance are also well known (219,220), no such mutations have been reported in brain tumors. To identify genetic determinants associated with resistance to EGFR therapy, and specifically to the TKI inhibitor, gefitinib, we carried out RNA-seq of gefitinib-resistant and gefitinib-sensitive clones and identified three proteins that were highly expressed as a result of the inhibition of the EGF receptor namely ROS1, DDR1 and PDGFRA. Of importance, all the three proteins are tyrosine kinase receptors themselves and regulate the same signaling pathways that are associated with EGFR activation. While PDGFRA upregulation has been associated with TKI resistance, to our knowledge, there are no reports of
ROS1 and DDR1 involvement in this process. We therefore followed up on DDR1 and ROS1 and confirmed that these proteins were upregulated at the protein level as well and not only at the RNA level. Blotting for ROS 1 protein also showed that several fusion proteins involving ROS1 were expressed. As indicated earlier, ROS1 is activated as a fusion protein with several proteins leading to a constitutively active chimeric protein. To further investigate the role of ROS1 overexpression in gefitinib resistance, we used the pyrazole ROS1 inhibitor that is very highly potent and specific and showed that this inhibitor sensitizes gefitinib-resistant cells to the drug at low concentrations. The mechanism by which ROS1 induces cell death in combination with gefitinib in both resistant and sensitive cells is through a cell cycle arrest in S phase and a subsequent cell death by apoptosis.

Given the recent success of molecularly targeted therapies in treating cancers driven by oncogenic kinases, there is acute clinical momentum to identify inhibitors that selectively target ROS1 fusions. Because the ROS1 and Anaplastic Lymphoma Kinase (ALK) domains are partially homologous, the Food and Drug Administration (FDA)-approved ALK/MET kinase inhibitor crizotinib is being investigated via phase I/II clinical trials for its efficacy in ROS1-driven lung cancer patients (221). The ROS1 inhibitor described in this study showed a stronger inhibitory effect on ROS1 rearrangements compared to crizotinib. More recently, foretinib (GSK1363089) and Gö6976 were also shown to be potent inhibitors of
ROS1 (222). Foretinib was shown to selectively suppress the growth of ROS1 fusion-driven cell lines as well as of FIG–ROS-driven tumors in mice (222).

These data indicate that a genetic screen of tumors that develop resistance to TKI can reveal alternative pathways that drive resistance to the drugs and these pathways can be targeted to achieve cell death. Developing inhibitors to the proteins identified during this study will provide alternatives to patients with recurrent brain tumors that are refractory to EGFR therapy.

2.5 Materials and Methods

2.5.1 Isolation of gefitinib-resistant clones

To isolate gefitinib-resistant clones, U87 GBM cells were transfected with a retroviral construct that expresses EGFR. Stable clones were isolated by selection with puromycin. EGFR Protein overexpression was confirmed by western blot. To isolate gefitinib-resistant clones, 70-80% confluent cells were treated with increasing concentrations of gefitinib starting from 0.75 μM for 2 weeks. The remaining viable cells were then treated with 1 μM gefitinib, and repeated the procedures until we reached 3 μM. Cells that were able to survive 3 μM were pooled together and used for RNA extraction and RNA sequencing.
2.5.2 RNA Isolation and RNA sequencing

RNA was isolated from gefitinib sensitive and gefitinib resistant U87-EGFR cells. These cells were grown as monolayer in DMEM media and lysed in the culture dish by addition of RNAzol®RT reagent (Molecular Research Center, Inc. Catalog No: RN 190). Culture medium was first removed and 1 ml of RNAzol®RT reagent was added per 3.5 cm culture dish (10 cm²). The lysate was then passed through a pipette several times and the RNA extracted according to the manufacturer’s protocol. RNA was solubilized in RNAase free water by vortexing 2-5 min at room temperature to attain a typical yield of 1 to 2 µg/ml total RNA.

2.5.3 RNA Sequencing:

RNA-seq was performed at the Cincinnati Children’s Hospital Medical Center (CCHMC) Genetic Variation and Gene Discovery Core. In short, a library of cDNA fragments is formed from the extracted RNAs, and then sequencing adaptors were added to the cDNA library. Several short fragment sequences were sequenced and the sequencing reads were aligned to the reference transcriptome.
2.5.4 MTS assay:

To evaluate the proliferation and survival, we used CellTiter 96® AQueous one solution cell proliferation assay from Promega that measures the metabolic activity of the cell lines under each treatment condition, according to the standard protocol (Promega).

2.5.5 Western Blots:

In order to confirm the protein expression of ROS1, DDR1, VAV3, we used western blotting following standard protocols. A combination of chemiluminescent detection and LI-COR detection was used in this study. For the detection of ROS1 protein expression, we used ROS1 (69D6, Cell Signaling Technology Inc. catalogue #3266) mouse mAb. For detection of Vav3 protein expression, we used Vav3 antibody, which recognizes endogenous Vav3 protein, (Cell Signaling Technology Inc. catalogue #2398). For DDR1 protein detection, we used DDR1 (D1G6, Cell Signaling Technology Inc. catalogue #5583) rabbit mAb antibody. Loading controls β actin (8H10D10) mouse mAb (#3700) and alpha tubulin antibody (#2148) were all from Cell Signaling Technology Inc. For apoptosis and downstream EGFR signaling, cells were treated with 3 μM Gefitinib for 24 hours and treated with 24nM Ros1 inhibitor for 12 hours. Antibody for p44/p42 MAPK (Erk1/Erk2) is for dually phosphorylated Erk1 (T202, Y204) and Erk2 (T185, Y187) or singly
phosphorylated at T202. We only detected Erk2 (T185, Y187). All antibodies are from Cell Signaling Technologies: p44/p42 MAPK catalogue #4370; PARP catalogue #9542; and pAKT1 catalogue #2965.

2.5.6 Flow cytometry for cell cycle profiling:

For cell cycle analysis, cell staining with propidium iodide was used to determine DNA content following the standard protocol. The profiles were obtained using Beckman Coulter™ Cell Lab Quanta flow cytometer. Data was analyzed using Cell Lab Quanta SC and Modfit Lt™ DNA data analysis software.

2.6 Acknowledgments and conflict of interest:

We would like to thank the University of Cincinnati, the Center for Clinical and Translational Science and Training (CCTST) and the UC Brain Tumor Center for providing funding for this project and Dr. Vallabhapurapu Subrahmanya Duttu for helping with the flow cytometry experiments. The authors declare no conflict of interest.
Figure 2.1: ROS1 and DDR1 mRNA is upregulated in gefitinib-resistant GBM cells. A. Outline of the experimental strategy used to isolate and characterize the gefitinib-resistant cells. B. A list of the most upregulated genes in the gefitinib-resistant clones. C. A ratio of ROS1/GAPDH and D. DDR1/GAPDH expression in untreated and gefitinib resistant U87 cells.
Figure 2.2: ROS1 and DDR1 protein expression in gefitinib-resistant cells.

To confirm that the increase in mRNA expression translates to an increase in protein expression, several clones were tested for the expression of ROS1 protein. A. Four resistant clones (RC) show the expression of different ROS1 fusion proteins (indicated by asterisk). The sensitive clone control (SC) did not show such fusions. B. Resistant clone #1 shows an overexpression of Vav3, a target of ROS1 and a potential ROS1 ligand as well. C. The gefitinib-resistant cell pool was also tested for the upregulated expression of the DDR1 protein.
Figure 2.3: ROS1 inhibitor sensitizes gefitinib-resistant cells to the gefitinib. A. Structure of pyrazole ROS1 inhibitor. B. Cell viability of gefitinib sensitive U87-EGFR cells and gefitinib resistant clones 36 hrs after treatment with ROS1 inhibitor and 1μM gefitinib. C1, C3, C5 and C8 indicate the four resistant clones that overexpress ROS1 fusions. P<0.05 vs untreated (*). P<0.05 vs untreated and vs gefitinib treated (**).
Figure 2.4: Gefitinib plus ROS1 inhibitor inhibits RTK signaling pathways and induces apoptosis in gefitinib-resistant cells. A. Pyrazole ROS 1 inhibitor sensitizes gefitinib-resistant cells to gefitinib through a prolonged S phase checkpoint arrest followed by cell death by apoptosis as indicated by the accumulation of cells in sub-G1 phase of the cell cycle. B. Increased PARP cleavage in cells treated with ROS1 inhibitor and gefitinib. C. Inhibition of pAKT1 and p-p42MAPK proteins following treatment with gefitinib and ROS1 inhibitor.
Figure 2.5. Supplemental Data: Upregulation of ROS1 and DDR1 in gefitinib resistant low passage tumor-derived GBM cell line. The 1048 GBM cell line (passage 7) was treated with increasing concentrations of gefitinib similar to what was described for U87 cells. The resistant clones were pooled and tested by real time PCR for the transcript levels of ROS1 and DDR1. Pooled gefitinib resistant U87 cells were used as a control. GAPDH expression was used for normalization.
Figure 2.6. Supplemental Data: Overall survival and progression free survival in GBM patients with upregulated ROS1 and DDR1 expression.

Survival data from TCGA indicates that GBM patients with upregulated ROS1 or DDR1 have shorter overall survival (OS) and progression free survival (PFS) times.
Figure 2.7. Supplemental Data: Treatment of gefitinib-resistant cells with **crizotinib**. Survival of gefitinib-sensitive cells and gefitinib-resistant clone C1 cells after crizotinib treatment.
Chapter 3: PLK1 inhibition enhances temozolomide efficacy in IDH1 mutant gliomas
PLK1 inhibition enhances temozolomide efficacy in IDH1 mutant gliomas

Robert F. Koncar¹, Zhengtao Chu¹, Lindsey E. Romick-Rosendale², Susanne I. Wells², Timothy A. Chan³, Xiaoyang Qi¹ and El Mustapha Bahassi¹∗

¹Department of Internal Medicine; Division of Hematology/Oncology, University of Cincinnati. 231 Albert Sabin Way, Cincinnati, OH. 45267-0508.

²Division of Oncology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA.

³Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Presented as published in (223):

3.1 Abstract:

Despite multimodal therapy with radiation and the DNA alkylating agent temozolomide (TMZ), malignant gliomas remain incurable. Up to 90% of grades II-III gliomas contain a single mutant isocitrate dehydrogenase 1 (IDH1) allele. IDH1 mutant-mediated transformation is associated with TMZ resistance; however, there is no clinically available means of sensitizing IDH1 mutant tumors to TMZ. In this study we sought to identify a targetable mechanism of TMZ resistance in IDH1 mutant tumors to enhance TMZ efficacy. IDH1 mutant astrocytes rapidly bypassed the G2 checkpoint with unrepaired DNA damage following TMZ treatment. Checkpoint adaptation was accompanied by PLK1 activation and IDH1 mutant astrocytes were more sensitive to treatment with BI2536 and TMZ in combination (<20% clonogenic survival) than either TMZ (~60%) or BI2536 (~75%) as single agents. In vivo, TMZ or BI2536 alone had little effect on tumor size. Combination treatment caused marked tumor shrinkage in all mice and complete tumor regression in 5 of 8 mice. Mutant IDH1 promotes checkpoint adaptation which can be exploited therapeutically with the combination of TMZ and a PLK1 inhibitor, indicating PLK1 inhibitors may be clinically valuable in the treatment of IDH1 mutant gliomas.
3.2 Introduction

Malignant gliomas are currently associated with a dismal prognosis and recurrence remains nearly inevitable despite a multimodal treatment strategy (224,225). Gliomas are histologically graded as I-IV and 70-90% of grades II-III gliomas and secondary grade IV glioblastomas contain a mutation in one Isocitrate dehydrogenase 1 (IDH1) allele, with R132H being the most common (86,226,227). IDH1 is found in the cytoplasm and peroxisomes where it converts isocitrate to alpha ketoglutarate (αKG). However, the mutant enzyme converts αKG into oncometabolite D-2-hydroxyglutarate (D2HG), which is structurally similar to αKG and a competitive inhibitor of αKG-dependent dioxygenases (106,111,112).

Treatment for gliomas typically consists of surgical resection, radiation therapy, and chemotherapy with the DNA alkylating agent, temozolomide (TMZ) (228). The cytotoxic effect of TMZ is mediated primarily through generating O-6-methylguanine (O6meG) lesions (26). If the methyl group is not removed by O-6-methylguanine-DNA methyltransferase (MGMT), an enzyme associated with TMZ resistance, O6meG mispairs with thymine during DNA replication, leading to futile rounds of mismatch repair and persistent G2 checkpoint arrest followed by apoptosis or senescence (35). MGMT promoter methylation and consequently low MGMT expression is typical in, but not unique to IDH1 mutant gliomas (229), which
generally respond better to TMZ than their \( IDH1 \) wild type (WT) counterparts (230,231). However, MGMT expression is not the sole determinant of TMZ sensitivity (62,64,65,232) and \( IDH1 \) mutant and wild-type gliomas have different molecular ontogenies, making comparisons between \( IDH1 \) mutant and wild type gliomas uninformative as to which tumor characteristics can be attributed directly to \( IDH1 \) mutation. Grade II-III gliomas lacking the \( IDH1 \) mutation are genetically distinct from \( IDH1 \) mutant gliomas and are more similar to primary grade IV glioblastomas. While genetic alterations such as \( EGFR \) amplification and \( CDKN2A \) deletion are common in \( IDH1 \) WT gliomas, they rarely occur in gliomas with mutant \( IDH1 \) (88). Despite being considered chemoresponsive IDH1 mutant gliomas commonly recur even after surgical resection and treatment with radiation and temozolomide, highlighting the need for new treatment options (233–235).

Recent evidence suggests that \( IDH1 \) mutant-mediated transformation promotes TMZ resistance and rapid G2 checkpoint exit due to increased homologous recombination capability (36). How IDH1 affects DNA repair and checkpoint signaling however, is unknown. The DNA damage checkpoint is a critical process that coordinates cell cycle progression with DNA damage repair. Thus, understanding how \( IDH1 \) mutation affects checkpoint signaling may reveal ways to further sensitize IDH1 mutant tumor cells to TMZ.

Polo-like kinase 1 (PLK1) is a key regulator of mitotic progression following DNA damage-induced G2 checkpoint activation. It is involved in checkpoint
recovery, which requires repair of damaged DNA, and checkpoint adaptation, in which cell division occurs with unrepaired DNA damage (38). PLK1 is commonly overexpressed or over-activated in cancer, and is the target of several promising drugs in late stage clinical trials (236).

In this study, we sought to elucidate the mechanism of TMZ resistance and to identify potential targets to enhance TMZ efficacy in IDH1 mutant tumors. To this end, we used immortalized astrocytes to ask whether mutant IDH1 promotes TMZ resistance as a consequence of D2HG production and whether checkpoint adaptation, mediated through PLK1 activation rather than swift DNA damage repair accounts for the early progression out of G2 arrest. We show that IDH1 mutant cells and tumors can be greatly sensitized to TMZ by inhibiting PLK1 in vitro, as well as in a xenograft mouse model.

3.3 Results

3.3.1 IDH1 mutant-associated D2HG promotes TMZ resistance

To study the effects of mutant IDH1, we used normal human astrocytes (NHA) which have been immortalized and described elsewhere (237). When transformed by expression of an exogenous mutant IDH1 gene, the NHA
epigenetically resemble IDH1 mutant gliomas (114). A hemagglutinin (HA) tagged WT or R132H mutant IDH1 gene was introduced into the NHA by retroviral transduction and gene expression was confirmed by Western blot (Figure 3.1A). WT and IDH1 R13H clones showing comparable levels of exogenous wild type and mutant IDH1 proteins were selected. The WT and mutant cell lines were additionally confirmed by Sanger sequencing (Figure 3.5A). NMR spectroscopy revealed increased 2HG concentrations in the IDH1 mutant cells (Figure 3.5B).

After confirming the presence of the IDH1 mutation and 2HG production by the astrocytes we used them to test the effect of IDH1 mutation on TMZ sensitivity by clonogenic survival. After treatment with TMZ (100 μM), mutant IDH1 NHA were significantly less sensitive to TMZ while WT NHA displayed an intermediate phenotype between the control and IDH1 mutant cells (Figure 3.1B), which is consistent with published data (36). Differences in TMZ sensitivity were not due to differential MGMT expression as all three cell lines were MGMT deficient (Figure 3.1C).

The response of an MGMT deficient cell line such as NHA to TMZ is prolonged G2 arrest (35,36). This characteristic arrest was seen in the control NHA which displayed sustained G2 arrest up to 7 days after TMZ treatment (Figure 3.1D). However, IDH1 mutant NHA displayed a much shorter G2 arrest and by day 3 post TMZ treatment, the percentage of cells in G2 was similar to that of untreated cells (Figure 3.1D). IDH1 WT NHA again displayed an intermediate
phenotype between the mutant and control NHA with sustained G2 arrest through Day 4 post TMZ.

Production of D2HG is considered to be the primary means by which mutant IDH1 promotes oncogenesis (111,112,238). D2HG is required for maintenance of oncogenic properties of IDH1 mutant cells and has been shown to inhibit the activity of αKG-dependent dioxygenases which may play a role in cellular transformation (106,111,238). To determine if TMZ resistance conferred by IDH1 mutation is mediated by D2HG production, we evaluated TMZ sensitivity in IDH1 mutant and parental NHA treated with D2HG, which has been shown to rapidly enter cells (239), at a concentration (5mM) within the range of what has been reported in IDH1 mutant gliomas (106,240). In parental NHA, D2HG alone resulted in a mild but significant decrease in survival which is consistent with reported effects of D2HG treatment on glioma cell lines (239). However, D2HG treatment resulted in increased survival of parental NHA treated with TMZ (Figure 3.1E), and there was a significant interaction between TMZ treatment and D2HG treatment (P=0.02), indicating D2HG production acutely promotes TMZ resistance. D2HG treatment had no effect on the IDH1 mutant astrocytes.
3.3.2 IDH1 mutation promotes premature G2 checkpoint exit following TMZ treatment.

DNA damage-induced G2 checkpoint arrest is followed by checkpoint recovery, checkpoint adaptation, or apoptosis. While checkpoint recovery allows cell cycle progression after repair of damaged DNA, checkpoint adaptation allows mitotic progression despite unrepaired DNA damage (38). To determine whether the shortened TMZ-induced G2 arrest in IDH1 mutant cells is due to efficient repair of damaged DNA or premature checkpoint override with residual unrepaired DNA damage, we measured total DNA damage by alkaline comet assay. IDH1 mutant astrocytes did not show statistically different levels of DNA damage than control and IDH1 WT astrocytes at days 1, 3, and 7 post TMZ (Figure 3.2 A,B). We next specifically examined the more lethal double-strand breaks by neutral comet assay, and again found no statistically significant differences between the three cell types (Figure 3.2 C,D), even at three and five days after TMZ treatment, when IDH1 mutant cells have already exited the G2 checkpoint (Figure 3.1D). These data indicate that IDH1 mutant astrocytes prematurely exit the G2 checkpoint with unrepaired DNA damage and that progression into mitosis is facilitated by checkpoint adaptation rather than checkpoint recovery.
3.3.3 Inhibition of Polo-like kinase 1 sensitizes IDH1 mutant astrocytes to TMZ

PLK1 regulates G2 checkpoint adaptation and progression into mitosis following DNA damage and acts antagonistically to the CHK1 signaling pathway which is essential for maintenance of G2 checkpoint arrest (41,45–47,241). Aberrant PLK1 activation can facilitate G2 checkpoint bypass and repress apoptotic signaling pathways, allowing cells to divide and survive despite failing to complete repair of damaged DNA (38,242,243). One mechanism by which PLK1 inactivates the G2 DNA damage checkpoint is through phosphorylation of the CHK1 regulatory protein Claspin, which targets it for degradation and leads to the inactivation of the ATR/CHK1 signaling pathway (47). Examination of PLK1 and CHK1 phosphorylation in TMZ-treated cells revealed elevated PLK1 activation and diminished CHK1 activation in the IDH1 mutant astrocytes (Figure 3.3A).

Several PLK1 inhibitors are currently in clinical trials as cancer therapeutics (236). To determine if inhibition of PLK1 sensitizes IDH1 mutant astrocytes to TMZ, we treated cells with BI2536, a potent and selective PLK1 inhibitor. Co-treatment of IDH1 mutant cells with TMZ and BI2536 resulted in more than a threefold decrease in clonogenic survival rate when compared to TMZ or BI2536 treatment alone (P<0.001) (Figure 3.3B). Compared to TMZ alone, treatment with BI2536 and TMZ also increased CHK1 activation at day 3 post TMZ (Figure
3.3C. Combination treatment of IDH1 WT cells resulted in a less dramatic, though significant reduction in clonogenic survival when compared to treatments of either TMZ or BI2536 alone (Figure 3.6).

3.3.4 A new in vivo model of IDH1 mutant glioma

Since inhibition of PLK1 sensitized IDH1 mutant NHA to TMZ in vitro, we sought to determine whether a combination of TMZ and BI2536 is more effective than either single drug in vivo. However, cells from the majority of IDH1 mutant gliomas do not grow in vitro and tumors passaged directly in mice do not consistently retain their original characteristics (130,244,245). We assessed the tumorigenic potential of the IDH1 mutant astrocytes in subcutaneous and orthotopic xenograft mouse models. Astrocytes expressing mutant IDH1 for either 15, 25, or 50 passages (P15, P25, P50, respectively) were injected subcutaneously in mice. The astrocytes were all of approximately the same total passage number, and differed only in the number of passages with the IDH1 mutant gene. All mice injected with the IDH1 mutant cells formed tumors. However, the time to tumor formation depended on the number of passages for which the cells expressed mutant IDH1 (Figure 3.7A). The P50 cells formed tumors by 11 weeks, while P25 cells averaged almost 13 weeks, and P15 cells averaged more than 18 weeks
to form tumors (Figure 3.7 A,B). Targeted sequencing revealed retention of wild-type and mutant IDH1 alleles in the tumors (Figure 3.7C).

P50 cells were also transduced with a constitutively active luciferase reporter and tested for orthotopic tumorigenicity in the mouse brain. The first tumor was detectable by six weeks after injection and was fatal by week 9 (Figure 3.7D). Of 12 mice injected, only three formed tumors that were clearly detectable by luminescence. We therefore chose to test the therapeutic treatments in the subcutaneous model.

### 3.3.5 BI2536 enhances TMZ anti-tumor efficacy in vivo

After establishing that IDH1 mutant astrocytes form tumors efficiently as subcutaneous xenografts, we tested TMZ and BI2536 as a combination treatment for subcutaneous, IDH1 mutant tumors in NOD- scid IL2Rgamma^{null} mice. Mice were injected twice, three days apart, with 80mg/kg of TMZ and 40mg/kg BI2536 either alone or in combination, or with vehicle. Tumor volumes were tracked for 28 days. The combination therapy had significantly greater anti-tumor efficacy than TMZ or BI2536 as monotherapies (day 28 P<0.001). Treatment with the combination of both drugs produced a remarkable reduction in tumor size. At day 28, tumors in TMZ or BI2536 treated mice were similar in size to those of the vehicle treated mice (Figure 3.4 A,B). Notably, five of eight mice treated with
both drugs exhibited complete tumor regression. In the three cases where tumor regression was not complete, the tumor shrank markedly (Figure 3.4B), with no tumor exceeding 20mm$^3$ at day 28. In contrast, each of the other three treatment groups had an average tumor volume exceeding 900mm$^3$ at day 28 (Fig 3.4A). Mice receiving the combination treatment showed no obvious signs of toxicity and lost no more than 10% body weight at any point in time (Figure 3.8).

3.3.6 PLK1 inhibitor plus TMZ combination is effective in IDH1 mutant patient-derived cells

After testing the combination therapy in IDH1 mutant astrocytes, we were able to obtain GBM164 cells which are patient derived cells that are maintained as xenografts but can be cultured for several passages in vitro (246). The cells were genotyped and confirmed to be IDH1 heterozygous mutant (Figure 3.9A). To confirm that the effect of combination treatment with BI2536 and TMZ is not unique to our astrocyte model, a cell viability assay was performed on GBM164 cells. Combination treatment resulted in over a seven fold decrease in cell viability compared to TMZ (P≤0.001) or BI2536 (P=0.002) alone (Figure 3.9B).
3.4 Discussion

In gliomas, when the *IDH1* mutation is present, it is typically found throughout the entirety of otherwise heterogeneous tumors, which makes targeting vulnerabilities conferred by mutant *IDH1* very appealing (86). In the current work, we report that the *IDH1* mutation promotes TMZ resistance through G2 checkpoint adaptation facilitated by PLK1 activation. Additionally, treatment with a PLK1 inhibitor dramatically improves TMZ efficacy while establishing the use of *IDH1* mutant astrocytes in a xenograft mouse model.

Our data indicate that in the context of an *IDH1* mutation, PLK1 activation promotes bypass of the TMZ-induced DNA damage checkpoint, limiting TMZ effectiveness. PLK1 can inactivate the DNA damage checkpoint by inactivating or facilitating degradation of target proteins such as Claspin, a mediator of CHK1 activation and G2 checkpoint maintenance (41,47). CHK1 and PLK1 act antagonistically as PLK1 can be inactivated in a CHK1 activity-dependent manner (46,241). Consistent with this model, our data confirm that CHK1 is inactivated and PLK1 is activated in *IDH1* mutant cells following TMZ exposure.

Interestingly, TMZ resistance has been linked to IDH1 mutant-mediated transformation, rather than the immediate activity of the mutant enzyme (36). Clearly, the long-term and immediate effects of IDH1 mutation are not mutually
exclusive and while the indirect effects of an IDH1 mutation likely play a role, we show that D2HG also acutely promotes TMZ resistance.

While the mechanism of PLK1 activation in IDH1 mutant tumors is still under investigation, we postulate that the immediate effect may be in part through the inhibition of αKG-dependent enzymes. D2HG produced by mutant IDH1 inhibits members of the TET and JmjC families of enzymes, which are regulators of DNA and histone methylation, respectively. TET inhibition is associated with DNA hypermethylation resulting in the CpG island methylation phenotype and altered gene expression profile. However, no significant changes in expression or DNA methylation occur at PLK1 or CHK1 loci in the IDH1 mutant astrocytes (114). Additionally, genes for upstream regulators of PLK1 and CHK1 such as Aurora A, Bora, and ATR also remain unaffected. A single CpG island locus in the ATRIP gene was reported to be slightly hypomethylated, though gene expression is not significantly affected (114).

Alternatively, it is possible that D2HG promotes PLK1 activation by inhibiting Egln3 activity. Hydroxylation of Tel2 by the αKG-dependent dioxygenase Egln3 is required for activation of the ATR/CHK1 checkpoint pathway which in turn leads to PLK1 inactivation (247,248). However, D2HG competes with αKG as an Egln3 substrate, possibly leading to inhibition of Egln3 activity and ultimately to CHK1 inactivation and PLK1 activation. Inhibition of Egln3 and the corresponding
decrease in Telo2 hydroxylation has also been shown to decrease apoptosis following DNA damage (247).

\[ \alpha \text{KG-dependent dioxygenases may also account for the mild TMZ resistance in the IDH1 WT NHA. While IDH1 mutant NHA display hypermethylation as a result of TET inhibition, Turcan et al. reported genomic hypomethylation in IDH1 WT NHA suggesting that } \alpha \text{KG production may enhance } \alpha \text{KG-dependent dioxygenase activity (114). It is possible that IDH1 WT NHA repair TMZ-induced DNA damage more efficiently by activating EGLN3 or members of the AlkBH family of } \alpha \text{KG-dependent DNA repair enzymes. Improved DNA repair capacity in the IDH1 WT NHA would account for the shortened G2 arrest (Fig 1D) and fewer double strand breaks at day 5 post TMZ (Fig 2C).}

Increased homologous recombination has also been proposed as the mechanism of TMZ resistance in mutant IDH1-transformed astrocytes (36). However, we demonstrate that \textit{IDH1} mutant cells have no less DNA damage than IDH1 WT and control cells, indicating checkpoint adaptation rather than recovery is responsible for early G2 checkpoint exit. While PLK1 has been shown to phosphorylate BRCA1 and RAD51 to promote homologous recombination, (249,250) it is possible that the \textit{IDH1} mutation and PLK1 activation not only promote homologous recombination, or at least activation of the homologous recombination machinery, but also premature mitotic progression prior to complete repair of damaged DNA.
To date, all work concerning the use of TMZ in combination with a PLK1 inhibitor has been in the context of primary grade IV glioblastoma, which rarely carries an IDH1 mutation (251–253). Compared to their wild type counterparts, IDH1 mutant gliomas have a favorable clinical response to TMZ and IDH1 has become an important prognostic factor(254). Importantly, IDH1 wild-type and mutant gliomas are molecularly very different and comparative clinical outcome does not indicate how mutant IDH1 affects a tumor’s response to treatment. Additionally, our work demonstrates the context-dependent effects of mutant IDH1 which has previously been reported to either increase or have no effect on TMZ sensitivity when expressed in U87 and U373 cell lines (255,256). In contrast, we and others have shown that the expression of mutant IDH1 in untransformed cells promotes cellular transformation and TMZ resistance (36).

The sensitivity of IDH1 mutant tumors to a PLK1 inhibitor plus TMZ combination may have implications beyond glioma treatment. IDH mutation is found frequently in acute myeloid leukemia, cholangiocarcinoma, osteosarcoma, and in central and periosteal chondrosarcomas which are nonresponsive to current chemotherapy regimens (257). Interestingly, a variety of PLK1 inhibitors are in clinical and preclinical development with two of them in late clinical trials (236). Our findings establish PLK1 as a very promising target in IDH1 mutant tumors and warrant consideration of clinical trials for TMZ and PLK1 inhibitor combinations.
3.5 Materials and Methods

3.5.1 Cell lines

Immortalized human astrocytes have been described elsewhere (237). HA-tagged wild-type and R132H mutant IDH1 were cloned into pBABE-neo retroviral vector. Astrocytes were transduced with retrovirus and underwent G418 selection. Clones were screened for expression of HA-IDH1. IDH1 mutant astrocytes used at passage 50 were generated as previously described (114). GBM164, MCF7, and all NHA cell lines were grown as adherent cells in DMEM, 10% FBS.

3.5.2 Western blotting

Western blotting was performed using Bio-Rad mini-protean® TGX™ gels and PVDF membranes. Primary antibodies used: anti-IDH1 (N-20;#sc-49996, Santa Cruz), anti-pPLK1 T210 (D5H7;#9062, Cell Signaling), anti-PLK1 (208G4, #4513, Cell Signaling), anti-pCHK1 S345 (133D3;#2348, Cell Signaling), anti-CHK1 (2G1D5, #2360, Cell Signaling), anti-β Actin (8H10D10, #3700, Cell Signaling), anti-HA (6E2, #2367, Cell Signaling), and anti-MGMT (OAAF03046, Aviva Systems Biology). Imaging was performed on a LI-COR Odyssey imager.
3.5.3 Nuclear magnetic resonance spectroscopy

Cell collections and extractions were performed as previously described (258). Briefly, the hydrophilic cell extract samples were dried in a SpeedVac centrifuge then resuspended in NMR buffer. One-dimensional $^1$H NMR spectra were recorded using Carr-Purcell-Meiboon-Gill pulse sequence with presaturation of the water peak on a 600 MHz INOVA spectrometer. Experiments were run with 4 dummy scans and 128 acquisition scans (acquisition time: 2.09s, relaxation delay: 4.0s, mixing time: 60ms). Spectral width was 26ppm, and 64K real data points were collected. Pure D2HG was used to confirm the metabolite of interest. All NMR data were processed using TopSpin3.1 (Bruker Analytik, Rheinstetten, Germany). All FIDs were subjected to exponential line-broadening of 0.3 Hz. Upon Fourier transformation, each spectrum was manually phased, baseline corrected, and referenced to the internal standard TMSP at 0.0ppm.

3.5.4 Clonogenic survival assay

Cells ($1 \times 10^3$) were plated on 10cm plates. When adhered, cells were treated with DMSO or 2nM BI2536 (Selleck) for 42hrs, 100\muM temozolomide (Cayman) for 18hrs, or 100\muM TMZ + 2nM BI2536 for 18hrs followed by an
additional 24hrs with 2nM BI2536 so that BI2536 was present during the second cell cycle following TMZ exposure. The day after TMZ was washed out was designated day 1 post TMZ. When D2HG treatment was included, cells were incubated with complete media plus 5mM D2HG from 8hrs prior to TMZ treatment until cells were fixed and stained. Cells were then grown in DMEM, 10% FBS until colonies clearly formed in DMSO-treated plates. The plates were washed, fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet.

### 3.5.5 Cell Viability assay

In a 96 well plate, 5 x 10^3 GBM164 cells were plated per well. Two days later cells were treated with 200μM TMZ and 4nM BI2536 and incubated for three days, after which treatment was washed out and cell viability was measured using cell counting kit 8 (Dojindo Laboratories, Rockville, MD) according to the manufacturer’s protocol.

### 3.5.6 Propidium Iodide staining/Cell cycle analysis

After 18hrs TMZ treatment, cells were washed and incubated with complete media until collection. Cells were trypsinized, washed, fixed with cold 70% ethanol, and propidium iodide stained. Flow cytometry was performed on a BD-
LSR-Fortessa flow cytometer. Analysis was performed on FCS Express4 with ≥10^4 events per time point.

3.5.7 Comet Assay

The Trevigen Comet assay kit (4250-050-K) was used according to manufacturer’s protocol. All samples for a given time point were run together in duplicate. DNA was stained with SYBR Gold (LifeTechnologies), viewed with an Olympus-BX51 microscope, imaged with a SPOT-RT-KE camera (Diagnostic Instruments), and ≥40 comets were scored for each sample using OpenComet(259).

3.5.8 Testing drug efficacy in vivo

Five-week-old female NOD-\textit{scid} IL2Rgamma\textsuperscript{null} mice were injected with 2.5x10^6 cells subcutaneously in the right flanks. Treatments were administered when tumors reached 300mm^3, and again three days later. Size was determined with calipers and volume was calculated by: V=LxWxH/2. Temozolomide (80mg/kg) was injected intraperitoneally in 10% DMSO. BI2536 (40mg/kg) was dissolved in 0.02N HCl and administered by tail vein injection.
3.5.9 Intracranial xenograft

Intracranial xenografts were established as previously described (260). Briefly, five-week-old female NOD-scid IL2Rgammnull mice were anesthetized, fixed in a stereotactic apparatus, and 10⁵ cells constitutively expressing luciferase were injected with a Hamilton syringe 2mm lateral to the bregma point at a depth of 3mm. Mice were imaged with a Bruker In-Vivo MS FX PRO. All mouse work was in accordance with a protocol approved by the University of Cincinnati Institutional Animal Care and Use Committee.

3.5.10 Statistical Analysis

Significance was set to P≤0.05 for all experiments. One-way ANOVA was used to compare between groups. To test for treatment interactions, two way ANOVA was used. The Holm-Sidak method was used for post-hoc testing. All statistical analyses were performed with SigmaPlotV13.
3.6 Acknowledgments and conflict of interest:

We would like to thank Dr. Jann Sarkaria from Mayo Clinic for generously providing the GBM164 cell line, Dr. Changchun Xie from the Division of Biostatistics and Bioinformatics, Department of Environmental Health at the University of Cincinnati for his help with statistical analysis, Kathleen LaSance from the University of Cincinnati imaging core for her assistance with in vivo imaging, and Dr. Peter Stambrook from the Department of Molecular Genetics at the University of Cincinnati for the critical reading of this manuscript.

With regard to the data presented in this manuscript, the authors declare no conflict of interest. The funding agencies had no involvement in the design of the experiments or in data interpretation.

3.7 Funding support:

Funding for this project was provided by the University of Cincinnati, the UC Brain Tumor Center, and the National Center for Advancing Translational Sciences of the National Institutes of Health, award number 1UL1TR001425-01 (EMB). This work was also supported in part by 1R01CA158372-01A0 (XQ).
**Figure 3.1. IDH1 mutation promotes resistance to TMZ by D2HG production.**

A. Western blot confirming expression of exogenous HA-IDH1 (red) and endogenous IDH1 (green). B. Clonogenic survival of empty vector control, IDH1 WT, and IDH1 mutant astrocytes after treatment with 100µM TMZ. C. MGMT expression was not detectable by Western blot in astrocytes regardless of IDH1 status. MCF7 cells were used as a positive control. D. Impact of mutant IDH1 on cell cycle profiles in response to TMZ treatment. Yellow boxes indicate >30% of cells in G2/M. E. Clonogenic survival of parental astrocytes (top) and IDH1 mutant astrocytes (bottom) cultured with or without 5mM D2HG and treated with TMZ. There was a statistically significant interaction between D2HG and TMZ treatments in the NHA (P=0.02) but not in IDH1 mutant astrocytes. Error bars represent SEM. P<0.05 (*); P<0.01 (**)
Figure 3.2. IDH1 mutation does not affect levels of DNA damage following TMZ treatment. A. Alkaline comet assay reveals no significant difference in total DNA damage between control, IDH1 WT, or IDH1 mutant astrocytes at day 1, 3, or 7 post TMZ treatment. B. Representative images of alkaline comet assay three days after TMZ treatment. C. No significant difference in double-strand DNA breaks was detected by neutral comet assay. D. Representative images of neutral comet assay three days post TMZ. Box plots represent median, first, and third quartiles. Bars show 10th and 90th percentiles. Circles show 5th and 95th percentiles.
Figure 3.3. Inhibition of PLK1 sensitizes IDH1 mutant cells to TMZ. 

A. Western blot using phospho-specific antibodies shows increased PLK1 activation and decreased CHK1 activation in mutant IDH1 astrocytes after TMZ treatment compared to control and WT astrocytes. 

B. Clonogenic survival of IDH1 mutant astrocytes following treatment with TMZ and a PLK1 inhibitor, BI2536. 

C. Western blot for total and activate CHK1 in IDH1 mutant astrocytes treated with TMZ and BI2536. Error bars represent SEM. n=3. P<0.001 (***).
Figure 3.4. Combination of TMZ and BI2536 leads to a marked tumor regression in IDH1 mutant tumors.  

A. Average subcutaneous tumor volumes of vehicle, TMZ, BI2536, and TMZ+BI2536-treated mice over 28 days.  

B. Representative images of excised subcutaneous tumors 31 days after treatment began.  Scale bars=1cm  

Error bars represent SEM.  P<0.001 (**).  n≥5 mice per treatment group
Figure 3.5. Supplemental Data: Confirmation of IDH1 WT and R132H mutant cell lines.  

A. Targeted sequencing of IDH1 cDNA sequence from gDNA of IDH1 WT (top) and R132H mutant transduced astrocytes (bottom). Codon 132 is highlighted in blue.  

B. NMR spectroscopy was unable to detect 2HG in control (red) and IDH1 WT astrocytes (blue). 2HG is clearly detected (seven peaks highlighted in blue) in R132H mutant astrocytes (green) and positive control (black).
Figure 3.6. Supplemental Data: Effect of TMZ and BI2536 combination treatment on IDH1 WT NHA.
Figure 3.7. Supplemental Data: In vivo model of IDH1-mutant glioma.

A. Time to tumor formation indicates that tumor formation rate is dependent upon how long mutant IDH1 has been expressed in the cells. Time to tumor formation was determined upon formation of a 100mm³ mass.

B. Two representative tumors formed from P50 IDH1 mutant astrocytes 14.5 weeks after injection.

C. Primers for the IDH1 gDNA (top) or cDNA (bottom) sequence were used to amplify endogenous and exogenous IDH1 from tumor gDNA. Targeted sequencing revealed retention of the WT (top) and R132H mutant (bottom) alleles in the subcutaneous tumors. Codon 132 is highlighted in blue.

D. Orthotopic tumor progression monitored via luminescence. Week 4, 6, 7, and 8 scans were 20 min. Week 9 scan was 5 min.
Figure 3.8. Supplemental Data: TMZ+BI2536 combination therapy does not cause significant weight loss in mice. Changes in percent body weight over time of mice treated with vehicle, TMZ, BI2536, or TMZ+BI2536. Error bars show S.D.
Figure 3.9. Supplemental Data: BI2536 sensitizes IDH1 mutant patient-derived xenograft cells to TMZ. A. Targeted sequencing of IDH1 reveals GBM164 cells are heterozygous mutant at codon 132 (R132H mutation). The mutation site is highlighted in blue. B. Cell viability of GBM164 cells treated with TMZ and BI2536 singly or in combination. Error bars show SEM.
Chapter 4: Discussion
Chapter 4: Discussion

The objective of this dissertation was to further our understanding of treatment resistance in adult gliomas and to rationally identify therapeutic targets for combination therapy to improve treatment efficacy. While EGFR is altered in the majority of primary GBMs, IDH1 is mutated in more than three quarters of lower grade gliomas and progressive secondary GBMs. Acknowledging the molecular differences between primary and secondary GBMs, we attempted to identify and target features unique to each disease. Chapter 2 focuses on identifying and overcoming resistance mechanisms to the EGFR TKI, gefitinib, in primary GBMs. Chapter 3 focuses on enhancing TMZ efficacy in lower grade gliomas and secondary GBMs by exploiting vulnerabilities conferred by mutant IDH1.

4.1 ROS1 mediates gefitinib resistance in GBM cells

4.1.1 Summary

EGFR is the most frequently altered gene in GBM. However, few GBM-specific mechanisms of resistance to EGFR inhibition have been identified. Chapter 2 describes work examining resistance to the EGFR TKI, gefitinib, in GBM cells. We
artificially overexpressed EGFR in U87 cells as a model for study because cell lines derived from GBMs with EGFR amplification do not retain their high EGFR copy number (261). After generating gefitinib resistant GBM cells through treatment with incrementally increasing concentrations of gefitinib, we showed that cells resistant to gefitinib overexpress the orphan RTK ROS1. Significantly, we verified that early passage primary GBM cells with EGFR amplification also develop gefitinib resistance through overexpression of ROS1, confirming that this mechanism of EGFR TKI resistance is not unique to a single cell line or model. The work in chapter 2 also demonstrated that the pyrazole ROS1 inhibitor decreases AKT and MAPK activation and promotes cell death only in the gefitinib resistant cells, suggesting that it is specific and not overtly toxic. The ROS1 inhibitor was also more effective against gefitinib resistant cells and at much lower concentrations than crizotinib, which was recently FDA approved for treating ROS1 positive NSCLC. While ROS1 has been reported to mediate resistance to EGFR inhibitors in other cancers, this is the first report of ROS1-mediated EGFR TKI resistance in glioblastoma cells. (262,263).

4.1.2 Future directions

Our identification of ROS1 as a key mediator of gefitinib resistance in GBM cells and our demonstration that a recently developed pyrazole ROS1 inhibitor efficiently
kills the gefitinib resistant cells raises several important questions which are discussed below.

*Does ROS1 mediate resistance to EGFR inhibitors in human GBM tumors?*

While we used primary GBM 1048 cells to confirm that ROS1-mediated resistance to gefitinib is not unique to our U87-EGFR model, it will be important to demonstrate that this resistance mechanism applies to GBM patients. EGFR amplification is not retained well *in vitro* and traditionally cultured cells fail to represent the heterogeneity of the tumor (264). Patient derived xenografts (PDX) in mice better recapitulate the characteristics of the original tumor, especially for GBMs with EGFR alterations (265–268). The PDX model would also allow for the screening of primary GBMs with different genetic backgrounds to determine whether ROS1-mediated resistance occurs ubiquitously in gefitinib treated GBMs or if it is specific to tumors with certain genetic alterations. Using a PDX model, we could expand tumors in mice subcutaneously, dissociate the tumors, and inject the cells into the brain to form an orthotopic tumor. We could then generate gefitinib resistant tumors *in vivo* by treating mice with gefitinib. Generating resistance in this manner would better mimic the microenvironment and growth factor concentrations of GBMs and more faithfully model the clinical experience (269).
Definitive determination that ROS1 drives resistance to EGFR inhibitors in patients would require examination of primary, treatment-naïve GBM tumors and tumors that recur in the same patients after EGFR targeted therapy within a clinical trial. Hegi et al. reported that while GBMs of patients preoperatively treated for 5 days with gefitinib exhibited effective inhibition of EGFR, no decrease in activation was detected for any of the 12 downstream pathway components evaluated (168). This supports our finding that an alternative RTK, rather than a downstream effector, mediates gefitinib resistance since another RTK would be more capable than a constitutively active effector of maintaining activation of multiple diverging pathways. The same study also compared the effects of gefitinib on patient tumors, an established GBM cell line *in vitro*, and subcutaneous xenograft tumors formed in mice from the established cell line. While the tumors from patients and the subcutaneous xenografts displayed inactivation of EGFR but persistent activation of downstream effectors in response to gefitinib, the *in vitro* model showed inactivation of EGFR as well as its signaling pathway components, further emphasizing the need to validate our findings *in vivo*.

*Does the pyrazole ROS1 inhibitor have clinical potential for treating GBM patients?*

The ROS1 inhibitor used in our study was far more effective and at a much lower dose than crizotinib, which was recently FDA approved for the treatment of
ROS1 positive NSCLC, in killing gefitinib resistant cells. Therefore, it would be worthwhile to assess the potential clinical utility of the drug. As previously mentioned, there can be substantial discrepancy between the in vivo and in vitro drug efficacy. Additionally, gliomas present a unique treatment challenge because therapies must be able to pass through the blood brain barrier to reach the tumor.

Our initial tests indicate that the drug is well tolerated by mice at doses of 100mg/kg and there are no obvious signs of toxicity. While collection of CSF or brain tissue can determine whether the drug crosses the blood brain barrier, orthotopic xenografts can be used to evaluate whether the drug penetrates the tumor, inhibits activation of ROS1 and downstream targets, and prolongs survival or affects tumor growth. In vitro, the ROS1 inhibitor was most effective when used in combination with gefitinib and the combination should be tested in mice for both toxicity and efficacy. The advantage of using gefitinib over other EGFR TKIs to treat GBMs is that gefitinib crosses the blood brain barrier and accumulates in the tumor remarkably well (168) in comparison to others such as erlotinib, which is able to cross the blood brain barrier but does not penetrate the tumor well (161).
What pathways downstream of ROS1 and EGFR are most essential for
tumor growth?

Based on phylogenetic analysis, ROS1 is not closely related to any other RTKs and mechanistically, little is known about wild type ROS1 signaling (270). No definitive ligand has been identified and it is unknown whether dimerization is necessary for ROS1 activation. Like EGFR, ROS1 can activate the PI3K/AKT and MAPK pathways (271) and in the single clone tested, treatment with the ROS1 inhibitor resulted in attenuation of AKT and MAPK signaling. This finding is notable because the PI3K/AKT pathway was reported to be more important than the MAPK pathway for ROS1-mediated transformation (272). For ROS1-mediated GBM resistance to EGFR TKIs, the relative importance of these two pathways is unknown. Additionally, we detected ROS1 at a different molecular weight in each of our gefitinib resistant clones which is indicative of different ROS1 fusion proteins. ROS1 has been reported to preferentially activate different pathways depending upon its N terminal fusion partner (271). Therefore, it may be beneficial to identify the ROS1 fusion partners in the current gefitinib resistant U87 clones, as well as any gefitinib resistant clones generated from the PDX model described earlier, and determine which downstream pathways and effectors are differentially activated between specific ROS1 fusion proteins and which effectors are common to all fusion variants.
Determining which ROS1 fusions occur in GBM and the signaling characteristics of each fusion could aid in the selection of a downstream effector to target, not only to prevent ROS1 inhibitor resistance, but possibly TKI resistance in general. Previous attempts to use combination therapy to inhibit EGFR and a downstream effector have not been clinically effective in treating GBM. Two phase II studies examined drug combinations targeting EGFR and mTOR but neither gefitinib plus everolimus (273) nor erlotinib plus temsirolimus (274) produced a durable response. Understanding how ROS1 signals in GBMs would aid in selection of downstream targets to further improve treatment.

4.2 PLK1 inhibition enhances TMZ efficacy in IDH1 mutant gliomas

4.2.1 Summary

IDH1 mutation is the most frequent and earliest alteration detected in grades II-III oligodendroglial and astrocytic tumors and secondary GBM. Other than global DNA and histone hypermethylation, little can be definitively attributed to mutant IDH1 since IDH1 mutant and WT gliomas are genotypically too different to attribute any differences in phenotype to a specific genetic alteration.
Furthermore, artificial expression of mutant IDH1 in established glioma cell lines fails to recapitulate the transformative effects of the mutant protein (36). Chapter 3 describes the evaluation of the IDH1 mutation’s effect on TMZ sensitivity using isogenic, immortalized astrocytes ectopically expressing WT or R132H mutant IDH1. Astrocytes expressing IDH1 R132H were less sensitive to TMZ than the astrocytes transduced with WT IDH1 or an empty vector despite no differences in MGMT expression and D2HG administration was associated with improved survival of parental astrocytes following TMZ exposure. After TMZ treatment, the vector control, WT, and R132H mutant astrocytes arrested in G2, however the IDH1 R132H astrocytes progressed into mitosis more rapidly than the other two cell lines, despite displaying similar levels of DNA damage. Increased PLK1 activation and decreased CHK1 activation were detected in TMZ-treated IDH1 mutant astrocytes which were sensitive to the combination of TMZ and BI2536, a PLK1 inhibitor. In a subcutaneous xenograft mouse model, the combination of TMZ and BI2536 achieved marked tumor shrinkage and was significantly more effective than either drug alone and produced no obvious signs of toxicity. We used IDH1 mutant PDX cells in vitro to verify that the efficacy of the combination therapy is not unique to our astrocyte model.
4.2.2 Future Directions

While gliomas are extremely heterogeneous, IDH1 mutation occurs very early in gliomagenesis and is usually found throughout the entirety of the tumor (84,86). Because IDH1 mutation is common to all cells in the tumor, targeting a weakness specific to IDH1 mutant cells has the potential to effectively eradicate the tumor and prevent recurrence. Combination treatment of TMZ and a PLK1 inhibitor has been proposed for use against primary GBMs, which are molecularly distinct from IDH1 mutant lower grade gliomas and secondary GBMs (251). We are the first to report that IDH1 mutant glioma patients may benefit from treatment with a PLK1 inhibitor in conjunction with TMZ. This finding raises the following questions to be addressed.

**Does the combination of PLK1 inhibitor and TMZ extend survival in orthotopic PDX models?**

With our recent acquisition of IDH1 mutant PDX cells, it is important to validate our findings in a survival study using patient derived orthotopic mouse models since reduction in tumor size is not always predictive of whether a treatment extends overall survival time (275). Additionally, the PLK1 inhibitor we used, BI2536 is no longer in clinical trials due to the development of a more specific second generation PLK1 inhibitor, BI6727 (volasertib), which has improved
pharmacokinetics and has been shown to cross the blood brain barrier, and it would be more relevant to test PLK1 inhibitors currently in clinical development (276).

The astrocytes we used for isogenic comparison of mutant and WT IDH1 express telomerase and the E6 and E7 viral oncogenes which inactivate P53 and RB. There is evidence that cells lacking functional P53 are especially sensitive to PLK1 inhibition, though there are also reports that P53 status is not predictive of response to PLK1 inhibitors (277–281). Interestingly, IDH1 mutant gliomas most often stratify into two categories: 1) P53 mutant, telomerase deficient astrocytomas and 2) P53 WT, telomerase-expressing oligodendrogliomas (88). Therefore, it would valuable to determine whether our findings broadly apply to IDH1 mutant tumors or to a subclass of IDH1 mutant tumors with a specific P53 or telomerase status. Establishment and characterization of several PDX lines from IDH1 mutant tumors with varying genetic profiles would allow for survival studies to further clarify what, if any, secondary genetic alterations predict combination treatment efficacy.

By what mechanism does IDH1 R132H promote TMZ resistance?

We demonstrated that D2HG can acutely contribute to the IDH1 mutant TMZ resistance phenotype but the mechanism is still unknown. Chapter 3
discussed hydroxylation of TELO2 by EGLN3 as a potential link between mutant IDH1 and the DNA damage G2 checkpoint and this idea warrants further testing. If inhibition of EGLN3 mediates the TMZ resistance phenotype we described, then we should be able to recapitulate the phenotype through either knockdown of EGLN3 or expression of an EGLN3 mutant lacking hydroxylase activity.

While inhibition of EGLN3 activity is a plausible mechanism for TMZ resistance, the EGLN proteins are some of the least sensitive αKG-dependent enzymes to D2HG and their role in mutant IDH1 mediated transformation is controversial. The TET family of DNA demethylases and the JmjC family of histone demethylases are known to be inhibited in IDH1 mutant tumors and the JmjC family of demethylases are hundreds of times more sensitive to D2HG than the EGLN proteins (112–114). Epigenetic alterations undoubtedly play a role in IDH1 mutant mediated transformation and possibly in TMZ resistance. However, our finding that D2HG treatment promotes TMZ resistance in the parental astrocytes suggests epigenetic alterations do not fully account for the resistance phenotype. At least 12 -15 passages are required before a significant difference in DNA and histone methylation can be detected in IDH1 mutant astrocytes (113,114). It is unlikely that time allowed for significant epigenetic alterations to accumulate since the astrocytes had only been exposed to D2HG for 8 hours at the time of TMZ treatment.
While regulation of histone methylation by $\alpha$KG dependent hydroxylases is well established, it was not until recently that protein methylation was recognized as a widespread post-translational modifications in non-histone proteins (282,283). Proteins including P53, RB, 53BP1, E2F1, VEGFR, STAT3, and most interestingly, PLK1, have been identified as substrates for lysine methylation (284–286). How methylation affects each protein’s degradation, activation, or stability is not yet understood but JmjC histone lysine demethylases have been reported to demethylate lysine and arginine on non-histone proteins (287,288).

Dysregulation of signaling pathways due to hypermethylation of non-histone proteins such as PLK1 would also explain the finding that treating mice bearing IDH1 mutant tumors with a mutant IDH1 inhibitor can lower D2HG concentrations and inhibit tumor growth without reducing DNA or histone methylation since global epigenetic remodeling is slow in comparison to posttranslational modification of signal transduction proteins (113,114,127). It would also explain why high doses of the inhibitor further decrease D2HG levels and reverse histone hypermethylation without affecting DNA methylation or improving anti-tumor efficacy (127) and also account for the acute effect of D2HG we report in chapter 3. Given the sensitivity of JmjC histone lysine demethylases to D2HG, the relationship between the IDH1 mutation and the methylproteome is in need of exploration.
References


33. Ochs K, Kaina B. Apoptosis induced by DNA damage O6-methylguanine is Bcl-


41. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, et al. Chk1 is an


64. Roos WP, Nikolova T, Quiros S, Naumann SC, Kiedron O, Zdzienicka MZ, et al. Brca2/Xrcc2 dependent HR, but not NHEJ, is required for protection against O6-methylguanine triggered apoptosis, DSBs and chromosomal aberrations by a process leading to SCEs. DNA Repair (Amst). 2009;8:72–86.


80. Aoude LG, Wadt KAW, Pritchard AL, Hayward NK. Genetics of familial...


144. Gan HK, Cvrljevic AN, Johns TG. The epidermal growth factor receptor variant III (EGFRvIII): Where wild things are altered. FEBS J. 2013. page 5350–70.


167. Padfield E, Ellis HP, Kurian KM. Current Therapeutic Advances Targeting EGFR
and EGFRvIII in Glioblastoma. Front Oncol. 2015;5:5.


251. Pezuk JA, Brassesco MS, Morales AG, de Oliveira JC, de Paula Queiroz RG, Machado HR, et al. Polo-like kinase 1 inhibition causes decreased proliferation


266. Pandita A, Aldape KD, Zadeh G, Guha A, James CD. Contrasting in vivo and in


283. Markolovic S, Wilkins SE, Schofield CJ. Protein hydroxylation catalyzed by 2-


