Preclinical Development of Dual EGFR/DNA Targeting Agent in Glioblastoma

Zeinab Sharifi

Division of Experimental Medicine, Faculty of Medicine, McGill University, Montreal, Canada

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ABSTRACT

Cancer in general is an extremely complex, heterogeneous disease that is regulated by many oncogenic drivers and multiple signaling pathways. Glioblastoma (GBM) is one of the most frequent, invasive and devastating primary brain tumors with a median overall survival rate of about 15 months despite aggressive multimodality treatment with surgery, concurrent radiation therapy (RT) and chemotherapy (TMZ). Characteristics such as heterogeneity, invasion, acquired resistance, presence of tumor stem cells that lead to recurrence of these tumors are among the reasons that has made GBM the most difficult brain cancer to treat and has left scientists and clinicians with limited therapy options. Importantly, overcoming the complexity seen in GBM with single-targeted drugs has proven to be challenging. It is in this context that we are using a multi-targeted approach termed "combi-targeting" or "combi-molecules" to target more than one oncogenic driver in tumors cells. Epidermal growth factor receptor (EGFR) dysregulation plays a critical role in GBM progression and DNA repair. Likewise, targeting DNA in tumor cells has been of a great interest in clinical management of solid tumors. We therefore investigated the strategy to combine a quinazoline ring (EGFR inhibitory arm) along with a chloromethyl group (DNA damaging arm) in one combi-molecule. By using this approach, we aim to not only enhance the potency of chemotherapeutic agents by simultaneously damaging DNA and inhibiting the EGFR pathway but also we aimed to target DNA repair by inhibition of the EGFR pathway which is known to be involved in DNA repair.

Here, we showed that oral administration of the dual EGFR-DNA damaging combi-molecule ZR2002 at doses up to 150mg/kg using either alternate or continuous treatment schedule was safe in athymic mice that have intact DNA repair pathway. ZR2002 was detected in the brain and plasma of mice using HPLC and LC/MS analysis. Interestingly, MALDI IMS confirmed the

presence of ZR2002 and its metabolite (ZR01) in the brain of nude mice with intracranial tumors. Given the central role of GBM stem cells (GSCs) in tumor progression, chemo- and radioresistance and tumor relapse, we used patient-derived GSC neurosphere cultures, as a model to also examine the effects of ZR2002. ZR2002, inhibited neurosphere formation of GSCs cultures and induced significant DNA damage in these cell lines starting at a low concentration (1 μM). Interestingly, this novel combi-molecule hindered the proliferation of TMZ-sensitive and resistant mesenchymal *in vivo* derived GSC sublines. We also tested for the first time and elucidated the mechanism of action of ZR2002 and studied its ability to kill GBM established cell lines with different EGFR levels. ZR2002 induced potent submicromolar growth inhibitory effects in U87/EGFR isogenic cell lines and hindered their clonogenic potential. ZR2002 was also able to induce significant DNA damage in these cell lines at a low concentration (0.6 μM). ZR2002 inhibited EGF-induced autophosphorylation of EGFR/EGFRvIII and downstream Erk1/2 phosphorylation, significantly increased DNA strand breaks and induced wild-type *TP53* activation in the established and stem cell lines tested. Its cytotoxic effects were mediated through a p53-dependent mechanism. Most importantly, oral administration of ZR2002 significantly increased survival in both the U87/EGFRvIII and TMZ-resistant GSC intracranial models. In sum, ZR2002, a unique binary EGFR/DNA combi-molecule with submicromolar potency imparts direct inhibition of EGFRvIII-induced proliferation and tumor growth. Its broad anti-proliferative, DNA-damaging activity in GSCs and *in vivo* anti-tumor activity provide proof-of-concept for its clinical evaluation in GBM.

RÉSUMÉ

Le cancer en général est une maladie extrêmement complexe et hétérogène régie par de nombreux facteurs oncogènes et par des voies multiples de signalisation. Le glioblastome multiforme (GBM) est l'une des tumeurs primitives du cerveau les plus fréquentes, invasives et dévastatrices, avec un taux de survie global médian d'environ 15 mois, malgré un traitement multimodal agressif ayant recours à la chirurgie, radiothérapie et chimiothérapie. Des caractéristiques telles que l'hétérogénéité, l'invasion, la résistance acquise, la présence de cellules ressemblant à des cellules souches qui conduisent à la récurrence de ces tumeurs sont parmi les raisons qui expliquent la complexité du traitement du GBM et limitent les options de traitement. Notamment, surmonter la complexité observée dans le GBM avec des médicaments à cible unique s'est avéré difficile. C'est dans ce contexte que nous utilisons une approche multi-ciblée appelée «ciblage combiné» ou «molécules combinées» pour cibler plus d'un facteur oncogénique dans les cellules tumorales. Depuis, la dérégulation du récepteur du facteur de croissance épidermique (EGFR) joue un rôle essentiel dans la progression du GBM et la réparation de l'ADN. Cette cible a également suscité un grand intérêt pour la prise en charge clinique des tumeurs solides. Nous avons donc investigué la combinaison d'un cycle quinazoline (bras inhibiteur de l'EGFR) et un groupe chlorométhyle (bras pour endommager l'ADN) dans une molécule combinée. En utilisant cette approche, nous visons non seulement à renforcer la puissance des agents chimiothérapeutiques en endommageant simultanément l'ADN et en inhibant la voie de l'EGFR, mais également à cibler la réparation de l'ADN par inhibition de la voie de l'EGFR, connue pour son implication dans la réparation de l'ADN.

Au cours de cette étude, nous avons montré que l'administration orale de ZR2002 à des doses allant jusqu'à 150 mg / kg a été tolérée suivant une posologie alternée ou continue chez des souris

athymiques chez lesquelles la voie de réparation de l'ADN était intacte. ZR2002 a été détecté dans le cerveau et le plasma de souris à l'aide d'analyses HPLC et LC /MS. Notamment, MALDI IMS a confirmé la présence de ZR2002 et de son métabolite (ZR01) dans le cerveau de souris nues atteintes de tumeurs intracrâniennes. Le traitement de ZR2002 chez des souris NSG a montré une toxicité qui prouve le fait que ce médicament pourrait agir par ADN-PKcs. Étant donné le rôle central des cellules souches de GBM (GSC) dans la progression tumorale, la chimiorésistance et la radiorésistance et les rechutes tumorales, nous avons utilisé des cultures de neurosphères de GSC dérivées de patients, comme modèle pour examiner également les effets de ZR2002. ZR2002, inhibe la formation de neurosphères dans les cultures de GSC et induit des dommages importants et significatifs à l'ADN dans ces lignées cellulaires à partir d'une faible concentration (1 μM). Il est intéressant de noter que cette nouvelle molécule combinée a empêché la prolifération de lignées de GSC dérivées in vivo de cellules souches mésenchymateuses résistantes et sensibles à TMZ. Nous avons également testé pour la première fois et élucidé le mécanisme d'action de ZR2002, une molécule combinée double qui endommage l'ADN et l'EGFR, et nous avons étudié sa capacité à tuer des lignées cellulaires établies de GBM avec différents niveaux d'EGFR. ZR2002 a induit de puissants effets inhibiteurs de la croissance submicromolaire dans les lignées de cellules isogéniques U87/EGFR et entravé leur potentiel clonogénique. ZR2002 a été capable d'induire des dommages importants à l'ADN dans ces lignées cellulaires à partir d'une faible concentration (0,6 μM). ZR2002 a inhibé l'autophosphorylation induite par EGF de la phosphorylation d'EGFR / EGFRvIII et Erk1 / 2 en aval, a considérablement augmenté les ruptures de brins d'ADN et induit l'activation de *TP53* de type « sauvage » non muté dans les lignées de cellules souches établies et testées. Ses effets cytotoxiques ont été médiés par un mécanisme dépendant de p53. Plus important, l'administration orale de ZR2002 a significativement augmenté la survie dans le modèle

intracrânien de la GSC résistant à U87/EGFRvIII et au TMZ. En résumé, ZR2002, une molécule combinée unique pour cibler l'ADN/EGFR de manière binaire avec une puissance submicromolaire confère une inhibition directe de la prolifération et de la croissance tumorale induites par EGFRvIII. Sa large activité antiproliférative, détruisant l'ADN dans les CSG et son activité antitumorale in vivo, consolident le concept de procéder à son évaluation clinique dans le GBM.

ACKNOWLEDGEMENTS

My journey in the field of cancer research and drug discovery and development started before my PhD. I would like to express my appreciation to Dr. Siham Sabri and Dr. Bassam Abdulkarim for their trust in me and giving me an opportunity to join their translational oncology laboratory and complete a 7-month internship. I would also like to express my sincere gratitude not only for their continuous support, patience, and thoughtful guidance during the internship but also for allowing me to pursue my PhD in their laboratory. Dr. Abdulkarim and Dr. Sabri, not only provided financial support throughout years of my PhD studies as an international student in Canada, but also providing amazing opportunities during my PhD that helped me grow as an independent scientist.

Also I would like to thank the members of my thesis committee, who guided me through all these years: my academic advisors Dr. Giovanni DiBattista, Dr. Martine Culty, Dr. Bertrand Jean-Claude, Dr. Kevin Petrecca, Dr. George Shenouda, Dr. Carlos Telleria for their insightful comments and encouragement, and for challenging questions.

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I thank all the past and current lab members, people who were there for me and were always ready to help and discuss projects that helped me to find solutions to challenging situations that allowed me to move forward with projects. A special thanks goes to Brian Meehan, who was a mentor for me in personal and professional matters. I learned a lot from you Brian, thank you for

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

This thesis is presented in accordance with the manuscript-based thesis guidelines of McGill University. It contains a research article in preparation for submission and a submitted peer-reviewed original research article (Chapter 3). The thesis consists of an introduction (Chapter 1), results (Chapter 2 and 3) and general discussion (Chapter 4). Each research chapter contains sections covering an abstract, introduction, material and methods, results, and discussion as well as its own reference section and supplementary results.

Chapter 1 provides a literature review of Glioblastoma (GBM), its molecular subtypes, the problem of tumor heterogeneity and the role of cancer stem cells in GBM, EGFR, its structure and its role in cancer, targeted therapy for cancer treatment, resistance to DNA-damaging agents and tyrosine kinase inhibitors, design, synthesis and mechanism of action of combi-molecules, and underlying rationale for combi-targeting of EGFR and DNA. Chapter 2 reports the safety, blood brain barrier permeability and pharmacokinetic/pharmacodynamics of ZR2002, and its efficacy in a subcutaneous TMZ-resistant glioblastoma stem cell (GSC) xenograft model. Chapter 3 investigates the cell-context dependent effects of ZR2002 (DNA damage and EGFR inhibition) on GBM stem cells (GSCs) from patients newly diagnosed with GBM and TMZ-sensitive and resistant mesenchymal *in vivo* derived GSC sublines and U87/EGFR isogenic cell lines stably expressing EGFR/wild-type or EGFRvIII, the ligand-independent activated *EGFR* mutation. The mechanism of ZR2002 was also assessed on EGF-induced autophosphorylation of EGFR and EGFRvIII and downstream Erk1/2 phosphorylation. Oral bioavailability and *in vivo* anti-tumor properties of ZR2002 was also determined in an orthotopic EGFRvIII and TMZ-resistant orthotopic mouse model. The thesis has been organized and written by myself. The contributions of myself and of the co-authors are described in the following section.

Contribution to knowledge:

- Manuscript "**Pharmacokinetic and Pharmacodynamic study of a combi-molecule with dual EGFR/DNA targeting properties**" is ready for submission to journal of Pharmacokinetics and Pharmacodynamics.
- Manuscript "**Antitumor activity of a binary EGFR/DNA targeting strategy overcomes resistance of glioblastoma stem cells to temozolomide**". The revised version has been submitted to Clinical Cancer Research (journal of the American Association of Cancer Research).

CONTRIBUTION OF AUTHORS

Unless otherwise stated below, I performed experiments, designed and coordinated the experiments not directly conducted by myself, performed analysis of results and wrote the manuscripts that constitute this thesis with guidance from my supervisor Dr. Bassam Abdulkarim and my co-supervisor Dr. Siham Sabri. Dr. Bertrand Jean-Claude (Director, Cancer Drug Research Laboratory and the Drug Discovery platform) designed ZR2002, provided mentorship and support at different stages of this work including for pharmacokinetic experiments and MALDI imaging mass spectrometry. The manuscript submitted to Clinical Cancer Research (Chapter 3) has been approved by all authors before submission and the revised version is now under review.

Chapter 2 is reproduced from my manuscript **"Pharmacokinetic and pharmacodynamic study of a combi-molecule with dual EGFR/DNA targeting properties"** to be submitted to the journal of Pharmacokinetics and Pharmacodynamics. In this chapter, Mr. Brian Meehan (Research Associate in Dr. Janusz Rak's lab) performed the subcutaneous injections of glioblastoma stem cells (GSCs). Mr. Elliot Goodfellow and Dr. Martin Rupp (both PhD students in Dr. Bertrand Jean-Claude's lab) prepared ZR2002. Dr. Nidia Lauzon (Research Assistant at the Drug discovery platform (Research Institute of McGill University Health centre-Glen site) performed the MALDI imaging mass spectrometry. Dr. Janusz Rak provided the mesenchymal TMZ-sensitive (1123IC12S) and TMZ-resistant mesenchymal *in vivo* derived GSCs sublines (1123IC7R and 1123IC8R).

Chapter 3 is reproduced from my manuscript "A**ntitumor activity of a binary EGFR/DNA targeting strategy overcomes resistance of glioblastoma stem cells to temozolomide**" which was revised and re-submitted to "Clinical Cancer Research" (journal of the

American Association of Cancer Research). In this chapter, Mr. Brian Meehan (Research Associate in Dr. Janusz Rak's lab) assisted with the *in vivo* study of ZR2002 (intracranial injections). Dr. Julie Schmitt (Post-doctoral fellow) produced ZR2002. Dr. Paul Daniel (Post-doctoral fellow) performed p53 knockdown of OPK49. Dr. Kolja Eppert and Ms. Heather M. Duncan (current PhD student at Dr. Kolja Eppert's lab) provided luciferase-BFP dual gene vector and assisted with lentiviral transduction. Dr. Kevin Petrecca provided GSC lines from patients newly diagnosed with GBM. Dr. Janusz Rak provided the TMZ-sensitive (1123IC12S) and TMZ-resistant mesenchymal *in vivo* derived GSCs sublines (1123IC7R and 1123IC8R).

OTHER CONTRIBUTIONS

1) Differential Response to Ablative Ionizing Radiation in Genetically Distinct Non-Small Cell Lung Cancer Cells. Ayman Oweida, **Zeinab Sharifi**, Hani Halabi, Yaoxian Xu, Siham Sabri and Bassam Abdulkarim. ***Cancer Biology & Therapy***, 2016

2) Sensitivity to PRIMA-1MET is associated with decreased MGMT in human glioblastoma cells and glioblastoma stem cells irrespective of p53 status. Mariia Patyka, **Zeinab Sharifi**, Kevin Petrecca, Jose Mansure, Bertrand Jean-Claude, Siham Sabri. ***Oncotarget***, 2016

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LIST OF ABBREVIATIONS

AUC- area under the curve

AML- acute myeloid leukemia

APL- acute promyelocytic leukemia

AR- amphiregulin

APE1- apurinc/apyrmidinic endonuclease 1

ABD- amino-terminal adaptor-binding domain

ATP- adenosine triphosphate

ATM- ataxia telangiectasia mutated kinase

ATR- ataxia telangiectasia and Rad3 related kinase

BMPs- Bone Morphogenetic Proteins

BER- Base-excision repair

BBB- blood-brain barrier

BTICs- brain tumor initiating cells

BTC- betacellulin

Bax- Bcl2-associated X protein

CSCs- cancer stem cells

CD133-/+- CD133 negative/positive

CNS- Central Nervous System

CML- chronic myelogenous leukemia

CDK2- cyclin-dependent kinase 2

PKA- cAMP-dependent protein kinase

DDR- DNA-damage response

DNA-PK- DNA dependent protein kinase

DSBs- double-strand breaks

EGFR- epidermal growth factor receptor

EC- endothelial cells

EGF- epidermal growth factor

EPR- epiregulin

EORTC- European Organisation for Research and Treatment of Cancer

Exo- exonuclease 1

FGFR- fibroblast growth factor receptors

FGF- fibroblast growth factor

Flap endonuclease-1- FEN1

GBM- Glioblastoma

GFs- growth factors

GTP- guanine triphosphate

Grb2- growth factor receptor-bound protein 2

GOF- gain of function

GADD45- growth arrest and DNA damage

GPCRs- protein-coupled receptors

HGFRs- hepatocyte growth factor receptors

IDH1- isocitrate dehydrogenase

IGFRs- insulin growth factor receptors

IHC- immunohistochemical

IRS- insulin receptor substrate

MAPKs- mitogen-activated protein kinases

mTOR- mammalian target of rapamycin

mAbs- monoclonal antibodies

NSC- Neural Stem Cell

NRGs- neuregulins

NSCLC- Non-Small Cell Lung Cancer

NCICCTG- National Cancer Institute of Canada Clinical Trials Group

OS- overall survival

O6-BG- O6-benzylguanine

O6-BTG- O6-bromotenyl guanine

PDGFRA- platelet derived growth factor receptor

PDGFRs- platelet-derived growth factor receptors

PDGF- platelet-derived growth factor

p-SH2- phosphotyrosine-Src homology 2

PTB- phosphotyrosinebinding

PDGFRA- Platelet Derived Growth Factor Receptor Alpha

PKB- Protein Kinase B

PI3K- phosphatidylinositol 3-kinase

PIP2- Phosphatidylinositol 4,5-bisphosphate

PIP3- phosphatidylinositol (3,4,5)-trisphosphate

PH- pleckstrin homology

PTEN- phosphatase and tensin homolog

PIKKs- Phosphatidylinositol-3 kinase-related kinases

PUMA- p53 upregulated modulator of apoptosis

PCNA- proliferating cell nuclear antigen

PARP1- poly (ADP-ribose) polymerase 1

RT- radiotherapy/ radiation therapy

RA- retinoic acid

RVS- rous sarcoma virus

RBD- Ras-binding domain

ROS- Reactive oxygen species

RFC- replication factor-C

RPA- replication protein A

SCID- Severe Combined Immunodeficiency

SH2- Src homology 2

STATs- signal tranducers and activators of transcription

SOS- Son of Sevenless

SH2- Src homology 2 domains

SSBs- single-strand breaks

SMG1- suppressor with morphological effect on genitalia 1

STAT- signal transducer and activator of transcription

SAR- structure activity relationship

SHH- Sonic hedgehog

TMZ, Temodal®- temozolomide

TKs- tyrosine kinases

TCGA- The Cancer Genome Atlas Project

TGF-α- transforming growth factoralpha

TKI- tyrosine kinase inhibitors

TRRAP- transformation/transcription domain associated protein

TADs- transcriptional activation domains

VEGF- Vascular Endothelial Growth Factor

VEGFRs- Vascular endothelial growth factor receptors

wt p53- Wild-type p53

WHO- World Health Organization

XRCC4- X-ray cross complementing protein 4

XLF- XRCC4-like factor

XRCC1- X-ray repair cross-complementing protein 1

Chapter 1.

LITERATURE REVIEW & INTRODUCTION

1.1. GLIOBLASTOMA

Brain tumors affect both adults and children and in Canada, brain tumors are the fourth leading cause of cancer death for adults (age range 30-49, Canadian Society of Cancer). The most common type of brain tumors are those originating from the astrocytic glial lineage, referred to as astrocytomas (gliomas) [1, 2]. Glioblastoma (GBM) is the most malignant and common form of gliomas, which accounts for approximately 45-50% of all primary brain tumors in adults. GBM is classified by the World Health Organization (WHO) as grade IV astrocytoma [3] that arise from astrocytes, the most abundant glial cells in the brain, closely associated with neuronal synapses and mediating important supportive functions of the brain tissue [4]. This aggressive and intractable nature of GBM is largely due to highly infiltrative growth pattern of GBM, high mitogenic activity of tumor cells, resistance to virtually all therapies and variety of cell shapes and sizes within the same tumor along with increased angiogenesis and necrosis [5]. Angiogenesis is the physiological process through which new blood vessels form pre-existing vessels and this process is pivotal for tumor growth more than a couple of millimeters in diameter.

The 2016 World Health Organization Classification has particularly advanced over its 2007 predecessor in using molecular parameters in addition to histology to define many tumor entities. Some notable changes of the diffuse gliomas incorporate new entities that are defined by both histology and molecular features such as glioblastoma IDH-wildtype and glioblastoma, IDH-mutant. Overall, the 2016 WHO edition has added newly recognized neoplasms, and has deleted some entities, due to no longer having diagnostic and/or biological relevance [3].

GBM can develop as secondary GBM from lower grade gliomas or arise *de novo as* primary GBM that accounts for 95% of all GBM [6]. The most common symptoms of GBM are paresis and aphasia [7], but also include seizures, headaches, cognitive or personality changes, eye

weakness and nausea or vomiting and development of high intracranial pressure is the most threatening feature of GBM [7]. The tumor is often located in the cerebral hemispheres with occasionally contralateral invasion and in association with the lateral ventricles and the basal ganglia [8, 9] (Figure 1.1), and due to its invasive growth pattern total resection is often not possible [10].

Due to the heterogeneous population it has been attempted to divide GBM patients into groups depending on how they are expected to benefit from a certain treatment. As an example it has been shown that GBM patients with methylation of the O-6-methylguanine-DNA methyltransferase (*MGMT*) promoter to a higher degree benefit from TMZ treatment and as such have a better prognosis [10, 11]. Despite the aggressive treatment (described in section 1.1.4), prognosis for patients diagnosed with GBM remains extremely poor with a median survival of only about 14.6 months [11].

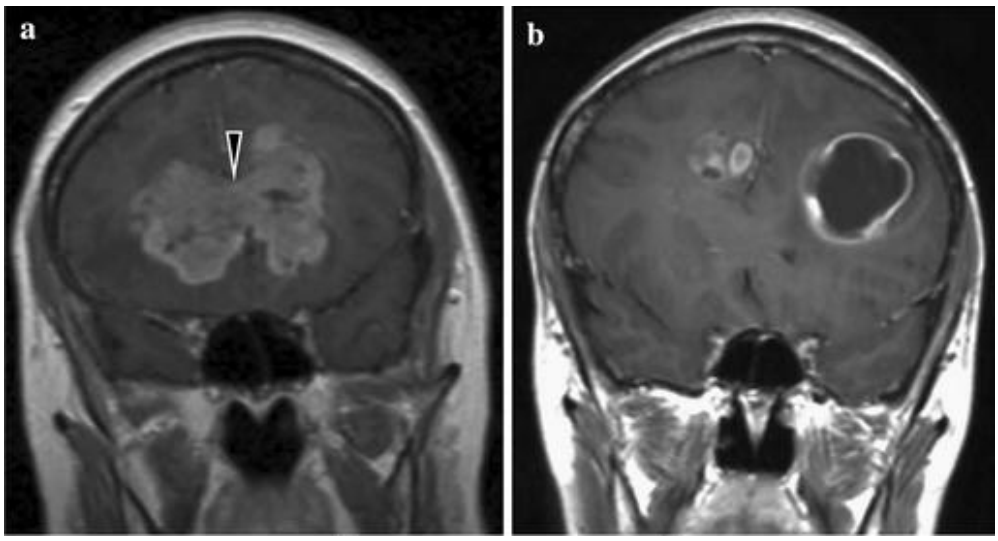


Figure 1.1. Examples of MR images in two GBM patients. In patient 1 (**a**), the T1-weighted image reveals bifrontal Gadolinium enhancement of a tumor that crosses the corpus callosum (*arrowhead*), resulting in a so called "butterfly glioma". In the second patient (**b**) shows multiple, independent lesions (adapted from [12]).

1.1.2 Primary and Secondary Glioblastoma

The majority (~ 95%) of adult GBMs arise via the primary GBM pathway (*IDH-wild type)*, without a preceding low grade lesion (*de novo*), and typically occur in older patients (around 60 years) [3]. Primary GBMs have rapid development of about 4 months and are associated with a distinct set of genetic lesions, which includes amplification of loss of heterozygosity (LOH) of chromosome 10q, alterations in phosphatase and tensin homolog (PTEN), overexpression of the epidermal growth factor receptor (EGFR) as well as changes in (RB) (CDKN2A/p16), receptor tyrosine kinases (PDGFR) and *TP53* mutations (~27%) [3, 13-16] (Figure 1.2).

Secondary GBM (~10% of cases) that corresponds to *IDH-mutant is* characterized by progression from lower grade (II, III) gliomas over the period longer than 6 months. It mainly affects younger adults (below the age of 45) [17, 18]. Common genetic events in secondary GBM are p53 mutations (~81%) in addition to losses in chromosome 1p/19q and a LOH involving chromosome 10q [3, 15, 19, 20] (Figure 1.2).

Comparing GBM patients with different *IDH1* status has shown that patients with mutant *IDH1* have a significantly longer median overall survival (OS) compared to patients with wild-type *IDH1*- 24 months *versus* 9.9 months with Radiation therapy (RT) or 31 months *versus* 15 months with RT+ chemotherapy [3, 21]. Based on evidence such as differences in the types of gene alterations, clinical outcome of patients, localization of the tumors in the brain, etc it has been

suggested that primary and secondary GBM should be considered as distinct tumor types.

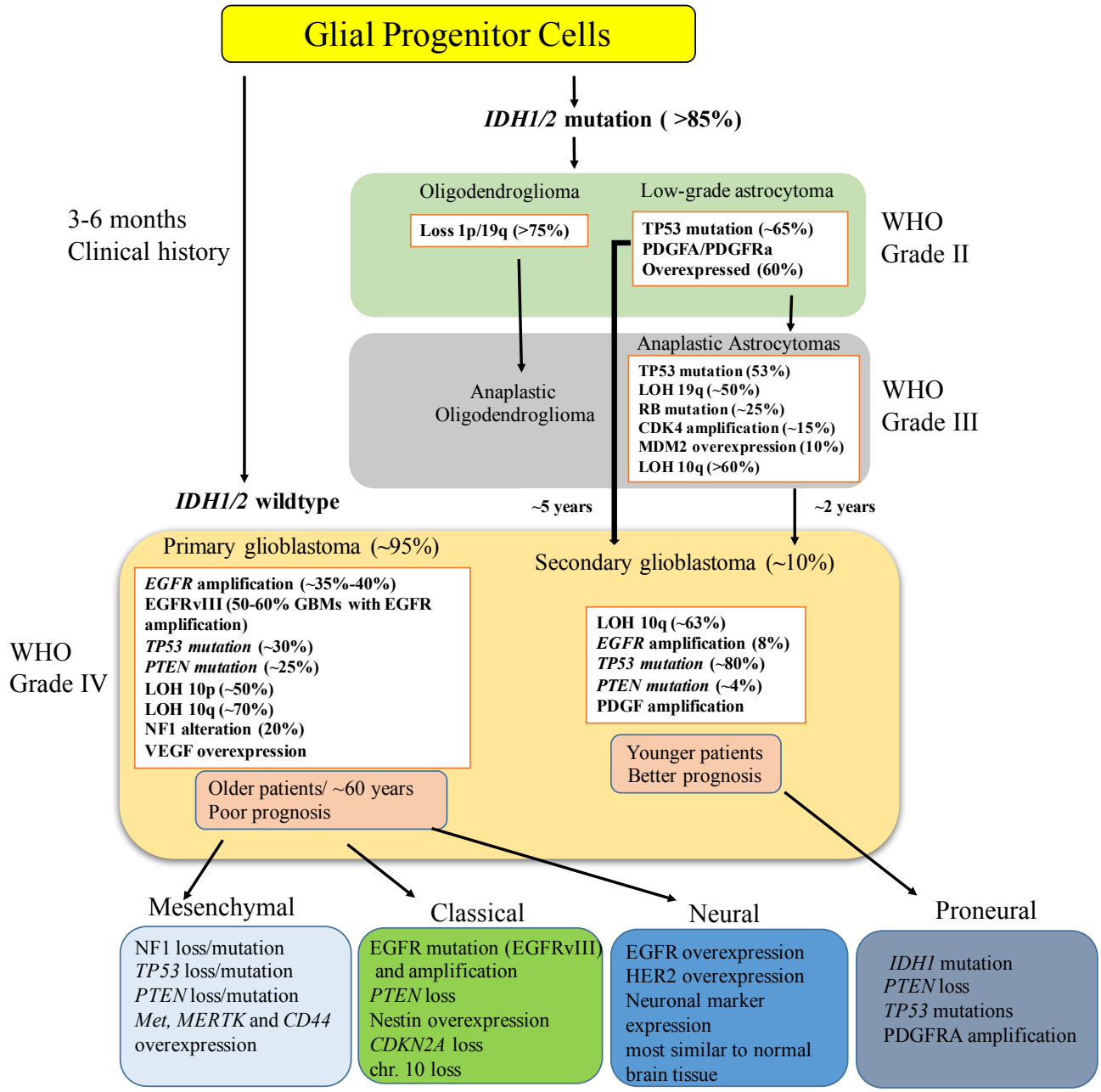


Figure 1.2. Development of primary and secondary GBM.

1.1.3 GBM subtypes and intratumoral heterogeneity

Phillips and colleagues were the first to reveal that gene expression patterns were not homogenous in a large cohort of GBM samples, but instead clustered around at least three distinct profiles [22] that were later referred to as sub-types or subgroups of GBM. They also showed that these sub-types represent different disease causing mechanisms, in spite of similar anatomical location, histology and clinical properties [22].

The Cancer Genome Atlas Project (TCGA: (www.cancergenome.nih.gov) [23]) has cataloged the genetic alterations underlying the entirety of major human malignancies in which GBM is among the first human cancers molecularly characterized by this database and today it provides publically available multimodal data of more than 500 GBM cases. Verhaak *et al*. analyzed 200 GBM samples from TCGA and identified four distinct molecular GBM subtypes: Proneural, Neural, Classical, and Mesenchymal [24]. The Classical sub-type is characterized by high epidermal growth factor receptor (*EGFR*) amplification, loss of the *CDKN2A* gene (cyclin-dependent kinase inhibitor 2A encoding both the *p16INK4A* and *p14ARF* tumor suppressor genes, *PTEN* loss and lack of *TP53* mutations. Chromosome (chr.) 7 amplification together with chr. 10 loss were also detected in 100% of the classical sub-type tumors. The Mesenchymal sub-type is named so due to the high expression of mesenchymal markers such as *CD44* and *MET* together with the astrocytic marker *MERTK*. Mutations in the neurofibromin 1 (*NF1*) and *PTEN* genes are also frequently observed in this sub-type.

TP53 mutations and LOH were frequent events in the proneural sub-type. Alterations of alpha-type platelet derived growth factor receptor (*PDGFRA*) amplification, isocitrate dehydrogenase (*IDH1*) point mutations and loss of heterozygosity are also frequent in this sub-type. Of note, since the proneural sub-type is enriched for IDH1 mutations, as is the case for

secondary GBM, this group contains the highest percentage of young patients [15]. Finally, the neural sub-type is not well defined but displays the most similar characteristics compared to normal brain tissue. As a summary, Verhaak and colleagues concluded that aberrations and gene expression of *EGFR*, *NF1* and *PDGFRA*/*IDH1* each defined the Classical, the Mesenchymal and the Proneural sub-types, respectively. This group also showed that there is a trend towards an increased survival for patients with the Proneural sub-type.

Sturm et al. [25] investigated a cohort of GBM tumors (children and adults) and by correlating their genome-wide DNA methylation patterns with mutations, DNA copy-number alteration and gene expression characteristics they identified six epigenetic GBM subgroups: RTK II (Classic), Mesenchymal, RTK I (PDGFRA), IDH, K27 and G34. RTK II (Classic) and Mesenchymal clusters corresponded to Classical and Mesenchymal gene expression profiles, respectively. As its name represents the IDH group was enriched for tumors with *IDH1* mutations and demonstrated Proneural gene expression pattern. In addition, this group along with K27 and G34 subgroups were highly enriched in *TP53* mutations. In accordance with Verhaak classification *PDGFRA* amplification and Proneural expression displayed characteristics of RTK I (PDGFRA) cluster.

Further extending the analysis, Brennan and co-workers defined three groups based on the expression and activation of distinct pathways and named the groups accordingly: the EGFR core, the PDGF core and the NF1 core [26]. This group compared the expression of a pre-defined panel of glioma relevant proteins in 27 GBM surgical specimens and relating them to the TCGA data. First, the EGFR core showed increased levels of total- and phosphorylated EGFR. Other characteristics of this group are chr. 7 gain, *EGFR* amplification and mutation as well as deletion of *Ink4a*/*ARF* and either chr. 10 loss or *PTEN* mutations. This group also displayed high levels of

the activated intracellular Notch-1 domain (ICN-1), the Notch ligands Jagged-1 (Jag-1) and Delta-like 1 (Dll-1) and the Notch downstream target hairy/enhancer of split-1 (Hes-1) that resembles the Classical subtype from Verhaak *et al*. The PDGF core showed increased level of PTEN as well as increased activation of the Ras pathway as evident by increased levels of phospho ERK and MEK. It also showed an up regulation of PDGFB, phospho-PDGFRβ and phospho-NFKB. In summary, there was a correlation between PDGF core and Proneural sub-type from Verhaak *et al.* The NF1 core resembles the Mesenchymal sub-type from Verhaak *et al*. in strongly being associated with low levels of NF1 and showed over expression of YKL40.

In a more recent study Brown et al. analyzed the relationship between expression of extracellular stem cell markers and known molecular subtypes of GBM [27]. Today, it is widely accepted that a subpopulation of GBM cells with stem cell properties is one of the main drivers of GBM tumor recurrence. This group showed that CD133 (the most commonly used stem cell marker) was enriched with Proneural molecular subtype and negatively correlated with the CD44 (stem cell marker) module that was enriched in the Mesenchymal subtype of GBM. Overall, a deeper understanding of the molecular subtypes of tumors and implementing them in the clinic has instigated a paradigm shift towards personalized or customized therapy and better therapeutic strategies to achieve maximum clinical response (reviewed in Woehrer *et al*. (2013) [28]).

It should be noted that several issues have hampered GBM classification. First, in many cases patient tumor samples used for molecular profiling only represent a small portion of the whole tumor mass and since GBM tumors are highly heterogeneous we could conclude that different sub-types might co-exist within the same tumor [29, 30]. Also, intratumor heterogeneity seen in GBM is associated with lack of complete drug response mediated by different acquired or preexisting genetic signatures within the tumour leading to drug resistance [31]. Moreover,

transition between sub-types has been seen upon tumor recurrence [22, 32]. In summary, although sub-types partially can be correlated to prognosis and treatment outcome but full scale sub-typing of GBM patients might be overstated in terms of stratifying patients to the most optimal treatment (Figure 1.3)

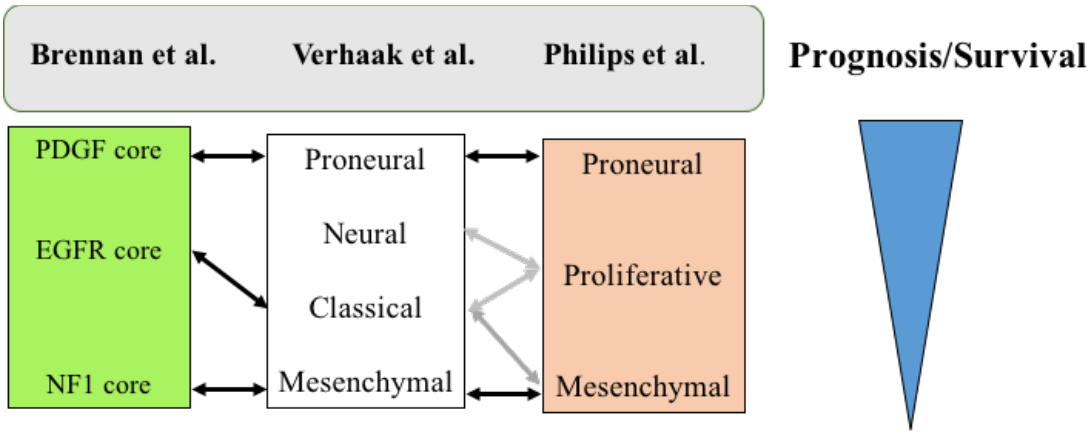


Figure 1.3. Comparison of subtype studies between Verhaak *et al*., Phillips *et al*. and Brennan *et al*. There is a good agreement between Verhaak's and Phillip's Proneural sub-type and Brennan's PDGF core as well as for the Mesenchymal subtype from Verhaak and Phillips and the NF1 core from Brennan, demonstrated by the top arrows. There is also a good correlation between Brennan's EGFR core and Verhaak's classical subtype, arrows down. However, there is concordance for Proliferative and Neural/Classical sub-types between Verhaak and Phillips, represented by the middle gray arrows. Patient survival decreases from the Proneural towards the Mesenchymal sub-type. Modified from [28].

1.1.4 Standard treatment

The standard treatment for GBM patients today is known as the "Stupp-regime", which consists of debulking surgery, followed by radiotherapy (RT) plus concomitant and adjuvant temozolomide (TMZ, Temodal®, an alkylating agent) [11, 33]. Although surgical removal comes with many risks there are data that suggests that a more aggressive surgical resection may potentially increase the efficacy of subsequent chemotherapy and radiation [34, 35] and therefore improve the outcome of GBM therapy [36, 37]. TMZ is an oral alkylating agent that methylates DNA in guanine rich regions of DNA at N^7 and, most importantly, at O^6 positions, but also methylates N^3 adenine (Figure 1.4). Alkylation of the O^6 site on guanine leads to the insertion of a thymine instead of a cytosine opposite the methylguanine during subsequent DNA replication, and this can result in DNA strands breaks, prevention of replication and G2/M cell cycle arrest in the second cycle after exposure to TMZ, and eventually leading to cell death by apoptosis. Of note, TMZ has relatively low toxicity and good penetration across the blood brain barrier (BBB).

In the late 1970s, RT was introduced to the standard treatment and the survival of GBM patients improved for the first time. In 2005 Stupp et al. compared concomitant administration of TMZ (75 mg/m^2 per day) with fractionated radiotherapy (2 Gy/day, 5 days/week for 6 weeks, total dose of 60 Gy) followed by up to 6 cycles of adjuvant TMZ (150-200 mg/m^2 per day, 5 days/week every 28 days) in patients with newly diagnosed GBM and showed that the median survival of patients receiving RT with TMZ was 14.6 months compared to 12.1 months for patients treated with RT alone [11] and the five year overall survival increased from 1.9% to 9.8% [33].

Interestingly, a recent open-label, randomized, phase 3 trial compared combination of lomustine and TMZ versus TMZ standard therapy in patients newly diagnosed with GBM with methylated *MGMT* promoter and reported that combination of lomustine-TMZ improved survival

compared with TMZ standard therapy (Median overall survival was improved from 31·4 months with TMZ to 48·1 months with lomustine-TMZ) [38]. Another trial in patients with newly diagnosed GBM reported a favorable long-term survival with acceptable toxicity in neoadjuvant TMZ followed by hypofractionated accelerated radiation therapy with concurrent and adjuvant TMZ (the median OS 22.3 months and progression-free survival was 13.7 months) [39].

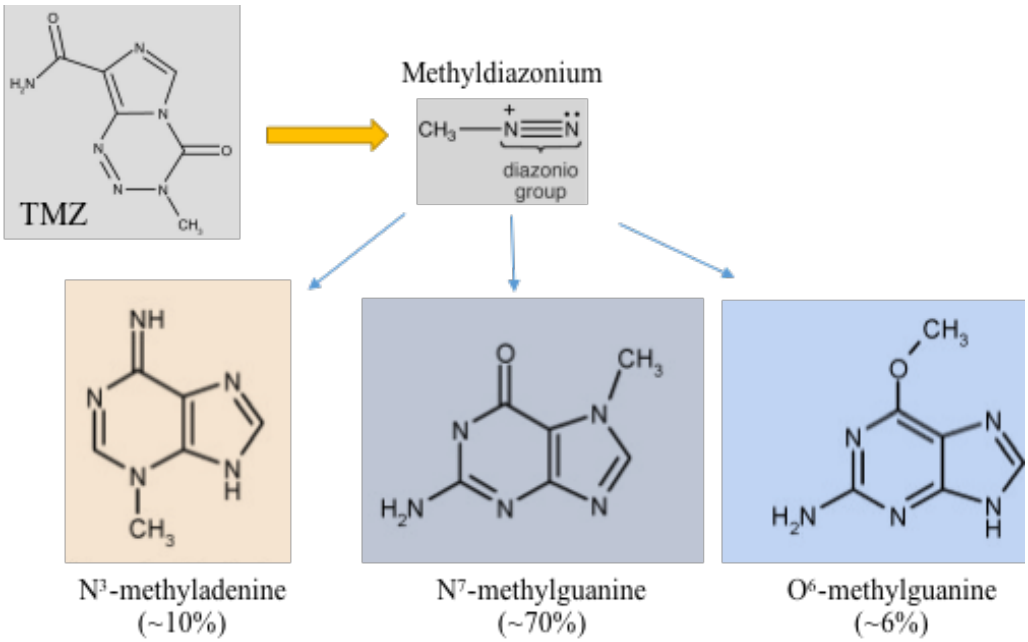


Figure 1.4. TMZ mechanism of action. TMZ can undergo hydrolysis under physiological conditions that leads to formation of methyldiazonium ion, which then can alkylate DNA. Alkylation of DNA occurs at O^6, N^7-position of guanine and N^3-position of adenine.

1.2 BRAIN CANCER STEM-LIKE CELLS

1.2.1 Definition and origin of GSCs

A wealth of data today shows malignant tumors are initiated and maintained by a population of tumor cells with similar biological properties as normal adult stem cells ("stem-like" properties) [9, 40-43]. In particular progression of GBM with various features of heterogeneity observed in this disease of which a subset of cells called tumor initiating cells (also called cancer stem cells (CSCs), brain tumor initiating cells (BTICs) or more specifically glioma stem cells (GSCs) is of particular interest [44-49]. Prior to describing CSCs and GSCs we need to describe the clonal evolution model and compare it to the cancer stem cell model.

Based on clonal evolution model when normal cells undergo spontaneous somatic mutation(s) they become "neoplastic" and will have the growth advantage over surrounding cells [50]. In this process, some cells occasionally have additional selective advantage and therefore would be selected [51]. The sequential selection of different subclones will lead to heterogeneous cell populations within the same tumor.

According to the cancer stem cell hypothesis, CSCs are able to self-renew and give rise to daughter stem cells as well as differentiated progenies [52, 53]. The progenitor will then proliferate and eventually form a heterogeneous tumor mass [54]. They can also display plasticity by reversibly transitioning between stem and non-stem cell states [55, 56]. Based on this model only CSCs are able to remain tumorigenic and other cells in the tumor mass will eventually become proliferatively exhausted and begin to terminally differentiate while the clonal evolution model assumes that all cells in the tumor have similar potential for regenerating tumor growth [57] (Figure 1.5). Lapidot et al, in 1994 [58] were the first to demonstrate the validity of the cancer stem cell model in acute myeloid leukemia (AML) and subsequently these cells were found in

several types of tumors such as gliomas and GBM [59, 60]. GSCs are characterized by surface markers such as CD44, nestin, CD133, SSEA1, NESTIN, SOX2, BMI1 and MUSASHI or ABC transporters. It should however be noted that CD133 may not serve as a distinct stem cell marker as also CD133 negative GBM neurosphere cells are able to form xenograft tumors [61-63].

As mentioned above these cells are able to maintain their own population through self-renewal along with being able to give rise to cells of the three neural lineages (neurons, oligodendrocytes and astrocytes) [64]. One main characteristic of GSCs is the ability of small (clonal) cell numbers to form tumors resembling the parental tumor when transplanted onto immunocompromised mice and to also drive tissue/tumor repopulation [65-67]. They are able to form neurospheres, when grown in the presence of growth factors (EGF, FGF2 and heparin) and under serum-free conditions [57, 68]. These cells have the ability to stay dormant for long periods of time or invade surrounding normal brain tissue and cause death [69]. While GSCs represent a small number of cells, due to many reasons such as slow cycle progression, they have unlimited self-renewal capacity. Both experimental models and clinical studies indicate that CSCs survive many commonly employed cancer therapeutics [69] and should be considered as a target in GBM therapy. Of note, GSCs express a number of genes that are similar to either proneural or mesenchymal GBM.

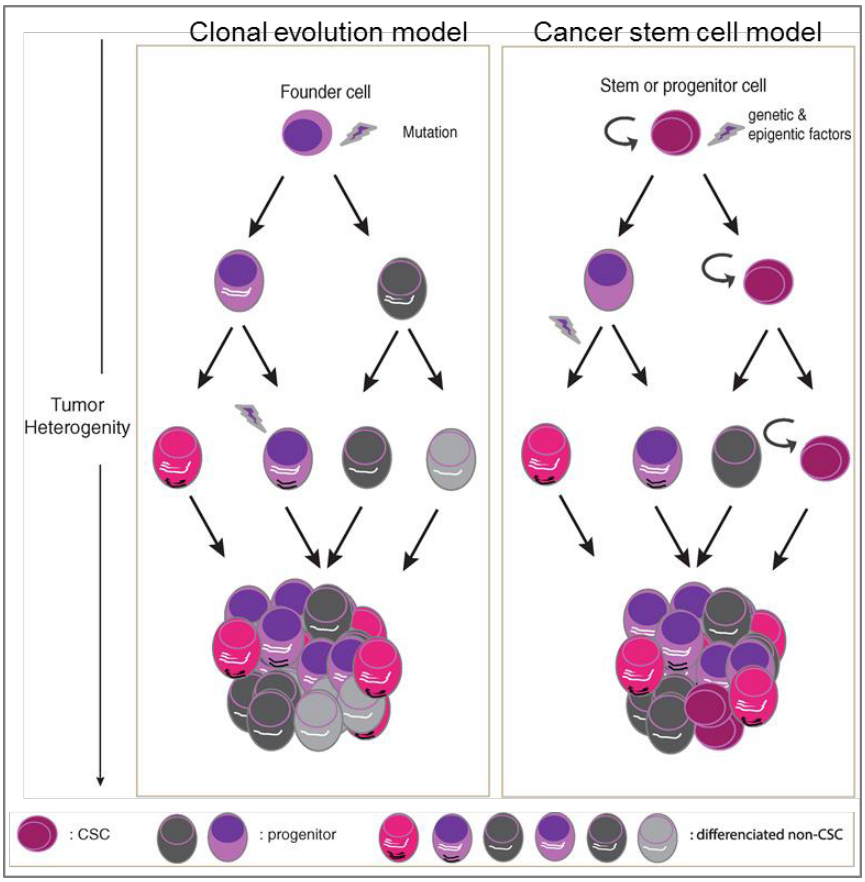


Figure 1.5. Clonal evolution (left) and cancer stem cell model (right) are models of tumorigenesis (adapted from [70]).

1.2.2 Role of GSCs in GBM

As mentioned above, GSCs play an important role in treatment failure of GBM patients. This failure has been suggested to be due to the fact that standard treatment in GBM only kills the bulk of the tumor, whereas the tumor initiating cells escape treatment and are able to cause relapse [71]. One explanation is that most cytotoxic treatments are aimed at fast-dividing cells, while the bCSC are normally quiescent or slowly cycling [72-74]. The slow cell proliferation and growth of CSCs also has been shown to contribute to their radioresistance, as IR mostly targets cells that are G2/M phase (high proliferation rate) while it minimally effects cells in S phase of the cell cycle [75]. GSCs have also been shown to decrease apoptosis by activating the DNA damage checkpoint response and increasing their DNA damage repair activity through phosphorylation of ataxia-telangiectasia-mutated (ATM), Rad17, Chk1 and Chk2 checkpoint proteins to a greater extent compared to non-stem tumor cells [76]. Furthermore, CD133+ cells also have elevated expression of DNA mismatch repair genes and multi-drug resistance genes as compared to CD133- cells, which can potentially allow for increased survival [77, 78].

In the context of tumor recurrence, Tamura et al. [79] have reported that CD133+ GSCs were enriched in GBM specimens obtained after high-dose irradiation suggesting that traditional anti-GBM treatment selects for a GSC population and it could be speculated that these cells are responsible for recurrence of the tumor after therapy with increased aggression. Indeed, RT has been reported to generate new mutations and epigenetic modifications and therefore, killing radiosensitive cells and selecting for cells with acquired radioresistant properties such as the GSC population [80].

In contrast to the studies above, other studies show that GSCs are more sensitive to GBM treatment due to their reduced double-strand break repair capacity [81] or their dependence on

Chk2 for glioma response to IR [82]. These studies use CD133 as the universal GSC surface marker to isolate and study radioresistance, however as mentioned above the use of CD133 as a reliable marker for GSC selection is controversial [83]. Moreover, another factor that could explain the controversy of these studies is the fact that GBM cells are characterized by distinct molecular profiles and may differ in their DNA damage repair capacity [84].

One characteristic of neural stem cells (NSC) in adult mammalian brain is their ability to migrate away from the stem cell niche and undergo terminal differentiation in a different central nervous system (CNS) area [85]. This ability has also been seen in GSCs as they are able to migrate throughout the brain parenchyma and initiate tumor formation in adjacent brain regions [42]. This causes these cells to be present in areas in which tumor resection is not possible and as a consequence some GSCs are able to avoid chemo- and radiation therapy and cause tumor recurrence. Indeed, studies have shown that in patients with recurrent gliomas tumors cells are seen not only at the tumor site but also throughout the brain parenchyma, including the ventricles [86-88].

GSCs are also known to be involved in tumor angiogenesis by secreting vascular endothelial growth factor (VEGF) as a pro-angiogenic factor [89, 90]. Bao *et al*. [89] showed that when GSCs were transplanted into severe combined immunodeficiency (SCID) mice massive angiogenesis, necrosis and hemorrhage was detected that was found to be due to elevated levels of VEGF. When they further cultured endothelial cells (EC) in stem conditioned media, they also showed a drastic increase in EC migration and tube formation when compared to non-stem conditioned media.

It has been reported that low O_2 levels inhibit differentiation of the stem cell population, and it is also important in stem cells and progenitor cells [91-93] and finally it has a crucial role in

glioma growth and tumorigenecity [94-98]. In toto, this showed the importance of GSCs and substantiates the need for developing GSC anti-GBM therapy.

1.2.3 Implication in treatment of GBM

As described above GSCs are responsible for tumor initiation, progression, chemo- and radio resistance leading to tumor recurrence. These cells are also known as the "mother population" of the tumor and serve as a potential powerful target for GBM treatment. In other words, GSC populations are responsible for maintenance of tumor bulk, and targeting these cells will eventually lead to elimination of tumor. Several factors are important in treatments used or designed for GBM: first, traditional chemo- and radiation therapy target the bulk of the tumor and are not able to kill the GSC population leading to tumor relapse. In addition, if the treatment only targets the GSC population, there is a possibility of dedifferentiation of tumor bulk to stem cell population and causing regrowth of the tumor. Finally, the treatment used for GBM should not only lead to killing the tumor bulk but also to be able to target the GSC population for a complete elimination of tumor (see Figure 1.6).

Several approaches have been used to kill stem cell populations and prevent them from regenerating the heterogeneous tumor mass. One approach is to force the GSC population to differentiate and hinder them from re-populating the tumor bulk. This therapy has been termed "differentiation therapy" and has been used in acute promyelocytic leukemia (APL). In APL patients, poorly differentiated leukemia cells populate the bone marrow and hinder the production of normal blood cells. By using *all*-trans-retinoic acid (RA) the poorly differentiated cancer cells are forced to differentiate and thereby lose their malignant potential [71, 99]. Another study has also showed that U87 derived neurosphere cultures differentiate when treated with low

concentrations of RA [100]. Lee et. al, demonstrated that by culturing GBM neurosphere cells in the presence of RA they express differentiation markers at similar levels as GBM cells grown in serum-containing media [101]. Another approach to target the GSCs is through signaling pathways such as Sonic hedgehog (SHH), Wnt, TGF-β, BMP Bone Morphogenetic Proteins (BMPs), Notch and epidermal growth factor receptors (EGFRs). EGFR, known to be important for the maintenance of stem cell population is described in section 1.3.

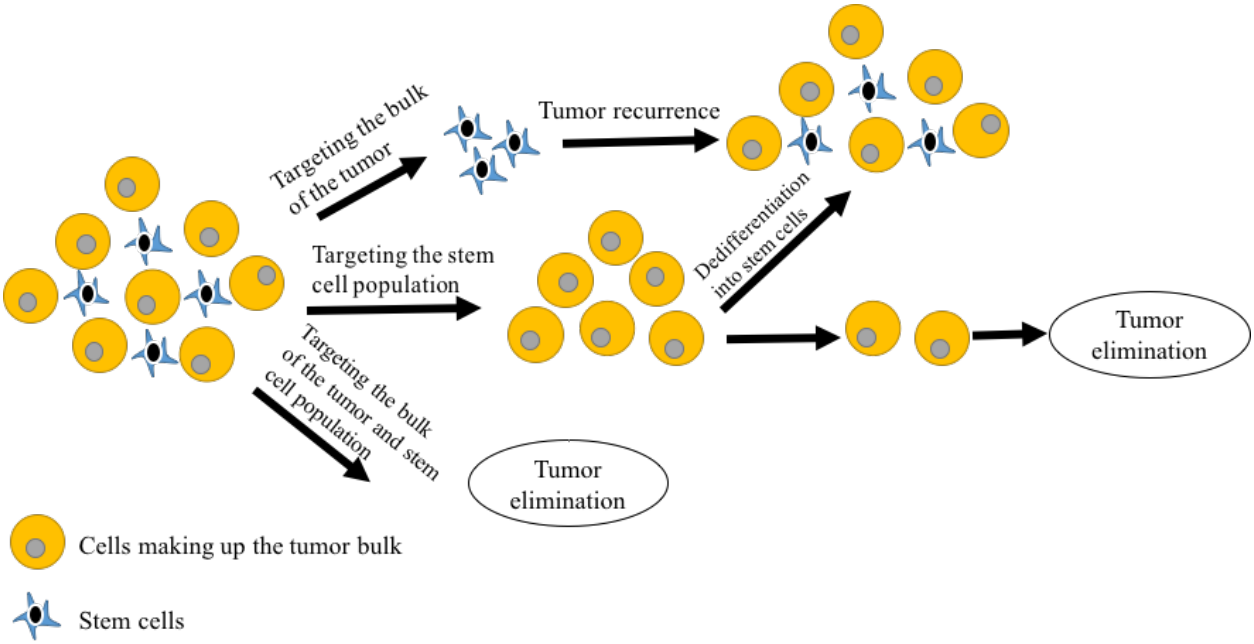


Figure 1.6. Targeting the GSCs in GBM therapy. First row shows that if we only target the bulk of the tumor bulk leaving the bCSC this might cause the re-generation of the tumor by the small population of stem cells and cause relapse. Targeting the bCSC (middle row) will lead to elimination of the tumor but there is still the risk of dedifferentiation of more mature tumor cells into new stem cells. The best strategy is to target both the bCSC population and the tumor bulk cells (bottom row) in order to eliminate the tumor completely and prevent relapse.

1.3 RECEPTOR TYROSINE KINASES (RTKs)

One main characteristic of multicellular organisms is the ability of each individual cell to be able to communicate via a cellular network. Cells are able to receive various external signals and connect with surrounding cell environment, and therefore perform important cellular functions. These cellular functions play a critical role in the regulation of a number of cellular processes including, differentiation, cell survival, cell-cell interactions, proliferation, metabolism, cell development and migration and are mainly regulated by growth factors (GFs) and their receptors [102, 103]. One of the main signaling events is carried out by a specialized class of enzymes termed protein kinases that are able to phosphorylate/dephosphorylate the hydroxyl-groups of serine, threonine and tyrosine in a reversible manner [104, 105]. The enzymatic properties of this large family of phosphotransferases is due to their ability to transfer the γ-phosphate from an ATP molecule to a hydroxylamine residue on the substrate protein [105-107]. Based on the phosphorylation site protein kinases are classified as serine/threonine or tyrosine kinases (TKs).

Although very little was known about signal transduction pathways, today receptor tyrosine kinases (RTKs) are among the best described growth factor regulated pathways which have with comparable structural features. Vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), fibroblast growth factor receptors (FGFRs), insulin growth factor receptors (IGFRs), hepatocyte growth factor receptors (c-Met, HGFRs), and epidermal growth factor receptors (EGFRs) are among the most important RTKs that have been studied in different type of cancers [108, 109]. Since, EGFR is one of the main focus of this thesis it will be discussed in detail.

1.3.1 Historical timeline of the discovery of receptor tyrosine kinases (RTKs)

Several landmark discoveries have brought us to our current state of understanding of cellular signaling and the importance of RTKs, which will be discussed in this section. The first phosphate group on a protein was identified in 1906 and the late 1930s marked the discovery of glycogen phosphorylase the enzyme that catalyses the rate-limiting step of glycogenolysis [110]. In 1950, Earl Sutherland was the first to demonstrate that a hormone could influence the activity of a specific enzyme, cAMP-dependent protein kinase (PKA) and the finding that it activated phosphorylase kinase [110-112]. This was the first example of 'cascade' events in which one protein kinase activates another.

In 1960-1962, pioneering discoveries of nerve growth factor and epidermal growth factor (EGF) revealed the importance of these two growth factors and it soon became clear that they are able to bind to cell-surface receptors and be involved in neuronal differentiation and cell proliferation [113, 114]. By late 1960s phosphorylation was still believed to be a specialized and controlled mechanism largely confined to the regulation of glycogen metabolism. A landmark finding was also shown in 1970s revealing proteins that are phosphorylated on two or more residues by more than two kinases, termed multisite phosphorylation [115]. Another surprising finding was in the late 1970s by Ray Erikson that showed that v-Src, the protein encoded by the transforming gene of rous sarcoma virus (RVS), was a kinase [116] that was later shown to be able phosphorylate tyrosine residues on other proteins, implying that it possessed intrinsic kinase activity [105, 117]. Soon after, EGFR was also shown to be a protein tyrosine kinase, and it was shown that it not only can become activated upon binding to EGF [118-120] but also it is able to "self" phosphorylate specific tyrosines which then stimulate 'downstream' pathways to mediate the effects of the signal transduction pathways. These findings stimulated researchers to search for

the physiological substrates of protein kinase activity. Surprising finding showed that the receptors were the main cellular substrates that frequently become phosphorylated at multiple tyrosine residues. This was later explained by showing some proteins contain a Src homology 2 (SH2) domain and are able to recognize particular phosphotyrosine-containing sequences and phosphorylated growth factor receptors [121-123].

Studies by Howard Temin (1966, 1967) [124, 125] suggested that cancer cells produce and use their own GFs. This discovery was followed by the discovery of many GFs that stimulate cell proliferation by binding to receptors at the cell surface. For example, designated platelet-derived growth factor (PDGF) [126, 127] isolated from human platelets, fibroblast growth factor (FGF) that is a growth factor isolated from bovine brain [128] and insulin-like growth factor 1 (IGF-1). These findings along with many other studies since the 1980s highlighted the link between growth factor receptors and that they play numerous important roles during development, during normal cellular processes as well as in pathologies such as cancer, diabetes, atherosclerosis, bone disorders, and tumor angiogenesis.

In early the 1990s a new family of protein tyrosine kinases, JAK kinases, was emerged from a PCR-based screen. Once this pathway is activated, it leads to phosphorylation of signal tranducers and activators of transcription (STATs) that mediate transcription directly [129]. In addition, MAPK and the PI3K pathways were identified in the late 1980-1990s. These discoveries showed the importance of signal transduction that are underlying mechanisms of cell growth, proliferation, survival and cell death.

1.3.2 EGFR structure and functions

The EGFR family of receptors, also called ErbB family of RTKs are among the twenty receptor tyrosine kinase classes found in human [119, 130]. This family of receptors consist of four human epidermal receptors: 1) ErbB1 (EGFR or HER1), 2) ErbB2 (neu or HER2), 3) ErbB3 or HER3 and 4) ErbB4 or HER4 which show strong structural homology. In the normal brain, EGFR is expressed in neurogenic areas and in many tissues of epithelial, mesenchymal and neuronal origin [131-133] and plays essential and fundamental roles in development, proliferation, differentiation, survival and transformation of cells. Also, it has been reported that EGFR is involved in regulation of the developmental and adult stage of NSC migration, proliferation, and differentiation [134-139]. More importantly, EGFR has been shown to be overexpressed in a large number of solid tumors, including head and neck, ovarian, brain, breast, bladder, colon, prostate and lung cancers (Figure 1.7) [140-145].

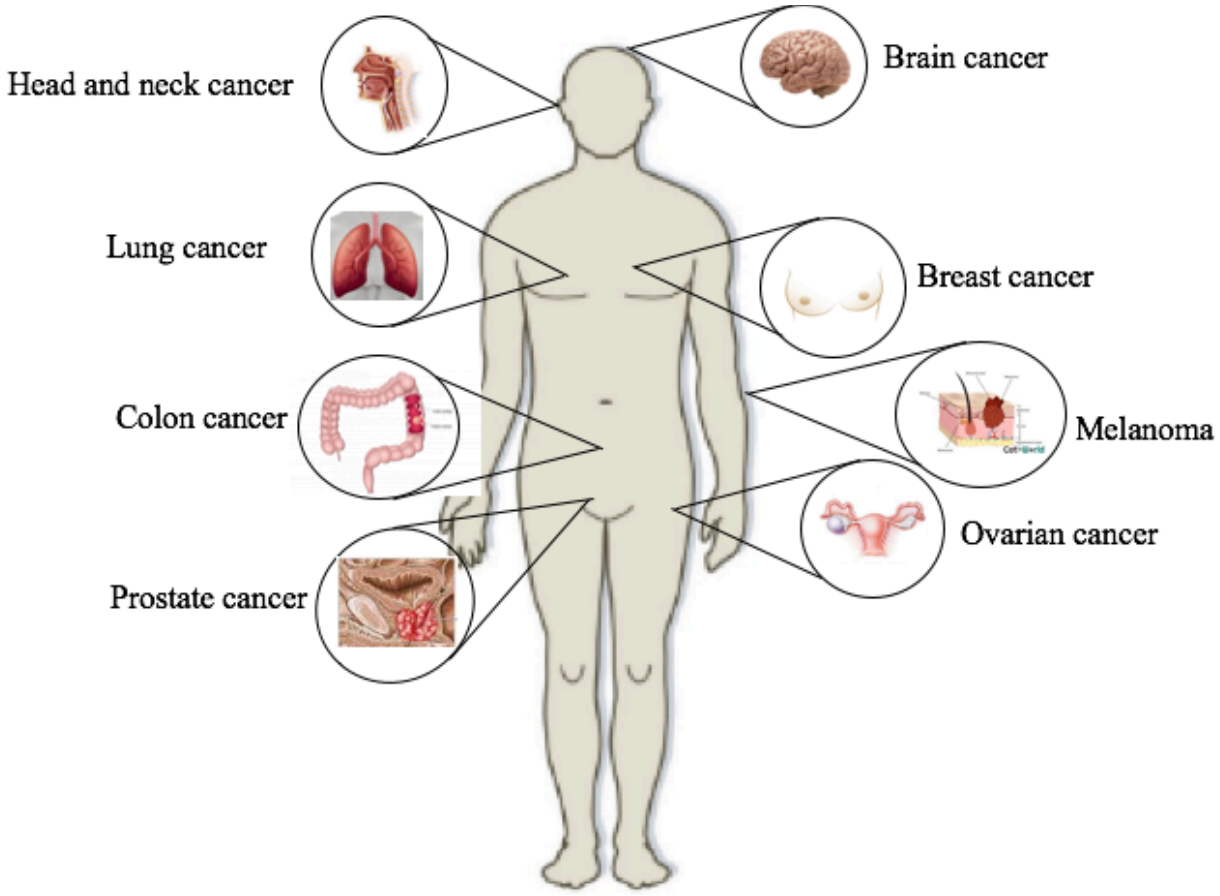


Figure 1.7. EGFR deregulation in different human cancers. This figure shows deregulation of EGFR in different human cancers. This deregulation in EGFR, including its overexpression or mutation, is implicated in many solid tumors such as: gliomas and breast carcinomas.

Stanley Cohen of Vanderbilt University was the first to discover EGFR and later he shared the 1986 Nobel Prize in Medicine with Rita Levi-Montalcini of University of Turin [146, 147]. EGFR is a 170-kD glycoprotein and consists of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and an intracellular tyrosine kinase (TK) domain. The extracellular ligand-binding domain is also referred to as domains I-IV and consists of four subdomains namely L1, CR1, L2 and CR2 in which L stands for leucine rich and CR means cystein-rich domain [130, 148, 149]. Domains I and III share 37% amino acid identity, while

domains II and IV are homologous Cys-rich domains, CR1 and CR2, respectively [150] (Figure 1.8.A) The transmembrane domain is mainly α-helical and is involved in receptor down regulation and ligand-dependent internalization. The kinase domain is essential because it harbors the ATP-binding site of the receptor between the N-terminal and C-terminal lobes (Figure 1.8A).

As shown in figure 1.8.B once ligands such as EGF, transforming growth factor alpha (TGF-α), amphiregulin (AR), heparin-binding EGF, betacellulin (BTC), neuregulins (NRGs), and epiregulin (EPR) bind to these receptors they get activated [130, 151-153] and form, "dimerization loop". ErbB family of RTKs (HER1, 3 and 4) exist in their inactive monomer form which does not allow homodimers and hetereodimers dimerization. However, when ligands bind to the receptors they force them to undergo conformational change in the extracellular region and allow the two ligand-bound monomeric receptors to interact with each other. Of note, HER3 lacks the tyrosine kinase domain and can only activate downstream signaling when it dimerizes with other HER family of receptors [154, 155]. This conformational change also results in activation of downstream signaling, which depends on the nature of the ligand receptor complexes [149, 156]. In fact, the dimerization of two monomers brings closer their tyrosine kinase domains and their tyrosine residues. Hence, the monomers transphosphorylate each other at their tyrosine residues. The C-terminus kinase domain of EGFR contains many important phosphorylation sites of which the most important ones are: Y845, Y992, Y1068, Y1086, Y1101, Y1148, Y1173) [157-159]. These sites can be either auto- or trans-phosphorylated upon dimerization and serve as substrates for other TKs to transmit the signal via phosphotyrosine-Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains, which mediate the activation of multiple downstream pathways (e.g., Ras-Raf-MAPK, PLC, PI3K and STATs) [130, 160-162]. In addition, this can

transduce signals down to the nucleus for transcription of a group of genes such as c-jun, c-fos, c-myc, c-myb, egr-1) [155].

Advanced findings in the structure of EGFR [148, 163, 164] and understanding the mechanisms regulating EGFR activity and the key amino acid residues involved using point mutants or deletion mutants of EGFR has paved the way for structure-activity studies with a series of EGFR tyrosine kinase inhibitors (TKI) [165-167].

Interestingly, several studies show the relation between GSCs and EGFR. First, these cells proliferate *in vitro* in response to growth factors such as EGF [101] and can be isolated based on expression of EGFR [168]. Second, glioma neurosphere cultures are sensitive to inhibition of EGFR signaling [169-171]. Of note, expression of EGFR together with the GSC signature has been linked to chemo- and radiation resistance [29].

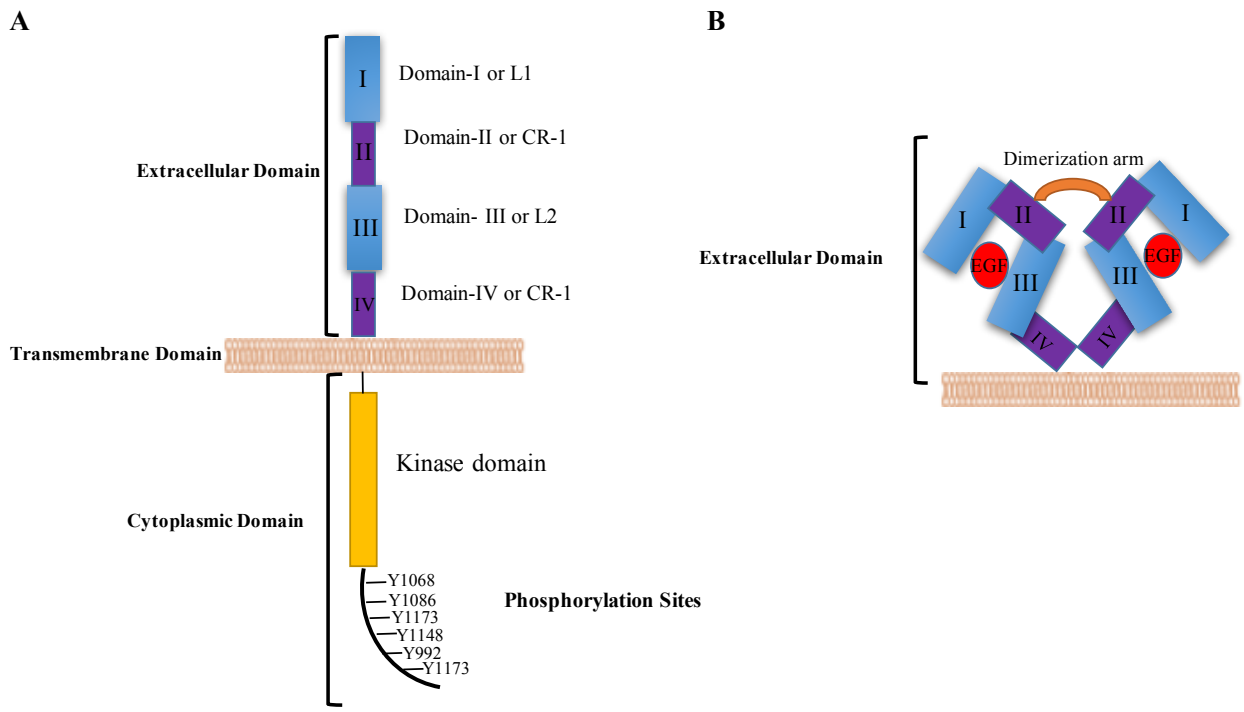


Figure 1.8. EGFR structure and receptor dimerization. **(A)** Receptor structure and domains of EGFR are shown in Figure A. **(B)** Ligand (EGF) binding and receptor dimerization seen in the extracellular region with the domains interacting with each other.

1.3.3 Mutations of EGFR and downstream mediators

The EGFR has previously been implicated in the malignant transformation of epithelial cells, including several solid organ malignancies: gastric, colorectal, prostate, bladder, esophageal, brain, lung, breast, head and neck, renal, pancreatic, and ovarian cancers [172-175]. Several mechanisms by which EGFR becomes oncogenic and cause constitutive EGFR activation are: (1) autocrine growth loop of the epidermal growth factor receptor (2) deletions of domains giving rise to constitutively active variants of EGFR (3) mutations that prevent EGFR internalization [172, 176-179]. As mentioned in section 1.1.3 alterations in RTKs are detected in ~60 % of primary (*de novo*) GBM tumors accounting for the heterogeneity of GBM tumor cells. More specifically, ~

57% of GBM samples harbored alteration in EGFR, 13% in Platelet Derived Growth Factor Receptor Alpha (PDGFRA) and 2% in MET [180]. EGFR overexpression can lead to increased activity of the EGFR promoter, amplification of the EGFR gene or deregulation at the translational and post-translational level [181, 182]. EGFR mutations are present in ~ 40-50% of GBM and at least nine EGFR mutation variants have been detected in this cancer [183, 184]. The most common EGFR mutation is EGFRvIII (31-58%), that is the result of in frame deletion of exons 2 to 7 (801 base pairs) in the EGFR gene [5, 185, 186] (Figure 1.9). This mutation is not expressed in normal tissue and causes the EGFR to lack the extracellular domain of the receptor (amino acid 6-273) and results in a truncated (145 kDa) receptor that is not able to bind to EGFR ligands [183, 187]. Although, this receptor has a distorted ligand binding area it exhibits a constitutively active tyrosine kinase activity and thus is able to activate downstream signaling pathways [188-191] (Figure 1.9) and in some cases it has even been reported that EGFRvIII has a higher signaling through certain pathways such as PI3-K/AKT pathway compared to EGFR wild type [155, 188, 192]. Despite the lack of ligand binding ability, EGFRvIII is still able to not only homodimerise with itself, but also form heterodimers when co-expressed with EGFR [188]. Of note, EGFRvIII expression mostly occurs in tumors with EGFR amplification (~40% of tumors with amplified EGFR) [188, 190]. In summary, tumors expressing EGFRvIII have been reported to be resistance to standard therapies such as cisplatin, paclitaxel and temozolomide due to many reasons such as increased proliferation, increased invasiveness (regulated by MMP-13), decreased apoptosis (due to 3-fold upregulation of Bcl-xL as well as inhibition of caspase-3-like activity), and increased angiogenesis (through NFκB signaling to VEGF and IL-8) [193]. It has been reported that TKIs such as Iressa®/gefitinib are ineffective against tumours expressing EGFRvIII due to their failure in blocking EGFRvIII phosphorylation completely and also they are not able to inhibit Akt

activation. In addition, EGFR inhibitors such as Tarceva® or gefitinib have less effect on patients that have EGFRvIII-positive tumors along with phosphatase and tensin homolog (PTEN) deficiency (PTEN mutant) while those with patients that have functional PTEN show a modest response to these treatment [194, 195]. Indeed, inactivation of PTEN due to *PTEN* mutations or loss, can increase EGFR signaling and therefore contributes to the abnormally high activity of the PI3K/AKT pathway, often seen in primary GBM [196-198] and has been correlated with the dismal prognosis of patients with GBM [199, 200]. Moreover, monoclonal antibodies cetuximab and mAb528 that are designed to target wt EGFR are also able to inhibit EGFRvIII *in vitro* but they have unfortunately shown no effect in *in vivo* studies [201]. On the other hand monoclonal antibodies such as Y10 and L8A4 were used to target EGFRvIII tumors and the results of this study showed promising results in a preclinical setting in mice and rats and treatment with monoclonal antibody 806 (mAb806) resulted in increased apoptosis and decreased angiogenesis [193].

The EGFRvIII has been also reported in breast cancer (27-78%), ovarian cancer (75%), head and neck squamous cell cancer (42%), NSCLC (15-41%) as well as metastatic prostate cancer [193, 202-205]. These mutations occur either in: (1) the intracellular domain, (2) the extracellular domain leading to loss of sensitivity to its ligand, and (3) specific residues at the tyrosine kinase domain [177, 206]. Another alteration downstream from EGFR is increased RAS-activity which is a frequent phenomenon in GBM possibly due to increased activation of the upstream RTK [207, 208]. Taken together, multiple alterations of the EGFR signaling pathway have been reported in GBM and other cancer types, and this pathway thus serves as a potential target for GBM therapy.

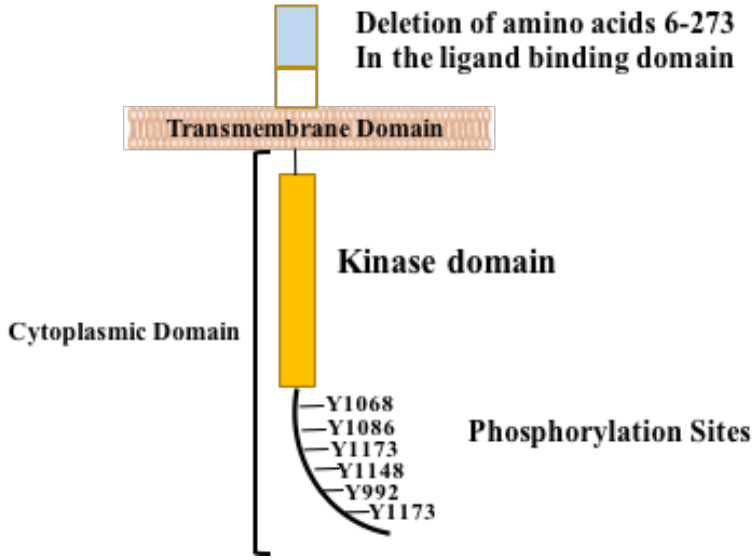


Figure 1.9. Structure of EGFRvIII.

1.3.4 Signal Transduction Pathways Activated Downstream of EGFR

As described in (section 1.3.2) when ligands bind to EGF receptors they undergo conformational changes and cause activation of the tyrosine kinase domain, which leads to catalyzing the transfer of a phosphatase group from donor adenosine triphospate (ATP) to an acceptor hydroxyl group of tyrosine residues residing near the catalytic site on the dimer neighbor [109, 209]. This leads to phosphorylation of additional tyrosine residues in the tail of the cytosolic EGFR domains [107, 210]. The phosphorylated tyrosine residues then serve as docking sites for adapter and signaling molecules leading to numerous EGFR downstream signaling pathways, generally leading to cell survival, proliferation, transformation and invasion [211-214]. In this thesis and due to this project specific interests only in MAPK and AKT pathways are discussed (Figure 1.10).

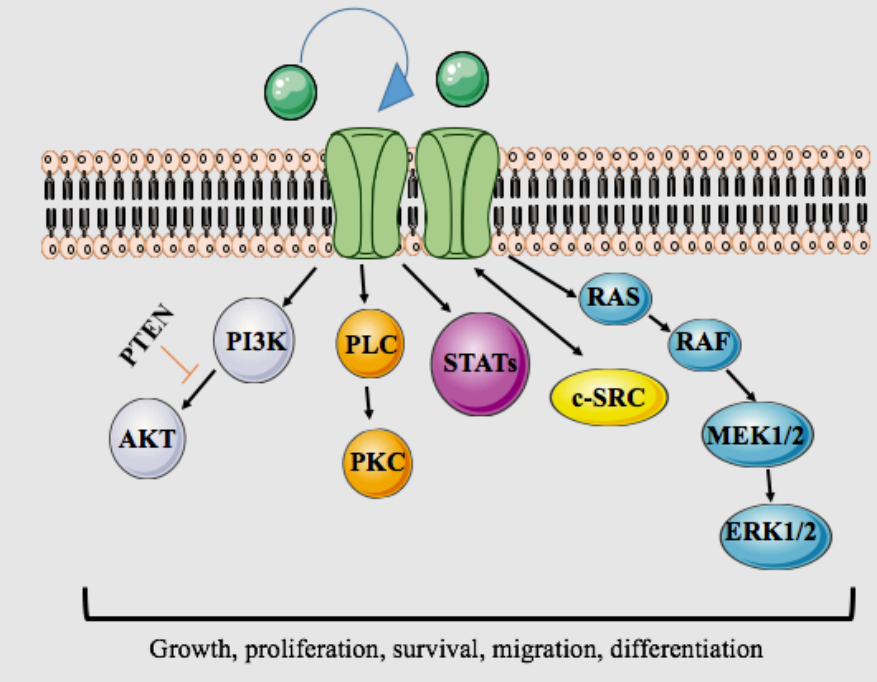


Figure 1.10. Activation of epidermal growth factor receptor (EGFR). Upon ligand binding, EGFR dimerizes and autophosphorylates itself that leads to the activation of downstream signaling pathways resulting in migration, survival, proliferation, etc.

1.3.4.1 Activation of the Mitogen Activated Protein Kinase (MAPK) pathway

The most extensively studied and best characterized EGFR pathway is cell signaling of mitogen-activated protein kinases (MAPKs) as shown in Figure 1.11. To date, there are six groups of known MAPKs that include extracellular signal-related kinase (ERK1/2), ERK3/4, ERK5, ERK7/8, JNK1/2/3 and p38 isoforms. Extracellular signal-regulated kinase, ERK pathway [215, 216], which is the most studied pathway, is mainly involved in growth and proliferation and found to be deregulated in cancers. MAPK can get phosphorylated and activated by a MAPK-kinase (MAPKK), which in turn is phosphorylated and activated by a MAPKK kinase (MAPKKK) that is activated by upstream proteins [217, 218]. This multistep process (Ras/Raf/MEK/ERK1/2 cascade) begins with stimulation of RTKs and phosphorylation of tyrosine residues within the

EGFR cytosolic domain and recruitment of growth factor receptor-bound protein 2 (Grb2) which is an adaptor protein. This facilitates the binding of Son of Sevenless (SOS), a guanine exchange factor, that exchange the RAS-bound guanine diphosphate (GDP) for guanine triphosphate (GTP) and thus activate the G-protein RAS. Grb2 can either bind to phosphorylated Y1068 or Y1086 via its SH2 or indirectly by SH2 domain of Shc that binds to phosphorylated Y1148 or Y1173. Of note, the RAS family of small GTP-binding proteins able to co-localize with many proteins and facilitates the interactions between several cascades, thus transmitting signals [219-222]. Active RAS then is able to phosphorylate the serine/threonine kinase Raf-1, a MAPKKK, that in turn is able to phosphorylate and activate MEK1 and MEK2 [223-225]. This leads to activation and phosphorylation of the last "tier" of this kinase module, ERK1/2, a MAPK, which then translocates to the nucleus which is then able to activate many proteins such as nuclear transcription factors (e.g., Elk-1, Sp1, E2F and AP-1) that subsequently activate transcription of target genes e.g., c-myc, c-myb c-jun and c-fos [214, 216, 223, 226-228].

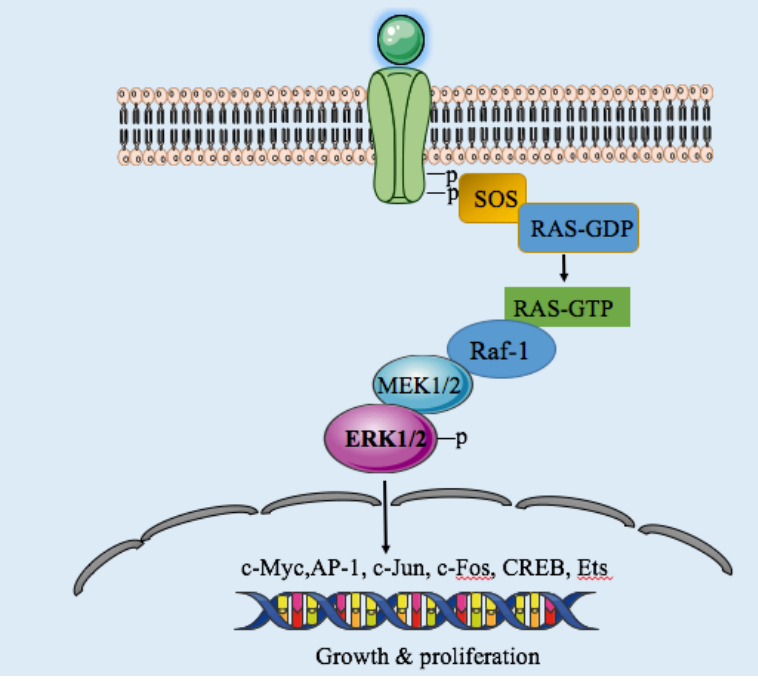


Figure 1.11 Activation of the MAPK pathway.

1.3.4.2 Activation of the Phosphotidylinositol-3-Kinase (PI3K) pathway

Another pathway downstream of EGFR is the phosphatidylinositol 3-kinase (PI3K) pathway that involves AKT (Akt), also known as Protein Kinase B (PKB), which is a serine /threonine kinase. Activation of this pathway can lead to cell proliferation, survival, growth, apoptosis, motility and metabolism [229-231]. AKT "AK" was a temporary classification name for a mouse strain developing spontaneous thymic lymphomas. Other members of this family are AKT1/PKBα, AKT2/PKBβ, AKT3/PKBγ [232, 233]. Identification of this highly conserved pathway began in the early 1980s through vigorous attempts to characterize insulin receptor signaling [234, 235]. Based on the substrate specificity, structure, function and mechanisms of activation of PI3Ks they are classified into classes I, II and III [231, 236].

Wealth of literature exists on the structure and functions for this founding class of the PI3K family [237]. This class is the only class that has been linked to cancer and can get activated by protein-coupled receptors (GPCRs) and Ras. In addition, it is a lipid, heterodimeric kinase that consists of two domains. A catalytic subunit (p110) and a regulatory domain (also referred to as an adaptor domain), p85 [231]. The catalytic subunit has four isoforms designated as p110α, p110β, p110γ and p110δ [236, 238]. These subunits have common domains such as amino-terminal adaptor-binding domain (ABD), Ras-binding domain (RBD) in the N-terminal extension, protein-kinase-C homology-2 (C2), a helical domain acting as a scaffold for other domains of p110, and a carboxyl-terminal kinase domains [230, 231]. ADR and RBD domains are involved in providing interaction surfaces and mediating the interaction between p110 and Ras-GTP, respectively [236]. The regulatory domain of PI3K class I, p85 is able to bind receptors and non-receptors through its Src homology 2 domains (SH2) domains (nSH2, cSH2) [230]. Another role

of p85 is that it also negatively regulates the kinase activity of p110α through helical domain interaction. Since, p110γ does not have a p85-binding domain forms it heterodimers with the regulatory subunits p101 or p84 and exclusively gets activated by GPCRs [238].

PI3K/AKT, same as the MAPK pathway starts with ligand binding, receptors dimerization and phosphorylation of tyrosine residues specific for the activation of this pathway. Activation of PI3K typically occurs through stimulation via its regulatory subunit bound to the activated receptor or through the small G protein Ras or through adapter molecules (e.g. insulin receptor substrate (IRS) proteins). This leads to binding of the regulatory subunit, p85, to an activated receptor and thus activation of the p110α catalytic subunit [231]. This triggers conversion of phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [230]. PIP2 and PIP3 act as docking sites for other regulatory proteins with a pleckstrin homology (PH) domain such as PDK1 (phosphorylates Thr-308 residue of AKT) and PDK2 (phosphorylates Ser-473 of AKT) [238]. AKT is then translocated to the plasma membrane allowing it to bind to PIP3 at the plasma membrane via its PH-domain leading to its partial activation via PDK1. For complete activation of AKT, serine 473 needs to get activated, which can be done by the mammalian target of rapamycin (mTOR) complex protein, mTORC2 [239]. One negative regulator of this pathway is phosphatase and tensin homolog known as PTEN, which antagonizes the action of PI3-K, by dephosphorylation of PIP3 to PIP2 [240]. Therefore, the length of the signals mediated by PI3K/AKT is regulated by the lipid phosphatase PTEN and deficiency of PTEN therefore promotes inappropriate and prolonged PI3K/AKT signals. Mutations or inactivation in PTEN activity are seen in many human cancers, which results in constitutive AKT activation [241, 242].

1.4 DNA AS TARGET FOR CHEMOTHERAPY

1.4.1. Types of DNA Damage

DNA, like any other molecule is subject to damage. DNA strands are easily damaged which can be the result of either endogenous (reviewed in [243]) reaction such as replication stress, reactive molecules created during normal cellular metabolism, alkylation or from exogenous sources such as DNA damaging agents (chemotherapeutics) and ionizing radiation. Many anticancer therapies are potent inducers of DNA damage. These include chemotherapy and radiotherapy. Some examples of different types of DNA damage are intra- and interstrand cross-links, base lesions, single-strand breaks (SSBs) and double-strand breaks (DSBs) that each form depending on the source and extent of DNA damage [244]. When DNA is damaged it can interfere with DNA replication, transcription that leads to mutations and chromosomal aberrations of DNA.

SSBs arises from disruption of the DNA backbone and breakage in the sugar phosphate backbone of only one strand of the DNA duplex. On the other hand, although DNA DSBs are less frequent, they are the most toxic DNA damage and occur when two complementary DNA strands are broken simultaneously.

Reactive oxygen species (ROS), such as O_2^-, H_2O_2, and $^{\bullet}OH$ are produced during cellular processes and are related to other endogenous reactive molecules [245, 246]. ROS can generate over one hundred different oxidative DNA adducts- that are critical in producing SSBs, DSBs, and DNA-protein cross-links [247]. Alkylation is another important type of DNA damage related to endogenous type of DNA damage in which the primary sites of alkylation are the O^- and N^- atoms of nucleobases.

The DNA damage resulting either from endogenous or exogenous agents can activate DNA repair mechanisms in the cell that will be described in section 1.5.4.

1.4.2 DNA targeting agents

DNA-damaging agents have a long history of use in cancer chemotherapy. One major limitation of DNA-damaging agents is the toxicity seen in patients such as treatment with cisplatin, which causes nephrotoxicity [248]. One reason is, when DNA-damaging agents were discovered most attention was given to the effects of these drugs on cancer cells and minimal attention was attributed to non-cancer cells. The toxicity seen in patients also suggested the lack of selectivity of these drugs. Today, however, cancer treatment still largely relies on use of chemotherapeutic drugs to reduce tumor growth and eliminate cancer cells.

1.4.3 Alkylating Agents

The alkylating agents were one of the earliest classes of drugs discovered and used effectively to treat cancer [249]. The story behind the recognition of the anti cancer effects of these compounds is an outstanding one. During World War I, sulfur mustards (gas) were used as military weapons. Subsequently, in World War II, US warship carrying a cargo of nerve gas off the coast of Italy was sunk in Italy. In both World War I and II, it was observed that people who were exposed to sulfur mustard showed bone marrow suppression and lymphoid aplasia [250] and therefore sulfur mustard was examined as an antitumor agent [251]. The results of clinical trials on patients with lymphoma showed promising antitumor activity [252, 253].

The alkylating agents can either react directly with biologic molecules or they first form a reactive intermediate that can then bind to biologic molecules. Major sites of reaction of these drugs are in the double helix structure of DNA and include the phosphate-sugar backbone and various hydrogen acceptor/donors to form covalent bonds. One main characteristic of these drugs is their ability to affect cancer cells during all phases of the cell cycle, although their biggest impact

is in the S-phase of the cell cycle. By adding alkyl groups to DNA these classes of agents cause breakage of the DNA strands and eventually, cancer cell death.

Many alkylating agents have been tested in the clinic and some are described in this thesis: 1) nitrogen mustards and nitrosoureas, 2) triazines, 3) alkyl sulfonates, and 4) ethylenimines.

1.4.3.1 Nitrogen mustards and nitrosoureas

The nitrogen mustards (e.g. 2-chloroethyl-sulfide) and nitrosoureas are among the first bifunctional alkylating agents that were used to treat cancer [254, 255] (Figure 1.12). The first clinical trial with a nitrogen mustard was reported in 1963 [256]. They damage DNA by forming guanine-guanine and guanine-adenine interstrand crosslinks. Bendamustine, mechlorethamine (the original "nitrogen mustard"), cyclophosphamide, ifosfamide, melphalan, and chlorambucil are commonly used [257] . This thesis also reports the effects of a hemi-mustard drug (ZR2002), tested for the first time for the treatment of GBM. Nitrogen mustards consist of a bischloroethyl group and react through aziridinium rings by intramolecular displacement of the chloride by the amine. Thus, it can react with the N-7 position of guanine leading to formation of N-7 chloroethyl adducts that can further cross-react with another DNA strand to form interstrand DNA cross-links that enhances the potency of nitrogen mustard.

Cyclophosphamide, marketed as Cytoxan® or Neosar® has shown less gastrointestinal and hematopoietic toxicity than other alkylating agents and therefore has been used in many cancers such as Hodgkin's and non-Hodgkin's lymphoma, chronic myelocytic leukemia, acute myelocytic leukemia, acute lymphocytic leukemia, retinoblastoma, rhabdomyosarcoma, breast chronic lymphocytic leukemia, multiple myeloma, neuroblastoma, testicular, endometrial, lung, and ovarian cancers, etc. [256, 258]. Chlorambucil (Leukeran®), is another well known and widely

used nitrogen mustard to treat giant follicular lymphoma, chronic lymphocytic leukemia, malignant lymphomas including lymphosarcoma, and Hodgkin's disease. It has also been successfully used to treat breast, ovarian, and testicular cancer, Waldenstrom's macroglobulinemia, thrombocythemia, and choriocarcinoma. Mechlorethamine (Mustargen®), Ifosfamide (Ifex®) and Melphalan (Alkeran®) are also well known nitrogen mustards that have been used in the treatment of many cancers such as Hodgkin's disease and non-Hodgkin's lymphoma and lung cancer (Mechlorethamine and Ifosfamide) and treat multiple myeloma, ovarian cancer, neuroblastoma, rhabdomyosarcoma, and breast cancer (Melphalan).

As mentioned above nitrosoureas are also among the most potent and widely used chloroethylating agents that add chloroethyl groups to the N7 and O6 of guanine [259]. Interestingly, once *O*6-chloroethylguanine are formed by reacting with cytosine they form guanine-cytosine interstrand crosslinks and cause cytotoxicity [259]. Like other drugs nitrosoureas have side effects such as bone marrow depression, nausea and vomiting. In addition, although they have been tested in brain tumors, such as GBM because of their ability to cross the BBB due to their lipophilic properties, they are not effective on tumors expressing MGMT.

Lomustine (CCNU or CeeNU®), Nimustine (ACNU), Carmustine (BiCNU® or BCNU), and Streptozocin (Zanosar®) are some well-known nitrosoureas. Lomustine, has been used in melanoma, lung, colon, brain tumors and Hodgkin's disease. Carmustine, on the other hand has been used in GBM, metastatic brain tumors, astrocytoma and ependymoma. Finally, streptozotocin has been used to treat islet cell pancreatic cancer.

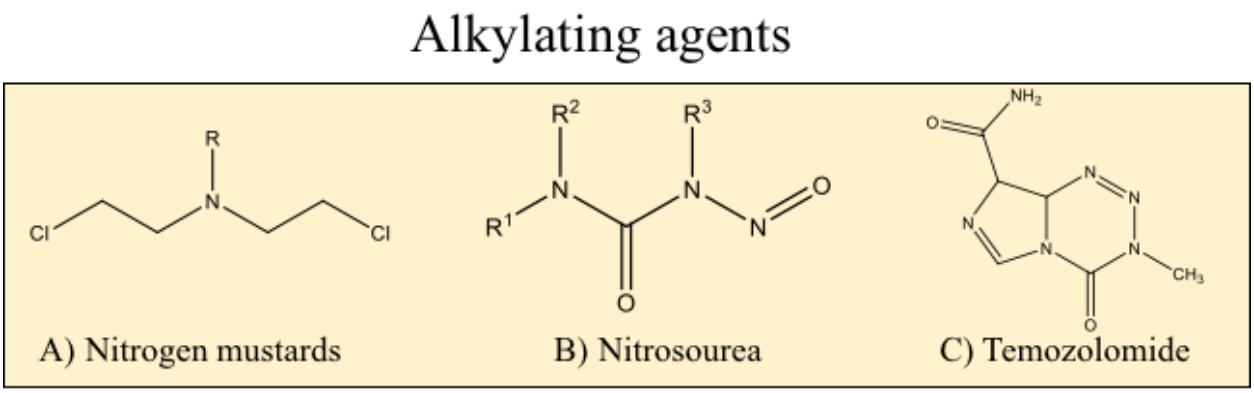


Figure 1.12. Structure of DNA-damaging agents. **(A)** The general structure of a nitrogen mustard alkylating agent; **(B)** The general structure of a nitrosourea alkylating agent; **(C)** Temozolomide, a methylating agent. Adapted from [260].

1.4.3.2 Triazines and Hydrazines

Triazines and hydrazines are a class of nitrogen-containing (three adjacent nitrogen atoms/ $C_3H_3N_3$) heterocycles and are the platform of numerous synthetic transformations [261]. Triazines (e.g., procarbazine, dacarbazine (DTIC-Dome®), TMZ (Temodar®) are also classified as monofunctional methylating agents. Once they are metabolized they produce alkyldiazonium ions (e.g., methyldiazonium) that can alkylate DNA. The cytotoxicity of Methyldiazonium ions is linked to their ability to react with several nucleophilic sites in DNA and produce O6-methyl guanine (5%), N3-methyl adenine (9%) and N7-methyl guanine (~65-80%) and therefore cause cytotoxicity [262, 263]. Despite the low proportion of O6-methyl guanine (5%) it is the most lethal monoadduct formed because guanine (G) pairs with thymidine (T) instead of cytosine (C) and eventually leads to cell death [259, 264].

Triazines and hydrazines have great pharmacokinetic properties, great bioavailability, distribution and limited toxicity and have been in used in many diseases including cancers. For

example, dacarbazine is used to treat metastatic malignant melanoma, rhabdomyosarcoma, soft tissue sarcomas, neuroblastoma, and medullary thyroid carcinoma. TMZ (also described in section 1.1.4), a small molecule, has been used in the treatment of anaplastic astrocytoma and GBM. TMZ is one of the few drugs that is orally bioavailable and survives the chemical stresses of oral absorption. The advantage of TMZ over DTIC is that it crosses the BBB more efficiently. The first phase I clinical trial of TMZ started in 1987, at the Queen Elizabeth Hospital, Birmingham, UK. Today, the current standard care of patients newly diagnosed with GBM is based on the trial of the European Organization for Research and Treatment of Cancer (EORTC)/ National Cancer Institute of Canada Clinical Trials Group (NCICCTG), that showed addition of TMZ during and after radiotherapy improved the overall survival (OS) from 12.1 months to 14.6 months compared to radiotherapy alone [11].

1.4.3.3 Platinum agents

Platinum agents (cisplatin, carboplatin, lipoplatin and oxaliplatin) are also used in cancer management. These agents are able to crosslink DNA strands resulting in ineffective DNA mismatch repair that eventually leads to cell apoptosis.

In the clinic setting, the clinical performance of platins has been disappointing [265, 266] due to many reasons such as the lack of selectivity of these drugs (leading to toxicity such as nausea/vomiting, sensory neuropathy and nephrotoxicity) or in the case of gliomas lack of efficient penetration into the CNS [267-269]. Although, there have been efforts to improve the effect of cisplatin by either testing local delivery to overcome the BBB issues with this drug or combination therapy of cisplatin and TMZ [270, 271] or using cisplatin encapsulated into liposomes [272] but none has showed any benefit to improve the clinical effect of this drug. Oxaliplatin has also been used with newly diagnosed GBM, however, early-phase clinical trials revealed peripheral sensory neuropathy as a dose-limiting toxicity with only modest responses in tumor treatment [273]. Platinum agents have been also tested in gastric, colon and pancreatic, breast, melanoma, prostate and other cancers.

1.5 CELL RESPONSE TO DNA DAMAGE

DNA is the repository of genetic information in each living cell, its integrity and stability are essential to life. Cells have evolved a number of mechanisms to detect and repair the various types of damage that can occur to DNA.

1.5.1 Phosphatidylinositol-3 kinase-related kinases (PIKKs)

Phosphatidylinositol-3 kinase-related kinases (PIKKs) also known as class IV PI3Ks (PI3Ks are described in section 1.3.4.2) are involved in DNA-damage response (DDR) [274]. They

include ataxia telangiectasia mutated kinase (ATM), ataxia telangiectasia and Rad3 related kinase (ATR), DNA dependent protein kinase (DNA-PK), and mammalian target-of-rapamycin (mTOR), suppressor with morphological effect on genitalia 1 (SMG1) and transformation/transcription domain associated protein (TRRAP).

1.5.1.1 Ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia and Rad3 related kinase (ATR)

ATM is mainly activated through DSB that causes phosphorylation of serine 1981 upon DNA damage [275]. Another important factor in ATM activation is the MRN complex that consists of Mre11, Rad50, and Nbs1 protein (MRN) [276]. Mutations in MRN complex can cause radiosensitivity, neurological abnormalities and cell cycle defects [277]. ATM facilitates the recruitment of homologous recombination (HR) proteins to damaged DNA sites leading to DNA repair [278]. In contrast to ATM, ATR responds to conditions that result in formation of SSBs and stalled DNA replication forks [279]. This PIKK DNA-damage-responsive kinase is not only activated by IR but also through replication inhibitors, such as hydroxyurea and also by methyl methansulfonate and cis-platinum [280]. ATM together with ATR are also involved in cell-cycle arrest and apoptosis and regulate a wide range of target molecules by phosphorylation. ATM mostly regulates the G2/M checkpoint, necessary for the cell-cycle arrest of the cells. ATM/ATR can inhibit activation of Cdc25C phosphatase that is essential for activation of cyclin B1 and cdk1 involved in progression of the cell-cycle [281]. Other substrates common for ATM/ATR are p53, MDM2 (described in section 1.5.2), CHK1/2 and H2AX [282, 283].

1.5.1.2 DNA-dependent protein kinase (DNA-PK)

DNA-PK is a nuclear serine/threonine protein kinase composed of 2 main subunits 1) a catalytic subunit (DNA-PKcs; 460 kDa) and 2) a DNA-binding component consisting of two subunits: Ku70 (70 kDa) and Ku86 (86 kDa), which is also referred to as Ku80 (reviewed in [284]). DNA-PK is important in non-homologous end joining (NHEJ) which is considered to be the major DSB repair pathway. Ku70/80 first binds to DSBs on the sugar backbone of DNA and then it recruits DNA-PKcs. The complex formed at the DSB consisting of DNA, Ku70/80, and DNA-PKcs is referred to as "DNA-PK" [285]. The Ku heterodimer also interacts with X-ray cross complementing protein 4 (XRCC4) [286], DNA Ligase IV [286] and XRCC4-like factor (XLF, also named Cernunnos) XLF.

As shown by Barnes et al., mice lacking DNA ligase IV gene show massive apoptosis in embryonic neural cells [287]. DNA-PKcs can be phosphorylated on more than 40 sites such as threonine 2609 (Thr2609). Hagan et al. [288], showed that upon ionizing radiation, EGFR is translocated to the nucleus and directly interacts with DNA-PKcs to form a complex that leads to increased activity of DNA-PKcs (a major component in NHEJ DNA repair) [289]. EGFR directly phosphorylates DNA-PKcs at T2609, which is required for NHEJ-mediated DSB repair. Studies have shown that phosphorylation of Thr2609 is essential in NHEJ and blocking phosphorylation of these site via substitutions such as alanine leads to severe radiosensitivity and non-functional DNA end-joining ability in vitro [290, 291]. Interestingly, it has been shown that cells that express L858R and d746-750 are defective in radiation-induced nuclear translocation of EGFR and therefore, cells with these mutations prevents the interaction between EGFR and DNA-PKcs and are sensitive to radiation treatment [292].

1.5.2 p53

P53 also known as the “guardian of the genome” was first described in 1979 as an antigen in mouse cancer models [293]. P53 was later described as transcription factor and a well-known tumor suppressor that prevents cells from becoming malignant [294]. The human p53 protein (53 kDa) is encoded by the 20 Kb *TP53* gene [295]. Wild-type p53 (wt p53) protein consists of a N-terminus, a central core and a C-terminal region. The N-terminus is divided into two subdomains 1) transcriptional activation domains (TADs: TAD1 and TAD2) and 2) proline-rich region. One main function of TADs is their interaction with MDM2, acetyltransferases p300 and CBP, etc. Proline-rich region plays a role in maintaining the stability of p53. The central core domain is also called the DNA-binding domain. The C-terminal region includes a tetramerization domain and negative auto-regulatory domain.

1.5.2.1 Activation of wtp53 and its regulation

P53 is activated through cellular stress such as DNA damage after exposure to IR and chemotherapy, hypoxia, ribosomal stress, infections, telomere shortening or oncogene overexpression, cell–cell contacts etc.

Once p53 is activated and is able to bind to DNA, depending on the extent and nature of p53 activation it can induce or suppress transcription of many genes, such as *Noxa*, *Bcl2 associated X protein* (*Bax)*, *Fas*, *p53* upregulated modulator of apoptosis *(PUMA)*, *CDKN1A*/*p21*, etc. leading to different responses such as cell-cycle arrest, regulation of oxidative stress, DNA repair, apoptosis, senescence, invasion, motility, autophagy, angiogenesis [296], differentiation, etc. [297, 298].

Upon DNA damage, cell-cycle arrest can occur in G1, S or G2-M to allow sufficient time for cells to repair DNA and to minimize the replication of damaged DNA. It is also important to mention that cells undergo apoptosis or repair based on the extent of DSBs. As mentioned when the damage is minor and repairable, p53 regulates cell-cycle arrest via G1/S checkpoint, which partially depends on the p53-regulated transcription of p21 protein (WAF1/Cip/Sdi1). Together, p21 protein with other proteins in this family (p27 and p57) can cause cell-cycle arrest via inhibition of cyclin E and cyclin-dependent kinase 2 (CDK2) [299]. When DNA breaks are high, cells mostly activate the transcriptional activation of proapoptotic factors such Bax, PUMA, growth arrest and DNA damage (GADD45), etc. [294, 300].

In a normal cell, p53 protein is almost non-detectable because of its very short half-life, ranging from 5 to 30 minutes [301]. P53, is negatively regulated by many factors that include MDM2, MDMX (MDM4), Pirh2, TOPORS, COP1, E6-AP, CHIP, ARF-BP1, TRIM24 and MKRN1 ubiquitin ligases [302]. Among these regulators, MDM2 is the primary negative regulator of p53 levels in the cell.

MDM2 suppresses p53 stability by ubiquitination dependent and proteasomal mediated degradation. Sequential activation of E1, E2 and E3 enzymes catalyze ubiquitination of p53 in cells. E1 first forms a thioester between the glycine of ubiquitin and its own cysteine. Ubiquitin is then transferred to E2 conjugating enzyme. Finally, E3 ubiquitin ligase, catalyzes the transfer of polyubiquitin chains to the protein substrate leading to proteasome degradation of p53.

Several pathways can become activated and stabilize the p53 protein by inhibiting the attachment of MDM2 and preventing its degradation. DNA damage and DNA breaks caused by IR or DNA damaging drugs is one such pathway that stabilizes p53 protein and prevents its degradation [297]. A wide range of studies have also shown that ATM and casein kinase II can

phosphorylate and activate TP53 and have linked this activation to DNA repair [303]. In the case of ATM, this phosphorylation (serine 20 and serine 15) make p53 resistant to the inhibitory effects of MDM2 [304]. In addition, $p14^{ARF}$ (ARF) is able to bind to MDM2 and transfer it to the nucleus, which leads to inhibiting the possibility of its interaction with p53.

1.5.2.2 p53 mutations

TP53 is the most commonly mutated gene in human cancer [305]. Some p53 mutations are frameshift, nonsense and missense mutations. Although frameshift or nonsense mutations result in the loss of p53 protein expression, missense mutations (substitution of a single amino acid) in p53 are frequently associated with stably expressed protein. Six "hotspot" amino acids (G245, R248, R249, R273, R175, and R282) in the DNA binding domain are mainly substituted, however, substitution can occur throughout the p53 protein [306]. Once p53 proteins are mutated, they acquire high stability that leads to their accumulation in the cell. Indeed, positive p53 via immunohistochemical (IHC) staining of cells is as an indicator of p53 accumulation associated with missense mutations. Today, it has become clear that p53 protein mutants are found in various cancer types and can give rise to more aggressive tumor profile promoting tumorigenesis and poor prognosis of patients [307].

Mutant p53 can also act as a dominant negative inhibitor over any remaining wild-type p53 by dimerization of mutp53 with wtp53 monomers that results in loss or diminution of the wild-type p53 activity [308]. Finally, mutant p53 proteins can acquire novel oncogenic functions, and are referred to as "gain of function" (GOF) mutants [309]. As reported in a recent review by Zhang, et al.[310], GOF mut-p53 is understudied in GBM and there has been a failure to distinguish between TP53 deletion and GOF mutations. Interestingly, one mechanism of resistance of GSCs

to treatment has been linked to a mechanism involving GOF mut-p53's status in GSCs which leads to differentiation and enhanced invasion capacity of theses cells. The GOF mut-p53's status in GSCs warrants further exploration.

1.5.3 γH2AX as a marker of DNA damage

The phosphorylation of histone H2AX at Ser139 in mammalian in response to DSB formation is called γH2AX [311]. It has been reported that H2AX is phosphorylated at a very early step in the DNA damage response by either PIKKs, ATM, ATR, DNA-PK and has a critical role in induction of DSB repair.

The critical role of H2AX phosphorylation on DNA damage/repair proteins and functions to promote genome stability has been reported by showing that γH2AX is involved in retention of some proteins participating in DNA repair and assembly of repair complexes at the damaged sites. Also, it has been confirmed that H2AX-knockout cells exhibit impaired recruitment of NBS1, 53BP1, and BRCA1 to irradiation-induced foci [312]. γH2AX and foci immunodetection is now well known as a quantitative marker of DSBs [313, 314]. γH2AX phosphorylation can be detected by western blotting of cell/tissue lysates and using antibodies that are specific for H2AX in individual cells and normalization to the total H2AX levels. Also, the presence of H2AX-containing nuclear foci can be ascertained by microscopy and flow cytometry [315]. Since γH2AX phosphorylation is an early and rapid event, timing of H2AX measurements is critical for interpretation of results.

1.5.4 DNA repair enzymes

DNA repair systems developed in cells to hinder the detrimental effects of DNA damage in cells and to maintain genome integrity. The DNA repair network is divided into distinct

mechanisms based on the type of DNA lesion. Major DNA repair pathways are 1) O6-alkylguanine-DNA-alkyltransferase (AGT/MGMT), (2) mismatch repair (MMR), (3) base excision repair (BER) and nucleotide excision repair (NER), (4) homologous recombination (HR) repair and (5) non-homologous end-joining (NHEJ). This section provides a brief discussion of each pathway's molecular mechanisms of recognition of DNA damage and repair of DNA damage. The major factor for the choice of repair pathway is the type of damage.

1.5.4.1 MGMT/AGT

MGMT, also sometimes referred to as AGT, is a small (21 kDa) DNA repair protein, that is specific for of O6-methylguanine lesions. O6-methylguanine lesions are highly toxic DNA damage that can be only repaired by MGMT. It acts by removing the O6-alkylguanine adducts in DNA and transferring it to the cysteine 145 residue (Cys 145) on its active site, forming S-methylcysteine [316]. Once S-methylcysteine is formed, MGMT is no longer able to be involved in the O6-methylguanine repair mechanism therefore this act is referred to as a suicidal action by this repair enzyme (Figure 1.13). Therefore, S-methylcysteine eventually becomes degraded via the ubiquitin proteolytic pathway [317].

While MGMT is a critical repair enzyme in normal cells, MGMT expression is variable in different cell types, tissues, and among individuals. For example, in a study the highest MGMT activity was detected in liver but to a lower extent in normal human brain tissues [318]. While MGMT is a critical repair enzyme in normal cells its presence (MGMT positive) impairs the cytotoxic effects of methylating agents such as TMZ (methylates O6 position of guanine) (also discussed in section 1.1.4) and therefore interferes with patient outcome. For this reason, MGMT is well known for conferring resistance in GBM patients and has been used as a predictive maker

to alkylating agents-based therapy in GBM [319]. Thus, modulating MGMT expression and activity has been tested and developed in the treatment of many cancers such as GBM to improve treatment response.

The MGMT gene is located on chromosome 10 and contains of 97 CpG sites within the CpG island and its proximal promoter region. MGMT is regulated by methylation of CpG sites and therefore *MGMT* gene silencing by its promoter methylation has been used in GBM. To this end results of studies show that GBM patients that have promoter methylation and received combination treatment of RT and TMZ show an overall survival of 21.7 months (RT alone; 15.3 months), however the same combination treatment on patients with unmethylated tumors only improved the survival to 12.7 months (RT alone; 11.8 months). Interestingly, *MGMT* gene silencing by its promoter methylation was shown to be associated with better prognosis for GBM patients compared to patients possessing tumors with unmethylated *MGMT* promoter (median overall survival of 18.2 months vs 2.2 months) [320].

O6-benzylguanine (O6-BG) and O6-bromotenyl guanine (O6-BTG) are two MGMT inhibitors that have been tested in the clinic and also in combination with alkylating DNA damaging agents [321] . O6-BG, interferes with the activity of MGMT by transferring its benzyl group to the cysteine residue at the active site of MGMT [322]. Phase I and II clinical trials using O6-BG showed that combination of O6-BG with TMZ did not have any benefit in the patients treated with these drugs and importantly half of the patients enrolled showed toxicity [323, 324].

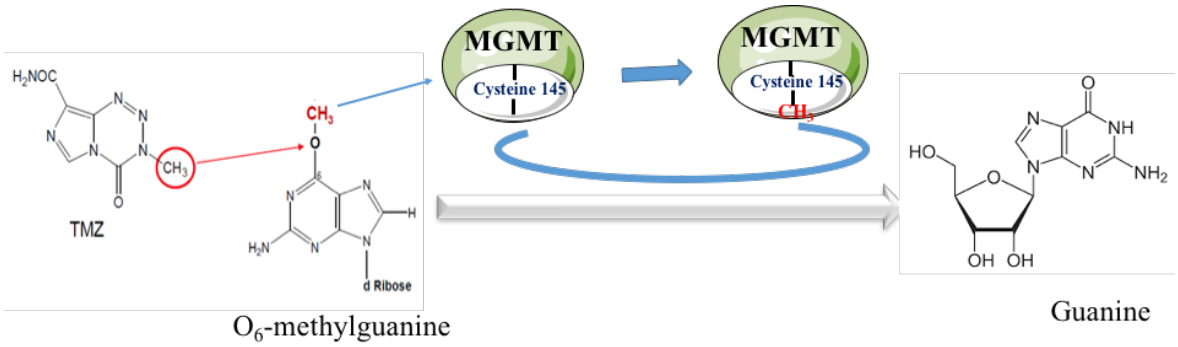


Figure 1.13. Mechanism of DNA repair by MGMT. MGMT is able to directly remove the O6-methyl lesions from guanine. This results in an unmodified guanine base. The methylated MGMT is subsequently degraded by proteases.

1.5.4.2 Base-excision repair (BER)

Base-excision repair (BER), which also includes several sub-pathways, is a highly coordinated pathway. Its role is in the repair of damaged DNA bases and major mechanism for SSB repair in the cell. The BER pathway is launched by hydrolytic removal of the damaged bases by DNA glycosylases. More than 12 DNA glycosylases have been identified [325] that act by cleaving the N-glycosidic bond that links the base to its corresponding deoxyribose. This leads to production of abasic sites or AP/s which are repaired by apurinc/apyrmidinic endonuclease 1 (APE1) that forms a single-strand break flanked by 3'-OH and 5'-deoxyribose phosphate (5'-dRP) end [326]. APE1 is an essential enzyme for the recognition and processing of the AP-sites. Other DNA glycosylases, by cleaving AP sites leave a 3'-phospho-α, β-unsaturated aldehyde and 5'-phosphate at the margins of the break. Therefore, regardless of the mechanism of action of DNA glycosylases, it leads to formation of intermediate strand breaks harboring 3'- and/or 5' -blocking lesions that prevent DNA polymerase and DNA ligase reactions in the BER repair process. Therefore, these ends need to be changed to conventional 3' –OH and 5' -phosphate ends by various

DNA end-processing enzymes that depends on the location of the blocking lesion (3' or 5'). For example, APE1 is not only an AP endonuclease but also is able to restore 3' -OH from 3'-phospho-α, β-unsaturated aldehyde.

This step is followed by DNA synthesis and ligation which is divided into 1) short-patch, which includes 80–90 % of all BER, and 2) long patch. In the short-patch, as the name implies, a single nucleotide gap is filled and ligated with either DNA ligase I or the complex of DNA ligase III and XRCC1. On the other hand, long-patch BER is associated with insertion of several nucleotides at the DNA strand break site [327]. This BER sub-pathway consists of proteins such as DNA polymerase ε or δ, PCNA (proliferating cell nuclear antigen), RFC (replication factor-C), FEN1 (flap endonuclease-1), and DNA ligase I.

Other proteins such as X-ray repair cross-complementing protein 1 (XRCC1) and poly (ADP-ribose) polymerase 1 (PARP1) are also involved in BER. XRCC1 does not have any enzymatic role in BER but rather plays a facilitative role for assembly of enzymatic components involved in this pathway. For example, XRCC1 interacts with APE1, DNA polymerase β, ligase III, PNKP, Tdp1, and APTX. Among the mentioned proteins XRCC1 is critical for ligase III activity as defects in XRCC1 were linked to low ligase III activity in the BER pathway [328, 329]. Of note, XRCC1 is not only upregulated upon radiation-induced DNA damage but also its changes are modulated in response to EGFR activation [330, 331]. Poly (ADP-ribose) polymerase 1 (PARP1) is a key component of a functional BER pathway. PARP1 interacts with nicotinamide dinucleotide (NAD^+) and catalyzes poly (ADP-ribosyl) ation (PAR) of itself which results in release of PARP-1 from DNA due to negative charge of PAR. This allows access of repair proteins to the DNA damage site [332]. In addition, PARP-1 can also physically interacts with XRCC1. Importantly, BER can repair N7 position of guanine and N3 positions of adenine that have been

methylated by TMZ. The mentioned adducts, although less important in the cytotoxicity induced by TMZ, can become important when O6-MeG are efficiently repaired by MGMT or are not recognized due to MMR deficiency, thus BER along with MGMT can be targeted to increase the efficacy of TMZ cytotoxicity.

1.5.4.3 Mismatch Repair (MMR)

The MMR system plays an essential role correcting mismatched bases and insertion/deletion loops (IDLs) generated during DNA replication. Major steps in MMR pathway are 1) recognition step where the the mismatch is recognized, 2) excision step where the error is removed leading to formation of a gap, 3) repair synthesis step in which the gap on the DNA is filled in this step. MutS, MutL, exonuclease 1 (EXO1), replication protein A (RPA), PCNA, DNA polymerase δ and DNA ligase I are the main proteins involved in the MMR pathway [333]. In humans, assembly of the MutS-MutL-DNA complex activates exonuclease 1 (Exo1), which performs the degradation of the mismatched pair through its 5' to 3' exonucleolytic activity [334]. RPA protects the gap generated by the excision and, together with PCNA, is required for the DNA re-synthesis step. Finally, DNA ligase I is responsible for sealing of the remaining nick.

Interestingly, the MMR complex (expression of MSH2, MSH6, and PMS2) has been linked to repairing DNA damage caused by alkylating agents. A relevant example to this thesis is the temozolomide-induced O6-MeG adducts. If O6-MeG lesions are not repaired first by MGMT enzyme it leads to the mismatch pairing of O6-MeG (Guanine) with thymine. This triggers the activation of the MMR pathway that eventually results in DNA strand breakage and cell death. However, if the MMR pathway is impaired, cancer cells become tolerant to O6-MeG lesions which can result in completion of DNA replication, cell cycle progression and avoidance of apoptosis.

Overall, in GBM, MMR-deficient tumors are resistant to temozolomide even in the absence of MGMT expression [335]. Alternations in, MSH6 has been reported in ~30% of GBMs after treatment with alkylating agents and MSH6 deficiency has been linked to temozolomide resistance in *in vitro* studies [335, 336]. Accordingly, restoration of MSH6 expression resulted in a more chemosensitive phenotype [336].

In the case of recurrent GBMs, many studies have reported reduced expression of several MMR proteins compared with the corresponding primary tumor[337]. Additionally, in a study done by the Cancer Genome Atlas Research Network showed that in recurrent GBMs, MMR mutations mainly occurred in *MGMT* promoter methylated tumors which suggests that although these tumors have initial TMZ sensitivity caused by *MGMT* silencing they become TMZ-resistant by selecting for MMR deficiency [336].

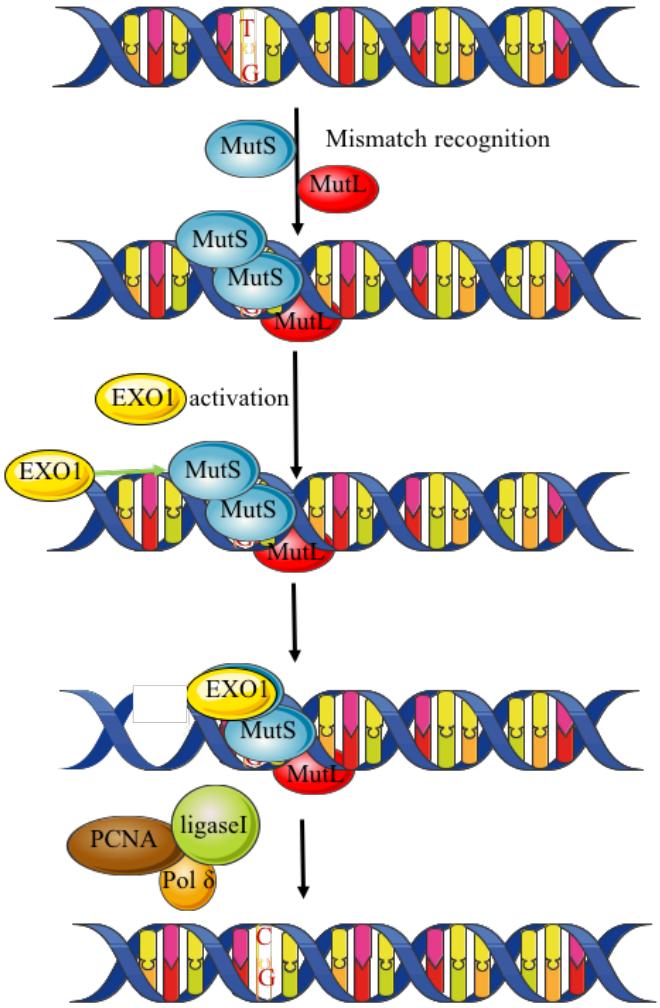


Figure 1.14. Mechanism of DNA repair by MMR. Single base-base mismatch is recognized by MutSα and MutLα which leads to the formation of MutSα-MutLα complex. This then causes the activation of EXO1 that cuts the error-containing strand. At the final step the DNA synthesis and ligation steps are performed by DNA polymerase δ and DNA ligase I, respectively.

1.5.4.4 Nucleotide-excision repair (NER)

NER is involved in repair of bulky lesions such as pyrimidine dimers and cisplatin-DNA intrastrand crosslinks. Mechanism of NER pathway is similar to BER however it involves more proteins. Major steps in the NER pathway are 1) recognition of DNA damage and binding of multiprotein complex, 2) opening of DNA around the lesion, 3) excision of a short single-strand segment of DNA containing the lesion, 4) synthesis and ligation of DNA. The NER repair pathway is initiated by XPC/HR23B/CEN2 (XP complementation group C/Rad23 homolog B/Centrin-2) or RNA polymerase II (RNAPII). Once the DNA damage is recognized either the XPC complex or CSB and CSA recruit the multi-subunit (ten protein complex) and the multi-functional transcription factor TFIIH to the damaged site. XPB and XPD that are also known as TFIIH-associated/ATP-dependent helicases unwind the DNA helix followed by complete extension and subsequent stabilization of pre-incision complex which is preformed by RPA (replication protein A). This is followed by excision of the lesion-containing nucleotides by XPG and XPF/ERCC1 at positions 3' and 5' to the damage, respectively. Finally, DNA polymerase ε or δ resynthesize the resulting gap and DNA ligase seals the nick on the repaired strand.

Importantly, activation of the DNA repair response is mediated by a stress response pathway and has been linked to resistance to DNA damaging drugs [338]. Many studies today have focused on inhibiting DNA repair enzymes through inactivating signaling pathways that are involved in activation of DNA repair. As mentioned before, ERCC1 and XRCC1 are two major components involved in NER and BER repair pathways, respectively. Studies have shown that by decreasing ERCC1-mediated repair through combination of DNA-damaging agents such as cisplatin with EGFR inhibitors (e.g., gefitinib) and MEK inhibitors (e.g., PD98059) we can improve cytotoxicity of drugs [330, 339].

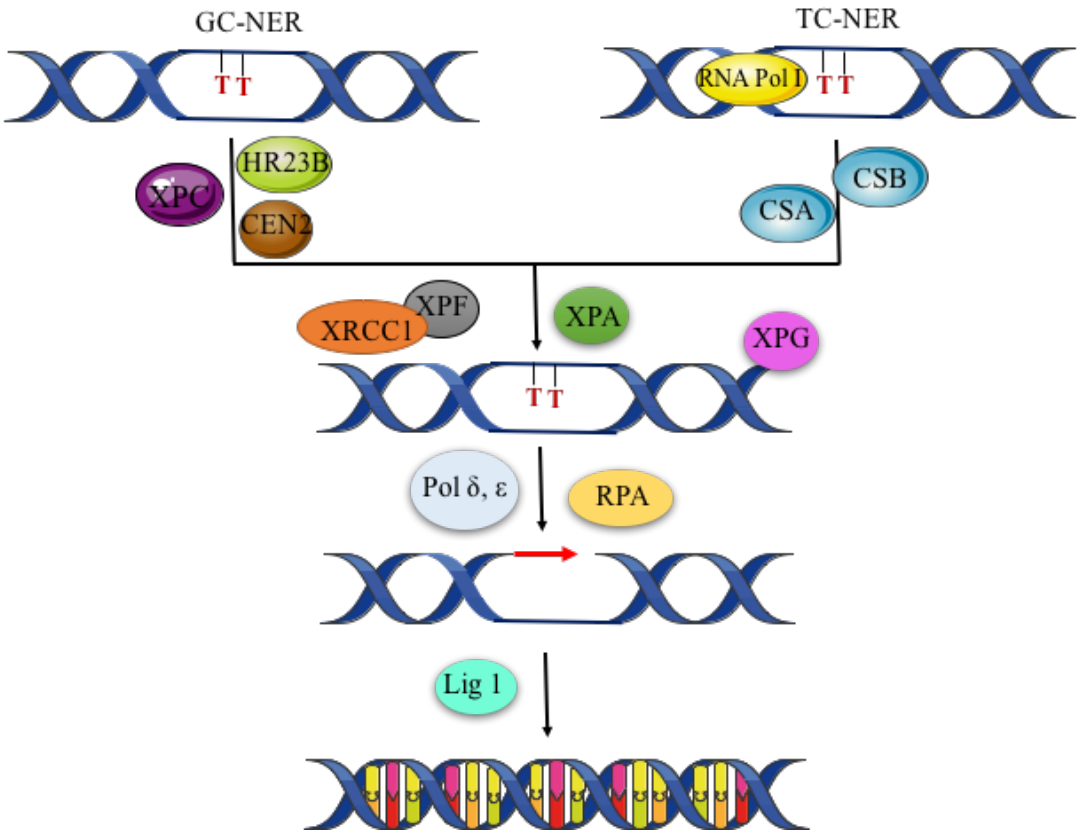


Figure 1.15. Mechanism of DNA repair by NER. DNA damage is recognized by XPC/HR23B/CEN2 protein complex global genomic-NER (GG-NER) or transcription coupled-NER (TC-NER). Although these pathways differ in how they recognize DNA damage but they share the same process for lesion incision, repair, and ligation. Briefly, they are able to recruit many proteins that facilitates the unwinding of DNA. Then the DNA strand that contains the lesion is removed by XPG and XPF-ERCC1. Finally, DNA is re-synthesized and ligated by DNA polymerase δ/ε and DNA ligase I, respectively.

1.5.4.5 Double strand break (DSB) repair

As discussed in section 1.6.1. DSBs repair is critical for cell survival and genomic integrity of cells. Homologous recombination (HR) and non-homologous end-joining (NHEJ) are two main mechanisms by which mammalian cells repair DSBs. These two pathways are similar in the use of the Mre11-Rad50-Nbs1 (MRN) complex however they have major differences as well. First, NHEJ can be functional in all phases of the cell cycle, while HR is restricted to the late-S and G2 phases of cell cycle. Another difference is NHEJ is an error-prone mechanism because it repairs DNA by direct ligation of the broken ends but since HR repair uses the genetic information contained in the undamaged sister chromatid as a template it is mainly an error-free mechanism. These two repair pathways are briefly described below:

1.5.4.5.1 Homologous Recombination (HR)

First step in HR is to generate 3'-single-stranded tails which involves the MRN complex. The MRN complex recruits the ATM protein kinase [340] which then can phosphorylates several proteins such as BRCA1, H2AX, CHK2, p53 [341]. Phosphorylation of BRCA1 by ATM leads to formation of the BRCA1-CtIP-MRN complex, which facilitates the 5’ to 3’ end resection of the DSB end [342]. This step is followed by attachment of Rad51 along with its mediator proteins (e.g. Rad52, BRCA2) and Rad51 paralogs (RAD51D, XRCC2, RAD51B, RAD51C, and XRCC3) [343]. The Rad51-coated single-stranded DNA tail invades the undamaged sister chromatid and uses it as the template DNA duplex. Finally, DNA polymerase synthesises DNA from the 3' -end of the invading strand followed by successive ligation by DNA ligase I resulting in the error-free correction of the DSB.

1.6.4.5.2 Non-Homologous End-Joining (NHEJ)

The initial step in the NHEJ repair is attachment of the MRN complex which is followed by binding of Ku70/Ku80 heterodimer (Ku) to the DSB. This leads to recruiting DNA-PKcs (discussed in section 1.5.1.2) to the damaged site. DNA polymerases μ and λ are involved in resynthesis of missing nucleotides in NHEJ repair process. Alternatively, DNA-PKcs interacts with the NHEJ specific nuclease Artemis to form the DNA-PKcs-Artemis complex [344]. Last step is ligation of the DNA ends that is done by DNA ligase IV/XRCC4 along with XLF which promotes DNA ligation [345]. Of note, DNA-PKcs are involved in V(D)J recombination for antibody production and interestingly its loss leads to an immuno-deficient state as reported for NOD scid gamma mouse (NSG) mice [346].

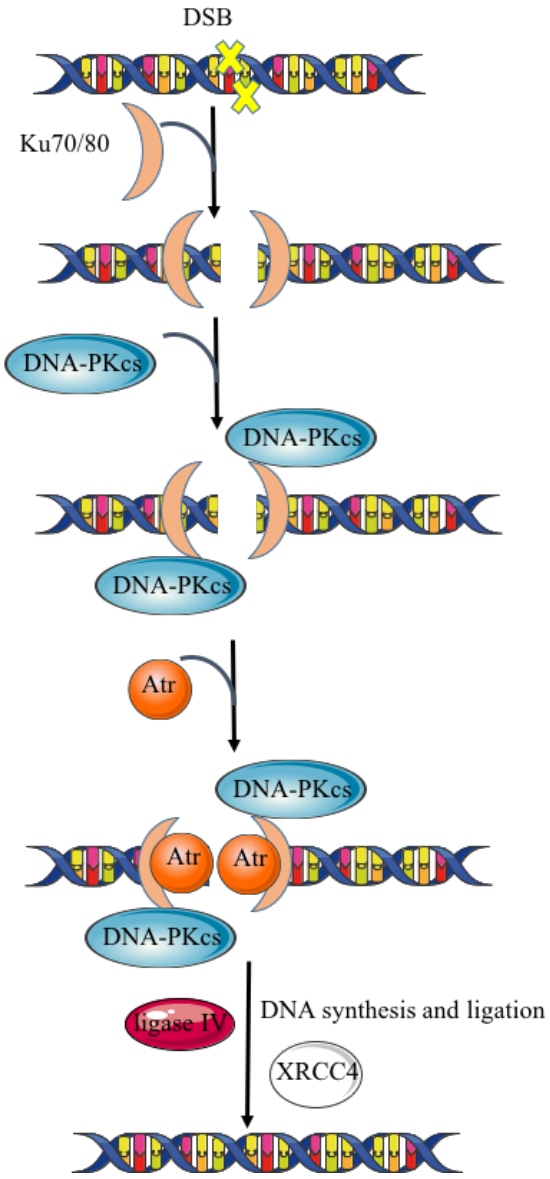


Figure. 1.16. Mechanism of DNA repair by NHEJ. The Ku70/Ku80 heterodimer first binds to the DSB ends. The Ku70/Ku80-DNA complex then recruits DNA-PKcs. The NHEJ- specific nuclease Artemis processes the DNA ends and finally the DNA is sealed by XRCC4/Ligase IV.

1.6. TARGETED THERAPY FOR CANCER TREATMENT

Today, most of the studies on improving the therapy for GBM focus on targeted delivery of the tested drugs by which they aim to selectively eradicate the tumor cells with minimal effects on normal brain cells. Such drugs are immunotherapy, anti-angiogenesis, PDGFR and EGFR inhibitors (reviewed in [347]). However, we will only focus on EGFR because of its relevance to this thesis [348, 349].

1.6.1 Epidermal Growth Factor Receptor (EGFR) in cancer

Cancer is a disease in which accumulation of mutations in genetic material of cells encourages a cancer state by enhancing expression of genes that have growth potential and either upon mutation(s) become oncogenes or when mutated attenuate or silence tumor suppressor genes [350, 351]. As described in section 1.3, EGFR is required for maintaining normal cellular activities such as cell growth, cell division and cell viability, however different mechanisms of alterations in EGFR expression and signaling exists and these alterations can play a role in the transformation of cells from a normal phenotype to a cancerous one. In the cancerous state of cells, overexpression of receptor-mediated signaling leads to increased growth, proliferation, invasion and survival of cells.

Today it is well known that EGFR is deregulated in several human cancers including breast, colon, head and neck, brain, prostate, lung, liver and pancreatic, and therefore it has been used as a target for the development of many anti-cancer agents [352-354]. Some of the major mechanisms leading to EGFR deregulation include: 1) point mutations, 2) in-frame deletion (e.g. EGFRvIII in GBM, refer to section 1.3.3), 3) structural alterations, 4) gene amplifications and 5) increased transcriptional activation that result in receptor and/or ligand overexpression and constitutive receptor activation [214]. The oncogenic potential of EGFR has been demonstrated by many

studies with the observation that cells overexpressing the receptor became transformed when stimulated by growth factors such as EGF [123] and this has been confirmed by a large number of animal studies [355]. EGFR expression has also been associated with risk of recurrence, promotion of migration, metastatic potential, invasion, and therefore correlates with poor clinical outcome [356-359]. EGFR can also cooperate with EGFRvIII and therefore phosphorylate signal transducer and activator of transcription (STAT) proteins and potentiate malignant transformation [360]. EGFR deregulation has been also seen in non-small cell lung cancer (NSCLC) which is a subtype of lung cancer. The two most frequently occurring mutations are the in-frame deletion in exon 19 (del E746-A650) and the point mutation in exon 21 (L858R) that occur in the kinase domain for EGFR. However, these mutations are not present in GBM. In breast, pancreatic and bladder cancer EGFR heterodimerizes with HER2, which can cause the progression of these cancers [361, 362].

1.6.2 EGFR as a paradigm of targeted therapy

Although cancer is a complex disease and many signaling events are involved in cancer development and progression, cancer cells might be dependent on one oncogene, which is termed as “oncogene addiction”, and blocking this oncogenic pathway can lead to inhibition of growth and induction of apoptosis in cancer cells [363]. As highlighted in section 1.1.3 increased EGFR expression, mutations or their downstream effectors are seen in 90% of GBMs. EGFR is one such oncogene that has been served as a powerful target for the development of new drugs that were further used in clinical trials [364]. The last decade has marked an extensive interest in the search for therapeutic strategies targeting EGFR. One of the most important characteristics of targeted agents is that they are developed to only target EGFR overexpressing cells versus non malignant cells, however they have faced many challenges [365, 366]. Other targeted therapies have also been made for the treatment of other cancers [367, 368] and the most successful targeted therapy

was the development of the first kinase inhibitor against the Bcr-Abl fusion protein, imatinib, which showed remarkable clinical response in patients with chronic myelogenous leukemia (CML) [369].

The two clinically most advanced strategies for inhibition of abnormal EGFR signaling are: (1) small molecules EGFR-TK inhibitors that can target the intracellular tyrosine kinase domain by competing with ATP for the EGFR ATP binding site and inhibiting subsequent phosphorylation of substrates, and (2) use of monoclonal antibodies (mAbs) to target the extracellular EGFR ligand binding domain at its inactive conformation stage. Other inhibitors are reviewed in Nedergaard *et al*. (2012) [210] and Karpel-Massler *et al*. (2009) [370] and Eskilsson *et al.* (2017) [348, 349].

As mentioned above TKIs are ATP-competitive inhibitors . Due to the structural similarity with ATP, anilinoquinazoline and pyrido[d]pyrimidines have been developed as small TK inhibitors [371]. The quinazolines contains a pyrimidine ring that have three critical residues at the active ATP site of EGFR required for anilinoquinazoline binding at the ATP binding pocket of the receptor: (1) Met-769, (2) Thr-766 (3) Asp-776 [166]. Met-769 and Thr-766 are involved in hydrogen bonding with N1 and N3 of the quinazoline respectively [166]. In addition, quinazolines that have a reactive group coupled at their 6- and 7-position is able to bind to Cys-773 of EGFR and is essential for irreversible inhibition of the this receptor [166, 372]. This also, shows the importance of structure activity relationship (SAR) in inhibiting TK of EGFR receptor.

Gefitinib (Iressa®/ZD1839) and erlotinib (Tarceva®) are among the first generation TKIs that have been used in phase I and II clinical trials for high grade gliomas, as monotherapies or in combination with conventional chemotherapy. In 2003, the FDA approved gefitinib for the treatment of patients with advanced NSCLC who failed to respond to conventional chemotherapy

[373-375]. However, in t phase III trials (INTACT-1, INTACT-2 and ISEL) gefitinib failed to show any significant improvement of overall survival [376, 377]. Gefitinib has been also tested on NSCLC patients and remarkable clinical response to this drug was reported for a subset of patients that carry gain-of-function mutations (deletion E746-A750 in exon 19 and L858R in exon 21) in the EGFR kinase domain (10% of cases) [378, 379]. In GBM trials gefitinib did not result in improved overall survival in a phase I/II trial in combination with radiation in newly diagnosed glioblastoma [380] or when tested in phase II trial in recurrent glioblastoma [381]. Erlotinib is also a TKI that although it had shown benefit in preclinical studies, failed in Phase II clinical trails as a monotherapy in newly diagnosed glioblastoma [221]. Later studies also failed when they combined it with mechanistic target of rapamycin (mTOR) blockers or bevacizumab [348].

The structure of bound gefitinib to EGFR tyrosine kinase is depicted in figure 1.17.

Figure 1.17. Crystal structure of the binding of gefitinib to the EGFR kinase domain. From [downloaded from PDB, code: 2ITY].

The emergence of acquired resistance to the first-generation EGFR-TKIs initiated the development of second generation EGFR-TKIs. This generation of EGFR-TKIs drugs are able to bind to the EGFR kinase domain, which leads to the irreversible inhibition of EGFR phosphorylation [382]. The most well known mechanism of resistance to reversible EGFR-TKIs is the acquired T790M mutation in the EGFR kinase domain (exon 20) [383]. Lapatinib (Tykerb®) is one example of second generation TKIs that can inhibit both ErbB1and ErbB2 signaling. Second-generation TKI, Afatinib was tested as monotherapy and showed only limited efficacy in patients with recurrent glioblastoma [384]. Lapatinib also showed no efficacy as a single agent in recurrent glioblastoma and limited efficacy in combination with pazopanib (reviewed in [348]).

As mentioned above mAbs can bind to the extracellular domain of the EGFR. Cetuximab (Erbitux®) is a mAb of the IgG1 type that has a high affinity for the extracellular domain of EGFR and it also can prevent the formation of EGFR dimers [385, 386]. Other characteristics of this mAb is that it is able to recognize EGFRvIII that leads to down regulation of the receptors on the cell surface by promoting receptor internalization [386-389]. The results of *in vitro* and *in vivo* studies using Cetuximab on glioma cell lines with EGFR over expression and/or mutations has been contradictory. Hasselbalch et. al, showed that this drug was not able to inhibit the growth of glioma cell lines [390] while other studies show reduced cell viability upon Cetuximab treatment [391-393]. A more recent phase I clinical trial evaluated the safety of disruption of the BBB with mannitol followed by super-selective intra-arterial cerebral infusion of cetuximab in recurrent malignant glioma patients [348, 349, 394]. Some examples of anti-EGFR mAb–drug conjugate are ABT-414 and ABBV-221 that have been tested in efficacy studies and showed promising results [348, 349]. There were also some attempts to use biologics to target EGFR such as EGFR-targeted toxin TP-38 in a phase I clinical trial [395] and immunotoxin D2C7-IT by utilizing

convection-enhanced delivery (CED) strategy in which the results of these studies are encouraging [348, 349]. Recently tesevatinib, a TKI that can target multiple kinases has been evaluated in patients with recurrent GBM. Nimotuzumab, is another antibody that was developed to specifically target EGFR overexpressing cells and underwent phase II trials for high-grade glioma. However, in a phase III trial to test the efficacy of adding nimotuzumab to standard radiochemotherapy in patients newly diagnosed with GBM, it did not meet the inclusion criteria to prove such subgroup efficacy [348, 349].

Although these TK inhibitors are well tolerated in patients and show some modest effects, the results from many studies were conflicting (reviewed in Karpel-Massler et al. (2009) [370]). The effect of EGFR inhibition and therapeutic failure in GBM still needs to be clarified by understanding the resistance mechanism in the clinic. A more in depth understanding and categorization of patients that benefit from an EGFR targeted anti-GBM treatment could improve the outcome of clinical trials. Some of the resistance mechanisms are discussed in section 1.7.1.

1.6.3 Pharmacokinetics of EGFR inhibitor Iressa

The recommended dose of gefitinib is 250 mg once daily with or without food. Absorption of gefitinib from the gastrointestinal tract is moderately rapid, with median time to reach peak plasma concentration (*tmax*) values ranging from 3 to 7 hours in healthy subjects and patients with solid malignancies [396]. The absorption and bioavailability of gefitinib can be altered by changes in gastric/intestinal pH, gastric/intestinal motility and enzymatic and transporter activity in the intestinal wall. Gefitinib is stable in human plasma for 24 hours if stored at room temperature and for up to 12 months if stored at -20 °C. Gefitinib has extensive tissue distribution and moderate to high plasma protein binding 90%, with a median volume of distribution at steady state (Vss) of

1700L [397]. It is metabolized by CYP3A4 and to a lesser extent by CYP2D6 and CYP3A5 [398] and is excreted as both parent compound and metabolites, mostly O-desmethyl gefitinib, which is considered to be inactive [399]. 86% of unchanged drug and metabolites is excreted in the faeces with a minor proportion excreted in the bile. The most common adverse effects were diarrhea, rash, acne, dry skin [400], nausea, vomiting, pruritus, anorexia, and asthenia. In patients with central nervous system (CNS) involvement, gefitinib can penetrate the BBB and accumulate in brain tumors [401].

The pharmacodynamics of gefitinib were studied in two phase I trials. Based on this studies skin biopsies, cutaneous rash can serve as a surrogate tissue for detecting EGFR tyrosine blockade [401]. Substances that are inducers of CYP3A4 activity increase the metabolism of gefitinib and decrease its plasma concentration. For example, rifampin, a CYP3A4 inducer, reduced the mean area under the curve (AUC) of gefitinib by 85% [402]. Substances that are potent inhibitors of CYP3A4 activity decrease gefitinib metabolism and increase its plasma concentration. For example when itraconazole a CYP3A4 inhibitor, is concomitantly administered with gefitinib, the mean AUC of gefitinib was increased by 88% [403]. At the level of drug absorption, gefitinib exposure may be altered by coadministration of drugs that increase gastric pH and, therefore, decrease solubility of gefitinib. In fact, concomitant administration of gefitinib with high doses of an H2-receptor antagonist, ranitidine decreased gefitinib AUC by 44%.

1.7. COMBINATION THERAPY FOR TREATMENT OF SOLID TUMORS

1.7.1 Resistance to DNA-damaging agents and tyrosine kinase inhibitors

As previously described, various DNA damaging compounds are used in the clinic however results from clinical trials showed prominent limitations such as high toxicity, drug resistance, lack of selectivity and poor pharmacokinetic properties [404, 405]. As shown by Roos et al. [406], TMZ can induce O6-methylguanine lesions, the main cytotoxic lesions caused by this drug, which lead to apoptosis and cell death if they are not repaired. MGMT is today known as a clinically approved biomarker of resistance to TMZ in GBM treatment [320, 407, 408] and when human cells are transfected with MGMT they become resistant to TMZ treatment [409]. It has also been reported that MMR (explained in section 1.5.4.3) plays an essential role in the TMZ-induced mechanism of cell death. As previously mentioned, TMZ can induce O6-methylguanine lesions however TMZ-induced O6-methylguanine lesions do not inhibit DNA replication and transcription and therefore are not lethal by themselves. When O6-methylguanine lesions are formed (meG) they incorrectly pair with thymine instead of cysteine (G-C→meG-T) during DNA replication. This leads to the activation of MMR, which removes thymine and reincorporates it opposite to the lesion leaving the methylguanine lesions intact. Continuous futile cycles of MMR lead to DNA strand breaks and apoptotic cell death [410]. Finally, the BER pathway is another mechanism of resistance to TMZ which repairs N7-methylguanine and N3-methyladenine lesions as previously described in section 1.5.4.2.

Gene amplification of driver oncogenes such as EGFR and Bcr-Abl have been observed in different tumors that render them resistant to targeted therapies and tyrosine kinase inhibitors [411]. Secondary mutations in tumors treated with specific targeted therapy are also another cause of resistance to targeted therapy such as T790M mutation in EGFR that causes resistance to

gefitinib/erlotinib [412], T315I mutation in Bcr-Abl that leads to resistance to imatinib [413] and T670I mutation in c-Kit that is important in resistance to imatinib [414]. Last, studies have also shown that some mutations in the downstream signaling pathways causes the constitutive activation of these pathways independent of upstream regulation by their receptor tyrosine kinases such as mutations in K-Ras, B-Raf (downstream of MAPK pathway) or PTEN (negative regulator of the PI3K/Akt pathway) therefore causing resistance to drugs targeting these receptors [195, 415-417].

Since cancer is a complicated disease and involves multiple proteins, enzymes, signaling pathways, targeting only one pathway (TKIs) causes the activation of an alternative signaling pathway or bypass mechanisms, which can not only lead to sustained survival and growth of the tumor but also it can lead to a more aggressive and resistant tumor particularly in GBM. For instance, when RTKs such as EGFR, HER2, c-Met, etc. are blocked by TKIs downstream pathways such as MAPK and PI3K/Akt are blocked, however cells in order to grow and survive activate alternative pathways such as JAK/STAT pathway which causes resistance to the targeted therapy [418, 419]. In other cases, therapeutic blockade of one oncogenic pathway overcomes by the activation of another, which is referred to as redundant signaling [420-423]. This is reported in many publications as the most likely cause of the failure of individual RTK targeting in clinical trials for glioblastoma. Indeed, in GBM it has been also reported that the effect of TKIs might be substituted for by other RTKs such as PDGFR and MET [195, 424].

As mentioned earlier, gefitinib and erlotinib are two approved EGFR TK inhibitors in clinical cancer treatment and have been used in the treatment of many human solid tumors, including lung, breast, prostate and colorectal cancer. However, since most EGFR TK inhibitors have a reversible mechanism of action in order to inhibit tumor growth *in vivo* they need to be used

continuously and over a long period of time. Also, due to high intracellular ATP concentration, sustained EGF inhibition in needed in order to inhibit signal transduction in tumor cells, which is another main obstacle in EGFR TK inhibitors. Resistance to treatment can also occur due to poor pharmacokinetics of drugs resulting from low ADME (absorption, distribution, metabolism and excretion).

Another possibility is that radiation therapy can cause resistance by killing radiosensitive cells and selecting for cells with radioresistant properties [425]. GSCs have been shown to have a more effective DNA repair mechanism [76] by early and rapid activation of checkpoint proteins such as ATM, Rad17, Chk1 and Chk2 [426] therefore causing cells to undergo cell cycle arrest and a more efficient DNA-damage repair leading to increased survival. Also, activation of signaling pathways that cause the cells to become resistant to apoptosis and promote cell survival. It has been also reported that these agents may not cross the BBB, therefore there is not sufficient drug at the tumor site to inhibit the growth of the tumor or kill the cancer cells. It has also been suggested that the cellular background such as the status of PTEN may modulate the dependence on EGFR in GBM and unknown mutations may provide resistance to TKIs [76]. In GBM it has been also reported that the effect of TKIs might be substituted by other RTKs such as PDGFR and MET [195, 424].

Despite all these issues and many reports on resistance mechanisms of targeting, EGFR still remains as a potential target for treating glioblastoma. One possible strategy to overcome the resistance seen is the use of combinatorial approaches [427] and multi-targeted therapy.

1.7.2 A shift in the drug discovery paradigm: emergence of multi-targeted therapy

Conventional and rational cancer drug design primarily seeks to identify very specific drugs, however, cancer is a complex disease, which is driven by several different oncogenic drivers. Therefore, single-targeted drugs may not overcome the complexity observed in many tumors. Today, two main strategies are used to overcome the resistance to DNA-damaging agents and tyrosine kinase inhibitors by reducing the side effects and improving the chemotherapeutic effects.

First, is the use of multi-drug combination chemotherapy in which more than one drug is given to the patients in concomitant or sequential manner. Multi-drug combination strategy is designed to increase of the number of targets to have a higher chance of killing the cancer cells and to reduce the possibility of development of drug resistance. Moreover, as cancer in general is a complex disease, it is impossible to control it using a single monofunctional drug especially in advanced cancers. Many studies have shown promising anti-cancer activity of this strategy in clinical trials [195] however its major disadvantage is systemic drug toxicity seen in patients. Therefore, individual drug properties, biodistribution, pharmacokinetics, solubility, etc., should be taken into account in order to minimize drug toxicity with the best potency of the combinations used.

In order to overcome challenges and resistance seen by the use of drug combinations a second strategy termed “polypharmacology” has been utilized to develop single drugs that can bind and inhibit two or more targets in the cell. The goal of polypharmacology is to generate drugs with optimal therapeutic effects and predictable pharmacokinetic (PK) profiles while having minimal toxicity. In order to choose the best candidates for polypharmacology based drug discovery, an important step is to identify the best biological targets to effectively abrogate all the main players involved in cancer progression and delay onset of resistance mechanisms.

1.7.3 Combi-molecules: design, synthesis and mechanism of action

Another strategy that has been also used to hit several targets in the cell is termed "combi-targeting" strategy. This strategy was developed in 2000 in which novel compounds termed "combi-molecules" were designed to have two bioactive pharmacophores a single molecule with dual mode of action [428-430]. Two bioactive moieties are linked either directly or by a linking unit. Combi-molecules are designed not only to have multiple mechanisms of action but also to have better pharmacokinetic and pharmacodynamic properties and to be less prone to resistance, when compared with the parent compounds. The advantage of using this class of compounds such as having superior anticancer activities and exhibiting less cytotoxic effects to normal tissues has been proven in many studies [431-435]. The main difference between combi-targeting strategy and polypharmacology strategy is the fact that the latter is done by random screening of best candidates. Polypharmacology also focuses on designing inhibitors capable of targeting two or more molecular targets (combinational therapies of individual drugs); or to design a single pill with different mechanisms of action or to combine multiple drugs that act on different targets (multi-targeted therapeutics) [436, 437].

1.7.3.1 Underlying rationale for combi-targeting of EGFR and DNA

As mentioned before classical cytotoxic agents, such as TMZ, are widely used in the clinic for the treatment of solid tumors [438-440] however, due to many reasons such as toxicity and chemoresistance associated with these agents, often the clinical benefit of these drugs is not achieved. On the other hand, EGFR is overexpressed in a large number of solid tumors and although EGFR inhibitors are less toxic compared to other cytotoxic chemotherapies [11, 441] they are generally limited due to their reduced anti-proliferative effect. Another limiting factor in

using chemotherapy is the increased activation of DNA repair genes. When EGFR is activated in response to genotoxic stress, it is translocated into the nucleus where it has been associated with increased induction of several DNA repair genes such as XRCC1 and ERCC1 [442]. EGFR has been also shown to interact with several DNA repair proteins, including DNA-PKcs, RAD51, ATM and BRCA1 [330, 331]. Therefore, inhibiting the EGFR pathway by using TKIs can lead to downregulation of these DNA repair genes [443-446]. Thus, by using combi-molecules that can inhibit EGFR and damage DNA we can benefit from the synergistic inhibition of EGFR and DNA damage in order to have a better anti-tumor activity in inhibiting growth and proliferation of cancer cells and inhibiting the repair of cancer cells at the same time. In this regard studies [447] have shown that combi-molecule with EGFR-DNA damaging properties is able to induce more DNA damage in cells that express higher EGFR levels compared to their wild-type counterparts and prove the strong rationale for designing EGFR-DNA combi-molecules.

With the idea to rationally design combi-molecules the laboratory of Dr. Jean-Claude for over a decade, has thoroughly studied, designed and developed classes of combi-molecules (I-Tz) which are categorized as type I [447], type II or type III. These molecules have divergent inhibitory profile (e.g. I targeting EGFR and Tz damaging DNA) that when combined leads to additive or synergistic antitumour effects. The combi-molecules connected by a linker have distinct biological targets such as EGFR and DNA, c-Abl and DNA, as well as EGFR, MEK and DNA.

Type I combi-molecules contain a cleavable linker and require hydrolysis to generate their two targeted metabolites (I +Tz) while type II combi-molecules have noncleavable linkers and exert their dual mechanism of action without the requirement for hydrolysis (I-Tz) within the cell. More recently, Dr. Jean-Claude's laboratory has developed type III combi-molecules which combine the characteristics of both type I and II molecules. In fact, type III combi-molecules not

only are able to target and inhibit two different kinases in intact form but also act when they do under go hydrolysis (i.e. EGFR-c-Src combi-molecules) [448, 449].

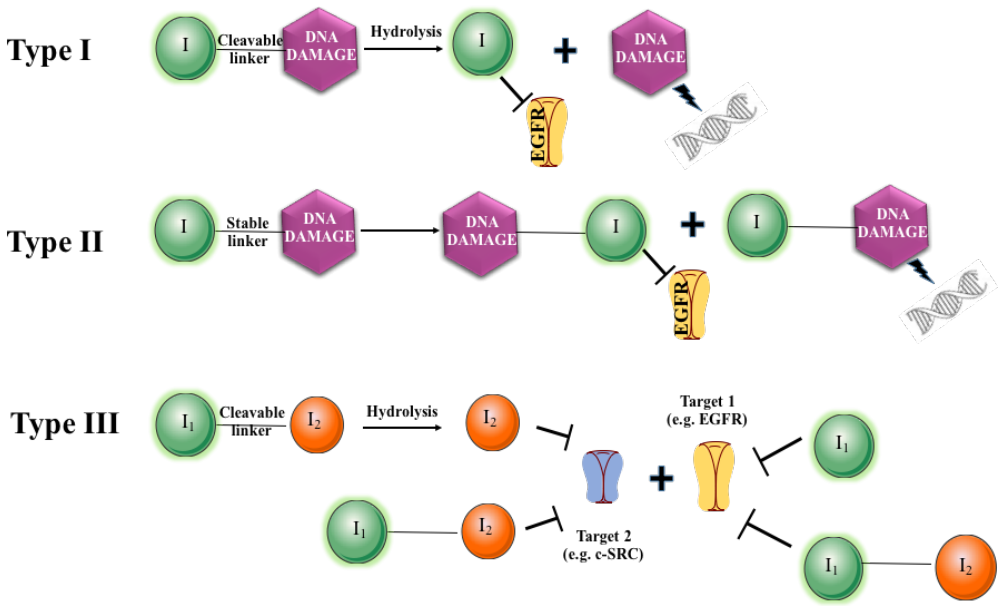


Figure 1.18. The combi-targeting concept. There are three types of combi-molecules (I, II and III). Type I combi-molecules upon hydrolysis release two inhibitors in which each of them is acting on its own target. Type II combi-molecules possess a stable, non-hydrolysable linker and are able to inhibit both their targets in their intact form. Last, type III combi-molecules have characteristics of both type I and II combi-molecules and inhibit their targets in both their intact form and after hydrolysis.

1.7.3.2 Type-I combi-molecules

In 2001 the feasibility of development of combi-molecules and their effectiveness was marked. SMA41 was the first type I combi-molecule (TZ-I) synthesized, which has mixed EGFR-DNA targeting properties. As a Type I combi-molecule, SMA41 needs to under go hydrolysis to generate a quinazoline based inhibitor of EGFR, SMA52, that competes with ATP for the ATP binding site of EGFR, and a DNA damaging moiety which consists of 3-methyl-1,2,3-triazene as the precursor of a methyldiazonium ion (the DNA-damaging agent TMZ) [450]. Although SMA41 showed significant EGFR (IC50=1.0 μM) and DNA damaging properties (alkaline comet assay) it had some challenges such as short half-life and poor water solubility. To overcome the above-mentioned challenges and to improve the EGFR inhibitory potency of SMA41, new prototypes of this combi-molecule were synthesized. ZRBA1 was one such compound that by appending a polar N- N-dimethylaminoethyl group at the alkylating moiety this compound was more water-soluble and than SMA41 had improved half-life (108 minute). In addition, an extra hydrogen bond with the acidic Asp-776 residue at the ATP site of EGFR led to a significantly ($p < 0.05$) superior potency of SMA41 against MDA-MB-468 breast cancer xenograft model [436, 451, 452]. Interestingly, ZRBA1 was also effective on cells with high MGMT levels, which was probably due to the fact that N,N-dimethylaminoethyl-guanine lesions formed from ZRBA1 were not a favored substrate of MGMT. BJ2000 [453], is another type I combi-molecule that hydrolyses into 6-amino-4-anilinoquinazoline FD105 [454] that has provn to have stronger EGFR TK inhibitory activity compared to SMA41. Of note, BJ2000 has a partially irreversible mechanism and is able to selectively block EGF or TGF-α in NIH3T3HER14 cells.

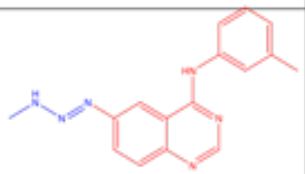
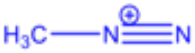
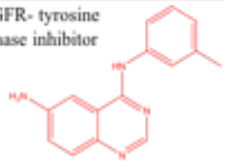
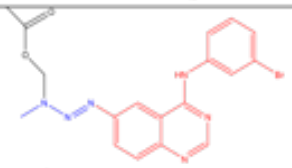
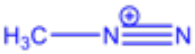
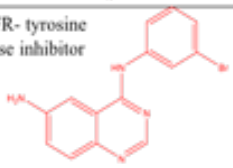
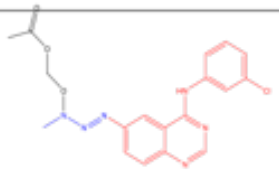
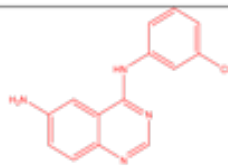
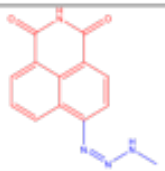
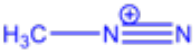
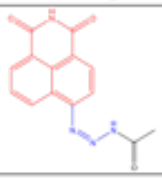
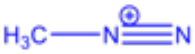
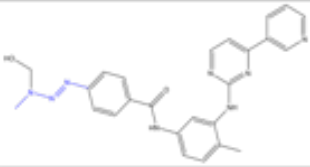
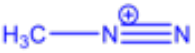
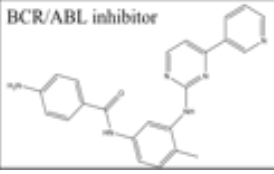
Type I Combi-molecules	Targets	Structure	Inhibitory arm 1	Inhibitory arm 2	Ref
SMA-41	DNA-EGFR		DNA damage 	EGFR- tyrosine kinase inhibitor 	[451]
RB24	DNA-EGFR		DNA damage 	EGFR- tyrosine kinase inhibitor 	[462]
ZRS1	DNA-EGFR		DNA damage		[448]
EG22	DNA-PARP		DNA damage 	PARP inhibitor 	[447]
ZSM02	DNA-PARP		DNA damage 	PARP inhibitor 	[447]
ZRCM5	DNA-BCR/ ABL		DNA damage 	BCR/ABL inhibitor 	[454]

Table 1.1. Published structures of type I combi-molecules.

1.7.3.3 Type- II combi-molecules

The group of Dr. Jean-Claude Bertrand (Drug Discovery lab, in our institution) also synthesized combi-molecules that without requirement for hydrolytic cleavage were able to generate EGFR inhibitory and DNA targeting properties [455]. The first type II combi-molecule synthesized was JDD36, which was designed to simultaneously inhibit EGFR by its 6-position of

4-anilinoquinazoline scaffold and damage DNA by its chloroethyltriazolinium function [456-458]. Then, a series of small hemi-mustard compounds that contain a chloroethyl group appended to the quinazoline moiety such as ZR2002 and ZR2003 [459], ZR2008 and ZR2009 [458] were designed. Of note, ZR2002 has a bromide substitutnt instead of chloride in its structure compared to ZR2003 [460]. Indeed, the hemi-mustard type II combi-molecules are strikingly potent (nM concentration) and have irreversible EGFR inhibitory effect.

EGFR/DNA damaging agents are an example of combi-molecules able to inhibit EGFR TK activity and to damage DNA at the same time. This strategy mimics the targeting mechanism of classical alkylating agents, (e.g., TMZ) and EGFR TK inhibitors (e.g., Iressa). The Iressa-like structure of the aminoquinazoline moiety has high affinity for the ATP-binding site of EGFR. On the other hand, the DNA-targeting triazene moiety of this combi-molecule is capable of alkylating DNA and is able to diffuse towards the nuclear DNA. ZR2002 [461] was one of the first type II combi-molecules designed and tested on breast cancer cell lines. ZR2002 was able to simultaneously block both EGFR and ErbB2-mediated activity and its downstream signaling and induce DNA damage in an irreversible manner [456, 462, 463]. Interestingly, the irreversibility and striking effects of ZR2002 was also suggested to be due to its effects on inhibiting EGF stimulated autophosphorylation, EGFR-mediated downstream signaling pathways and its ability to simultaneously downregulate DNA repair activity of cells therefore increasing the cytotoxic effects of the DNA damaging moiety of this drug. In ZR2003 and ZR2009 the chloroethyl group responsible for damaging DNA was attached to the 6- and 7-position of the quinazoline skeleton in ZR2003 and ZR2009, respectively [456]. ZR2003 was 10-fold more potent compared to ZR2009 in damaging DNA and inhibiting EGFR TK, which suggested that appendage of the chloroethyl group to the 6-position of quinazoline was optimal for DNA damage and led to a more

favorable result. Fortunately, not only were these compounds extremely potent in inhibiting EGFR and damaging DNA but also they showed fluorescence, which correlated with EGFR levels. This special fluorescent characteristic of the combi-molecules could be employed as non-invasive small molecule probes for the detection of EGFR, and to establish the biodistribution profiles of these compounds in addition to their colocalization with corresponding cellular targets [458]. The linear correlation (R = 0.7) between EGFR levels and fluorescence intensity of the above-mentioned combi-molecules was more significant in ZR2002 with lower concentrations. However, when ZR2003 was tested *in vivo* it showed less potency than gefitinib, which suggested lower uptake of the drug in the model tested compared to gefitinib [457].

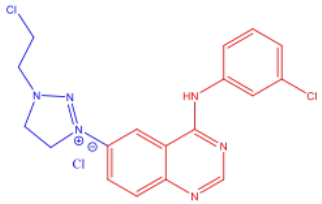
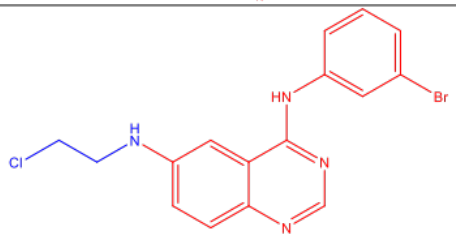
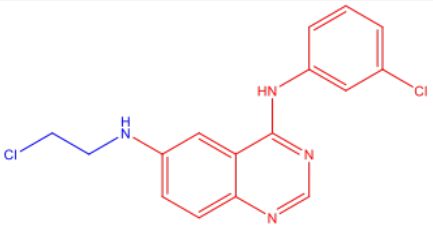
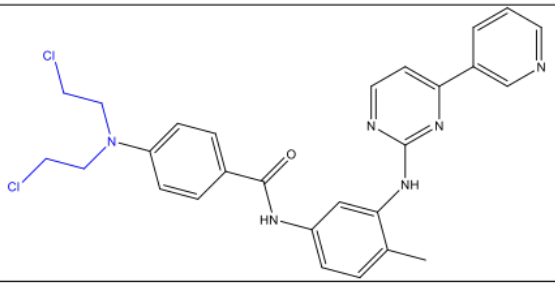
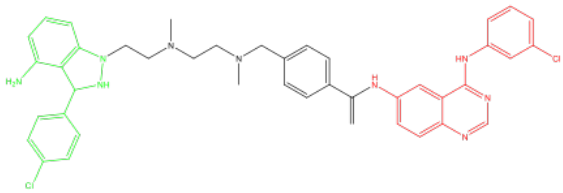
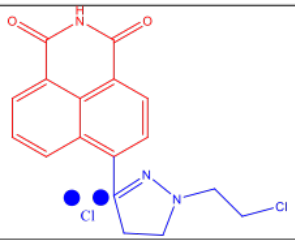
Type II Combi-molecules	Targets	Structure	Ref
JDD36	DNA-EGFR		[458]
ZR2002	DNA-EGFR		[455]
ZR2003	DNA-EGFR		[463]
AK04	DNA-BCR/ABL		[454]
SB163	c-Src-EGFR		[464]
EG40	DNA-PARP		[447]

Table 1.2. Published structures of type II combi-molecules.

1.7.3.4 Type-III combi-molecules

AL776 was the first first type III combi-molecule designed to target multiple kinases associated with oncogenic signaling, EGFR and c-Src. It was shown that AL776 can inhibit these targets in both its intact form and after undergoing hydrolysis, while forming two kinase inhibitors (EGFR inhibitor and c-Src inhibitor) acting on their specific targets. Similar to ZR2003, although AL776 showed strong inhibition of phosphorylation of EGFR and c-Src in *in vitro* models, it failed to show any response in breast cancer 4T1 subcutaneous mouse model [464, 465].

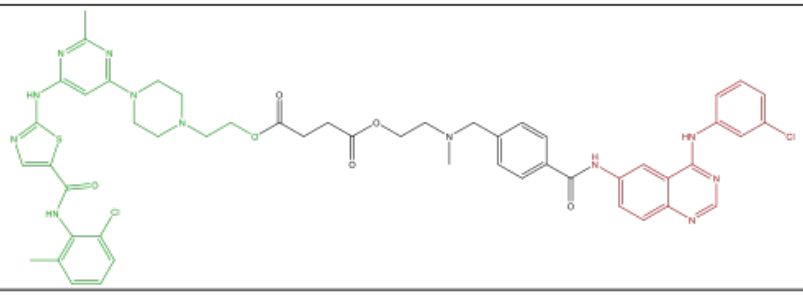
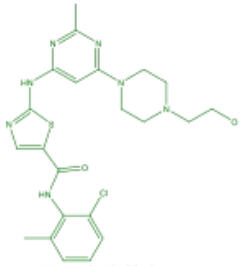
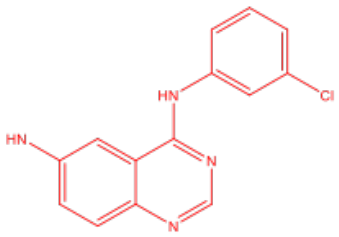
Type III Combi-molecules	**Targets**	Structure	Ref
AL776	**c-Src-EGFR**		[449]
		Arm 1:  c-Src inhibitor	Arm 2:  EGFR- tyrosine kinase inhibitor

Table 1.3. Published structures of type III combi-molecules.

1.8 HYPOTHESIS AND EXPERIMENTAL AIMS

Cancer is one of the most complex human disease. For many years, cancer treatment has been leading toward more targeted and personalized treatment modalities. Despite rapid advances in cancer treatment and discovery of novel therapeutic strategies, the complexity of this disease has pushed scientists to find new approaches, which deal with many of the challenges posed by this disease. GBM treatment has proved elusive, despite decades of research. It is in this context that the work in my thesis was oriented. In my work I aimed to verify and study a novel multi-targeted combi-molecule on GBM to be able to overcome the complexity and resistance seen in the GBM patients through several mechanisms of actions. This novel therapeutic is able to damage DNA and inhibit EGFR oncogenic signaling pathway in tumor cells. Certainly this strategy has to be further investigated; however, we are excited about the novel findings in the context of GBM and about investigating a unique mechanism of action and the potential to overcome the challenges seen in GBM therapy.

The first purpose of our study was to evaluate the safety, blood brain permeability and pharmacokinetic/pharmacodynamics of ZR2002, a type II combi-molecule, and its efficacy in a subcutaneous TMZ-resistant glioblastoma stem cell (GSC) xenograft. Second, we aimed to test if this EGFR-DNA damaging combi-molecule is able inhibit EGFR signaling and is able to damage the DNA in cells at the same time. Molecular analyses were performed to investigate the effect of each arm of these combi-molecule namely, DNA damage and EGFR targeting of cells with different EGFR and p53 levels. We also elucidated its mechanism of action compared to TMZ as the standard treatment of GBM patients and gefitinib. In our next step, we investigated the effect of this drug on brain tumor stem cells from patients newly diagnosed with GBM. We also, tested the effects of this drug on cells that are resistant to TMZ treatment. Finally, the effect of ZR2002

was also tested in an intracranial mouse model of U87/EGFRvIII and TMZ resistance.

Hypothesis:

ZR2002 has greater anti-proliferative activity compared to gefitinib and TMZ alone through concomitant inhibition of EGFR-induced signaling pathways and increased DNA strand breaks in GBM.

The objectives of my work are summarized below and reported in chapter 2 and 3:

Objective 1: To study the safety and ability of EGFR-DNA combi-molecule, ZR2002, to cross the blood brain barrier (pharmacokinetic study) and to verify its optimal dose profile (Chapter 2).

Objective 2: To preform western blotting and immunohistochemistry to evaluate the pharmacodynamics (PD) of ZR2002. Also to evaluate its *in vivo* activity in a subcutaneous TMZ-resistant GSC model (Chapter 2).

Objective 3: To study the effect of ZR2002 on brain tumor stem cells from patients newly diagnosed with GBM. We planned to study the ability of this combi-molecule to overcome the resistance seen in a clinically relevant TMZ-resistant GBM mouse model (Chapter 2 and 3).

Objective 4: To test the effect ZR2002 as a novel combi-molecule on GBM established cell lines with different EGFR levels and elucidate its mechanism of action on both GSCs and GBM established cell lines (Chapter 3).

Objective 5: To provide the proof-of-concept for the *in vivo* efficacy of ZR2002 in an intracranial U87/EGFRvIII and TMZ-resistant GSCs mouse model (Chapter 3).

The reported objectives resulted in original and novel findings, which are summarized and discussed in the contribution to knowledge.

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Chapter 2.

Pharmacokinetic and pharmacodynamic study of a combi-molecule with dual EGFR/DNA targeting properties

Zeinab Sharifi, Bassam Abdulkarim, Brian Meehan, Janusz Rak, Nidia Lauzon, Elliot Goodfellow, Martin Rupp, Bertrand Jean-Claude and Siham Sabri*

Division of Experimental Medicine (Z.S., M.R., E.G. N.L.), Research Institute of McGill University Health Centre (B.M., B.A., J.R., B.J, and S.S.), Department of Medicine (B.J.), Department of Oncology (B.A.), Department of Pediatrics (J.R.), Department of Pathology (S.S.) McGill University, Montreal, Quebec, Canada.

***Corresponding author:**

Siham Sabri, PhD, The Research Institute of the McGill University Health Centre, 1001 Decarie Blvd, Montreal, Quebec, H4A 3J1
Office: EM2.3218; Phone: 514-934-1934 Ext.: 44686
siham.sabri@mcgill.ca

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2.1 ABSTRACT

Background:

The limited clinical success of traditional DNA-damaging chemotherapeutics, such as temozolomide (TMZ) prompted the quest for novel strategies in glioblastoma (GBM). Targeting the epidermal growth factor receptor (EGFR), known to harbor genomic alterations in half of GBMs failed to overcome this therapeutic deadlock. The purpose of our study was to evaluate the safety, blood brain permeability and pharmacokinetic/pharmacodynamics of ZR2002, a type II of combi-molecule, and its efficacy in a subcutaneous TMZ-resistant glioblastoma stem cell (GSC) xenografts.

Methods:

We first determined the safety of ZR2002 by testing two treatment schedules and evaluating morbidities, clinical signs, body weights, complete blood count (CBC), biochemistry assessment of enzymes and post mortem evaluation including gross examination for all the animals at the terminal necropsy. We further provide the proof-of-concept for ZR2002 delivery across the blood brain barrier (BBB) by MALDI imaging mass spectrometry (MALDI IMS). We then, performed preclinical assessments of brain penetration of ZR2002 using HPLC and LC/MS analysis. Western blotting and immunohistochemistry were used to evaluate the pharmacodynamics of ZR2002. Finally, we evaluated its *in vivo* activity in a subcutaneous TMZ-resistant GSC model.

Results:

Oral administration of ZR2002 at doses up to 150 mg/kg/day for 5 days on- 5 days off -5 days or 21 continuous days in athymic mice that have intact Non-Homologous End-Joining (NHEJ), a major, DNA repair pathway was safe. ZR2002 was detected in the brain using HPLC but the plasma concentration of ZR2002 was lower than its detection range when given orally. Therefore,

we used LC/MS to confirm the presence of ZR2002 in the plasma of mice. Interestingly, MALDI IMS confirmed the presence of ZR2002 and its metabolite (ZR01) in the brain of nude mice with intracranial tumors. Western blotting revealed that treatment with ZR2002 significantly ablated phosphorylation of EGFR (Tyr1068), Erk 1/2 and Akt (p-Akt/Ser473). However, treatment of ZR2002 in NSG (NOD scid gamma) mice showed toxicity that suggests that this drug might work through DNA-PKcs. Preliminary preclinical evidence of ZR2002 potency in subcutaneous TMZ-resistant GSC model was also confirmed.

Conclusions:

ZR2002 is a safe drug that is able to cross the blood brain barrier. Further investigation of ZR2002 *in vitro* and *in vivo* is warranted to understand the mechanism of action of this drug and to assess the efficacy of ZR2002 in a GBM intracranial mouse model (chapter 3).

2.2 INTRODUCTION

Glioblastoma (GBM), is one of the most aggressive and malignant brain tumors in humans with the median survival of ~15 months for patients who enroll in clinical trials [1]. Temozolomide (TMZ) is a clinical alkylating agent that induces O6-methyl guanine N7-methylguanine and N3-methyladenine lesions [2, 3] and is well known as the standard treatment of GBM.

Overexpression of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) are observed in many human cancers including brain [4], bladder cancer [5], colon carcinoma [6] and breast and non-small cell lung carcinomas [7, 8]. Agents capable of blocking disordered growth signaling, mediated by the tyrosine kinase (TK) activity of these receptors, are now used or are in clinical trials. As an example Herceptin (trastuzamad), a humanized antibody against erbB2, showed a 22% response rate as a single agent in metastatic breast cancer [9]. Iressa (gefitinib, ZD1839), 4-(3-chloro-4-fluorophenylamino)-7-methoxy-6-(3-(4-morpholinyl) propoxy) quinazoline is an orally (P.O.) administered EGFR-TKI of the quinazoline class with demonstrated *in vitro* efficacy against lung, colorectal, prostate, ovarian, and breast cell lines and *in vivo* growth delay in a subcutaneous (s.c.) human tumor xenografts that overexpress EBFR. In 2003, the FDA granted a fast-tracked approval for gefitinib for the treatment of patients with advanced non-small cell lung cancer (NSCLC) who failed to respond to conventional chemotherapy [10-12]. However, in the phase III trials gefitinib did not show any significant improvement of overall survival, which lead to restriction of gefitinib use by the FDA [13-15].

It has now been shown that EGFR induces expression of DNA repair enzymes including excision repair cross-complementation group 1 (ERCC1) and X-ray repair cross-complementing protein 1 (XRCC1), two DNA repair enzymes that are involved in nucleotide excision (NER) and base-excision repair (BER) of alkylated adducts. Studies have shown that EGFR causes resistance

through activation of mitogen-activated protein kinase (MAPK) pathway [16, 17] and through activation of the phosphatidylinositol-3 kinase (PI3K) pathway it can lead to reduced sensitivity to anti-tumor drugs [18]. Dysregulated expression of receptor tyrosine kinases has been associated with resistance to radiation and DNA damaging drugs such as cisplatin and TMZ and its dysregulation correlates strongly with poor prognosis [19, 20].

Studies have also been designed to combine EGFR TK inhibitors with classical cytotoxic drugs and potentiate the action of drugs [21-23]. With the same idea in mind, Jean-Claude et al. has developed a novel tumor targeting strategy, termed "combi-targeting", that seeks to develop novel drugs designated as "combi-molecules" capable of blocking growth factor-mediated signaling while inducing cytotoxic DNA damage. The "combi-targeting" strategy consists of combining a cytotoxic DNA damaging function with an EGFR inhibitory into a single molecule i.e. "combi-molecule". Type II combi-molecules are designed to have cytotoxic DNA damaging function and the EGFR inhibitory function without the need for hydrolysis. This in turn is expected to lead to synergistic killing of EGFR-overexpressing cells.

ZR2002 [24], is type II of combi-molecule that does not require hydrolysis to generate the EGFR and DNA targeting properties. By appending the chloroethyl group to the 6-position of the quinazoline backbone, ZR2002 was designed to inhibit EGFR TK via its quinazoline moiety and to damage DNA by its alkylating chloroethyl function [25] (the chemical structure of ZR2002 is shown in Fig. 2.1A).

ZR2002 has been tested in breast cancer in *in vitro* studies in previous studies [25] and it may be a valuable component to be tested in GBM. For successful treatment of brain tumors, a drug must first cross the blood brain barrier (BBB). Many factors might affect this BBB permeability such as molecular weight [26], drug's affinity for the ATP-binding cassette efflux

transporters, permeability glycoprotein (P-gp), and breast cancer-resistance protein (BCRP) [27-30]. Therefore, we investigated the *in vivo* safety and brain permeability of ZR2002 in GBM by MALDI imaging mass spectrometry (MALDI IMS). We also, sought to determine the *in vivo* efficacy of ZR2002 against subcutaneous glioma stem cell tumors (GSCs) with high EGFR expression and known to be resistance to TMZ and compare its effect to gefitinib and TMZ treatment.

2.3 MATERIALS AND METHODS

2.3.1 Investigational agent

ZR2002 is a quinazolin combi-molecule EGFR and DNA damaging irreversible inhibitor, which was kindly provided by Dr. Bertrand Jean-Claude (McGill, CA) [24] and TMZ from Tocris Bioscience. For oral (Per os /P.O./orally) or intravenous (IV) administration to animal subjects, drugs, were dissolved in 20% ethanol and 20% cremophore and 60% dextrose at concentrations of 12.5, 50, 75, 100, and 150 and 200 mg/kg (ZR2002).

2.3.2 GBM stem cells (GSCs) culture

GSCs (1123P and 1123IC7R) were established and characterized in the laboratory of Dr. J. Rak [31]. Low passage number (<5) GSCs were maintained in NeuroCult NS-A Basal Medium (STEMCELL Technologies) with NeuroCult NS-A proliferation supplements including heparin, EGF (20 ng/ml) and fibroblast growth factor 2 (FGF, 20 ng/ml).

2.3.3 Animals used for dose finding study

To verify the most effective schedule, 2 dose finding study designs was evaluated in this study. Six to eight-week-old nude mice were purchased from Charles River Laboratory. Animals were randomly assigned to dose groups based on body weight. This strain of mice has been historically used in safety evaluation studies. In addition, mice were commonly used in the efficacy studies of cancer drugs. Animals were group housed in solid bottom polycarbonate cages (3–5 animals/cage) and provided with pelleted food that was pre-irradiated via Co60 by the Supplier. The food was provided ad libitum unless otherwise specified. Animals were also provided with sterilized pure water ad libitum. No known contaminants were present in the water at levels that might interfere with this study. Environmental controls for the animal room were set to maintain 20–25◦C, a relative humidity of 50–70%, a minimum of 10 air changes/h, and a 12-h light/12-h dark cycle. The light/dark cycle might be interrupted for study-related activities. The numbers of animals, study design, and treatment of animals were reviewed, and approved by McGill University and Goodman Animal Facility. All procedures in this protocol are in compliance with Glen and Goodman vivarium housing regulations. The medical treatment necessary to prevent unacceptable pain and suffering, including euthanasia, was the responsibility of the attending laboratory animal veterinarian. Discretionary medical treatment has been carried out based upon consensus agreement between the Study Director and the attending Laboratory Animal Veterinarian. In the event of severe toxicity, in which decisions were to be made regarding treatment or euthanasia of study animal(s), the Veterinarian and the Study Director reserve the right for subsequent action.

2.3.4 Assessment of Toxicity

To evaluate systemic toxicity, athymic immunosuppressed female nude mice (nu/nu) mice without tumors received ZR2002 at doses of 100 mg/kg/day or 150 mg/kg/day or 200 mg/kg/day or the carrier as a control over 21 days. Toxicity was monitored by daily weights and neurological examinations. Mice were euthanized with CO_2 on varying days based on different treatments and immediately autopsied, with organs fixed with formalin.

2.3.4.1 Study design number 1

In order to verify this schedule, 8 female nu/nu were assigned to the study. The animals received dose formulation containing ZR2002 and the control article at various dosages via P.O. injections for 5 days. Then the mice were given a 5-day break and then the second 5-day cycle was started. The MTD in this study is defined as the highest dose that will be tolerated and not produce major life-threatening toxicity for the study duration. The starting dose level (100 mg/kg) for ZR2002 study has been selected based upon the findings of an efficacy study of the compound in nude mice (data not shown). Detailed group design is listed in table 1. The following parameters and endpoints were evaluated for 60 days in this study: mortality, changes in body weights, skin toxicity, biochemistry assessment of blood levels of liver enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), complete blood count (CBC) and post mortem evaluation included gross examination for all the animals at the terminal necropsy.

2.3.4.2 Study design number 2

In order to verify this schedule, 6 female nu/nu were assigned to the study. The animals received dose formulation containing ZR2002 and the control article at dose of 150 mg/kg via P.O. injection for a continuous 21 days including weekends. Detailed group design is listed in table 2.

The following parameters and endpoints were evaluated for 60 days: mortality, changes in body weights, skin toxicity, CBC and post mortem evaluation included gross examination for all the animals at the terminal necropsy.

2.3.4.3 Termination

Unscheduled sacrifices and deaths

Animals exhibiting weight loss (>20%), any signs of acute pain or distress, which were unable to eat, walk, groom normally, or moribund, or have other signs of severe systemic toxicity were euthanized immediately. At the end of each experiment all surviving animals were euthanized.

2.3.5 Chemicals and materials for Pharmacokinetic study

ZR2002, RB10 (internal standard) and RB10-N (internal standard) were synthesized in Dr. B. Jean-Claude's (Montreal, Quebec) laboratory (purity: 99.98%). HPLC-grade methanol was purchased from Fisher Scientific, Inc (Canada). HPLC-grade water was obtained from a Milli-Q system (Canada). All reagents were purchased at the analytical grade.

2.3.5.1 Pharmacokinetic studies in mice

Adult CD-1 and Balb/c mice (females, 20-23g) were purchased from Goodman Animal Facility (McGill). All animals were housed under controlled conditions (22–23°C-humidity ~70%). Two sets of pharmacokinetic (PK) studies were performed. In the first set 57 mice were given a single dose of 12.5 mg/kg ZR2002 by intravenous injections (IV) or a single dose of 50 mg/kg orally (P.O.) (Supplementary Fig. diagram S2.1). Blood samples were collected into heparinized tubes at 0, 5 min, 15 min, 30 min, 1 hr, 3hr, 8 hr, 24 hr and 0, 15 min, 30 min, 1 hr, 3hr, 8 hr, 24 hr for IV and P.O. injections respectively (N=4/Time point). Blood was immediately

processed to extract plasma by centrifugation at 13000×g for 10 min. Then the plasma and brain were frozen at −80 °C for future HPLC analysis.

In the second set, 10 mice were given a single dose of 75mg/kg of ZR2002 orally. As described before brain and plasma samples were collected at 0, 30 min and 1hr (Balb-c & CD-1) (N=3/time point). Plasma and brain were frozen at −80 °C for future HPLC analysis.

2.3.5.2 Brain and plasma sample preparation

Briefly, brain samples were transferred from -80 freezer to dry ice. Brain tissues were removed from their vials to record their mass using a digital balance. Once adequately thawed a scalpel was used to cut the tissue into small pieces, transferred to a mortar half-filled with liquid nitrogen, snap-frozen then crushed into a fine powder. Once finely ground, the powdered sample was transferred into a 50mL conical tube and well homogenized with methanol (2 parts of methanol to 1 part of powdered sample). Next, the samples were snap-frozen in liquid nitrogen for 10 minutes. This procedure was followed by sonicating in an ice water bath, snap freezing the sample and sonicating again for 10- 15 minutes. The samples were centrifuged at 13,000 rpm for 20 minutes and the supernatant collected into a 15mL conical tube (from the snap-freezing the samples till the 20 min centrifuge was repeated). The samples were evaporated overnight in the SpeedVac and concentrated into one 1.5mL eppendorf tube. Finally, the dried powder was spiked with RB10 or RB10-N as internal standards to a final concentration of 50 or 100 µM. The supernatant (20 µL) was used to measure drug concentration by HPLC.

After the frozen plasma samples (300 µl) were thawed on ice, to preform the extraction 300 µl methanol (MeOH) was added to the samples and mixed completely. The mixture was

centrifuged at 13,000 rpm for 5 min (4º C). The solution was transferred to a clean tube; to repeat the extraction for the second time the pellet was mixed with an additional 300 µl MeOH then vortex-mixed and finally centrifuged as mentioned earlier (total 3 rounds of extraction). The samples were evaporated overnight in the SpeedVac and concentrated into one 1.5mL Eppendorf tubes. Finally, the dried powder was spiked with RB10 or RB10-N as internal standards to a final concentration of 50 or 100 µM. The supernatant (20 µL) was used to measure drug concentration by HPLC.

2.3.5.3 Instrument and chromatography conditions

Blood and plasma samples were analyzed on a UV HPLC system containing a Surveyor MS pump, a Surveyor PDA detector, an auto sampler and a reversed-phase column (Agilent TC-C18, 250×4.6 mm, 5 µm). Separation of ZR2002 and internal standard was performed using a mobile phase consisting of MeOH (20%) delivered at a flow rate of 0.5 mL/min. Solutions of plasma were prepared on the day of analysis by appropriate dilution of internal standard with MeOH. The stock solutions were kept at 4 °C. The internal standard (RB10 and RB10-N) was prepared as a solution (50 or 100 µM) in methanol.

2.3.5.4 Preparation of stock solutions, quality control samples and plasma/brain samples for a reproducibility assay

Calibration curve samples for measuring ZR2002 in plasma and brain were prepared at concentrations of 250, 125, 62.5, 31.25. 15.61and 7.81 µM for ZR2002 and 50 or 100 µM for RB10 and RB10-N. Plasma and brain tissue ZR2002 concentrations were calculated using calibration curves. The calibration curves in plasma were assessed by the correlation coefficients that were obtained with the following equation: $Y=A\times X+B$ (the weighting factor was 1/X), where

Y was the ratio of the relative peak area of ZR2002 to that of the internal standard RB10, X was the concentration of ZR2002, A and B were the values from a least squares regression analysis. The maximum plasma concentrations (C_{max}) and their time of occurrence (T_{max}) were calculated from the data. The results for tissue levels of ZR2002 were expressed as μg/g of brain tissue, and the levels were calculated by the following equation:

$Ct\ (\mu g/g) = Cs\ Vs/W$

where Ct is the tissue concentration (μg/g), Cs is the supernatant concentration, Vs is the supernatant volume, and W is the weight of the tissue sample. The mean tissue concentration as a function of time curves were obtained, and pharmacokinetics parameters were determined using the equations that are described for plasma.

2.3.6 Pharmacodynamic study

Mice were implanted with GSC (1123P) subcutaneously; after 2 weeks mice were randomized into treatment groups (N=2 per group): control, ZR2002 (150 mg/kg), all 4 mice in each drug-treated group were given a single dose of their respective drugs by oral gavage at the same time. At 2 hours, after injection, all mice from each group were sacrificed and their tumors were harvested. Tissue samples were stored at −80°C until further analysis by western blotting.

For western blots tumor tissues were lysed with RIPA buffer (Boston BioProducts) supplemented with 0.2 mM sodium orthovanadate, protease (Sigma-Aldrich) and phosphatase (Roche Diagnostics) inhibitor cocktail. Proteins (30 μg, Pierce BCA protein assay kit, Thermo Fisher Scientific Inc.) were electrophoretically separated in 12% SDS-PAGE and transferred onto PVDF membranes. Membranes were probed for phosphorylated EGFR (p-EGFR/Tyr1068), total EGFR, phospho-p44/42 MAPK (phosphorylated Erk1/2) (p-Erk1/2), total Erk1/2, phosphorylated Akt (p-Akt/Ser473) (Cell signaling), total Akt1/2/3 (H-136) (Santa Cruz Biotechnology), and β-

actin (Sigma-Aldrich), according to the manufacturer's recommendations. Appropriate horseradish peroxidase–conjugated secondary antibodies (Life Technologies) and chemiluminescence detection were used (Amersham, GE Healthcare).

For immunohistochemistry, GSC (1123P) tumor tissue was fixed with 4% buffered formalin overnight. Brains were then embedded in paraffin and sectioned for immunohistochemical analysis. Sections were processed manually using phosphorylated EGFR (p-EGFR/Tyr1068) and phospho-p44/42 MAPK (phosphorylated Erk1/2) (p-Erk1/2) antibodies according to the manufacturer's recommendations.

2.3.7 MALDI imaging mass spectrometry (MALDI IMS)

Experiments were performed in accordance with a protocol approved by our Institutional Animal Care Committee (McGill University Health Centre Research Institute and McGill University). 1123IC7R GSC cells were lentivirally transduced with a luciferase-BFP dual gene vector (Luc2 pSMALB; Luc2 cloned from pGL4.51 (Promega) into the pSMALB backbone described previously) [32] to monitor tumor growth using bioluminescence imaging (BLI). For orthotopic injection, cells were dissociated to single-cell suspensions, and 20,000 cells were stereotactically injected into brains of 6 to 8-week-old nude mice (Charles River). Mice were randomized into vehicle (control) (N=1) or ZR2002/150mg/kg (N=1) given orally. Mice were treated for 21 days and at the terminal end point of the experiment (upon significant weight loss (>20%) or presentation of neurologic symptoms necessitating euthanasia) mice were given a final dose of 150 mg/kg ZR2002 before their euthanasia to measure brain tumor permeability of ZR2002 on tumor tissue collected from mice by MALDI imaging mass spectrometry (MALDI IMS). Snap frozen brain tumor tissue blocs were cut using a Leica CM1950 cryostat (Leica Microsystems

GmbH, Wetzlar, Germany). Sections for MALDI IMS (10 µm thickness) were thaw-mounted on an ITO-coated microscope glass slide (Delta Technologies Ltd, Loveland, CO). Tissue sections were washed 30 seconds in a container filled with hexane to remove lipids from the sections. Upon desiccation, the MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) was deposited onto the tissue sections using the HTX M3 TM-sprayer connected to an isocratic LC pump using the following parameters: CHCA solution 5 mg/ml in 50% ACN/0.1% TFA; nozzle temperature 55 °C; nozzle height 40 mm; nitrogen pressure 10 psi; flow rate 0.1 ml/min; z-arm velocity 1200 mm/min; moving pattern VV; track spacing 3 mm; number of passes 14; and drying time 0 s. IMS of the tissue sections were performed on a MALDI TOF/TOF Ultraflextreme mass spectrometer equipped with a SmartBeam II Nd:YAG 355 nm laser operating at 2000 Hz, using the medium laser focus setting (Bruker Daltonics, Billerica, MA, USA). IMS data were acquired using 300 shots per pixel with spatial resolution of 50 µm in a mass range of 100-1000 Da. External calibration was carried out using CsI cluster ions. The MALDI images were displayed using the software SCiLS (2019b Premium 3D, Bremen, Germany). ZR2002 identification was confirmed by MS/MS using the LIFT-TOF/TOF instrument mode. The precursor ion for ZR2002 (m/z 377.0) was isolated using an isolation window of ±2 Da. The primary fragment (m/z 377.0 → m/z 341.0) coming from the neutral loss of HCl (-36 Da) was used to identify ZR2002 on-tissue.

2.3.8 Subcutaneous GSC xenografts

Experiments were performed in accordance with a protocol approved by our Institutional Animal Care Committee (McGill University Health Centre Research Institute and McGill University). GSC xenografts, 1123IC7R cells were dissociated to single-cell suspensions, and 1 x 10^6 cells were implanted into the right flanks of 6- to 8-week-old NOD scid gamma (NSG) mice. Mice were randomized after 7 days to oral treatment with vehicle (control), ZR2002 (150 mg/kg)

or TMZ (66 mg/kg) (N=3 each treatment arm). Tumor growth was measured every other day starting from day 7 (start of treatment) using calipers and the volume was calculated according to the following equation: $V = LxW^2/2$. Body weights were recorded as indicated in Figure 2-7B. Mice were sacrificed upon significant weight loss (>20%) or presentation of neurologic symptoms necessitating euthanasia.

2.3.9 Statistical analysis

PRISM 6, (GraphPad Software), was used to conduct all statistical analyses. Animals that died during anesthesia or as a result of oral gavages were excluded from survival analyses. Data are reported as mean +/- SEM and are representative of at least 3 independent experiments run in triplicate, unless otherwise stated. Statistics were performed using unpaired two-tailed Student's t-test. P-values <0.05 were considered statistically significant.

Table 2.1: Detailed group design for Schedule #1 (5 days on-> 5 days off-> 5 days on)

Dose Range Finding (DRF) study (escalating dose design)		
Group	**No. of animals**	**Dose level (mg/kg/day)**
1	2	Control
2	2	100
3	2	150
4	2	200

Table 2.2: Detailed group design for Schedule #2 (21 continuous days)

Dose Range Finding (DRF) study		
Group	**No. of animals**	**Dose level (mg/kg/day)**
1	3	Control
2	3	150

2.4 RESULTS

2.4.1 ZR2002 Dose Finding Study

To evaluate the safety and optimal dose of ZR2002 (structure of the novel ZR2002 is shown in Fig. 2.1A) to be used in *in vivo* efficacy studies, we performed a dose finding study testing two treatment schedules (#1 & #2). To evaluate schedule #1 (Fig. 2.1B), non-tumor-bearing nu/nu mice were treated every day for 5 days with ZR2002 at 100, 150 and 200 mg/kg/day or the carrier control (Dose Range Finding, DRF). After 5 days mice were given a 5-day break and the second 5-day cycle was started. We monitored the mice up to 60 days from the start of the treatment and we showed ZR2002 at doses up to 150 mg/kg (200 mg/kg/day resulted in 100% mortality) was safe shown by: no mortality, no mean weight loss >20% (Fig. 2.1C), no significant changes in CBC (Table 2.3) and no significant changes in biochemistry assessment of liver enzymes ALT/AST (Table 2.3). Post-mortem evaluation included gross examination for organs (spleen, brain, skin, kidney, lung and heart) of all the animals receiving doses up to 150 mg/kg and the terminal necropsy showed no signs of toxicity.

Also, to mimic TMZ treatment schedule in the clinic, we examined of oral gavage toxicity in mice when 150 mg/kg ZR2002 is given for 21 continuous days (Fig. 1B, schedule #2). We monitored the mice up to 60 days from the start of the treatment and we showed continuous

treatment with ZR2002 (21 days) caused no mortality (no mean weight loss >20%) (Fig. 2.1D), and no significant changes in CBC (Table 2.4). In addition, post-mortem evaluation included gross examination for organs (spleen, brain, kidney, lung and heart) of all the animals and the terminal necropsy showed no signs of toxicity.

One major toxicity seen after use of EGFR inhibitors is skin toxicity which is related to the inhibition of EGFR in the skin that has a crucial role in normal development and physiology of the epidermis. Although skin toxicity may not be life-threatening it causes physical and psycho-social discomfort [33, 34]. Mice were examined daily for skin toxicity and behavior changes in either treatment groups over an observation period of 60 days.

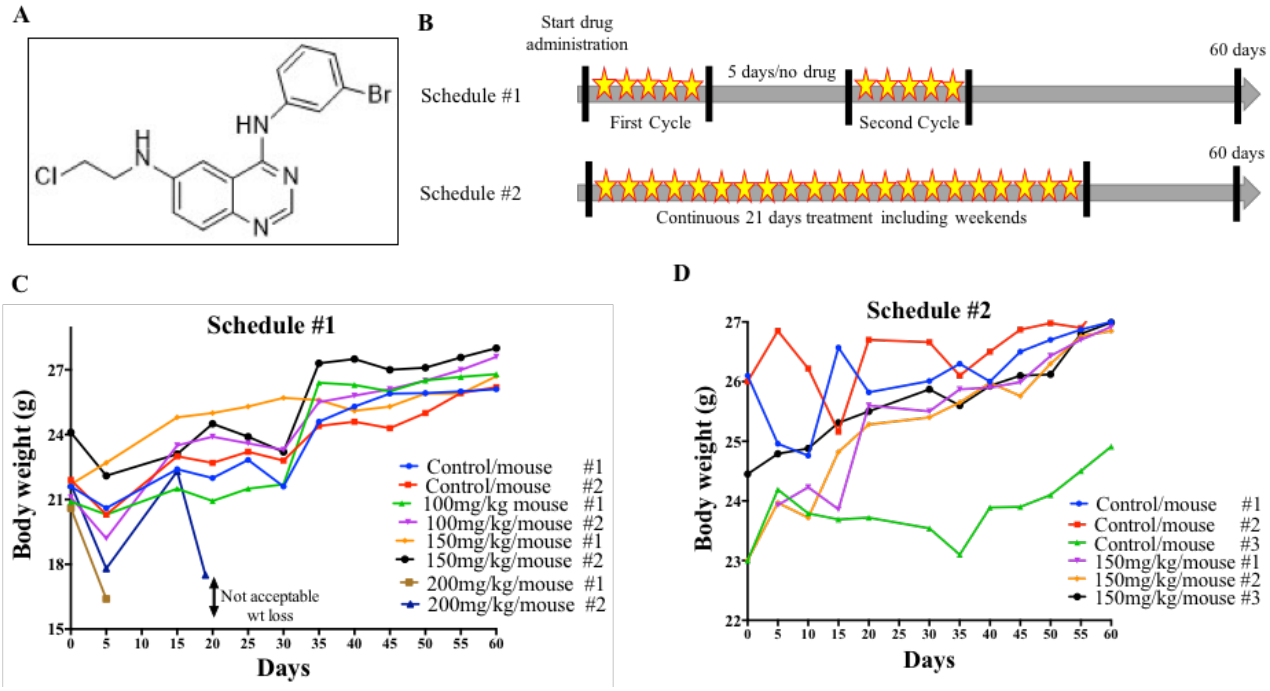


Figure 2.1 (A-D). ZR2002 Dose Finding Study. A. Chemical structure of ZR2002 (6-(2-chloroethylamino)-4-anilinoquinazoline) molecular structure (Molecular weight: 377.67 g/mol). **B.** Eight female nude mice were assigned to evaluate schedule #1 treatment. Mice received

ZR2002 (100, 150, 200 mg/kg) or control *per os* (p.o.) for 5-days (first treatment cycle, yellow stars indicate daily treatment). Mice were then given 5-days of rest, followed by second course of 5-days treatment period (second cycle) then monitored up to 60 days post-treatment. Six female nude mice were assigned to evaluate schedule #2 treatment. Mice received ZR2002 or control at a dose of 150 mg/kg p.o for 21 consecutive days (yellow stars indicate daily treatment) then monitored up to 60 days post-treatment. Mice were weighted every 5 days and data reported as graphs for schedule #1 **(C)** and schedule #2 **(D)**.

Table 2.3: Biochemistry assessment of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes and complete blood count (CBC) and for Schedule #1.

ZR2002 (mg/kg/day)	Units	Mouse normal range	Control#1	Control #2	100 #1	100 #2	150 #1	150 #2
ALT	U/L	28-132	54	39	56	45	41	41
AST	U/L	59-247	79	72	145	62	77	58

ZR2002 (mg/kg/day)	Control #1	Control #2	100 #1	100 #2	150 #1	150 #2
WBCs x 10^9/L	10.5	6.1	15.2	8.1	11.2	8.8
RBC x 10^9/L	9.28	8.87	11.85	10.03	7.90	8.32

Table 2.4: Complete blood count (CBC) for Schedule #2.

ZR2002 (mg/kg/day)	Control # 1	Control# 2	Control# 3	150 #1	150 #2	150 #3
WBCs x 10^9/L	14.2	12.1	11.4	8.2	8.2	9.8
RBC x 10^9/L	8.42	11.09	7.93	8.55	8.57	9.58

2.4.2 HPLC-UV method to measure ZR2002 in brain and plasma

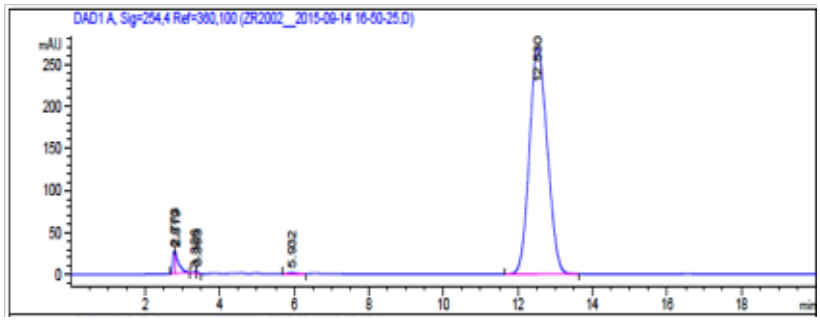
In our first step we verified the retention time of ZR2002 alone using HPLC-UV method. As shown in figure 2.2A the retention time of ZR2002 was 12.5 minute. We then used RB-10 as our internal standard and exhibited the baseline separation of ZR2002 and RB10 at 12.5 and 7 min retention times, respectively. No interference peaks from intrinsic substances were observed at the ZR2002 and RB10 retention times in homogenate samples from control mice. Representative HPLC-UV chromatograms for plasma samples are shown in Figure 2.2.

2.4.2.1 Pharmacokinetics of ZR2002

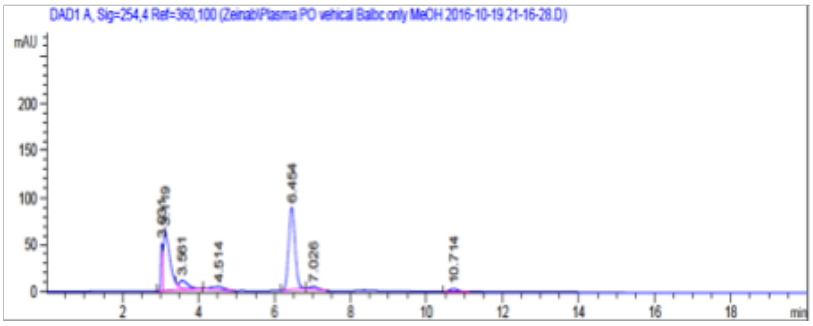
The concentration-time courses of ZR2002 in plasma and brain homogenates of mice are shown in Figure 2.3. The brain *Tmax* and *Cmax* for 50 (P.O.) and 12.5 (IV) mg/kg ZR2002 was 3hr/3.58µg/g and 5min/2.7 µg/g respectively. For plasma samples *Tmax* and *Cmax* for 50 and 12.5 mg/kg ZR2002 was 30min/0.6µM and 5min/107.93µM, respectively. When higher doses of ZR2002 (75 mg/kg, by gavage) was used at 1hour time point, 8.1 µg/g of ZR2002 was detected in the brain's of Balb/c mice that is 16-fold more when comparing it to the same time point of 50 mg/kg ZR2002. Of note ZR2002 was not detected in the brain after 8 hours when administered orally (Tables 2.5 & 2.6).

Since the concentration of ZR2002 was below the limit of quantification (BLQ) when HPLC was used as the method of quantitation of ZR2002 on plasma samples (oral gavage) collected from mice, we then chose the LC/MS method as a more sensitive method to analyze plasma samples collected from these mice. The data collected at 3 hours after administration of ZR2002 showed rapid plasma clearance with a peak ZR2002 concentration of 0.6 μM detected in plasma of mice (Supplementary Fig. S2.2).

A



B



C

D

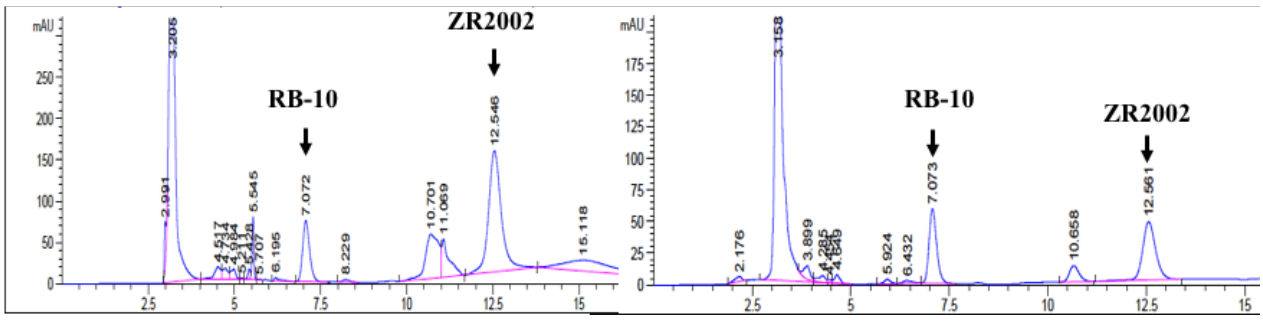


Figure 2.2 (A-D). HPLC-UV chromatograms of mice plasma. A. ZR2002 reference, **B.** Mice plasma blank, **C.** Mice plasma spiked with RB10 at 100 µM **(D)** Mice plasma spiked with RB10 at 100 µM. Plasma collected 0 **(C)** and 5 min **(D)** after a 12.5mg/kg IV dose of ZR2002 in mice. The retention times of RB10 and ZR2002 are 7 min and 12.5 min, respectively.

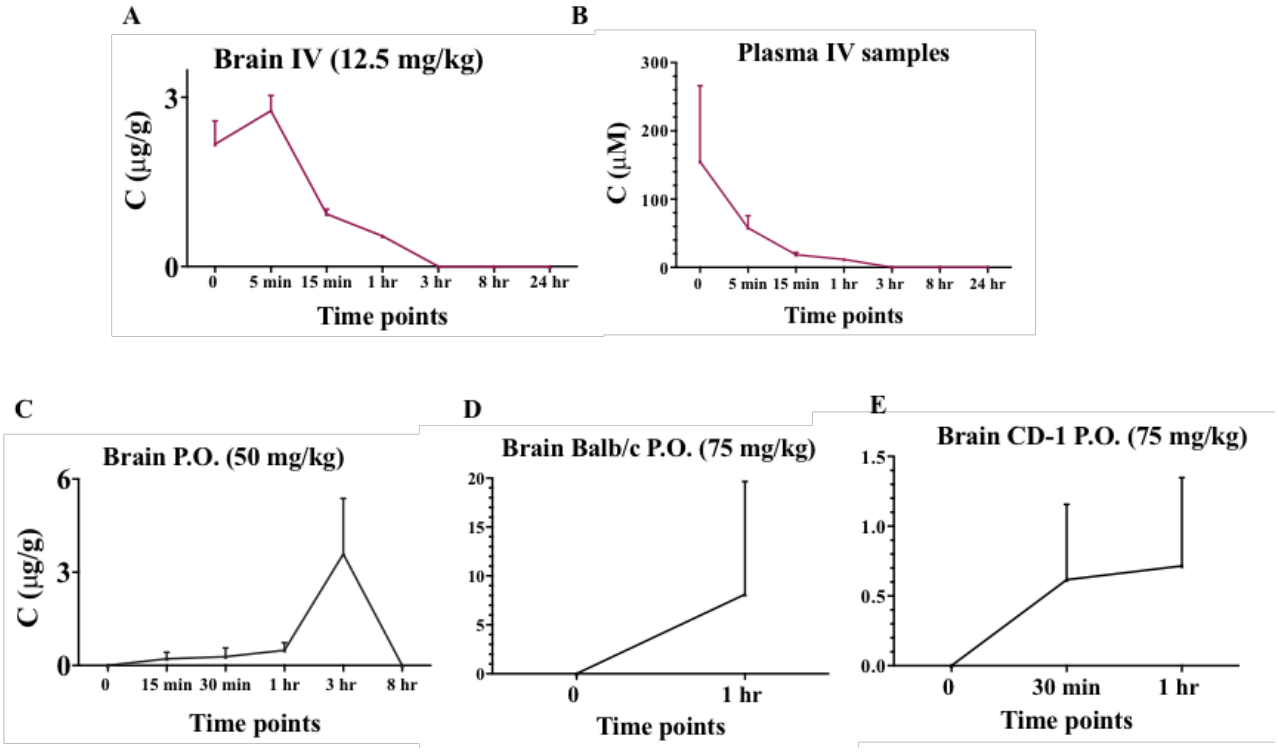


Figure 2.3 (A-E). Concentration-time curves for ZR2002 (A) in brain tissue and **(B)** in plasma after a 12.5 mg/kg IV dose of ZR2002 in mice. Each point represents the mean value from at least 2 mice. Concentration-time curves **C, D** and **E** shows the concentration of ZR2002 in brain tissue after a 50, 75 mg/kg P.O. dose of ZR2002 in different strains of mice. Each point represents the mean value from at least 2 mice.

Table 2.5: ZR2002 pharmacokinetics in plasma and brain, following intravenous (IV) administration of ZR2002 at 12.5 mg/kg to mice using HPLC.

HPLC results	C_{max} (µg/g)	T_{max}
Brain/IV-12.5 mg/kg	2.76 (µg/g)	5 min
Plasma/IV-12.5 mg/kg	107.93µM	0

Table 2.6: ZR2002 pharmacokinetics in brain, following oral (P.O.) administration of ZR2002 at 50, 75 mg/kg to Balb/c and CD-1 mice using HPLC.

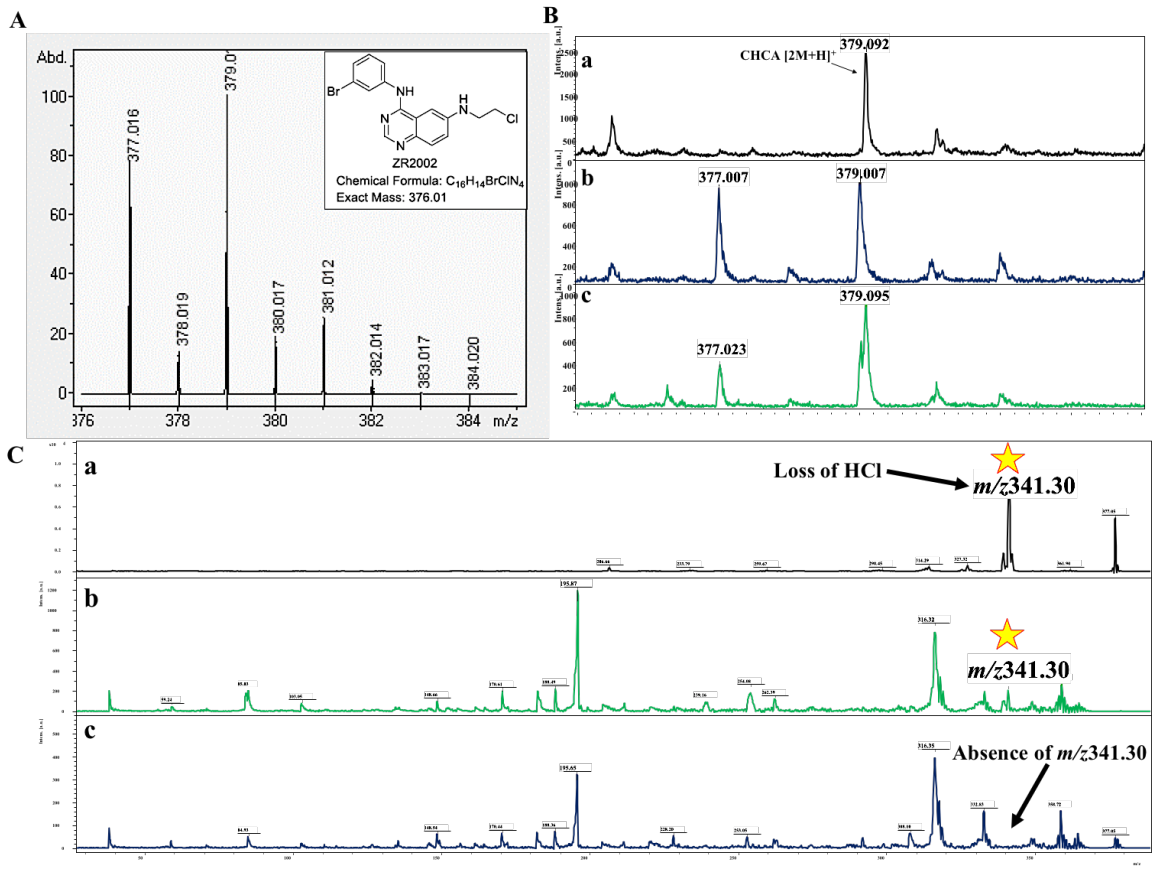
HPLC results	C_{max} (µg/g)	T_{max}
Brain/ P.O. (50 mg/kg)	3.58	3 hr
Brain/Balb/c/ P.O. (75 mg/kg)	8.11	NA
Brain/CD-1/ P.O. (75 mg/kg)	0.71	NA

Abbreviations: C_{max}, maximum plasma concentration; NA, not applicable; T_{max}, time to C_{max}.

2.4.3 ZR2002, is detected in brain of mouse with 1123IC7R intracranial tumor

In further studies, we assessed the brain permeability of ZR2002 in a preliminary experiment using an intracranial GSC (1123IC7R) xenograft mouse model. 1123IC7R GSC was injected intracranially into the brains of nu/nu mice (N=2). Tumor growth was monitored using BLI imaging. Once tumors reached a significant size mice were given a single oral dose of ZR2002

(150 mg/kg) or vehicle control and euthanized after 2 hours. Interestingly, analyzing brain tissue from non-treated mice and treated-mice using MALDI imaging revealed the presence and distribution of ZR2002 in the treated mouse brain. Isotopic pattern and MS/MS (*m/z*377.0→*m/z*341.0) were used to confirm the presence of ZR2002 on-tissue. As shown in figure 2.4 (A-C, this figure was included in the paper Submitted to Clinical Cancer Research) we utilized the primary fragment (m/z 377.0 → m/z 341.0) coming from the neutral loss of HCl to identify ZR2002 on-tissue. The yellow star on the histogram shows the presence of m/z 341.0 fragment in the treated mouse compared to its non-treated counterpart. Hematoxylin and eosin (H&E) staining confirmed the presence of tumor and its morphology (Fig. 2.4D). Optical scan of tissue after CHCA deposition revealed the brain margin on four serial brain sections (Fig. 2.4E, panel a). Distribution of ZR2002 is shown in the mouse brain treated with ZR2002 (turquoise signal), while absent in a mouse treated with vehicle control (Figure 4E, panel b). Heme (red, *m/z*616.18) was used as a marker for the lumen of blood vessels as shown previously [35, 36]. IMS (mass spectrometry) potentially detected ZR2002 dealkylated metabolite (ZR01 [25], *m/z*315.0), a potent EGFR inhibitor in the brain of treated mouse (Fig. 2.5 (A-C)). As expected, both signals of ZR2002 and ZR01 are co-localized on the treated tissue and absent from the control (overlay of ZR2002 and ZR01 signal as yellow color, Figure 2.5C). Supplementary figure S2.3 shows other possible metabolites of ZR2002. Of note, recent results suggest that tissue concentrations are not reflective of the amount of active drug present [37] therefore, further, studies are needed to verify whether treatment with ZR2002 can improve the survival of an intracranial GBM mouse model (Chapter 3).


A
Abd.
377.016
378.019
379.0
380.017
381.012
382.014
383.017
384.020
m/z
ZR2002
Chemical Formula: C16H14BrClN4
Exact Mass: 376.01
B
a
CHCA [2M+H]+
379.092
b
377.007
379.007
c
379.095
377.023
C
a
Loss of HCl
m/z341.30
b
m/z341.30
c
Absence of m/z341.30

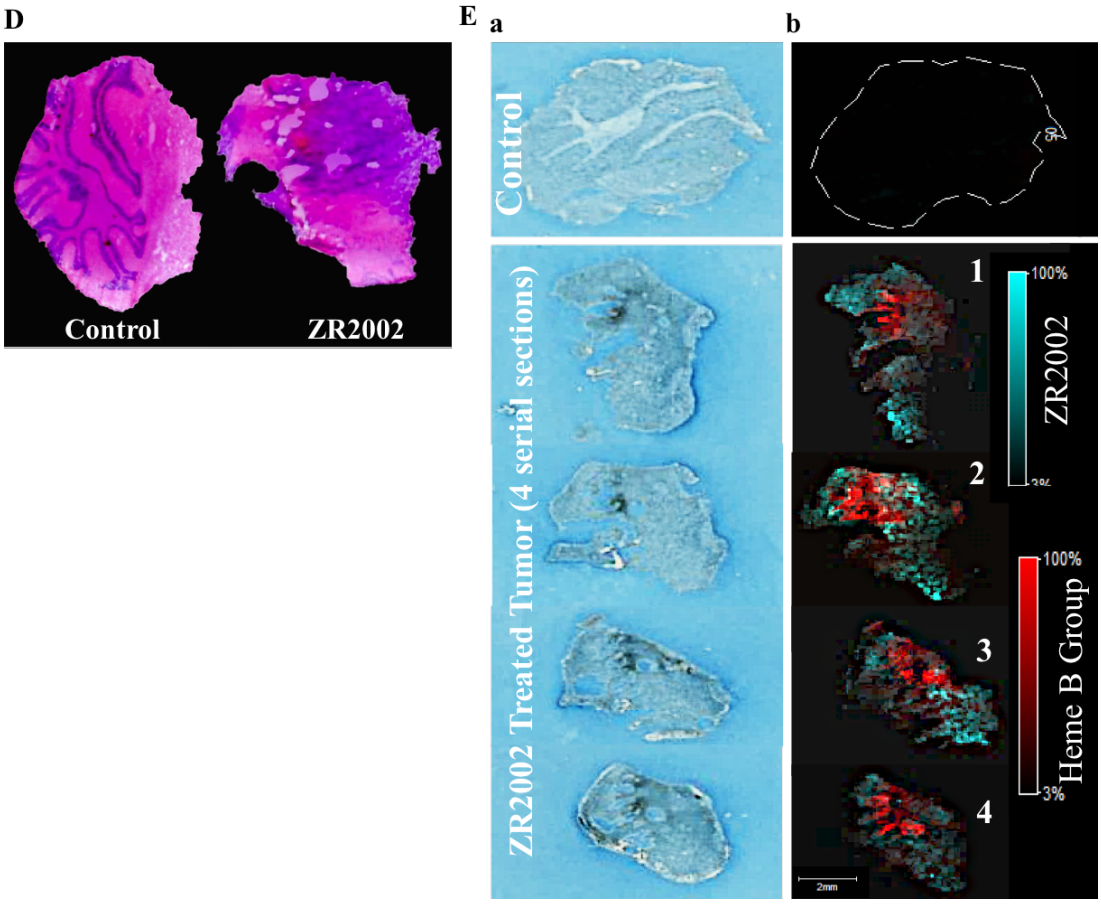


Figure 2.4 (A-E). **ZR2002, is detected in brain of mouse with 1123IC7R intracranial tumor using MALDI/Imaging Mass Spectrometry (IMS). A.** Theoretical isotopic pattern of ZR2002**. B.** MALDI isotopic pattern of ZR2002; a) TMZ-resistant GSC 1123IC7R tumor tissue from a mouse treated with vehicle control (absence of *m/z*377.0), b) control tissue manually doped with ZR2002 standard, c) TMZ-resistant GSC 1123IC7R tumor tissue from a mouse treated with ZR2002 (150 mg/kg) (presence of *m/z*377.0 and expected isotopes). **C.** Identification of ZR2002 by MS/MS; a) ZR2002 standard (primary fragment at *m/z*341), b) treated tumor (presence of the expected primary fragment at *m/z* 341) c) control tissue (background m/z signals). **D.** Hematoxylin and Eosin (H&E) staining confirms the presence of tumor and its morphology. **E. Panel a**. Optical scan of tissue after CHCA deposition revealed the brain margin on four serial brain sections. **Panel**

b. Distribution of ZR2002 in the mouse brain treated with ZR2002 (turquoise, *m/z*377.01) and absent in control mouse. Heme (red, *m/z*616.18) served as a marker for the lumen of blood vessels.

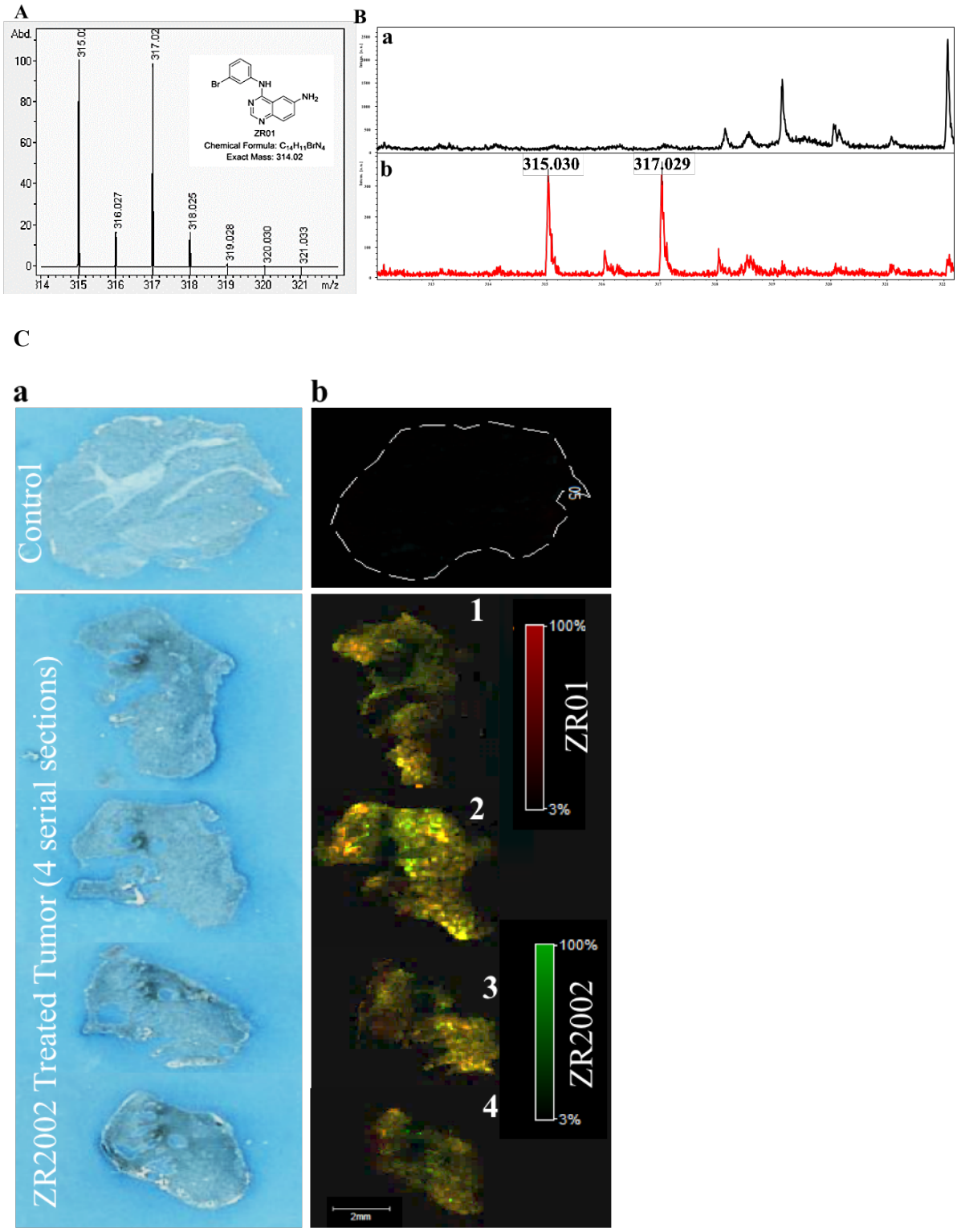


Figure 2.5 (A-C). ZR01 (a potent metabolite of ZR2002) is detected in the brain of mice with intracranial tumors. A. Theoretical isotopic pattern of ZR01**. B.** MALDI isotopic pattern of ZR01; a) TMZ-resistant GSC 1123IC7R tumor tissue from a mouse treated with vehicle control (absent), b) TMZ-resistant GSC 1123IC7R tumor tissue from a mouse treated with ZR2002 (150 mg/kg) (present). **C.** ZR01 was detected by MALDI IMS in the brain of the treated mouse at the same localization as ZR2002. **Panel a.** Optical image of four serial tissue sections (10 μm thickness) after CHCA deposition revealed the tumor margin on serial brain sections. **Panel b.** Overlay of ZR2002 (green) and ZR01 (red) signal is shown as a yellow color that is absent in control mouse tissue.

2.4.4 Pharmacodynamics of ZR2002 on EGFR signal transduction

To gain a better understanding of the underlying mechanism when cells are treated with ZR2002, compared with mice that are not treated, we examined the phosphorylation state of EGFR and downstream signaling mediators in tumor lysates derived from the single-agent treated xenografts. GSC line (1123ICP) was injected subcutaneously and after 2 weeks, 2 mice were treated with 150 mg/kg ZR2002 by oral gavage and 2 mice were given vehicle control substrate. Changes in phosphorylation levels of EGFR signal transduction pathway mediators were assessed in pharmacodynamics experiments at 2 hours, single administration of 150mg/kg ZR2002 (N=2) with immunoblotting analysis and compared with time-matched controls (N=2). Western blot on mice tissue indicated a drastic reduction P-EGFR, P-Erk1/2 and P-AKT after 2 hours treatment with ZR2002 (Fig. 2.6A). Furthermore, the effect of ZR2002 on phosphorylation of EGFR and its downstream signaling was also confirmed by immunohistochemistry results showing (Fig. 2.6B) a drastic effect of ZR2002 on inhibition of phosphorylated EGFR and Erk1/2 (Fig. 2.6B).

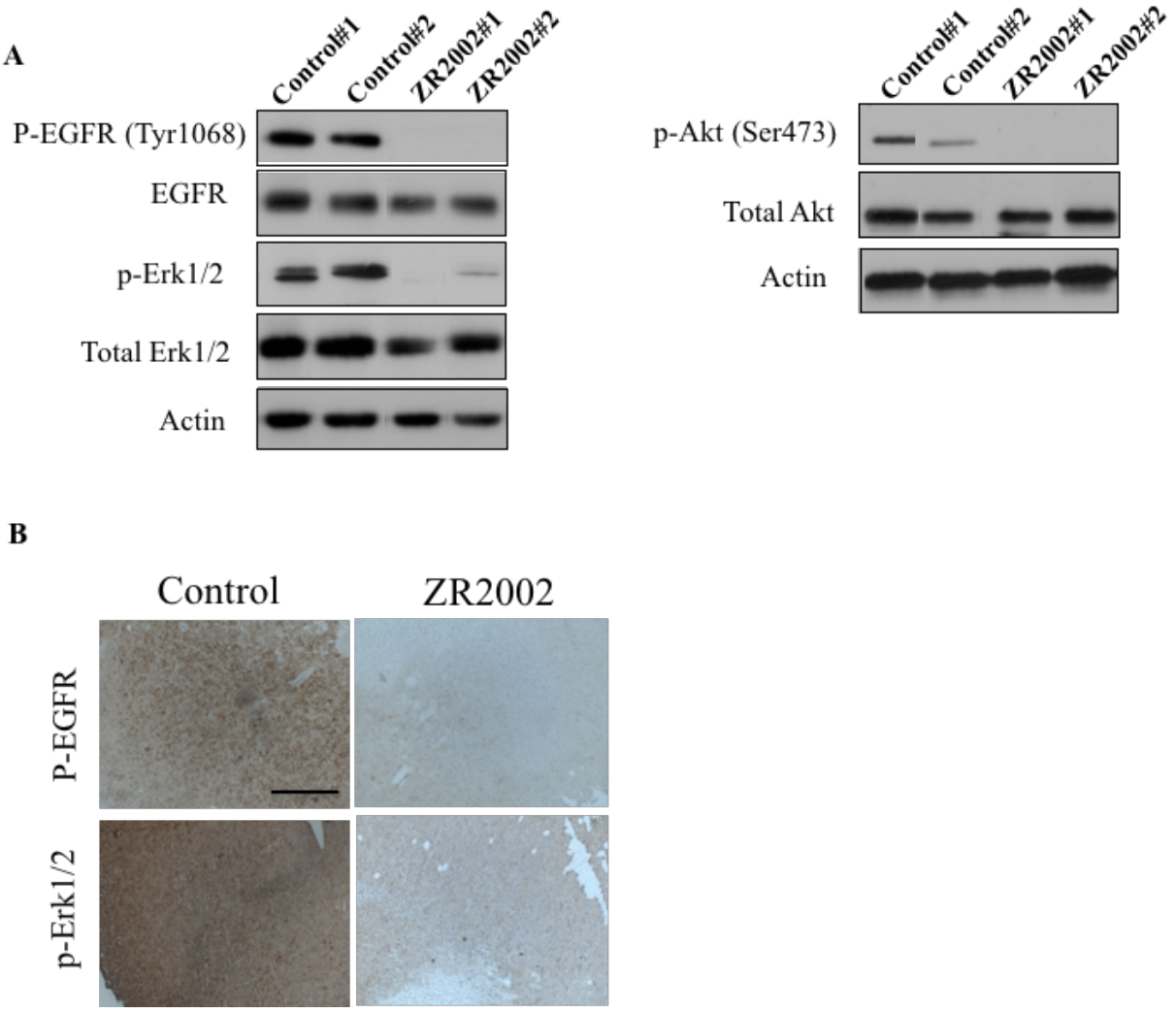


Figure 2.6 (A-B). Phosphorylation status of the epidermal growth factor receptor (EGFR) pathway during the pharmacodynamic study with ZR2002 using western blot analysis and immunohistochemistry. A. ZR2002 decreased phosphorylation of EGF Receptor which led to decreased EGFR Pathway Signaling. **B.** Immunohistochemistry analysis evaluating the effects of 2 hours incubation with 150mg/kg ZR2002. Scale bare= 100μm.

2.4.5 Systemic administration of ZR2002 reduces tumor growth in subcutaneous *in vivo* models

To further evaluate the efficacy of ZR2002 in a more clinically relevant model, NSG mice bearing intracranial 1123IC7R xenograft tumors were treated with vehicle control, ZR2002 (150 mg/kg/day), or TMZ (66 mg/kg) once daily in a subcutaneous NSG mouse model (Fig. 2.7A). On the 5th day after tumor implantation (to allow formation of a tumor mass) the anti-tumor efficacy of ZR2002 was measured by monitoring tumor volumes. As we anticipated (based on our dose finding results) the series of daily injections without break in between were well tolerated without unacceptable side effects.

ZR2002 induced a significant reduction in tumor size relative to the control group (p=0.021), while there was no significant difference between TMZ treatment and control group (p=0.19) (Fig. 2.7A). Despite tumor growth delay following ZR2002 treatment for 5 days (day 12 since GSC implantation), NSG mice had a rapid weight loss exceeding 20% of their initial body weights and required early euthanasia (Fig. 2.7B). Testing three different schedules to further evaluate toxicity did not delay tumor growth to the same extent as ZR2002 at 150mg/kg (Testing different dosing schedules of ZR2002 in NSG mice, supplementary Fig. S2.4). This deterioration evoked the phenotype of hypersensitivity to ionizing radiation in SCID mice known to be defective for DNA repair [38], due to deficiency in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), a key player in response to DNA damage. Nude mice with intact DNA-PKcs did not show any toxicity up to 150mg/kg (body weight measurement, cell blood counts and liver enzymes).

Collectively, our data highlight the striking effects of ZR2002 on mesenchymal TMZ-resistant GSC model. The potency of its DNA-damaging moiety might account for ZR2002-

mediated toxicity selectively in NSG mice defective for DNA-PKcs and subsequently for DNA repair.

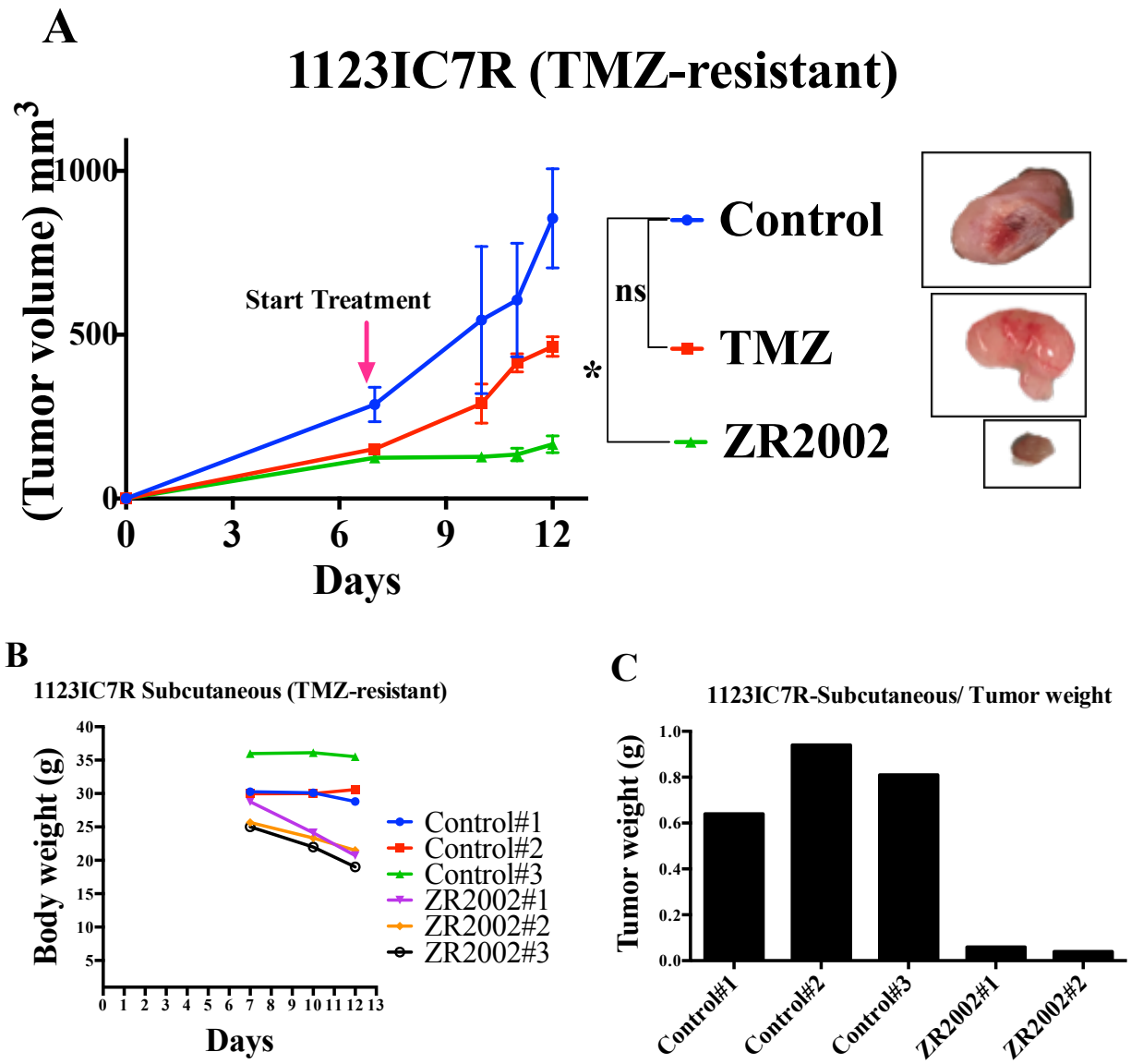


Figure 2.7 (A-C). Systemic administration of ZR2002 reduces tumor growth, in subcutaneous *in vivo* models. A. ZR2002 (150mg/kg; N=3), TMZ (66 mg/kg; N=3) or control (N=3) was given orally 7 days after subcutaneous tumor implantation for 5 days. Representative images of tumors for vehicle, TMZ or ZR2002 treatment group are shown. Each data point represents mean±SEM * (p-value=0.021). –ns (not significant) (p-value=0.19). **B.** Body weight

(g) of mice is shown in mice treated with Control or ZR2002. **C.** Tumor weight (g) of mice in the control and ZR2002 group are shown.

2.5 DISCUSSION

Several obstacles impede the drug discovery process for GBM treatment. First, the growth of refractory tumors is driven by multiple signaling disorders that often cannot be blocked by the use of a single drug. Numerous failed clinical trials suggest combination therapies will likely be the most promising method of GBM treatment. To overcome this obstacle, in this study aimed to test a "combi-molecule" strategy, we can inhibit the EGFR pathway that is frequently overexpressed in many cancers including GBM and damage the DNA simultaneously. ZR2002, is designed to inhibit EGFR TK via its quinazoline moiety and to damage DNA by its alkylating chloroethyl function [24, 25]. This new type of combi-molecule that does not require hydrolysis to generate the EGFR and DNA targeting properties is tested for the first time in GBM.

One other challenge in GBM therapy is identifying a therapy that is permeable to the BBB, and developing robust clinical trials to assess the effectiveness of the potential treatment. CNS drugs typically have a lower FDA-approval rate than non-CNS drugs. In a study of CNS drugs entered into clinical trials from 1990-2012, CNS drugs were 45% less likely to pass Phase III trials than non-CNS drugs, with 46% failing to show improved efficacy over placebo [39]. Despite decades of research in brain tumor drug discovery such as in the case of GBM, crossing the BBB remains a key obstacle in the development of drugs for brain diseases. This phenomenon suggests more rigorous preclinical research should be conducted before expensive clinical trials are initiated.

EGFR inhibitors are today well known for their strong *in vitro* effect however to date many clinical trials with these inhibitors failed to detect any improvement in outcome of patients in GBM [40]. Inefficient drug penetration and distribution in the CNS might be another major reason for failure of anti-EGFR therapies in clinical trials. For example, erlotinib and gefitinib are potent tyrosine kinase inhibitors (TKI) *in vitro*, but failed clinical trials due to limited brain exposure because of Pgp and ABCG2-mediated efflux [41, 42]. In addition, gefitinib inhibits signaling of EGFR proteins with mutations in exons 19 and 21 of the TK domain that are often absent in gliomas [43].

Indeed, gefitinib did not result in improved overall survival in a phase II trial in recurrent glioblastoma [44]. This drug also failed in a phase I/II trial in combination with radiation in newly diagnosed glioblastoma [45]. Clinical trials which have utilized EGFR inhibitors such as gefitinib in combination with traditional chemotherapy did not provide additional benefit to GBM patients. Our results suggest that the combination of a small chloroethyl cytotoxic function to damage DNA with an EGFR targeting quinazoline can be an effective strategy in GBM therapy.

Because of the importance of preclinical pharmacokinetic/pharmacodynamic studies in GBM, we investigated the safety and BBB permeability of ZR2002, necessary to achieve efficacy in orthotopic xenografts and facilitate clinical translation. Before the pharmacokinetic/pharmacodynamic investigations in tumor free mice, the safety profile of ZR2002 was obtained and showed that up to 150 mg/kg of this drug is safe in our pre-clinical stetting using nude mice with intact DNA repair pathway. Our study was in accordance with previous *in vivo* studies using gefitinib showing the highest nontoxic oral dose of 150 mg/kg [46, 47].

As most preclinical pharmacokinetic and pharmacodynamics studies in mice assume homogeneous drug distribution in the brain therefore they use measurements from whole brain homogenates and averaged drug concentration for interpretation of their data. Methods using HPLC and LC-MS/MS were developed for the quantification of ZR2002 in plasma and brain samples. These methods were successfully applied to investigate the pharmacokinetics of ZR2002 in mice after administration at various doses and roots of administration. The current study was designed to understand and characterized the pharmacokinetic and pharmacodynamic characteristics of ZR2002, and as a first investigation of this drug in GBM.

Our pharmacokinetic study testing different routes of administration in mice suggests that ZR2002 is able to cross the BBB by P.O. at the maximum dose (75mg/kg) tested. Quantitative analysis of ZR2002 showed that an average of 4μg/g of ZR2002 is detected in the mouse brain. These results suggest that ZR2002 can be delivered across the blood brain barrier and can become available in brain tissue soon after its absorption. Since there are few orally available administered chemotherapeutics currently available with demonstrated efficacy against brain tumors the results of this study are very interesting. In our next step (chapter 3) we aim to assess whether administration of ZR2002 at the dose 150 mg/kg in tumor bearing mice will be sufficient to achieve the potential therapeutic in GBM intracranial mouse models.

We did not use tumor bearing mice for our pharmacokinetic studies. However, it is important to take into account when measuring, the concentrations of ZR2002, vascularity, blood flow, and interstitial fluid pressure and genomic characteristics of tumors that could impact both pharmacokinetic and pharmacodynamic variability, and ultimately, personalized medicine [48]. Since ZR2002 is a combi-molecule it is extremely important to use up to date pharmacokinetic/pharmacodynamic tools as multidrug combinations designed that may be act on

the same and intersecting cell signaling pathways. Clear understanding of the pharmacokinetic/pharmacodynamics of drugs will also provide a unique tool to attain therapeutically relevant pharmacokinetic concentrations in the entire tumor that will produce optimal pharmacodynamic effects and to understand why drugs may be inactive and further to offer computational approaches to mitigate the effects of tumor heterogeneity. This will also provide a unique tool to attain therapeutically relevant pharmacokinetic concentrations in the entire tumor that will produce optimal pharmacodynamic effects. Treatment with ZR2002 was associated with marked dephosphorylation of EGFR and Erk1/2 and t p-Akt (Ser473) that proves the combi targeting activity of this drug.

Finally, we used a unique experimental GSCs model of TMZ-resistant in GBM to assess the ability of ZR2002 to overcome TMZ resistance in highly aggressive mesenchymal GSC lines. The potential for inhibition of the EGFR pathway in GSCs is based on previous studies that indicate that EGFR and EGFRvIII have been coupled to a CSC phenotype. For example, EGFR knockdown in EGFR-positive GBM neurosphere cultures led to differentiation and less malignant tumors in vivo [49] and in another study EGFR inhibition resulted in reduced sphere formation in the presence of EGF in EGFR positive neurosphere cultures [50].

It is well documented that EGFR and downstream proteins that are activated by it such as MAPK are involved in DNA repair or other mechanisms of resistance to DNA damaging agents [16, 51]. Thus, blockade of EGFR and MAPK activation using small molecule inhibitors such as ZR2002 may down regulate DNA repair activities required to rescue the cells, thereby enhancing the cytotoxic effects of the concomitantly induced DNA lesions. EGFR directly interacts with and enhances the activity of DNA-PKcs, which is known to play a major role in non-homologous end joining (NHEJ) of double strand break repair. Previous findings suggest that the alteration of

EGFR, excision repair cross-complementation group 1 (known as ERCC1) and basal expression of the X-ray repair cross-complementing group 1 (XRCC1) protein are also important for the repair of DSBs [52]. ZR2002 caused severe toxicity in NSG mice that are known to be defective for DNA repair highlighting the effect of ZR2002 on inhibiting the EGFR pathway therefore inhibiting DNA repair in which the effect of this drug is more on this strain of mice that is well known to be defective for DNA repair.

Collectively, the P.O. active ZR2002 is a safe drug and can cross the BBB. Our findings demonstrate ZR2002 has drastic effects on glioma stem cells and more interestingly has the potential to overcome TMZ resistance in GBM patients. Additionally, because there are relatively few P.O. administered chemotherapeutics currently available for treatment of brain tumors, ZR2002 represents a promising new agent for the treatment of these tumors. Further studies are warranted to verify the mechanism of action of ZR2002 and to test it in an intracranial mouse model with intact DNA repair system.

2.6 ACKNOWLEDGEMENTS

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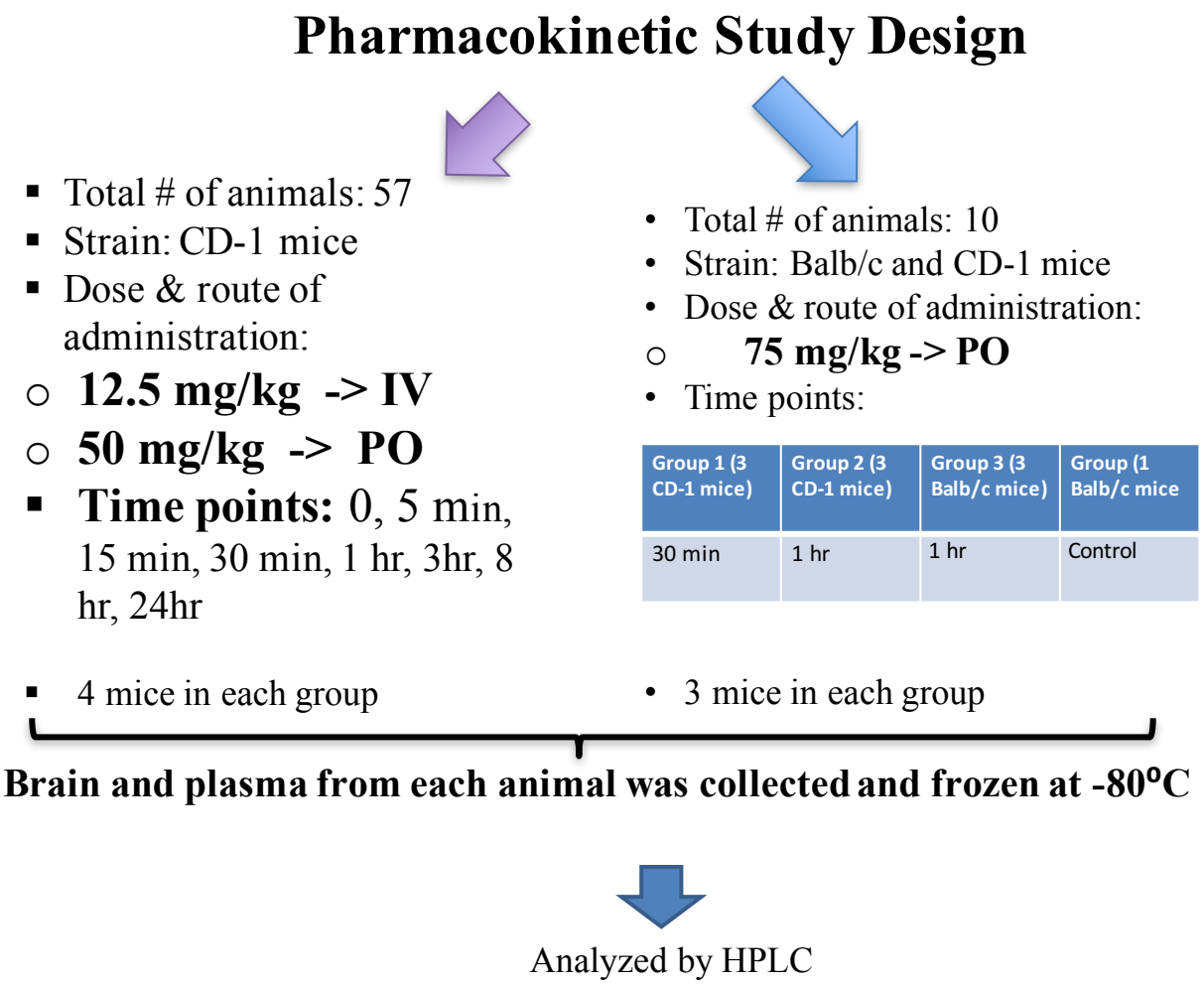
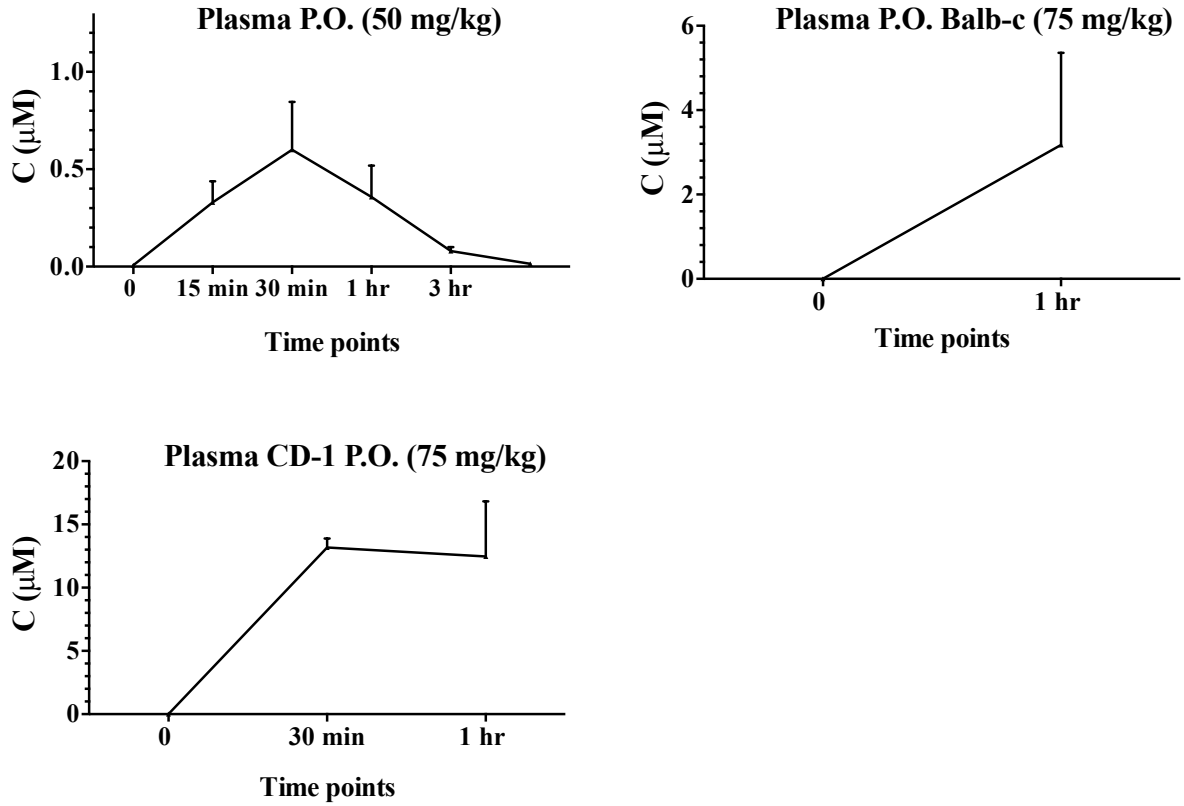
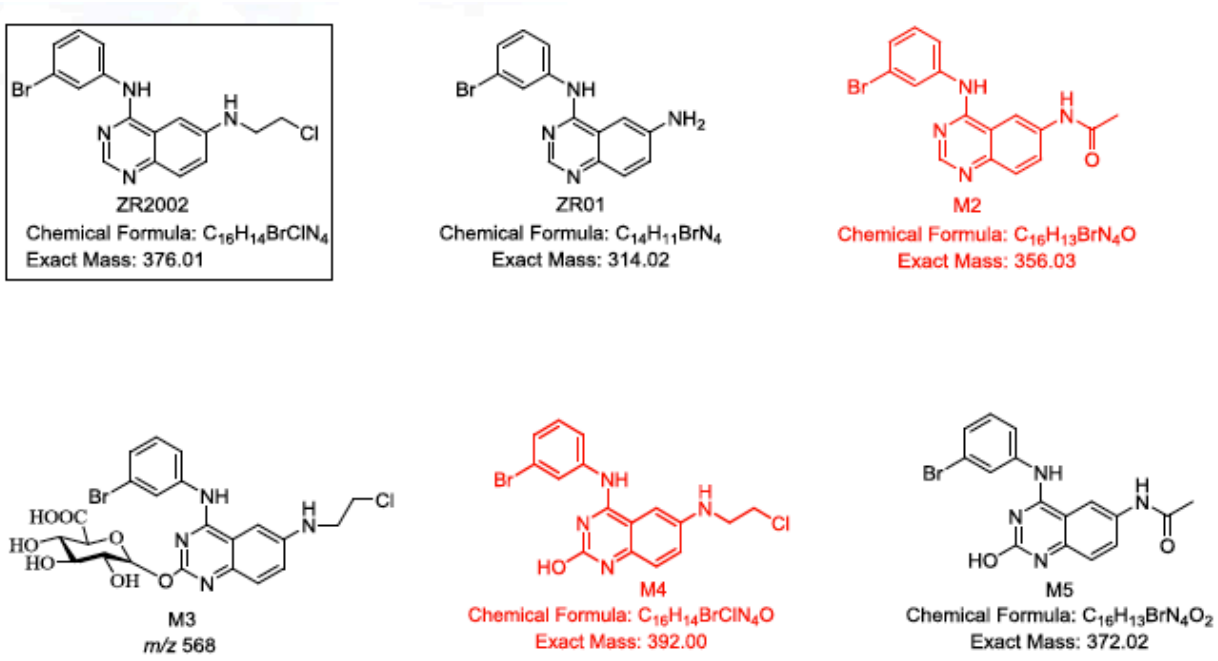
2.8 SUPPLEMENTAL FIGURES



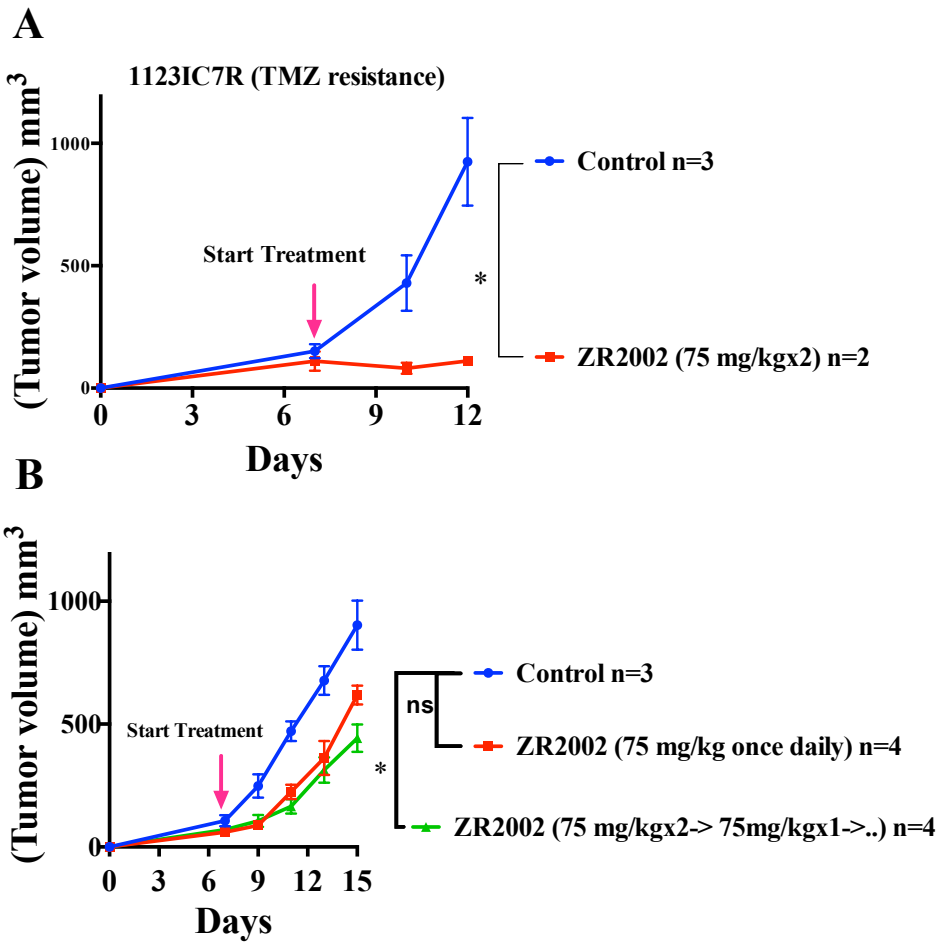
Supplementary diagram S2.1 Design of pharmanokinetic (PK) studies in mice. Two sets of pharmanokinetic (PK) studies were performed. In the first set 57 mice were given a single dose of 12.5 mg/kg ZR2002 by intravenous injections (IV) or a single does of 50 mg/kg orally (P.O.). Blood samples were collected into heparinized tubes at 0, 5 min, 15 min, 30 min, 1 hr, 3hr, 8 hr, 24 hr and 0, 15 min, 30 min, 1 hr, 3hr, 8 hr, 24 hr for IV and P.O. injections respectively (N=4/Time point).



Supplementary Figure S2.2. Concentration-time curves for ZR2002 in plasma after 50 and 75 mg/kg P.O. dose of ZR2002 in different strains of mice using LC/MS. Each point represents the mean value from at least 2 mice.



Supplementary Figure S2.3. The structures of ZR2002 and its possible metabolites are shown.



Supplementary Figure S2.4 (A-B). Testing different dosing schedules of ZR2002 in NSG mice. A. On day 7 after subcutaneous injection of 1123IC7R TMZ-resistant GSC, vehicle control (N=3) or ZR2002 (75 mg/kg; N=2) was given orally to NSG mice in 2 doses (8 hours apart) every day until day 12, wherein mice were sacrificed due to weight loss exceeding 20% of their initial body weights, which was not related to tumor growth. Each data point represents mean±SEM * (p-value=0.044). **B.** On day 7 after subcutaneous injection of 1123IC7R TMZ-resistant GSC, vehicle control (N=3) or ZR2002 were given to NSG mice by oral gavage: 75 mg/kg once daily (N=4); or alternate schedule with 75 mg/kg twice daily (8 hours apart) followed by one dose 75 mg/kg the next day (N=4). On day 15 mice were sacrificed due to excessive tumor growth

(ulceration of tumors reaching their max size. Each data point represents mean±SEM * (p-value=0.024). ns (not significant) (p-value=0.89).

CONNECTING TEXT

In the previous chapter, we evaluated the safety and optimal dose of ZR2002 to be used in *in vivo* efficacy studies (Chapter 2). We also showed the blood brain barrier permeability and oral bioavailability of ZR2002. In particular, we showed that ZR2002 at doses up to 150 mg/kg/day was tolerated following the use of 2 different schedules (5 days on- 5 days off- 5 days on or 21 continuous days treatment with ZR2002) in athymic mice that have intact DNA repair pathways. Brain permeability of ZR2002 was also confirmed by two routes of administration: oral gavage and intravenous injection. We also showed that the concentration of ZR2002 in the brain is slightly higher when given orally. Interestingly, MALDI IMS confirmed the presence of ZR2002 and its metabolite (ZR01) in the brain of nude mice with intracranial tumors. In this chapter, we also tested the pharmacodynamics of ZR2002 on EGFR signal transduction and showed that this drug drastically inhibits EGFR phosphorylation and its downstream signaling (Erk1/2 and AKT). These observations directed our interest to verify the potency of ZR2002 in a preliminary *in vivo* experiment in a subcutaneous TMZ-resistant *in vivo* model in which we showed that systemic administration of ZR2002 drastically reduces tumor growth compared to TMZ, which is well known as the standard treatment of GBM. Of note, the toxicity seen in NSG mice after treatment with ZR2002 in this subcutaneous model highlighted the importance of DNA repair in the context of ZR2002 treatment and proves the fact that this drug might work through DNA-PKcs.

Based on these promising results, in the next chapter (chapter 3) we focused on the cell-context dependent effects of ZR2002 in GBM stem cells and U87/EGFR isogenic cell lines with different EGFR levels. Also, we provide the proof-of-concept for *in vivo* anti-tumor properties of ZR2002 for further pre-clinical evaluation of this first-in-class drug in GBM. Specifically, ZR2002 inhibited EGF-induced autophosphorylation of EGFR and EGFRvIII and downstream Erk1/2

phosphorylation, significantly increased DNA strand breaks and induced wild-type *TP53* activation. Oral administration of ZR2002 significantly increased survival in an orthotopic EGFRvIII mouse model and it also improved survival of mice harboring intracranial mesenchymal TMZ-resistant GSC line and decreased EGFR, Erk1/2 and AKT in tumor tissue *in vivo* (chapter 3).

Chapter 3.

Antitumor activity of a binary EGFR/DNA targeting strategy overcomes resistance of glioblastoma stem cells to temozolomide

Zeinab Sharifi[1,2], Bassam Abdulkarim[3,2], Brian Meehan[2], Janusz Rak[4,2], Paul Daniel[3,2], Julie Schmitt[5,2], Nidia Lauzon[2], Kolja Eppert[4,2], Heather M. Duncan[1,2], Kevin Petrecca[6], Marie-Christine Guiot[7,2], Bertrand Jean-Claude[5,2] and Siham Sabri[7,2]*

[1]Division of Experimental Medicine, [2]Research Institute of McGill University Health Centre, [3]Department of Oncology, [4]Department of Pediatrics, [5]Department of Medicine, [6]Department of Neurology and Neurosurgery, [7]Department of Pathology; McGill University, Montreal, Quebec, Canada.

Running title: ZR2002 as a novel EGFR/DNA combi-molecule in glioblastoma

Keywords: Glioblastoma, EGFR, temozolomide-resistant, combi-molecule, glioblastoma stem cells

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***Corresponding author:** Siham Sabri, PhD, The Research Institute of the McGill University Health Centre, 1001 Decarie Blvd, Montreal, Quebec, H4A 3J1, Office: EM2.3218; Phone: 514-934-1934 Ext.: 44686 siham.sabri@mcgill.ca

Conflict of interest: The authors declare no conflict of interest

3.1 ABSTRACT

The limited clinical success of traditional DNA-damaging chemotherapeutics, such as temozolomide (TMZ) prompted the quest for novel strategies in glioblastoma (GBM). Targeting the epidermal growth factor receptor (EGFR), known to harbor genomic alterations in half of GBMs failed to overcome this therapeutic deadlock. Here, we investigated the effects of ZR2002, a 'combi-molecule' designed to inflict DNA damage through its chlorethyl moiety and induce irreversible EGFR tyrosine kinase inhibition with subsequent inhibition of EGFR-mediated DNA repair. We assessed cytotoxicity of ZR2002, its DNA-damaging properties and molecular effects using a panel of patient-derived GBM stem cells (GSCs) and U87/EGFR isogenic cell lines stably expressing EGFR/wild-type or EGFRvIII, the ligand-independent activated *EGFR* mutation. ZR2002 inhibited proliferation and neurosphere formation of GSCs including TMZ-sensitive and resistant mesenchymal *in vivo* derived GSC sublines, with negligible effects on normal human astrocytes. ZR2002 induced submicromolar growth inhibitory effects and hindered clonogenic potential of U87/EGFR isogenic cell lines. ZR2002 inhibited EGF-induced autophosphorylation of EGFR and EGFRvIII and downstream Erk1/2 phosphorylation, significantly increased DNA strand breaks and induced wild-type *TP53* activation. Its cytotoxic effects were mediated through a p53-dependent mechanism. Oral administration of ZR2002 significantly increased survival in an orthotopic EGFRvIII mouse model. ZR2002 also improved survival of mice harboring intracranial mesenchymal TMZ-resistant GSC line and decreased EGFR, Erk1/2 and AKT in tumor tissue *in vivo*. These findings provide the proof-of-concept for the blood brain barrier permeability, oral bioavailability and *in vivo* anti-tumor properties of ZR2002 for further pre-clinical evaluation of this first-in-class drug in GBM**.** Significance: ZR2002 is an effective EGFR/DNA combi-targeting strategy to overcome EGFRvIII-driven and temozolomide-resistant GSC growth in glioblastoma.

3.2 INTRODUCTION

Glioblastoma (GBM), a grade IV astrocytoma, is the most common and aggressive malignant primary brain tumors in adults [1]. The standard treatment of patients newly diagnosed with GBM, implementing surgical tumor resection, chemoradiation using the DNA alkylating agent temozolomide (TMZ) followed by adjuvant TMZ (Stupp-regimen), improved the median survival to 14.6 months [2]. Tumor recurrence is inevitable, poses major challenges for clinical management and leads to a fatal outcome. Several mechanisms account for GBM recurrence including activity of the O6-methylguanine-DNA methyltransferase (MGMT), which repairs TMZ-induced O6-methylguanine adducts [3]. Chemo- and radioresistance of a small population of self-renewing, tumorigenic cancer stem cells termed tumor-initiating cells or glioma stem cells (GSCs) [4] prompted the need for effective molecularly targeted therapies [5]. The epidermal growth factor receptor (EGFR), a key oncogene driver of chemo- and radioresistance displays gene alterations in more than half of primary GBMs [6]. Activation of EGFR by ligand binding (e.g., EGF) triggers a cascade of cellular signaling events associated with increased cell proliferation and survival through downstream effectors including PI3-K/Akt, Ras-Raf-MAPK and protein kinase C signaling pathways. The most common *EGFR* mutation, EGFRvIII (EGFR type III, de2-7, Δ EGFR) [7] results in a ligand-independent and constitutively active receptor. EGFR and EGFRvIII confers protective effects in response to DNA-damaging agents through several mechanisms including increased repair of DNA strand breaks (DSBs) [8]. Likewise, alterations of the tumor suppressor *TP53* pathway in 30% of patients newly diagnosed with GBM [9] affect DNA repair, cell cycle progression, apoptosis and senescence in response to various stress stimuli through transcriptional activation of multiple target genes, including $p21^{WAF1/CIP1}$ (p21) [10].

The EGFR pathway can be disrupted by EGFR tyrosine kinase inhibitors (TKIs), such as ZD1839 (Iressa, Gefitinib) an orally active, selective EGFR-TKI that blocks signal transduction pathways involved in cancer cell proliferation and survival [11]. However, treatment with adjuvant gefitinib did not significantly improve progression free survival (PFS) or overall survival (OS) in patients newly diagnosed with GBM [12] .

In this study, we anticipate that the combinatorial approach termed "combi-targeting" seeking to design a "combi-molecule" as a single agent with two mechanisms of action could be applied. The combi-targeting strategy has been described for breast [13], lung [14] and prostate cancers, while its use for GBM had scant attention [15]. Type II combi-molecules do not require hydrolysis to exert their dual mechanism of action [16]. Our group has characterized the dual EGFR/DNA targeting property of ZR2002 (6-(2-chloroethylamino)-4-anilinoquinazolines), a type II molecular prototype in breast cancer cell lines, wherein it irreversibly blocks EGFR-induced signaling and exerts its DNA-damaging function through its chlorethyl moiety [13]. We hypothesized that ZR2002 was designed to target EGFR and inflict DNA damage [13], might overcome the activation of intrinsic or adaptive DNA damage pathways involved in chemo- and radioresistance of GSCs [4]. Given the central role of GSCs in tumor initiation, chemo- and radioresistance and tumor relapse [17], we used patient-derived GSC neurosphere cultures to investigate the effects of ZR2002. Herein, we report the mechanism of action and the *in vivo* efficacy of a combi-molecule designed to possess mixed EGFR-TK inhibitory and DNA-damaging properties for the first time in GSCs and U87MG GBM cell lines isogenic for EGFR and EGFRvIII. We show that ZR2002 drastically suppressed GSC neurosphere growth in all GSC lines tested including a highly aggressive mesenchymal TMZ-resistant GSC line. ZR2002 induced cytotoxic effects in U87MG isogenic GBM cell lines stably expressing EGFR or EGFRvIII within

a submicromolar range. Mechanistically, ZR2002-induced cellular effects were associated with decreased phosphorylation of EGFR, Erk1/2-induced signaling, increased DNA damage and activation of wild-type (wt)*TP53*. Importantly, we showed that oral administration of ZR2002 significantly increased survival in U87/EGFRvIII and the TMZ-resistant GSC orthotopic xenografts mice models. In the latter, we further provide the proof-of-concept for its *in vivo* efficacy through decreased EGFR, Erk1/2 and AKT phosphorylation strongly supporting the potential clinical benefit of such combi-molecule in GBM.

3.3 MATERIALS AND METHODS

3.3.1 Cell culture, drug treatment and transfection

GSCs OPK111, OPK49, OPK161, 48EF and OPK257 GSC lines (isolated from patients newly diagnosed with GBM, provided by Dr. K. Petrecca) were characterized by our group [18]. TMZ-sensitive (1123IC12S) and TMZ-resistant mesenchymal *in vivo* derived GSCs sublines (1123IC7R and 1123IC8R) were established in the laboratory of Dr. J. Rak [19]. Low passage number GSCs were maintained in NeuroCult NS-A Basal Medium (STEMCELL Technologies) with NeuroCult NS-A proliferation supplements [18]. U87MG, and its isogenic counterparts stably transfected to overexpress EGFR (U87/EGFRwt) or EGFRvIII (U87/EGFRvIII) GBM cell lines (provided by Dr. B. Jean-Claude) [15] and normal human primary astrocytes (NHA) (provided by Dr. J. Rak) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and incubated in 5% CO2 atmosphere at 37°C. Cells were treated with DMSO (control), gefitinib/Iressa (Ark Pharm), TMZ (Tocris), or ZR2002 (designed and synthesized in the laboratory of Dr. B. Jean-Claude, Supplementary Fig. S3.1) at the indicated doses. PLKO.1 shp53 vector (Addgene #19119) was used to generate p53 knockdown [20] of OPK49 GSC line. Cells were tested for mycoplasma using the MycoAlert™ kit (Lonza).

3.3.2 GSCs growth assays and neurosphere formation assay

GSC neurosphere cultures were dissociated with Accumax™ (Millipore), then seeded in triplicate at 1,500 cells per 96-well for 24h before treatment with TMZ, gefitinib, ZR2002 or DMSO for 5 days. Cell viability was assessed using the alamarBlue® assay (Invitrogen) according to the manufacturer's instructions. Drug sensitivity was also assessed using the neurosphere assay. Cells were seeded overnight, treated the next day with TMZ, gefitinib, ZR2002 or DMSO, and the number of spheres over 50µm in size was counted 14 to 20 days later [18].

3.3.3 EGF-Induced autophosphorylation assay and western blot analysis

GSCs were seeded in NeuroCult™ (STEMCELL) medium overnight. U87/EGFR isogenic cell lines were allowed to attach overnight, then serum-starved for 24 hours. U87/EGFR isogenic cell lines or GSCs were exposed to the drugs for 2 hours rinsed with PBS, treated with EGF (50 ng/mL) for 20 minutes, then rinsed with ice-cold PBS to stop treatment before lysing. For immunoblotting analysis (Fig. 3.6A-B), cells were seeded overnight in standard medium, treated (drug or control) for 48 hours at the indicated concentrations, washed twice (established cell lines) or collected (GSCs) with ice-cold PBS, then lysed with RIPA buffer (Boston BioProducts) supplemented with 0.2 mM sodium orthovanadate, protease (Sigma-Aldrich) and phosphatase (Roche Diagnostics) inhibitors cocktails. Western blotting analysis on tumors excised from orthotopic mice xenografts was performed following brain tumor tissue homogenization, lysates preparation and analysis of the protein concentration, as described above. Proteins (30μg, Pierce BCA protein assay kit, Thermo Fisher Scientific Inc.) were electrophoretically separated in 12% SDS-PAGE and transferred onto PVDF membranes. Membranes were probed for phosphorylated EGFR (p-EGFR/Tyr1068), total EGFR, phospho-p44/42 MAPK (p-Erk1/2), total Erk1/2, p-Akt/Ser473, $p21^{WAF1/CIP1}$ (Cell signaling), total Akt1/2/3 (H-136), p53 (DO-1, mutant and wild-type forms) (Santa Cruz) and β-actin (Sigma-Aldrich). Appropriate horseradish peroxidase-conjugated secondary antibodies (Life technologies) and chemiluminescence detection were used (Amersham, GE Healthcare). Quantification of Western blotting data normalized to corresponding total antibodies and controls was performed using ImageJ software.

3.3.4 Alkaline comet assay

Cells were seeded, allowed to attach for 24 hours, treated with DMSO, TMZ or ZR2002 (0, 1, 5, 10, 20, 50 or 100μM) for 2 hours. Cells were harvested, washed twice in PBS, electrophoresed at 20 V, 400 mA for 20 min. Membranes were then, processed for staining with SYBR Gold (Molecular Probes). Comets (at least 50) were visualized at 400X magnification and DNA damage was quantified using Comet Assay IV software to calculate tail moments, as previously described [14].

3.3.5 MTT cell proliferation assay

Normal human astrocytes (NHA) and U87/EGFR isogenic cell lines (1000-1500 cells) were plated in triplicate in 96-well plates and allowed to adhere overnight. Cell viability and proliferation was measured following 5 days of treatment with indicated concentrations of each drug or DMSO using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Absorbance at a wavelength of 570 nm was measured on a microplate reader (Bio-Rad).

3.3.6 Clonogenic assay

Cells were plated in 6-well plates, allowed to adhere overnight and treated with ZR2002 or DMSO at varying concentrations in standard medium for 2 hours. The medium was replaced with drug-free medium and cells were incubated for additional 8-14 days or until colonies (more than 50 cells) were formed. Cells were then fixed with 10% formalin and stained using 1.5% methylene blue to count colonies. The surviving fraction was normalized to the plating efficiency of the corresponding controls [18, 21].

3.3.7 Intracranial U87/EGFRvIII-Luc2 and 1123IC7R-Luc2 xenografts

Experiments were performed in accordance to a protocol approved by our Institutional

Animal Care Committee (McGill University Health Centre Research Institute and McGill University). Kaplan–Meier survival studies, U87/EGFRvIII and 1123IC7R GSC line were lentivirally transduced with a luciferase-BFP dual gene vector (Luc2 pSMALB; Luc2 cloned from pGL4.51 (Promega) into the pSMALB backbone described previously) [22] to monitor tumor growth using bioluminescence imaging (BLI). For orthotopic injection, cells were dissociated to single-cell suspensions, and 20,000 cells were stereotactically injected into brains of 6 to 8-week-old nude mice (Charles River). Three days after U87/EGFRvIII-Luc2 cells implantation, mice were anesthetized with isoflurane and subsequently administered 15µg/mL of D-luciferin (D-luciferin potassium salt (Cedarlane) via intraperitoneal injection to perform BLI for pre-treatment time point. For mice injected with U87/EGFRvIII cell line, mice were randomized into vehicle (control) (N=7) or ZR2002 150mg/kg, p.o (N=6). For mice injected with 1123IC7R GSC line, mice were randomized into vehicle (control) (N=6) or TMZ/66mg/kg (N=6) or gefitinib/150mg/kg (N=6) or ZR2002/150mg/kg (N=6) given orally. Tumor growth was monitored by BLI [19] using an IVIS 200 scanner (PerkinElmer).

For intracranial tumor models, body weights were recorded and mice were sacrificed upon significant weight loss (>20%) or presentation of neurologic symptoms necessitating euthanasia.

3.3.8 Statistical analysis

Data are reported as mean +/-SD and are representative of at least 3 independent experiments run in triplicate, unless otherwise stated. Statistics were performed using unpaired two-tailed Student's t-test. A Kaplan–Meier survival test was used for survival studies in mice. Analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). P-values <0.05 were considered statistically significant.

3.4 RESULTS

3.4.1 Anti-proliferative effects of ZR2002 in GSCs

We first investigated the effect of ZR2002 in a panel of patient-derived primary GSCs (OPK161, OPK49, 48EF, OPK257 and OPK111), which exhibit different EGFR expression levels, as shown by immunoblotting analysis (Fig. 3.1A). ZR2002 reduced the viability (alamarBlue®) of OPK111, OPK49, OPK161, 48EF and OPK257 at the half maximum inhibitory concentration (IC50) concentrations of 0.69, 0.60, 0.50, 0.39 and 1.77μM, respectively (Fig. 3.1B, Supplementary Table S3.1). Of note, ZR2002 is highly effective against MGMT-positive GSCs (48EF, OPK111 and OPK161) and MGMT-negative (OPK257 and OPK49), previously characterized by our group [18] for their MGMT expression levels (Supplementary Table S3.1). Interestingly, all GSC neurosphere cultures were highly resistant to TMZ (IC50>100μM) and displayed IC50 concentrations ranging between 24 to 55μM in response to gefitinib treatment.

We subsequently verified the effects of ZR2002 on a mesenchymal TMZ-sensitive GSC (1123IC12S) and two TMZ-resistant variant GSC lines (1123IC7R and 1123IC8R) previously derived from NOD SCID gamma (NSG) immunodeficient mice harboring intracranial tumors of the parental GSC line (1123ICP) and treated *in vivo* with TMZ [19]. As expected, 1123IC12S was sensitive to TMZ (IC50:~21μM) and 1123IC7R and 1123IC8R were resistant to TMZ (IC50: >100μM) [19] .

Strikingly, our results revealed that 1123IC12S, 1123IC7R and 1123IC8R were highly sensitive to ZR2002, reaching IC50's in the submicromolar range (~0.6μM) regardless of their sensitivity to TMZ (Fig. 3.1C). Supplementary Figure S3.2 shows a representative image of the exquisite potency of ZR2002 against 1123IC7R cells compared to DMSO control. To ascertain whether ZR2002 anti-proliferative effects might affect normal brain cells, we used MTT proliferation assay to investigate its effects on NHA. ZR2002 exhibited at least ten-fold higher

IC50 for NHA compared to GSCs (~12μM, Fig. 3.1D), suggesting that its anti-proliferative effects on GSCs may not affect non-cancer cells. Collectively, our data demonstrate that ZR2002 had striking anti-proliferative effects on GSCs derived from patients newly diagnosed with GBM, a mesenchymal TMZ-sensitive and resistant GSC lines, but not on NHA.

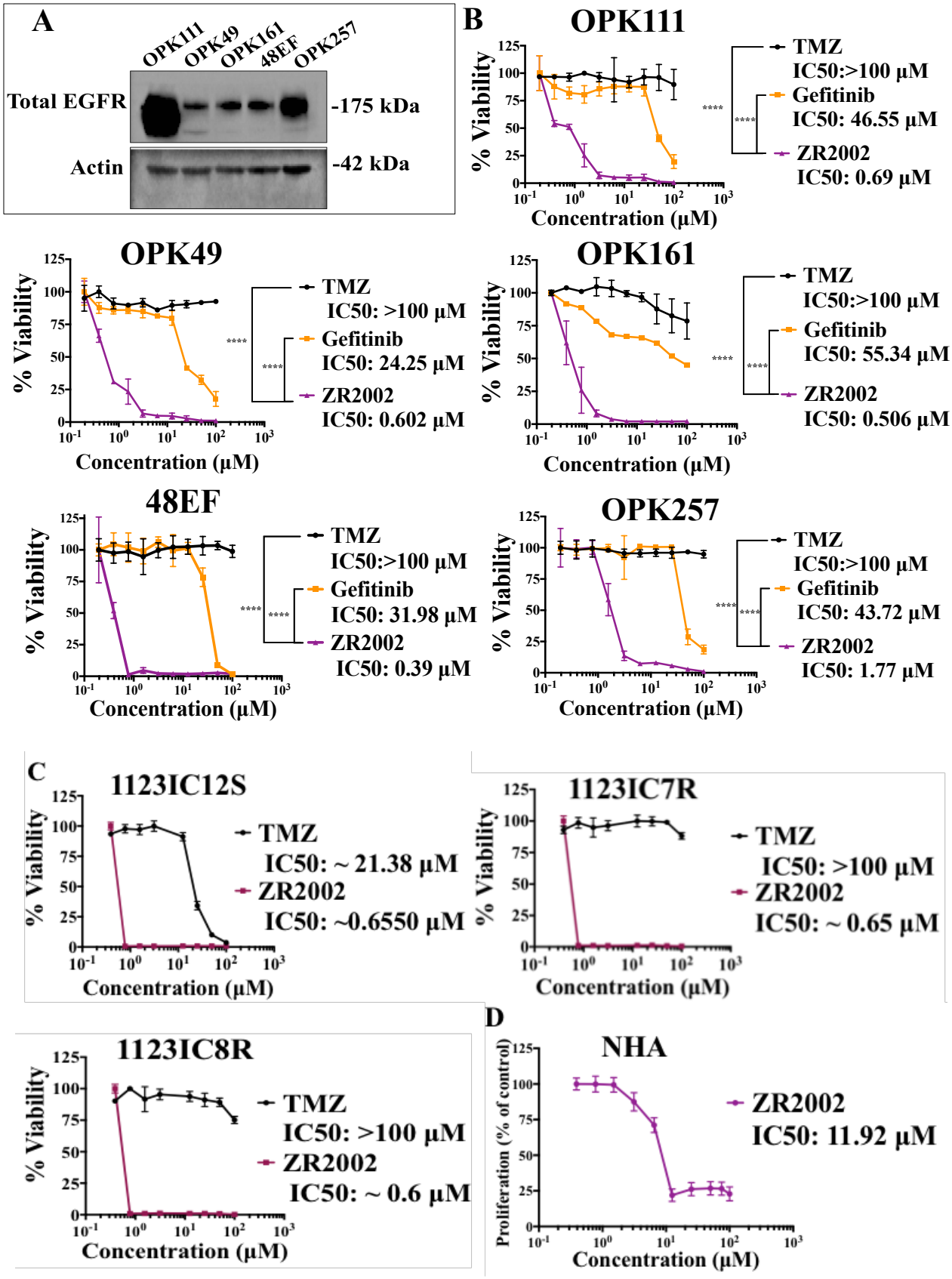

A
OPK111
OPK49
OPK161
48EF
OPK257
Total EGFR
-175 kDa
Actin
-42 kDa
B
OPK111
% Viability
Concentration (μM)
TMZ
IC50:>100 μM
Gefitinib
IC50: 46.55 μM
ZR2002
IC50: 0.69 μM
OPK49
TMZ
IC50: >100 μM
Gefitinib
IC50: 24.25 μM
ZR2002
IC50: 0.602 μM
OPK161
TMZ
IC50:>100 μM
Gefitinib
IC50: 55.34 μM
ZR2002
IC50: 0.506 μM
48EF
TMZ
IC50:>100 μM
Gefitinib
IC50: 31.98 μM
ZR2002
IC50: 0.39 μM
OPK257
TMZ
IC50:>100 μM
Gefitinib
IC50: 43.72 μM
ZR2002
IC50: 1.77 μM
C
1123IC12S
TMZ
IC50: ~ 21.38 μM
ZR2002
IC50: ~0.6550 μM
1123IC7R
TMZ
IC50: >100 μM
ZR2002
IC50: ~ 0.65 μM
1123IC8R
TMZ
IC50: >100 μM
ZR2002
IC50: ~ 0.6 μM
D
NHA
Proliferation (% of control)
ZR2002
IC50: 11.92 μM

Figure 3.1 (A-D). ZR2002 significantly inhibits proliferation of GSCs at doses that do not affect proliferation of NHA. A. Western blotting analysis of EGFR expression levels in a panel of 5 patient-derived primary GSCs. Actin was used as a loading control. **B.** GSCs were treated with various concentrations of DMSO, TMZ, gefitinib or ZR2002 for 5 days and cell proliferation was measured using alamar blue assay (****, $p<0.0001$). **C.** Effect of ZR2002 compared to TMZ treatment of TMZ-sensitive (1123IC12S) and TMZ-resistant (1123IC7R and 1123IC8R) GSCs was measured using alamar blue assay (5-day treatment). **D.** Normal human astrocytes (NHA) were treated with ZR2002 at various concentrations for 5 days and cell proliferation was measured using MTT assay. Graphs represent mean values±SD from at least three independent experiments in triplicate.

3.4.2 Inhibition of EGFR phosphorylation, neurosphere formation and increased DSBs in GSCs

We have previously shown that ZR2002 induced irreversible inhibition of EGF-stimulated autophosphorylation in breast cancer cell lines and irreversible inhibition of their cell growth [13]. Western blot analysis showed that ZR2002 treatment of GSCs at 1μM (Fig. 3.2A-B) was sufficient to attenuate EGFR tyrosine phosphorylation (Tyr1068) and to dramatically reduce Erk1/2 and Akt phosphorylation (Ser473), thereby blocking downstream signaling pathways in all tested GSC lines with varied levels of EGFR and MGMT expression. Interestingly, gefitinib induced similar effects at the IC50 20μM (lowest range for gefitinib IC50s in GSCs), while TMZ did not show any effects at a concentration as high as 100μM.

Congruent results were also obtained with the neurosphere assay. All GSCs tested failed to form neurospheres following 1μM ZR2002 treatment compared with DMSO control, while

gefitinib (20μM) only partially affected neurosphere formation and TMZ as high as 100μM had no effect (Fig. 3.3A-B). We subsequently tested the DNA-damaging potential of ZR2002 GSCs using the comet assay. ZR2002 treatment (1μM, 2hours) resulted in significantly higher levels of DNA damage in GSCs compared with DMSO control (Fig. 3.3C, $p<0.0001$), while in the assay treatment with TMZ induced barely detectable levels of DNA damage at concentrations as high as 100μM. Figure 3.3D shows representative images of DNA comets stained of ZR2002 (1μM) and compared to TMZ (100μM) in 48EF.

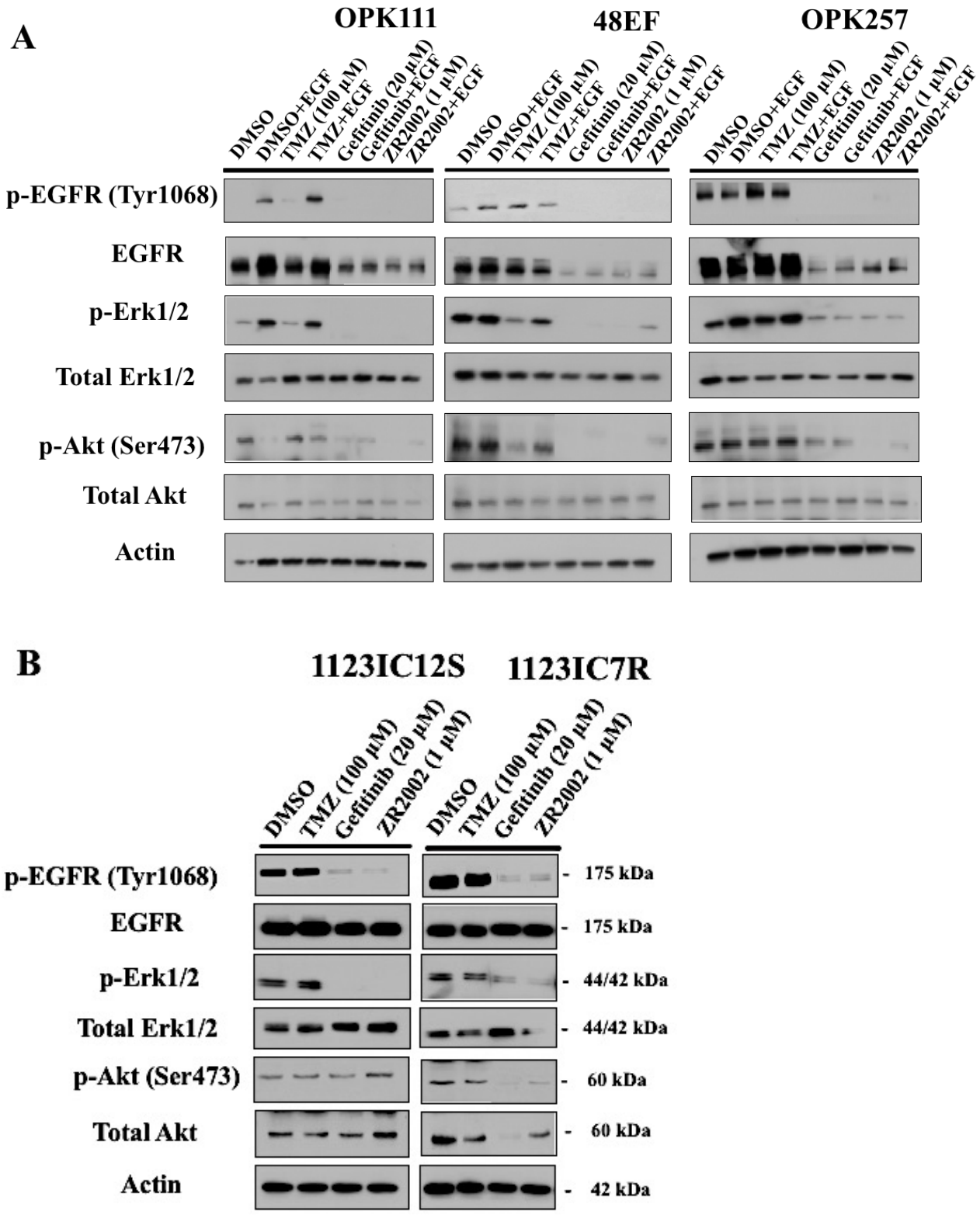

A
OPK111
48EF
OPK257
DMSO
DMSO+EGF
TMZ (100 μM)
TMZ+EGF
Gefitinib (20 μM)
Gefitinib+EGF
ZR2002 (1 μM)
ZR2002+EGF
p-EGFR (Tyr1068)
EGFR
p-Erk1/2
Total Erk1/2
p-Akt (Ser473)
Total Akt
Actin
B
1123IC12S
1123IC7R
DMSO
TMZ (100 μM)
Gefitinib (20 μM)
ZR2002 (1 μM)
p-EGFR (Tyr1068)
EGFR
p-Erk1/2
Total Erk1/2
p-Akt (Ser473)
Total Akt
Actin
175 kDa
175 kDa
44/42 kDa
44/42 kDa
60 kDa
60 kDa
42 kDa

Figure 3.2 (A-B). ZR2002 inhibits EGFR autophosphorylation and EGFR-induced downstream signaling in GSCs. OPK111, 48EF and OPK257 **(A)**, 1123IC12S and 1123IC7R GSCs **(B)** were treated with TMZ (100μM), gefitinib (20μM), ZR2002 (1μM) or DMSO control for 2 hours, stimulated or not with EGF (50 ng/mL) for 20 min, then probed for p-EGFR (Tyr1068), total EGFR, p-Erk1/2, total Erk1/2, p-Akt (Ser473), total Akt and actin as a loading control by western blotting.

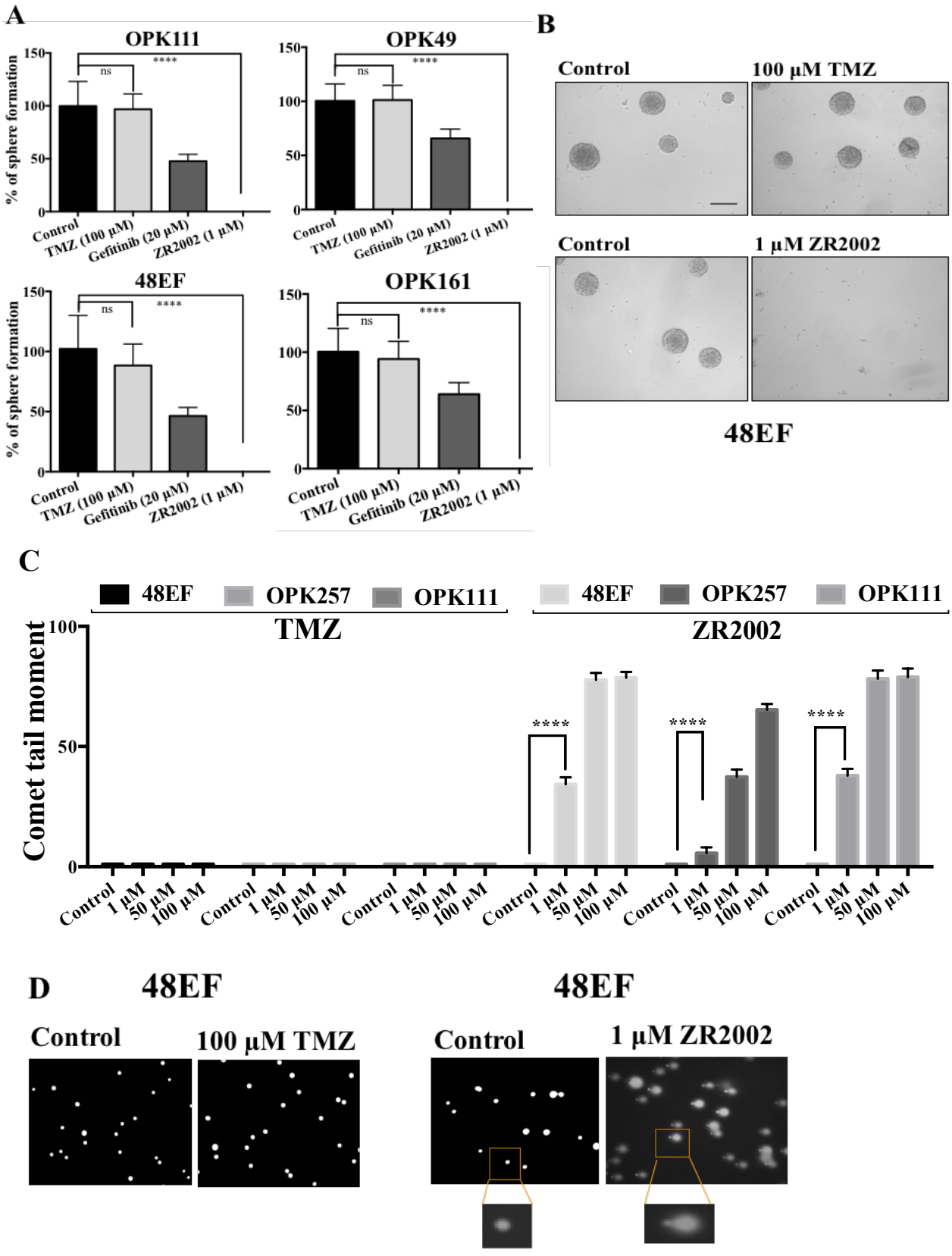

A
OPK111
OPK49
48EF
OPK161
% of sphere formation
ns
****
Control
TMZ (100 µM)
Gefitinib (20 µM)
ZR2002 (1 µM)
B
Control
100 µM TMZ
Control
1 µM ZR2002
48EF
C
48EF
OPK257
OPK111
TMZ
ZR2002
Comet tail moment
Control
1 µM
50 µM
100 µM
D
48EF
48EF
Control
100 µM TMZ
Control
1 µM ZR2002

Figure 3.3 (A-D). ZR2002 inhibits neurosphere formation ability of GSCs and inflicts DNA damage in GSCs. A. Sphere formation results for GSCs after treatment with TMZ (100μM), gefitinib (20μM), ZR2002 (1μM) or DMSO control. Ten random fields were photographed for both vehicle and drug-treated conditions and the number of spheres over 50 μm in size was scored 14 to 20 days later. **B.** Representative images of 48EF treated with TMZ (100μM) or ZR2002 (1μM). Scale bar=200μm. **C.** Cells were exposed to ZR2002 or TMZ for 2 hours, and assessed for drug-induced DNA damage using an alkaline comet assay. Average comet tail moments were calculated from 50 comets based on three independent experiments for each concentration. **D.** Representative images of DNA comets stained with SYBR Gold dye and visualized by fluorescence microscopy are shown for ZR2002 (1μM) and compared to TMZ (100 μM) in 48EF (****, $p<0.0001$; ns – not significant).

3.4.3 Responses of EGFR-driven glioma cells to ZR2002 treatment *in vitro*

To determine whether ZR2002 exhibits cytotoxicity for cells with EGFR overexpression (EGFRwt) or expressing the constitutively active variant EGFRvIII, we used GBM U87/EGFR isogenic cell lines. Immunoblotting analysis confirmed that U87MG cells had low EGFR expression levels while U87/EGFR isogenic cell lines had high expression of EGFRwt (U87/EGFRwt) or EGFRvIII (U87/EGFRvIII) (Fig. 3.4A).

We used MTT viability/proliferation assay to assess the cytotoxicity of ZR2002 and determine the IC50 of ZR2002, TMZ, gefitinib or DMSO control in U87/EGFR isogenic cell lines. ZR2002 treatment reduced cell viability in a dose-dependent manner and exhibited strikingly low IC50s for U87MG, U87/EGFRwt and U87/EGFRvIII (0.78, 0.76 and 0.6μM, respectively) (Fig. 3.4B). Importantly, ZR2002 displayed ~27, ~23 and ~41-fold superior anti-proliferative activity

over gefitinib in U87MG, U87/EGFRwt and U87/EGFRvIII cells, respectively. TMZ up to 100μM did not decrease the viability of these cell lines, as previously reported [23].

To further explore the cytotoxic effects induced by ZR2002, we performed a clonogenic assay to analyze the colony formation ability of U87/EGFR isogenic cells. ZR2002 at 1μM (short exposure for 2 hours then growth in drug-free medium for 8-14 days) reduced the clonogenic survival of all EGFR/isogenic cell lines tested (Fig. 3.4C). U87/EGFRvIII cells were significantly more sensitive to ZR2002 at 5μM compared to U87MG (p=0.0013) and U87/EGFRwt (p=0.0156). The plating efficiencies of U87MG, U87/EGFRwt and U87/EGFRvIII cells at 5μM ZR2002 (mean±SD) were 0.1±5, 0.11±10 and 0.06±4.3, respectively (Fig. 3.4C). Figure 3.4D shows a representative image of the drastic effects of ZR2002 (5μM) on U87/EGFRvIII compared to DMSO control.

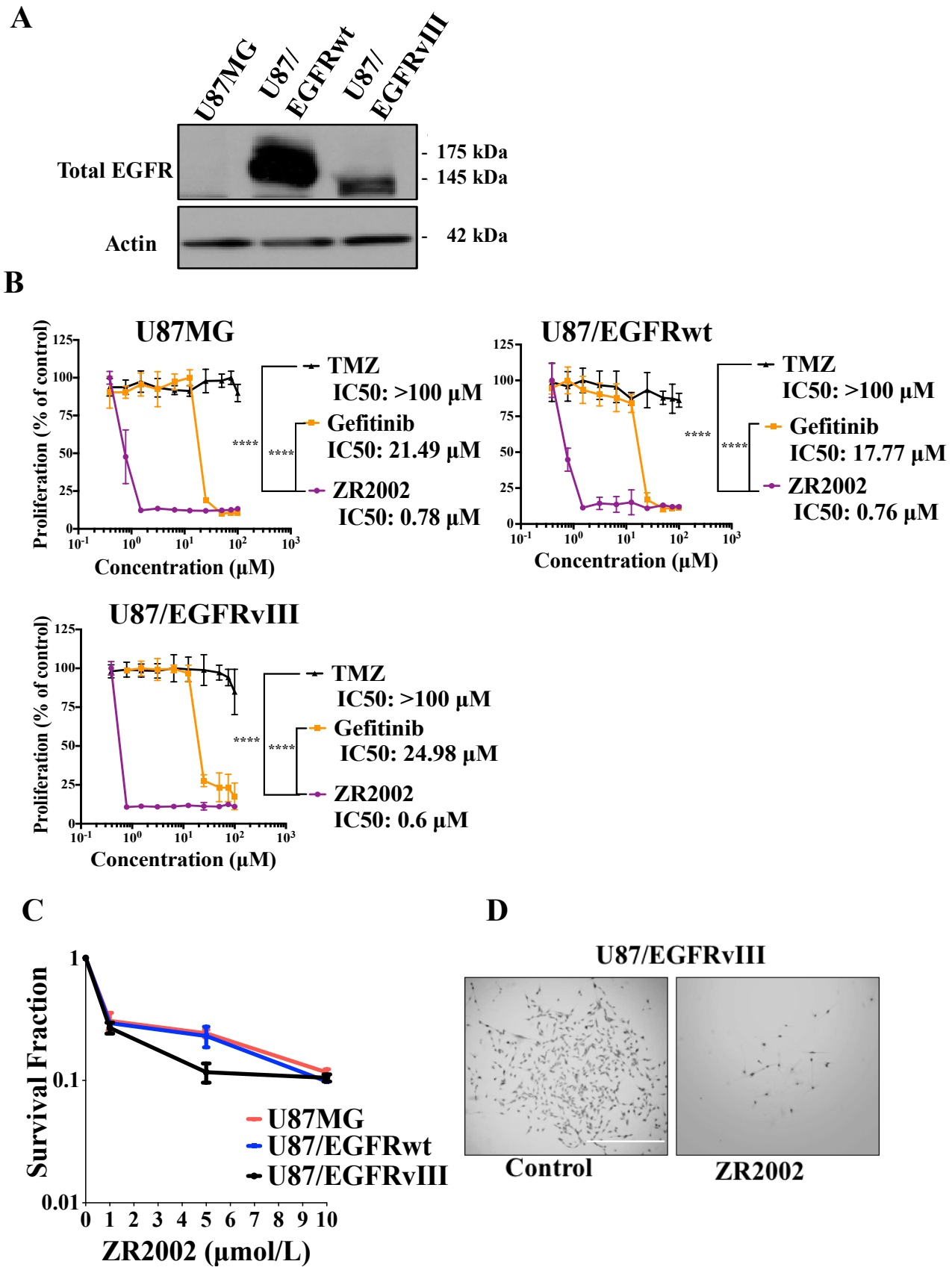

A
U87MG
U87/ EGFRwt
U87/ EGFRvIII
Total EGFR
175 kDa
145 kDa
Actin
42 kDa
B
U87MG
Proliferation (% of control)
Concentration (μM)
TMZ
IC50: >100 μM
Gefitinib
IC50: 21.49 μM
ZR2002
IC50: 0.78 μM
U87/EGFRwt
TMZ
IC50: >100 μM
Gefitinib
IC50: 17.77 μM
ZR2002
IC50: 0.76 μM
U87/EGFRvIII
TMZ
IC50: >100 μM
Gefitinib
IC50: 24.98 μM
ZR2002
IC50: 0.6 μM
C
Survival Fraction
U87MG
U87/EGFRwt
U87/EGFRvIII
ZR2002 (μmol/L)
D
U87/EGFRvIII
Control
ZR2002

Figure 3.4 (A-D). Responses of EGFR-driven glioma cells to ZR2002 treatment *in vitro.* A. Western blotting analysis of EGFR levels in U87/EGFR isogenic lines. **B.** Cells were treated with various concentrations of DMSO, TMZ, gefitinib or ZR2002 for 5 days. Cell proliferation was measured using Vybrant MTT Cell Proliferation Assay Kit. Graphs represent mean values±SD from at least three independent experiments in triplicate ****, $p<0.0001$. **C.** Colony formation assay results for U87/EGFR isogenic cell lines. fractions were normalized to plating efficiency of the corresponding DMSO controls. **D.** Representative image of the drastic effects of ZR2002 (5μM) on U87/EGFRvIII compared to its DMSO control condition. Scale bar=100μm.

3.4.4 Inhibition of EGFR autophosphorylation and DNA-damaging effects of ZR2002 in U87/EGFR isogenic cell lines

We investigated its ability to inhibit EGFR autophosphorylation in U87/EGFR isogenic cell lines (Fig. 3.5A). Cells were treated with or without EGF and the effects of ZR2002 on EGFR autophosphorylation and downstream signaling were analyzed by western blotting. ZR2002 treatment (1μM,2hours) induced complete inhibition of EGF-induced EGFR autophosphorylation), and downregulated Erk1/2 phosphorylation, suggesting a robust blockade of the MAPK pathway in all tested EGFR-expressing isogenic cell lines (Fig. 3.5A). As expected, Akt phosphorylation (Ser473) was not affected after ZR2002 treatment due to the PTEN (negative regulator of PI3K/Akt pathway) status (PTEN-deficient) of U87/EGFR isogenic cell lines [24].

We subsequently tested the DNA-damaging potential of ZR2002 on U87/EGFR isogenic cell lines using the comet assay. ZR2002 (5μM,2hours) significantly increased the levels of DNA strand breakage in single cells as reflected by comet tails analysis in all U87 isogenic cell lines compared to their respective controls (p<0.0001) (Fig. 3.5B-C). TMZ treatment (50μM,2 hours) did not significantly increase DNA damage in these cells (p=0.9) (data not shown).

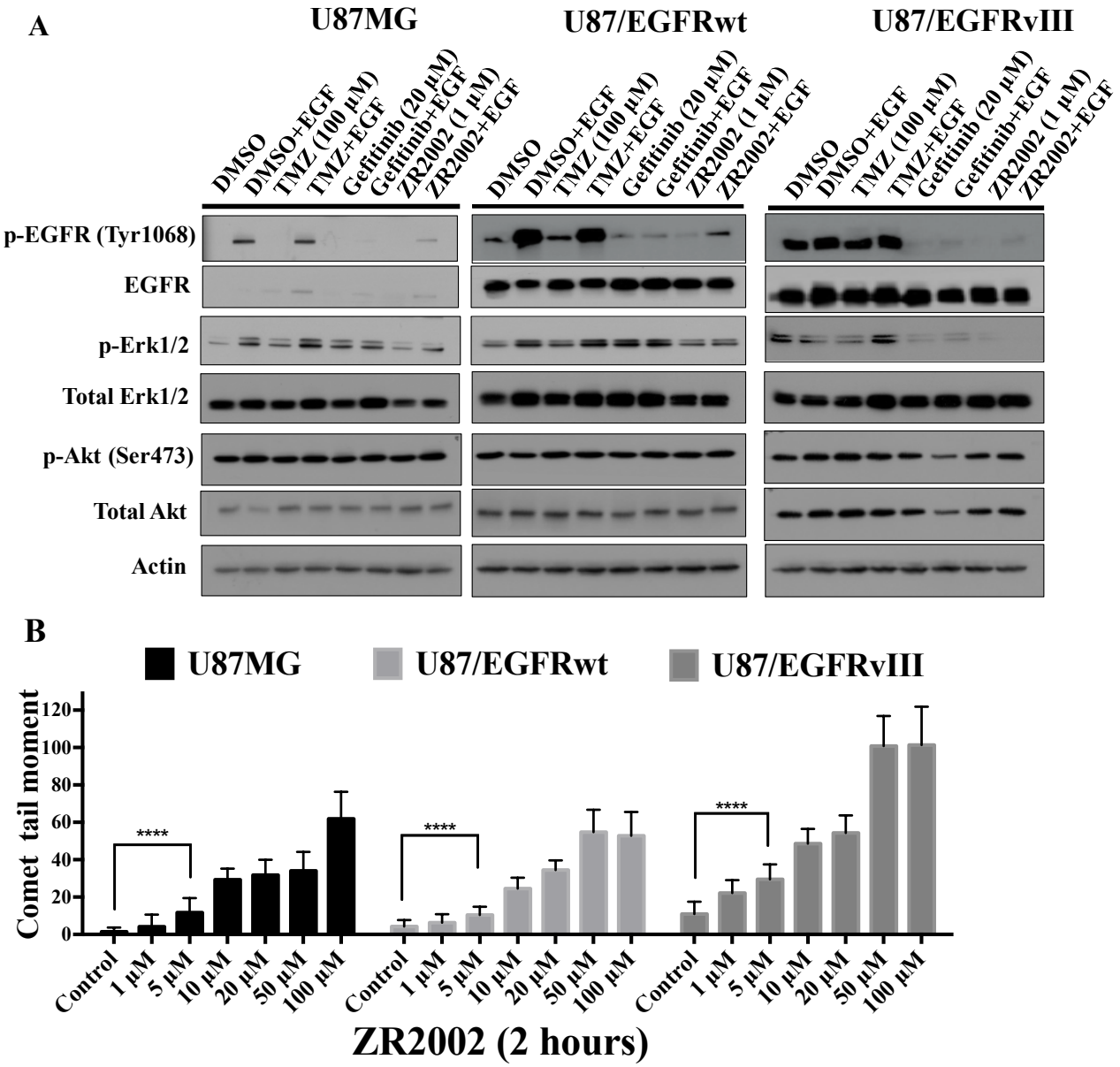

A
U87MG
U87/EGFRwt
U87/EGFRvIII
DMSO
DMSO+EGF
TMZ (100 μM)
TMZ+EGF
Gefitinib (20 μM)
Gefitinib+EGF
ZR2002 (1 μM)
ZR2002+EGF
p-EGFR (Tyr1068)
EGFR
p-Erk1/2
Total Erk1/2
p-Akt (Ser473)
Total Akt
Actin
B
U87MG
U87/EGFRwt
U87/EGFRvIII
Comet tail moment
120
100
80
60
40
20
0
****
****
****
Control
1 μM
5 μM
10 μM
20 μM
50 μM
100 μM
ZR2002 (2 hours)
C
U87MG
U87/EGFRwt
U87/EGFRvIII
Control
ZR2002 (5 μM)

Figure 3.5 (A-C). ZR2002 inhibits EGFR autophosphorylation and induces DNA damage in U87/EGFR isogenic cell lines. A. Serum-starved U87MG, U87/EGFRwt and U87/EGFRvIII cells were treated with TMZ (100μM), gefitinib (20μM), ZR2002 (1μM) or DMSO control for 2 hours, stimulated or not with EGF (50 ng/mL), lysed, then probed by western blotting for p-EGFR (Tyr1068), total EGFR, p-Erk1/2, total Erk1/2, p-Akt (Ser473), total Akt and actin as a loading control. **B.** Cells were exposed to for 2 hours, and assessed for drug-induced DNA damage using an alkaline comet assay. Average tail moments were calculated from 50 comets based on three independent experiments for each concentration. **C.** Representative images of DNA comets stained with SYBR Gold dye and visualized by fluorescence microscopy were shown for ZR2002 (5μM) and DMSO control in U87/EGFR isogenic cell lines (p-value for each condition compared to DMSO control is shown, ****, $p<0.0001$).

3.4.5 ZR2002 mechanism of action is mediated through wtp53 activation

Upon DNA damage, ataxia-telangiectasia (ATM), Rad3-related (ATR), and DNA-PK activate p53 through phosphorylation [25]. Treatment with TMZ (100μM), or ZR2002 (1μM) or gefitinib (20μM) induced a marked increase in p53 levels and was accompanied by induction of its known target p21 protein in U87/isogenic cell lines (wt*TP53*) and OPK161, OPK49, 48EF, OPK257 and OPK111 (wt*TP53*) with the exception of OPK257 (mut*TP53*) [18] (Fig. 3.6A-B). To further explore the effect of p53 inhibition on ZR2002 treatment, we used the OPK49 GSC line, which showed the greatest increase of p53 protein expression levels upon exposure to ZR2002 (Fig. 3.6B) and performed shRNA-mediated *TP53* knockdown. We achieved at least 90% decrease of p53 expression with a concomitant decrease of expression levels of p53 target protein, p21 (Fig. 3.6C). Next, we examined whether p53-knockdown affects the growth inhibitory effects of ZR2002. Silencing of p53 caused a significant increase in drug resistance in OPK49/shRNA (p <0.0001) (IC50:0.66 and 2.66μM in OPK49 and OPK49/shRNA, respectively) (Fig. 3.6D). To further explore the effect of p53 status on sensitivity to ZR2002 in OPK49, we performed a neurosphere formation assay. OPK49/shRNA was able to form neurospheres, despite treatment with ZR2002 at 1μM, whereas the same concentration completely inhibited neurosphere formation of OPK49 mock cell line (Fig. 3.6E).

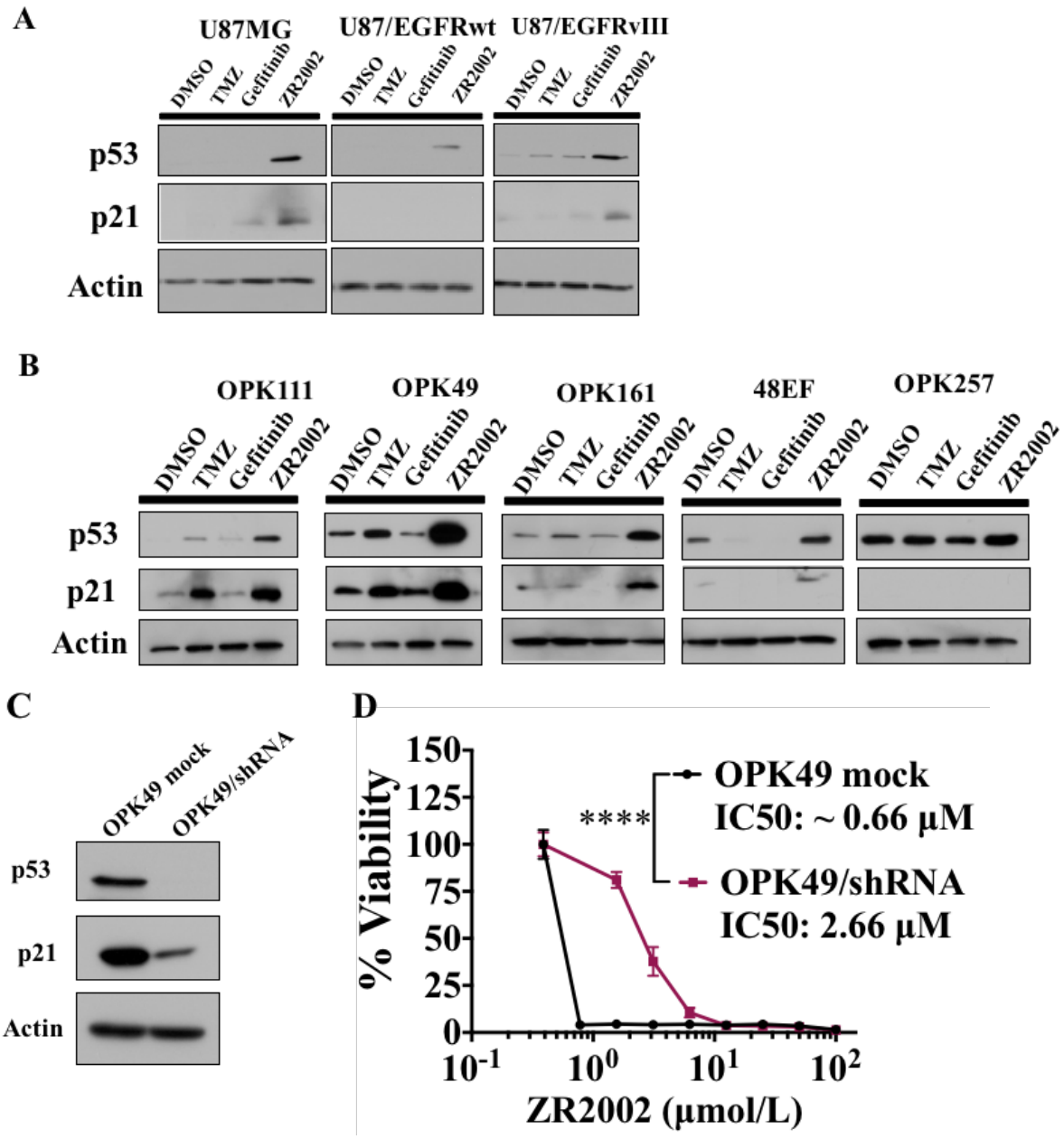

A
U87MG
U87/EGFRwt
U87/EGFRvIII
DMSO
TMZ
Gefitinib
ZR2002
p53
p21
Actin
B
OPK111
OPK49
OPK161
48EF
OPK257
C
OPK49 mock
OPK49/shRNA
D
% Viability
****
OPK49 mock
IC50: ~ 0.66 µM
OPK49/shRNA
IC50: 2.66 µM
ZR2002 (µmol/L)
E
Control
ZR2002
OPK49 mock
% of sphere formation
ZR2002 (1 µM)
OPK49sh/RNA

Figure 3.6 (A-E). ZR2002 mechanism of action is mediated through wtp53 activation. A-B. Western blotting analysis showing p53 and p21 expression in GBM established cell lines **(A)** and GSCs **(B)** treated for 48 hours with DMSO, TMZ (100μM), gefitinib (20μM) or ZR2002 (1 μM). Cell lysates were probed with p53 antibody, then re-probed for p21 and actin as a loading control. **C.** Western blotting confirmed p53-knockdown by at least 95% in OPK49/shp53. **D.** p53-knockdown induced resistance to ZR2002 compared to the parental OPK49, as shown in viability assay and **(E)** sphere formation assay (****, $p<0.0001$). Scale bar=200μm.

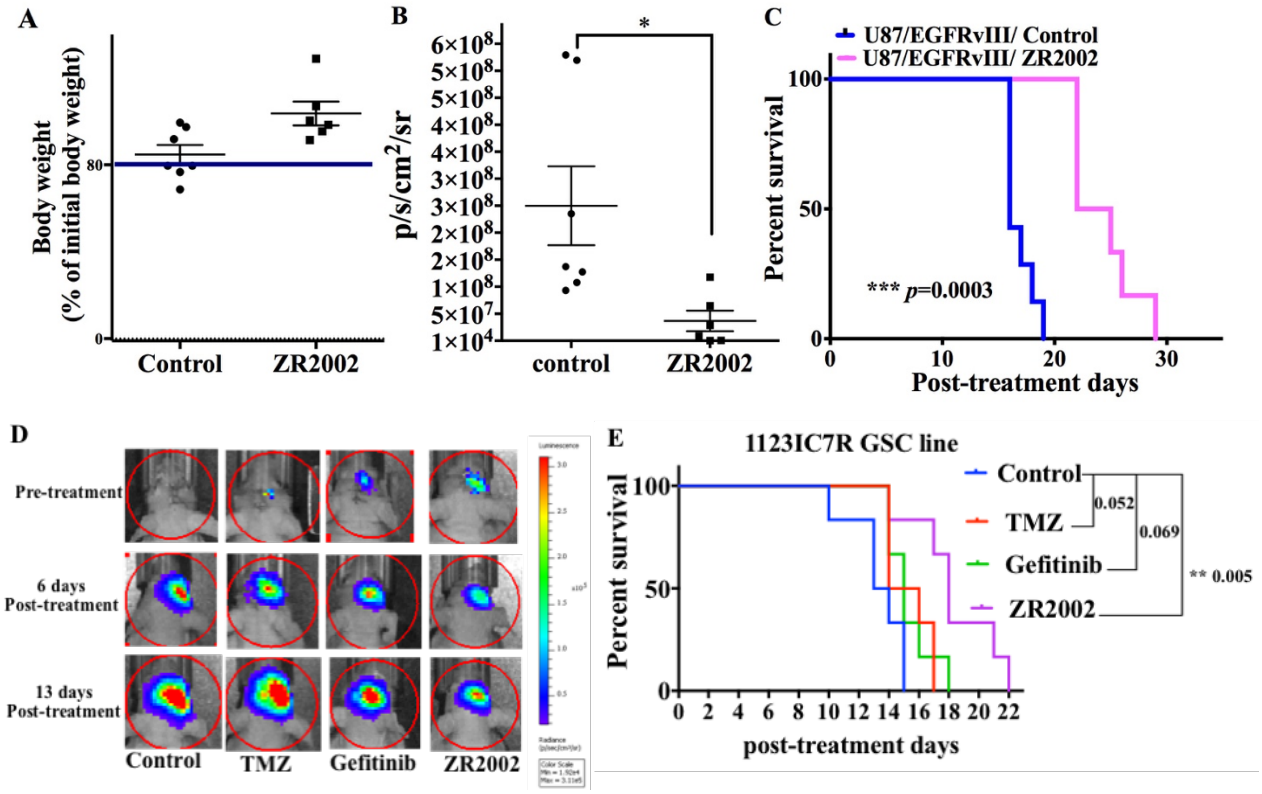
3.4.6 ZR2002 improves survival of mice with EGFRvIII and 1123IC7R intracranial tumors

To assess the *in vivo* efficacy of ZR2002 we used a mouse orthotopic U87/EGFRvIII GBM xenograft model known for its high rate of intracranial tumor growth and short median survival [26]. U87/EGFRvIII-Luc2 cells were stereotactically injected into the striatum of nude mice only 4 days before starting treatment. After the second 5-days treatment cycle, 4 out of 7 mice in the control group showed significant weight loss (>20%), while non of the mice in the ZR2002 group showed significant weight loss (>20%) (Fig. 3.7A). ZR2002 significantly reduced tumor BLI signal compared to control group at the same time point ($p=0.0262$) (Fig. 3.7B and Supplementary Fig. S3.3 (A-B)). Interestingly, ZR2002 at 150mg/kg significantly improved survival of mice compared to the control group ($p=0.0003$; Fig. 3.7C). Hence, ZR2002 exhibits anti-proliferative effects within a submicromolar range *in vitro* and anti-tumor activity in the highly aggressive U87/EGFRvIII orthotopic model without toxicity in nude mice.

We also assessed the *in vivo* efficacy of ZR2002 in the highly aggressive intracranial xenograft 1123IC7R GSC mesenchymal TMZ-resistant mouse model [19]. Three days following

stereotactic injection of 1123IC7R GSCs stably transfected with luciferase (1123IC7R-luc), mice were treated once daily with either vehicle control (3 weeks), TMZ (66mg/kg, 5 days) [27], gefitinib (150mg/kg, 3 weeks) [28] or ZR2002 (150mg/kg, 3 weeks) and monitored for tumor growth using BLI imaging. Figure 3.7D shows representative images of BLI signals from mice in control and treatment groups at different time points. Remarkably, ZR2002 treatment significantly prolonged the survival of mice compared to vehicle control (p=0.005) (Fig. 3.7E and Supplementary Fig. S3.3 (C-D)).

Mice were given a final dose of 150mg/kg (ZR2002 or gefitinib) and 66mg/kg TMZ prior to euthanasia to assess downstream signaling effectors on tumor tissue collected from mice by western blotting. ZR2002 dramatically reduced EGFR, Erk1/2 and AKT phosphorylation in 1123IC7R (Fig. 3.7F). Taken together, our data indicate that ZR2002 is well-tolerated in nude mice, crosses the BBB and improves survival of mice with EGFRvIII and 1123IC7R intracranial tumors.


A
Body weight
(% of initial body weight)
80
0
Control
ZR2002
B
p/s/cm2/sr
6×10^8
5×10^8
5×10^8
4×10^8
4×10^8
3×10^8
3×10^8
2×10^8
2×10^8
1×10^8
5×10^7
1×10^4
*
control
ZR2002
C
U87/EGFRvIII/ Control
U87/EGFRvIII/ ZR2002
Percent survival
100
50
0
*** p=0.0003
0
10
20
30
Post-treatment days
D
Pre-treatment
6 days
Post-treatment
13 days
Post-treatment
Control
TMZ
Gefitinib
ZR2002
E
1123IC7R GSC line
Control
TMZ
Gefitinib
ZR2002
0.052
0.069
** 0.005
Percent survival
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22
post-treatment days

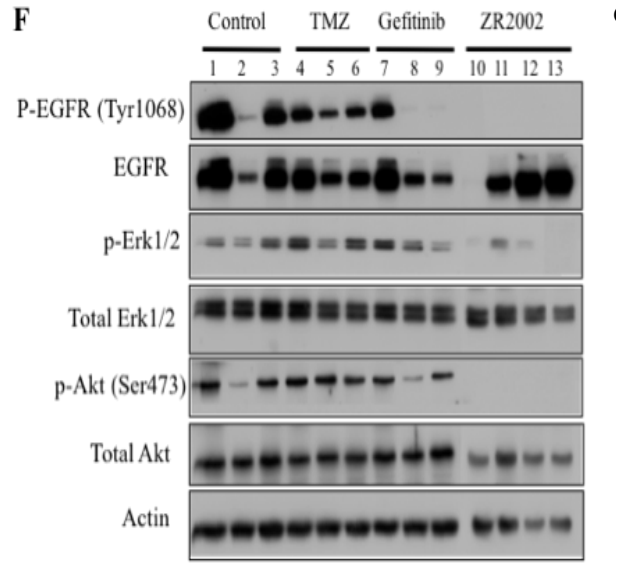

F
Control
TMZ
Gefitinib
ZR2002
1 2 3 4 5 6 7 8 9 10 11 12 13
P-EGFR (Tyr1068)
EGFR
p-Erk1/2
Total Erk1/2
p-Akt (Ser473)
Total Akt
Actin

Figure 3.7 (A-F). ZR2002 improves survival of mice with EGFRvIII and 1123IC7R intracranial tumors. A. U87/EGFRvIII-Luc2 was stereotactically injected into the brain of nude mice. After 4 days, mice were orally treated with control, (N=7) or ZR2002 150mg/kg, (N=6) according to schedule #1. Body weights of mice are shown for after the second 5-days treatment cycle. **B.** BLI signal of mice is shown for the same time point (after the second 5-days treatment cycle) (*denotes statistical significance control versus ZR2002 (p=0.0262). **C**. Survival curves were generated for EGFRvIII-Luc2 intracranial tumors; ***denotes statistical significance (p=0.0003). **D-E.** 1123IC7R-Luc2 GSCs was stereotactically injected into the brain of nude mice. After 3 days, mice were orally treated with control (N=6), TMZ/66mg/kg (N=6), gefitinib/150mg/kg (N=6) or ZR2002/150mg/kg (N=6) according to schedule #2 (explained in chapter 2). **D.** Tumor growth was monitored using BLI imaging and representative images of BLI signal are shown at pre-treatment, 6 days post-treatment and 13 days post-treatment for each treatment group. **E.** Kaplan-Meier survival curves were generated for 1123IC7R-Luc2 GSC intracranial tumors; *denotes statistical significance (p=0.005), ns – not significant (Control versus TMZ; p=0.0529), (Control versus gefitinib; p=0.069). **F.** Mice were given a final dose of Control, ZR2002 (150mg/kg), gefitinib (150mg/kg) or TMZ (66 mg/kg) before euthanizing them. Tumor tissue collected from mice brains was processed either for lysis to assess p-EGFR (Tyr1068), total EGFR, p-Erk1/2, total Erk1/2, p-Akt (Ser473), total Akt and actin by western blotting.

3.5 DISCUSSION

Despite compelling evidence demonstrating the potential of EGFR as a target in GBM, EGFR-targeted agents did not fulfill their promise in the treatment of patients newly diagnosed with GBM [12] or with recurrent disease [29]. In this study, we present novel findings for the potential clinical efficacy of ZR2002, a small molecule designed to block EGFR-mediated signaling but in contrast to other EGFR inhibitors, it carries a haloalkyl arm capable of reacting with the receptor itself and with DNA bases, and importantly was kept small enough to maintain brain penetrability. First, we provide experimental evidence for a unique growth inhibitory profile of ZR2002 in experimental settings that recapitulates the heterogeneity and aggressive nature of GBM disease. This includes (*a)* GSCs derived from newly diagnosed GBM patients, *(b)* an experimental GSC model for *in vivo* TMZ resistance and GBM recurrence with the highly aggressive TMZ-resistant mesenchymal *in vivo* derived GSC subline, and *(c)* GBM established cell lines isogenic for EGFR or EGFRvIII. Second, our study highlights the cytotoxic effects of ZR2002 through DNA damage (DSBs) shown by comet assay with concomitant inhibition of EGFR or EGFRvIII-induced downstream signaling. Importantly, its DNA damaging arm seems to act in a p53-dependent manner, as suggested by increased expression of p53 in all GSCs (except for mutantp53 OPK257) and the causal relationship between *TP53* activation and the anti-proliferative effects of ZR2002 in wtp53 GSC line. Third, we achieved a key step in pre-clinical development of ZR2002 and showed its safety, BBB permeability, oral bioavailability and *in vivo* anti-tumor properties with significant delay of tumor progression for either EGFRvIII-driven or mesenchymal GSC TMZ-resistant intracranial xenografts in nude mice. Thus, our results suggest DNA damage with concomitant irreversible inhibition of EGFR tyrosine kinase activity as a key vulnerability in GBM. The concept of EGFR oncogene “addiction” has gained a momentum based

on clinical evidence for the success of different EGFR-targeted therapies in different cancer types. Further experimental evidence revealed the role of EGFR as a key oncogene driver at the nexus of tumor metabolism and immunogenic cell death [30]. Oncogenic TKs orchestrate complex signaling pathways, cross-talk with each other, trigger similar signaling pathways that enable alternate compensatory mechanisms following inhibition with RTK inhibitor as a monotherapy. The potency of ZR2002 stems from its conceptual design to achieve divergent targeting of different cellular components (i.e, co-targeting a RTK and DNA) [13, 15]. Our findings showing the *in vivo* potency of ZR2002 support the concept of divergent targeting as an efficient and promising multi-targeting approach beyond co-targeting RTKs to inhibit downstream compensatory mechanisms.

Given the important role of GSCs as a disease reservoir in GBM, unraveling the molecular mechanisms involved in the maintenance of GSCs provided the rationale for pre-clinical and clinical testing of targeted therapeutic strategies aiming to eradicate GSCs [31]. ZR2002 displayed cytotoxic anti-proliferative effects with an IC50 within a submicromolar range and drastically obliterated neurosphere formation of GSCs. Our results are in accordance with studies showing that EGFR-knockdown in EGFR-positive GBM neurosphere cultures led to differentiation and less malignant tumors *in vivo,* and its inhibition resulted in reduced neurosphere formation in the presence of EGF [32].

Our study provides some mechanistic insights underlying the anti-proliferative effects and potency of ZR2002 to eradicate neurosphere forming ability and improve survival in a highly aggressive GSC model refractory to TMZ and gefitinib. Tyr1068, has been reported as one major EGFR autophosphorylation site [33], which is key in Ras-Raf-MAPK ERK1/2 pathway. Our *in vitro* and *in vivo* experiments show that ZR2002 treatment drastically down-regulated this tyrosine

kinase site. It has been previously reported that TMZ and ZR2002 are able to induce methylation of genomic DNA [23] and alkylating chloroethyl function [13], respectively. ZR2002 inflicts DNA damage inducing DSBs through its DNA-damaging moiety as shown by comet assay in our study and in breast cancer cell lines [13]. We surmise that its EGFR TK-targeting moiety irreversibly induces covalent damage to ATP site, which subsequently cutback EGFR-mediated DSB repair. Indeed, besides its canonical role as a cell surface receptor in signal transduction to downstream effectors, EGFR is shuttled to the nucleus [34]. Several studies provided convincing evidence for the role of nuclear EGFR in transcriptional regulation (cyclin D1) [35], DNA synthesis, and repair and showed its role in chemo- and radio-resistance and association with worst clinical prognosis [36]. Nuclear EGFR directly interacts with and enhances the activity of DNA-PKcs known for its major role in non-homologous end joining (NHEJ) of DSBs repair [37] in addition to its direct interaction with histone H4 affecting DNA synthesis and repair [38]. Furthermore, a study by Yakoub et al. demonstrated that EGFR is also involved in upregulation of DNA repair genes such as XRCC1 and ERCC1 [39]. Additional studies are needed to identify the specific mechanism(s) by which ZR2002 inhibits EGFR-mediated repair of DNA damage (decrease in DNA synthesis and repair in GBM), which might be critical for its *in vivo* efficacy.

In accordance with the important role of p53 in response to DNA damage, ZR2002 treatment increased expression of p53 to a variable extent in EGFR isogenic cell lines and GSCs, and this increase was more pronounced compared to TMZ or gefitinib. Accordingly, increased expression of its downstream effector p21, a readout of p53 activation known to mediate cell cycle checkpoints and apoptosis [40] might support ZR2002-induced cytotoxicity. Previous work has shown that loss of functional p53 increased the sensitivity of normal and neoplastic astrocytic cells to DNA alkylating agents [41]. Dinca et al.[42] demonstrated in an intracerebral xenograft model

that U87MG cells were sensitized to TMZ by pre-treatment with pifithrin-α (inhibitor of p53). Loss of functional p53 was previously shown to contribute to stemness and survival in GSCs [43]. The relationship between p53 and sensitivity to ZR2002 in wtp53 GSC line (OPK49), wherein p53-silencing by RNAi significantly conferred resistance to ZR2002 (Fig. 6D), suggests that ZR2002 might partially exert its effects on GSCs in a p53-dependent manner and extends on previous findings corroborating the role of functional p53 in GSCs in response to DNA damage.

ZR2002 exhibited *in vitro* and *in vivo* effects on U87/EGFRvIII cell line unveiling EGFR/DNA binary targeting as a novel strategy to directly inhibit EGFRvIII. Thus far, strategies targeting EGFRvIII-positive GBM tumors have failed in GBM [44, 45]. EGFRvIII has been shown to enhance DSB repair in a mouse orthotopic glioma model [46]. Co-expression of EGFRvIII and PTEN (negative regulator of PI3K/Akt pathway) in GBM cells is associated with heightened sensitivity to EGFR kinase inhibitors, while PTEN deficiency decreases response to EGFR inhibitors due to high levels of Akt activation [47]. Accordingly, ZR2002 induced marked dephosphorylation of EGFR and Erk1/2, but not p-Akt (Ser473) in PTEN-deficient [24] U87/EGFR isogenic cell lines.

Collectively, our findings demonstrate the drastic effects of ZR2002 on GSG neurosphere formation *in vitro* and its *in vivo* efficacy in a TMZ-resistant GSC model in addition to noticeable cytotoxic effects on EGFRvIII *in vitro* and *in vivo*. Our study highlights binary EGFR/DNA targeting strategy to induce irreversible inhibition of EGF-stimulated autophosphorylation, while increasing DSBs as a potentially attractive therapeutic strategy to overcome EGFR-induced compensatory DNA repair mechanisms in GBM. It also provides the proof-of-principle to suggest ZR2002 as a novel approach in GBM including for patients with recurrent TMZ-resistant GBM, for which effective therapeutic options are not currently available.

3.6 ACKNOWLEDGMENTS

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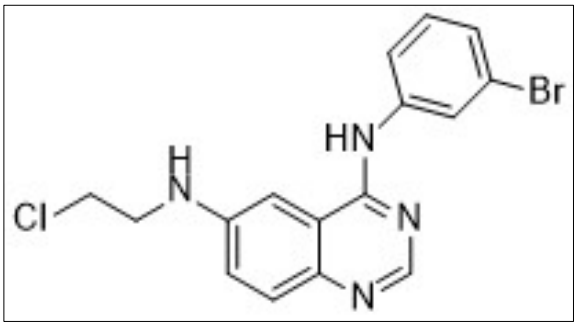
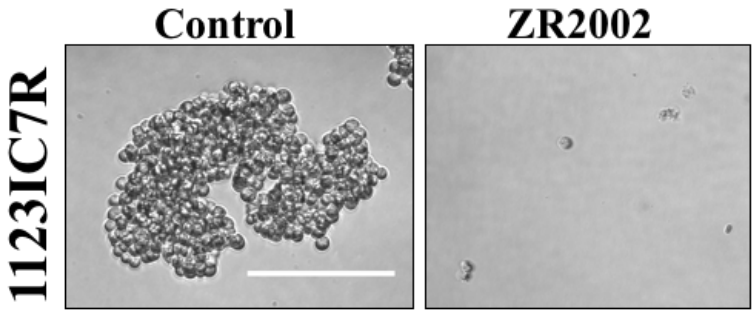
3.8 SUPPLEMENTAL FIGURES AND TABLES

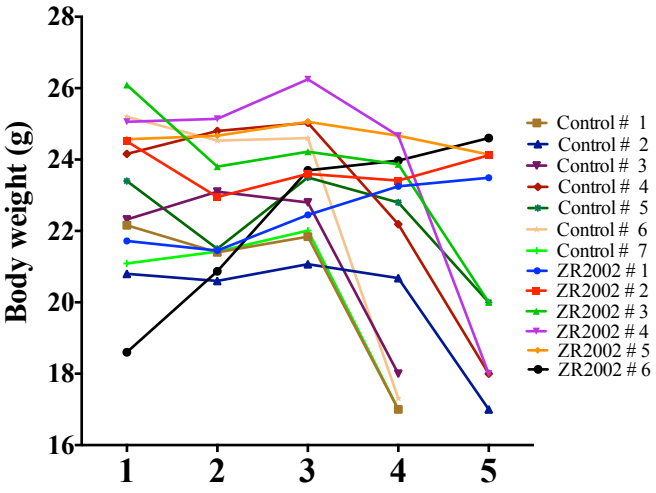


Supplementary Figure S3.1. ZR2002 (6-(2-chloroethylamino)-4-anilinoquinazoline) molecular structure (Molecular weight: 377.67 g/mol).

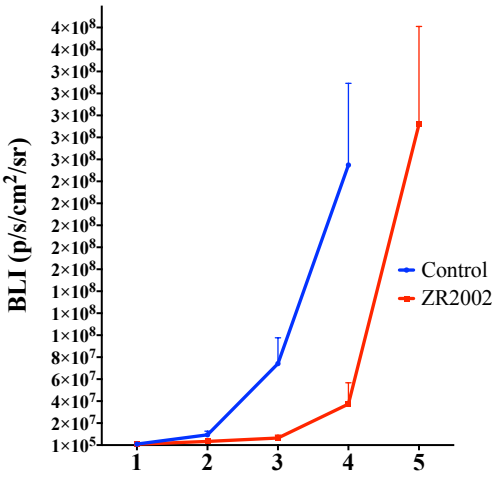


Supplementary Figure S3.2. Representative images of TMZ-resistant GSC 1123IC7R treated with ZR2002 (2μM) compared to DMSO control condition after 5 days of treatment. Scale bar= 100μm.

A



B



1: Pre-treatment

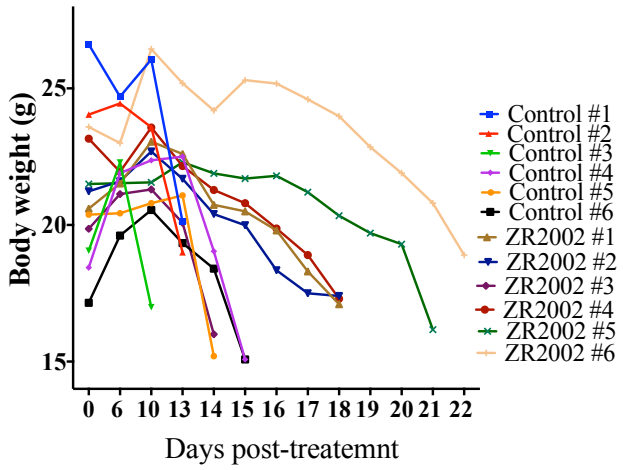
2: End of first 5-days treatment cycle

3: Before second 5-days treatment cycle

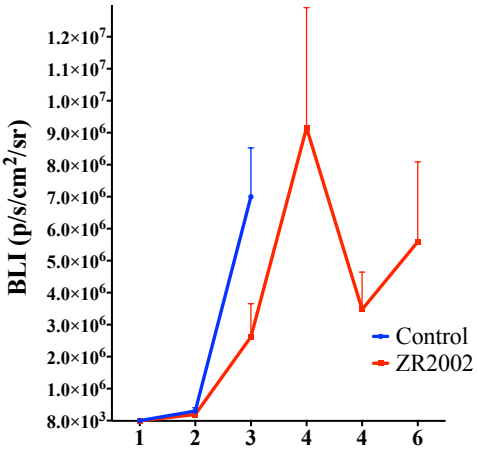
4: After second 5-days treatment cycle

5: 5 days after end of second 5-days treatment cycle

C



D



1: Pre-treatment,

2: 6 days post-treatment

3: 13 days post-treatment

4: 15 days post-treatment

5: 18 days post-treatment

6: 20 days post-treatment

Supplementary Figure S3.3 (A-D). ZR2002 improves survival of mice with EGFRvIII and 1123IC7R intracranial tumors. A-B. ZR2002 (150 mg/kg) or vehicle was given to nude mice orally 4 days after U87/EGFRvIII tumor implantation for 5 days (first treatment cycle) followed by 5-day rest and a second 5-day treatment cycle (second treatment cycle). The numbers from 1 to 5 indicate the time points used to assess changes in the mice body weights (**A)** and tumor growth by BLI (**B)**. **C-D.** ZR2002 (150mg/kg) or vehicle was given to nude mice orally 3 days after TMZ-resistant GSC (1123IC7R) tumor implantation for 21 continuous days. **C.** Changes in mice body weights in control (N=6) and ZR2002 (N=6) are shown (post-treatment days). **D.** Tumor growth

was monitored using BLI and reported as numbers from 1 to 6 that indicate the time points used to assess changes in BLI signal.

IC50 (µM)	OPK111	OPK49	OPK161	48EF	OPK257
MGMT status	+	-	+	+	-
TMZ	>100	>100	>100	>100	>100
Gefitinib	46.55	24.25	55.34	31.98	43.72
ZR2002	0.69	0.602	0.506	0.39	1.77

Supplementary Table S3.1. Summary of MGMT status and IC50's of GSCs from patients newly diagnosed with GBM. Top row indicates expression of MGMT (previously shown by western blotting by our group [18] for five GSCs from patients newly diagnosed with GBM. IC50's of TMZ, gefitinib or ZR2002 are shown for GSCs treated with various concentrations of DMSO, TMZ, gefitinib or ZR2002. Cell proliferation was measured using alamar blue assay (5 days treatment).

Chapter 4.

GENERAL DISCUSSION

&

FUTURE DIRECTIONS

Cancer is a complex disease that is driven by many oncogenic factors and redundant signaling networks. Among cancers of the CNS, GBM is recognized as the most common and lethal form of cancer. Despite radical treatment with maximum safe resection, TMZ, and RT, GBM remains the most challenging brain tumor to treat with dismal outcome.

Resistance to anticancer drugs and the resulting decrease in antitumor effects of these agents may be due to many factors such as development of alternative pathways for tumor growth, challenges in crossing the BBB (e.g. increased drug efflux), changes in the tumor microenvironment (hypoxia, acidic and metabolic stresses; oncogene and transcription factor activation such as Notch, NF-κB and EZH2) [1-7] and tumor heterogeneity. Also, when monotherapy is used it might target only rapidly growing cells and lead to toxicity, while immunosuppression mechanisms are heightened [8]. GSCs are a subset of cancer cells that escape chemo-RT and cause therapeutic resistance [1, 9, 10]. MGMT, a key player in TMZ resistance acts by reversing the mutagenic DNA lesion *O*-6-guanine (introduced by TMZ) back to guanine [11, 12]. Several strategies have been used to overcome challenges arising with resistance to TMZ, such as combinations of individual drugs that rely on complementary mechanisms of antitumor activity [13-15]. Thus, combination of two or more therapeutic treatments in cancer has been demonstrated to be more effective than monotherapy [8, 16-20]. Also, ideally if a combined treatment modality works in a synergistic manner, lower doses of each individual drug will eventually achieve a therapeutic effect while decreasing drug resistance and drug toxicity to normal non-malignant cells.

In this thesis, we aimed to study a pharmacological approach of single drugs capable of targeting multiple oncogenic targets in tumor cells [21-28]. Classical DNA alkylating agents are relatively not selective, which might cause toxicity [29]. On the other hand, TKI are targeted agents

aimed to inhibit oncogenes that drive tumor progression. In the context of the new approach termed the “combi-molecules”, we tested one combi-molecule that has alkylating function to target DNA (cytotoxic DNA-damaging moiety) and an EGFR inhibitor (targeting moiety).

This PhD thesis describes my novel findings obtained during the investigation of the potency of a type II combi-molecule with dual EGFR and DNA damaging properties, ZR2002, without the requirement for hydrolysis [30, 31]. We investigated the effects of ZR2002, a prototype of type II combi-molecule carrying a hemimustard DNA-damaging moiety in GBM cell lines and GSCs with different levels of EGFR, MGMT, *TP53* status (wild-type or mutant). I also described the brain permeability and *in vivo* efficacy of this drug in GBM.

In order to have a successful combination therapy in single molecules, ZR2002 should damage DNA of cells and inhibit the EGFR pathway. This is mainly important because when designing combi-molecules even minor structural changes in one targeting moiety can lead to a drastic loss of binding potency of one of the arms. Furthermore, in some cases dual targeting requires modifications, which lead to a bulky molecule, thereby affecting its cellular penetration. Another challenge was to prove that the combination is more effective than each individual drugs. Therefore, we compared the anti-proliferative effects of ZR2002 to TMZ (as the standard treatment of GBM) and gefitinib treatment (EGFR inhibitory arm) and showed that our dual-targeted molecules have remarkably stronger potency when compared to each drug individually. Finally, the biggest challenge of this thesis was to determine the brain permeability, safety and efficacy of the combi-targeting approach in a preclinical setting. While this combi-molecule was demonstrated to selectively target cells, the mechanism of selectivity still needs to be investigated.

4.1 Contribution 1 (Chapter 2)

Chapter 2 of my PhD thesis is of critical importance since drug delivery to the brain and safety of novel drugs still remains one of the biggest challenges in modern medicine and a major obstacle for modern pharmacotherapy. In this regard, several studies suggest rigorous preclinical research to be conducted before performing further extensive studies to initiate a clinical trial [32].

Since EGFR is highly deregulated in GBM, EGFR inhibitors/antibodies have been widely and effectively investigated in preclinical models of GBM. However, clinical trials using these inhibitors failed to detect any improvement in survival outcomes due to many reasons such as failure of EGFR antibodies to cross the BBB [33]. In order for a drug to be able to cross the BBB, there are several important characteristics that need to be taken into careful consideration before bringing drug candidates into clinical trials. First, the compound must be small, which is measured by its molecular weight and polar surface area. As reported by many studies [34] for CNS drugs the optimal molecular weight and polar surface area is < 450 g/mol and 90 $Å^2$, respectively. Generally, such drugs are relatively few and in fact 98% of all small molecules and approximately 100% of the large molecules do not cross the BBB [35]. ZR2002, is a small molecule that has a molecular weight of 377.67 g/mol [31]. Additionally, the optimal lipophilicity of CNS drugs is ClogP = 2, which is defined as the octanol-water partition coefficient of a compound (ClogP) 2 [37]. Finally, although brain tumors are known to have leaky blood vessels allowing for increased permeability most of the BBB remains intact, therefore the effect of Pgp efflux pumps must be also taken into careful consideration [38].

Many strategies have been used to overcome this problem such as intratumoral injections of drugs using an implanted catheter, however, although these strategies have shown promising effects they have raised many issues such as being time-consuming, unfamiliar to oncologists, and

pose biosafety concern [36]. One such drug that has been used by intratumoral injections is DNX-2401, an oncolytic adenovirus, in phase I clinical trial and has shown antitumor activity with no dose-limiting side effects [36]. TKIs such as erlotinib and gefitinib failed in the clinical trials due limited brain exposure, the effect of Pgp and ABCG2-mediated efflux [39, 40]. One recent study that highlights the obstacles faced when bringing a novel therapy to the market is the rindopepimut trial. Rindopepimut is a 14-mer peptide that spans the length of EGFRvIII, a mutant variant of EGFR, conjugated with an immunogenic carrier protein keyhole limpet hemocyanin, This drug showed promising efficacy in Phase I and II trials in combination with temozolomide by increasing the progression-free survival and median overall survival (OS) by 10–15 and 22–26 months, respectively, compared with 6 and 15 months in historical controls [41]. However, in the Phase III study, rindopepimut failed to meet OS endpoint criteria [42]. In more recent clinical trials on the third generation EGFR inhibitor osimertinib (AZD9291) and GDC-0084, more extensive BBB permeability studies has been done to avoid the major problem with current EGFR TKIs [43].

In chapter 2, I studied the safety and drug permeability of our dual EGFR targeting and DNA damaging agent. We assessed for the first time the safety of ZR2002 using two different schedules (5 days on- 5 days off -5 days on or 21 continuous days) in mice and we showed that this drug did not cause any toxicity including skin toxicity in nude mice. Of note, the skin toxicity seen after use of EGFR inhibitors is related to the inhibition of EGFR in the skin that has a crucial role in normal development and physiology of the epidermis. Although skin toxicity may not be life-threatening it causes physical and psycho-social discomfort [44, 45].

We verified the presence and concentration of ZR2002 using IV and P.O. routes of administration in the brain and plasma of non-tumor bearing mice by HPLC and LC-MS methods. We then took a step further and used MALDI IMS imaging [46] to confirm the presence of ZR2002

and its metabolite (ZR01) in the brain of mice with tumors. To our knowledge, this is the first report showing the distribution of a combi-molecule in brain of mice with TMZ-resistant stem cell tumors using MALDI IMS imaging. As shown in chapter 2, the highest dose used in the pharmacokinetic study was 75 mg/kg (concentration chosen based on previous experiments done in Dr. B. Jean-Claude lab). I am currently finalizing experiments to verify the concentration of ZR2002 in brain and plasma of mice following oral administration of ZR2002 at 150 mg/kg using LC/MS. Also, in collaboration with the drug development platform available in our institution (Research Institute of McGill University Health Centre), we are currently developing a 3D image of ZR2002 distribution in the brain of mice with intracranial tumors.

In the same chapter of my thesis, we also report toxicity of ZR2002 in NSG mice due to lack of DNA repair and more particularly lacking DNA-PKcs. DNA-PKcs plays a major role in DNA repair and many studies have shown that DNA-PKcs phosphorylation at the Thr2609 cluster causes conformational change of DNA-PKcs in a way that leads to the dissociation of DNA-PKcs from Ku70/80 heterodimer and release from DSB ends [47, 48]. Also, this conformational change in DNA-PKcs facilitates its association with other DNA repair molecules. Many findings demonstrated the critical function and requirement of DNA-PKcs and Thr2609 cluster phosphorylation in DNA repair. Our study is in accordance with studies that proposed a relation of DNA-PKcs and DNA repair and genomic stability. For example, Bogue et al. [49] reported extreme radio-sensitivity of the cells lacking DNA-PKcs. Interestingly, it is mentioned in the same study that mutations of DNA-PKcs or Ku in humans have lethal consequences [49]. However, Van der Burg et al. [50] reported identification of the first human DNA-PKcs gene mutation in an immunodeficient patient. This mutation only affected Artemis, an essential kinase required for nucleolytic processing of DNA ends that did not affect the activity of DNA-PKcs and therefore

resulted in mild radiosensitivity. Additionally, cells derived from DNA-PKcs^{3A} knock-in mutant mice show extreme sensitivity to stress agents and have impaired DNA repair pathways such as non-homologous end joining (NHEJ). The mice with DNA PKcs^{3A} knock-in mutant lack a functional Thr2605 cluster and also show bone marrow failure and early lethality [51]. Although my work suggests the importance of DNA-PKcs in the mechanism of ZR2002, the exact role of DNA-PKcs and other DSB responses in ZR2002 treatment is still unclear and deserves to be further investigated.

4.2 Contribution 2 (Chapter 3)

GBM is a complex disease notoriously known for alterations of several RTKs, such as EGFR, IGFR1, MET and PDGFRα/β, which account for sustained activation of downstream signaling pathways involved in proliferation and survival [52]. EGFR was the first molecule to be linked to oncogenesis in GBM [53] and overall, around 60% of glioblastoma patients have genomic alteration affecting the EGFR pathway [54, 55]. This includes constitutively active truncations and an in-frame deletion leading to constitutive activation of the intracellular tyrosine kinases [56]. It remains unresolved why EGFR targeting has not been successful for treatment of patients newly diagnosed with GBM [57] or with recurrent disease [58, 59] as it should be ideally suitable in the context of this disease.

One reason reported is that oncogenic TKs orchestrate complex signaling pathways, cross-talk with each other, trigger similar signaling pathways that enable alternate compensatory mechanisms following inhibition with RTK inhibitor as a monotherapy [60]. For a complex cancer such as GBM, our study addresses the need to extend the benefit of EGFR-targeted therapies benefit beyond EGFR-addicted tumors and propose novel and divergent combinatorial therapies.

Accordingly, the novelty and importance of ZR2002 stems from its conceptual design to achieve divergent targeting of different cellular components (i.e, co-targeting a RTK and DNA) [61, 62].

Here in the context of this study, we have thoroughly studied the cell-based properties and the dual mechanisms of action of the EGFR-DNA inhibitor, ZR2002. To the best of our knowledge, these investigations are the first to describe the effect of this combi-molecule on GBM exclusively. In our efforts to delineate the novel functional roles of ZR2002 in GBM, we used an *in vitro* model system consisting of the commonly utilized EGFR (low)/ MGMT (-) U87MG glioblastoma cell line [63] stably transfected with EGFRwt or its mutated form (U87/EGFRvIII). Comparing U87/EGFRwt and U87/EGFRvIII cell lines enabled us to accurately analyze the effect of ZR2002 on cells that have different EGFR levels. We also used a more clinically relevant model by using a panel of low-passage patient-derived primary GBM cell lines and an experimental GSCs model of TMZ-resistant in GBM to assess the ability of ZR2002 to overcome TMZ resistance in highly aggressive mesenchymal GSC lines. These low passage primary cells replicate the *in vivo* behavior of GBM more accurately than established cell lines [64].

Using these models, we first studied the ability of ZR2002 to inhibit both its targets. We showed that ZR2002 is able to act through inhibition of EGFR and its downstream signaling and damage DNA (shown by comet assay) at remarkably low concentration and early time point. We found that the range of potency of type II combi-molecule was in the submicromolar level on different cell lines and GSCs tested. Of note, one of the initial responses to DNA damage is phosphorylation of H2AX (γ-H2AX) that still needs to be investigated following ZR2002 treatment in our future experiments. ZR2002 has proven to be potent in tumor cells with high/low expression of MGMT and cells with PTEN-mutant status, which are biomarkers associated with resistance to TMZ and gefitinib respectively [65, 66].

EGFR has a low expression in the adult central nervous system and high degree expression in tumor cells in the brain and thus it becomes extremely important in terms of drug selectivity and optimal efficacy of EGFR-based therapies [67]. Our study also showed that ZR2002 is 10-fold less effective on normal brain cells and shows a selectivity towards EGFR-expressing cells (in both GSCs and EGFR isogenic cell lines). This therapeutic index was promising as it suggested the possibility of a therapeutic window without unacceptable dose-limiting toxicity. Other models such as EGFR knockdown models could be explored in future experiments to assess the safety, selectivity and potency of ZR2002 on cells that have no EGFR expression and do not display EGFR phosphorylation.

Our study also provides some mechanistic insights underlying the anti-proliferative effects and potency of ZR2002 *in vitro* and *in vivo*. First, Tyr1068, which has been reported as one major EGFR autophosphorylation site [68] that is key in Ras-Raf-MAPK ERK1/2 pathway [69], was drastically down regulated by ZR2002 treatment in our *in vitro* and *in vivo* studies. Second, the DNA damage seen in GBM cell lines and GSCs allows us to hypothesize that the combi-molecule could also reach the nucleus intact. In addition to our own work in which we show the localization of ZR2002 in perinuclear regions of cells using the florescent properties of this unique combi-molecule (data not shown in the thesis), there is ample evidence of the perinuclear and nuclear localization of EGFR [77]. One such study is by Lin et al.[70] that suggested a potential role of EGFR membrane receptor as a transcription factor. Later studies also showed the nuclear EGFR activity by interacting with several nuclear proteins [71-76]. Several studies provided evidence for the role of nuclear EGFR in transcriptional regulation of cyclin D1 [77], and assembly of DNA repair complexes with proteins such as DNA-PKcs [78, 79], in addition to its direct interaction with histone H4 affecting DNA synthesis [80] and showed its role in resistance to chemotherapy

and radiotherapy and association with worste clinical prognosis [79]. We propose a model that does not only consider EGFR in the perinuclear region but also in the nucleus. Interestingly, it has been reported that cyclin D1 is involved in GBM progression and its down regulation inhibits proliferation, induces apoptosis, and attenuates the invasive capacity of human GBM cells [81]. Third, a study by Yakoub et al. demonstrated that EGFR is also involved in upregulation of DNA repair genes such as XRCC1 and ERCC1 [82]. Furthermore, it has been reported that EGFR inhibition led to down regulation of a few DNA repair genes (e.g. XRCC1, ERCC1) [83]. We surmise that ZR2002 inflicts DNA damage through its DNA-damaging moiety, while its EGFR TK-targeting moiety irreversibly induces covalent damage to the ATP-binding site, which subsequently impairs EGFR-mediated DSB repair. Fourth, EGFR inhibitors such as gefitinib induce apoptosis in human glioma cells by targeting Bad/Bax, Bcl-2 and Bcl-xL signaling pathway [84, 85]. Additional studies are needed to identify the specific mechanism(s) by which ZR2002 inhibits EGFR-mediated repair of DNA damage (decrease in DNA synthesis and repair in GBM) and apoptosis, which might be critical for its *in vivo* efficacy and survival benefit over TMZ and gefitinib. Of note, the effect of ZR2002 on cyclin D1 and p27/Kip.1 is currently being investigated in our laboratory.

Today, it is well known that upon DNA damage, p53 can become activated and act as the guardian of cells [86, 87]. One interesting result of our *in vitro* validation was the drastic up-regulation of p53 after short exposure (2 hours) to ZR2002 treatment in EGFR isogenic cell lines and GSCs, and this increase was more pronounced compared to TMZ or gefitinib. Other studies using similar combi-molecules have also elicited activation as well as accumulation of p53 in all cell lines tested which suggest that these type of combi-molecules act through a mechanism involving p53wt activation [62]. TP53 protein is known to bind to damaged DNA and activate

cell-cycle arrest, apoptosis or DNA repair events through a number of mediators, the most widely studied of which is p27/Kip.1 [86, 88]. Cellular stresses including DNA damage may trigger a pro-apoptotic arm of the TP53 network in which our study also shows up-regulation of PARP after ZR2002 treatment in samples collected from our *in vivo* experiments (data not shown). The upregulation of PARP in response to ZR2002 treatment requires further mechanistic explorations and confirmation such as expression of annexinV, cleaved caspase 3 or by using TUNEL assay in different experimental settings.

Interestingly, ZR2002 was universally effective on all GSCs regardless of their endogenous EGFR and MGMT levels, except for the GSC line OPK257 previously characterized by our group for its mutant *TP53* status [92] had the highest IC50 among all GSC lines tested (0.6μM versus 1.7 μM). It is well known today that mutations in TP53 are seen in ~30% of primary GBM [93, 94] and these mutations can lead to deregulated response to stress, DNA damage such as radiation due to many reasons such as failure to activate p53 targets required for efficient DNA repair or apoptosis [95, 96]. As, we extend our observations on the relationship between p53 and sensitivity to ZR2002 to inhibit p53 function in GSC (OPK49) we showed that p53-silencing by RNAi significantly conferred resistance to ZR2002. Our results are in accordance with previous studies showing that p53 knockdown in GSCs inhibits the loss of stemness [97]. It would be interesting to explore this fascinating functional observation in an *in vivo* setting using OPK49 mock and OPK49/shRNA. As reported by many studies, the p27/Kip.1 has a role in inducing growth arrest, differentiation or senescence and can be stimulated by other pathways that are independent of p53 [89-91]. Using brain tumor tissue collected from mice injected with1123IC7R (*TP53* mutant GSC) and treated with TMZ, gefitinib and ZR2002 it is possible to unmask the effect of ZR2002 on p27/Kip.1 and to further reveal the underlying biology of the remarkably stronger potency of

ZR2002 when compared to each drug individually.

An important finding in our study is that ZR2002 exhibited strong *in vitro* and *in vivo* effects on U87/EGFRvIII cell line unveiling EGFR/DNA binary targeting as a novel strategy to directly inhibit EGFRvIII. Thus far, strategies targeting EGFRvIII-positive GBM tumors has failed to show efficacy in GBM [98, 99]. EGFRvIII has been shown to enhance DSB repair in a mouse orthotopic glioma model [100]. Mellinghoff et al.[101] reported that co-expression of EGFRvIII and PTEN (negative regulator of PI3K/Akt pathway) in GBM cells is associated with heightened sensitivity to EGFR kinase inhibitors. PTEN deficiency (as in PTEN-deficient U87MG model) decreases response to EGFR inhibitors due to high levels of Akt activation [101]. Accordingly, the anti-proliferative potency of ZR2002 was associated with marked dephosphorylation of EGFR and Erk1/2, but not p-Akt (Ser473) in U87/EGFR isogenic cell lines. Although we did not use gefitinib as one of the treatment groups when testing the effect of ZR2002 on U87/EGFRvIII, previous studies have shown that gefitinib is not effective on cells expressing vIII mutation [102]. Also, we did not compare ZR2002 to TMZ in our U87/EGFRvIII model because U87/EGFRvIII is a MGMT negative cell line and is not the right model for this comparison. Here, mice were treated for 5 days and then given a 5 day break and treated again for another 5 days. As ZR2002 is an EGFR inhibitor and U87/EGFRvIII is an aggressive cell line, on the days in which mice were not treated may have been opportunities for cells to grow and proliferate [103]. We would like to test a continuous dosing regimen of ZR2002 in a second survival study, and to see if there is benefit on the survival of mice compared to this alternative schedule. In addition, it would be interesting to validate our finding on the effect of ZR2002 on U87/EGFRvIII model using a GSC model that express the EGFRvIII mutation. This will hopefully serve as a guidance for future directions of the research on effects of ZR2002 on tumors that express EGFRvIII mutation.

Given the important role of GSCs as a disease reservoir in GBM, unraveling the molecular mechanisms involved in the maintenance of GSCs provided the rationale for pre-clinical and clinical testing of targeted therapeutic strategies aiming to eradicate GSCs [104]. Our results are in accordance with studies showing that EGFR-knockdown in EGFR-positive GBM neurosphere cultures led to differentiation and less malignant tumors *in vivo* and its inhibition resulted in reduced neurosphere formation in the presence of EGF [105]. ZR2002 demonstrated potency in our panel of glioma stem cells and a highly aggressive TMZ-resistant stem cell line (11231C7R) possessing an IC50 of around 0.6 μM despite its *TP53* mutant status (sequencing data not shown). A continuous 21 days' regimen of ZR2002 was well tolerated by tumor-bearing TMZ resistant mice and lead to a significant increase in median survival of mice in this group. This was especially impressive considering the highly aggressive nature of this cell line. We used an equimolar concentration of oral gefitinib (150 mg/kg) for continuous 3 weeks which been also reported as the highest non toxic dose of this drug in mice [106]. As expected mice in the TMZ group were in as equally poor condition as the vehicle-treated mice and had a similar median survival.

Since the 1123IC7R cell line has been reported to be MGMT negative [107] another, interesting future direction of this project would be to verify the mechanism of resistance of 1123IC7R to TMZ. Accordingly, it has been reported that in GBM, MMR-deficient (alterations in expression of MSH2, MSH6, and PMS2) tumors are resistant to temozolomide even in the absence of MGMT expression [108]. Alternations in, MSH6 has been reported in ~30% of GBMs after treatment with alkylating agents and MSH6 deficiency has been linked to temozolomide resistance *in vitro* studies [108, 109]. Restoration of MSH6 expression resulted in a more chemosensitive phenotype [109].

In toto, these studies demonstrate the potential of ZR2002 to be used as a novel approach to circumvent resistance to TMZ in patients with recurrent GBM and indicate that continuous once-a-day p.o. dosing may be a suitable therapeutic regimen. Overall, ZR2002 is a strong EGFR inhibitor and a potent DNA damaging agent and to our knowledge, this was the first report on a combi-molecule capable of inhibiting EGFR and damaging DNA in different GBM established cell lines including EGFRvIII. Remarkably, in addition to being a potent agent on established GBM cell lines, ZR2002 was capable of inhibiting the proliferation of GSCs from patients newly diagnosed with GBM and TMZ resistance GSC models. Thus, this molecule has the potential to be developed as a single agent not only to be used for patients that co-express EGFR and MGMT but also to overcome the resistance to TMZ.

4.3 CONCLUSIONS

Patients diagnosed with glioblastoma (GBM) have a generally poor prognosis despite treatment with surgery, radiation therapy and temozolomide (TMZ). Effective treatment strategies are needed to improve their dismal outcome. The central objective of this work was to test ZR2002 a dual EGFR and DNA damaging drug in GBM. Two strategies were employed to verify whether this drug meets the primary qualities to be carry forward into a clinical setting.

First, we achieved a key step in pre-clinical development of ZR2002 and showed its safety, BBB permeability. this work shows oral administration of ZR2002 at doses up to 150 mg/kg/day for 21 continuous days in athymic mice without tumors that have intact DNA repair pathways was safe. ZR2002 was detected in the brain using HPLC and confirmed by MALDI IMS imaging. Western blotting revealed that treatment with ZR2002 significantly ablated phosphorylation of EGFR (Tyr1068), Erk 1/2 and Akt (p-Akt/Ser473). However, treatment of ZR2002 in NSG mice showed toxicity that suggests the fact that this drug works through DNA-PKcs. Second, this thesis also contributed to describing the potency and mechanism of action and distribution of ZR2002. Type II combi-molecules carrying a hemi-mustard appended to a quinazoline moiety (EGFR inhibitor), possess strong mixed EGFR-DNA targeting potentials. We provide several lines of evidence supporting EGFR/DNA binary combi-targeting strategy using the combi-molecule ZR2002 in GBM *(i) in vitro* cytotoxicity against U87MG isogenic GBM cell lines stably expressing EGFR or EGFRvIII within a submicromolar range, *(ii)* significant increased survival of mice bearing intracranial U87/EGFRvIII xenografts orally treated with ZR2002 (p=0.0003), *(iii)* drastic inhibition of proliferation in patient-derived GBM stem-like cells (GSCs) beside reduced tumor volumes in a subcutaneous GSC TMZ-resistant mesenchymal *in vivo* derived GSC subline and *(iv)* concomitant inhibition of EGFR/EGFRvIII-induced signaling while inflicting

DNA damage and delaying DNA repair through a mechanism involving *TP53* activation. Our study highlights the potential of ZR2002 to overcome TMZ resistance and represents a promising novel therapeutic strategy in GBM.

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