Resistance to Targeted Anti-Cancer Therapeutics 11
Series Editor: Benjamin Bonavida

Amanda Tivnan *Editor*

Resistance to Targeted Therapies Against Adult Brain Cancers

Springer

Resistance to Targeted Anti-Cancer Therapeutics

Volume 11

Series Editor
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Amanda Tivnan
Editor

Resistance to Targeted Therapies Against Adult Brain Cancers

Springer

Editor
Amanda Tivnan
Centre for Systems Medicine, Department
of Physiology and Medical Physics
Royal College of Surgeons in Ireland
Dublin, Ireland

ISSN 2196-5501 ISSN 2196-551X (electronic)
Resistance to Targeted Anti-Cancer Therapeutics
ISBN 978-3-319-46504-3 ISBN 978-3-319-46505-0 (eBook)
DOI 10.1007/978-3-319-46505-0

Library of Congress Control Number: 2016959185

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This volume is dedicated to the memory of Mr. Joe Rylands (1966–2013), Dublin, Ireland whose battle against Glioblastoma, and the battle of all those facing brain tumours, inspires researchers to improve current therapies and standards of care.

Preface

According to the most recent figures, there are 650 newly diagnosed malignant brain tumours daily, severely threatening human health and quality of life. Almost 80 % of these tumours are referred to as gliomas, a broad class of neuroectodermal tumours arising from the sustentacular neuroglial cells in the brain which includes astrocytomas, ependymomas and oligodendrogliomas. Of these gliomas, over 75 % are astrocytomas which are classified as low-grade gliomas (LGGs, Grades I and II) or high-grade gliomas (HGGs, Grades III and IV). Grade IV astrocytoma is also known as glioblastoma (GBM) and is the most aggressive and lethal form of brain tumour which can be diagnosed. The first chapter of this book was written by Drs. Crilly and O'Halloran, who provide a structured overview of several of the major forms of brain tumours which arise in patients, including gliomas, along with clinically relevant targeted therapies which are currently under investigation. The authors discuss glioblastoma, oligodendroglioma, ependymoma and haemangioblastoma with regard to each of their mechanisms and pathways of resistance to currently used therapeutics. This extensive chapter provides a great introduction to the clinical and research challenges which arise in generating targeted therapies for a myriad of malignant brain tumours.

Several chapters of this book are focused on one particular type of glioma called glioblastoma (GBM). GBM is a highly invasive form of brain cancer with extremely poor prognostic outcome despite intensive treatment. Prognosis is reported as 'median survival' which, for adults with aggressive GBM treated with surgical resection, radiotherapy with concurrent and adjunct chemotherapy using the DNA alkylating agent, temozolomide (TMZ), is only 14.6 months. Notably, the absence of treatment typically yields a median survival rate of 3 months, and, despite treatment, the average 5-year survival rate for these patients remains at less than 5 %. The effects of this form of cancer in terms of total years of life lost, over 20 years on average, in addition to the socioeconomic and financial impact of the intense treatment protocols required render GBM the most lethal form of brain tumour with the highest impact on the patient's quality of life post-diagnosis. Although genetic alterations significantly contribute to the pathology of GBM, including self-sufficiency in growth signals through receptor tyrosine kinase signalling,

insensitivity to antigrowth signals, evasion of apoptosis, angiogenesis, replicative potential and activation of invasive/metastatic pathways, the true epidemiology of GBM occurrence has not yet been fully elucidated. In this regard, researchers are attempting to develop gene therapy approaches in order to improve patient's outcome for both initial GBM diagnosis and recurrent tumour presentation. Due to its aggressive nature, several chapters of this book are focused on discussing the current status of novel targeted therapies for this form of brain tumour. For example, Dr. Tivnan outlines the role each of the adenosine triphosphate-binding cassette (ABC) superfamily multidrug resistance proteins may play in providing chemoresistance to GBM, reviewing all clinical trials which are currently targeting these proteins and the resistance mechanisms by which GBM cells have developed in order to maintain survival. The standard clinical protocol for GBM treatment is known as the Stupp protocol, a clinical regimen involving surgical resection and adjunct and concomitant chemotherapy in addition to radiotherapy. Drs. Shen and Hau discuss the mechanisms of resistance to targeted radiotherapy in this brain tumour underlining the role of the microenvironment, hypoxia and the *HIF-1* gene in this process. Identification of each of these elements has, to date, provided researchers with potential avenues through which alternate therapeutics may be developed in order to eliminate radiotherapy resistance.

As is the case for all diseases of the brain, the efficient delivery of potential therapeutics beyond the blood-brain barrier (BBB) is a major hindrance. Drs. Kealy and Campbell describe the normal physiology of the blood-brain barrier, its biological components and its compromised structure in GBM patients. They specify how the BBB affects targeted therapeutic administration and outline methods through which researchers are attempting to improve clinical outcome through BBB modifications during treatment.

The crossover of drug use among various forms of cancer is examined by Dr. O'Neill whereby the use of small molecules as targeted therapies in adult brain cancers, and their potential resistance in these diseases, can be assessed through their prior use in various other forms of cancer, for, example, TRAIL, EGFR and VEGFR inhibitors. The concept of 'lessons learnt from various cancer types' is further developed by Dr. Hill et al. discussing the repurposing of several drugs for brain tumour treatment, which are clinically successful for other cancer types, in an attempt to circumvent chemotherapy resistance. Following from the introduction of small molecule inhibitor use in brain tumours, Ms. Pokorny et al. examine the use of small molecule inhibitors specifically in GBM and how pathways of resistance occur and may be circumvented in this form of brain tumour.

Connor et al. review the topic of imaging targeted therapy response in GBM and how traditional imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI) and intraoperative ultrasound were routinely used to monitor the therapeutic effects of cancer interventions. They mention how there are now many additional non-invasive imaging modalities available, each with unique advantages, disadvantages and applications. The authors highlight that, despite advances made in non-invasive imaging techniques for brain tumour assessment, there remains a lack of effective imaging modalities which allow visualisation of

conversion of a proliferative to an invasive glioma phenotype particularly after treatment with targeted therapeutics such as anti-angiogenic or anti-invasive drugs. In this book, Smith et al. contribute the last of the malignant primary brain tumour chapters, reviewing the physiology, prevalence and treatment of a rare form of malignant brain cancer called meningioma. These brain tumours are typically benign; however, those that require targeted therapies quite often display resistance patterns similar to aggressive gliomas, and, as considered by Smith et al., several genetic alterations have been identified which may contribute to this resistance. The penultimate chapter was contributed by Dr. Zakaria, in which the potential role, if any, that targeted therapies have had on low-grade glioma progression-free survival (PFS) and overall survival (OS) rates and the cognitive decline which is quite often noted in these patients during treatment regimens, is reviewed. The author of this chapter comments that they must realise that low-grade gliomas will recur despite surgical intervention and become more aggressive and resistant to treatment; hence, there will always be an urgent need for new active targeted therapeutic agents, and resistance to such will be a constant challenge.

The final chapter of this book details a much more prevalent form of brain tumour, a secondary or metastatic brain tumour. Drs. Langley and Fidler estimate that approximately 200,000 cases of brain metastases occur in the United States each year and between 20 % and 40 % of patients with disseminated cancers will develop brain metastases during the course of their disease, most frequently arising from tumours that originate in the lung (40–50 %), breast (15–20 %) and skin (5–10 %). The authors comment on the mechanism of establishment and the role which the host interactions may play in contributing to the development of metastatic brain tumours, drawing attention to this potential target for reducing acquired resistance in metastatic brain tumours.

Overall, this book provides a historical study overview detailing the current treatment options available to brain tumour patients; the identification of genetic alterations in several glioma types, especially glioblastoma; and the development of targeted therapy to circumvent chemoresistance and the inherent resistance to these newer therapeutic approaches. The chapters in this book provide an extensive point of reference for up-to-date research and clinical applications of a myriad of treatment regimens for various brain tumour types.

Dublin, Ireland Amanda Tivnan

Acknowledgements

To my husband, Paul, and two children, Analla Faye and Braoc Bán, whose support and love drives me and pushes me forward. Thank you.

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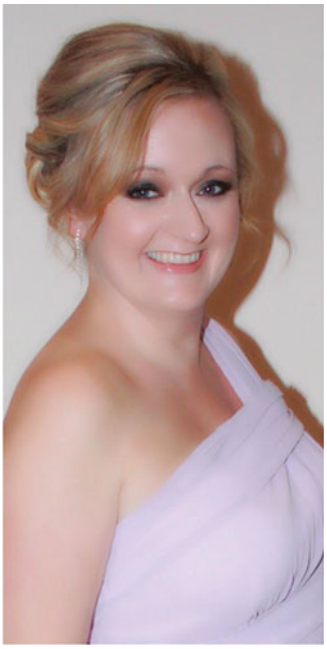

Dr. Amanda Tivnan began her career undertaking a Ph.D. at Trinity College Dublin where she studied transient RNAi-mediated suppression of the thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) to allow increased bioavailability of the neuroprotective peptide thyrotropin-releasing hormone (TRH) and subsequent transport across the blood-brain barrier (BBB) for treatment of central nervous system (CNS) disorders. This gave Amanda a keen interest in RNAi-based therapeutic development for the treatment of a myriad of diseases. Following this, she was employed as a postdoctoral researcher in the Royal College of Surgeons in Ireland, where she developed microRNA expression vectors for stable inhibition of gene targets in the paediatric cancer, neuroblastoma, resulting in the development of neuroblastoma-specific targeting using nanoparticle-based delivery systems for administration of microRNA molecules. Her research in Prof. Stalling's laboratory contributed to several first author and co-author publications on the role of dysregulated microRNAs in neuroblastoma tumour development and progression, highlighting their potential therapeutic applications in this form of cancer.

Additionally, at the Children's Cancer Institute and UNSW Australia, Dr. Tivnan was involved in projects aimed at identification and validation of new therapeutic targets in both neuroblastoma and glioblastoma. She used her knowledge of RNAi-based therapeutics to investigate the role of multidrug resistance proteins on chemotherapeutic drug resistance in both these aggressive forms of cancer. She gained

valuable experience in the clinical setting, liaising with prestigious Australian neurosurgeons to procure fresh glioblastoma patient tumour samples, adopting a bench-to-bedside approach which led to her involvement in several public-relation brain tumour awareness drives and charity events. This opportunity to work in the translational aspect of glioblastoma primed Dr. Tivnan to target her research exclusively towards glioblastoma, and she returned to work in the Royal College of Surgeons in 2013 upon receipt of the highly competitive Irish Cancer Society Research Fellowship to investigate the role of ABCC1 (MRP1) in chemoresistance in the aggressive brain cancer glioblastoma.

Dr. Tivnan is an independent and highly motivated researcher and future academic of the highest calibre. She is also a mother of two and has progressed her academic career at exceptional pace and with exemplary rigour and strength. Her studies have made use of an elegant blend of cancer research, neurooncology and neuroscience, as well as molecular biology and drug delivery research to yield novel data with substantial clinical and therapeutic significance.

Chapter 1
Targeted Therapies in Brain Tumours: An Overview

Shane M. Crilly and Philip J. O'Halloran

Abstract Chemotherapy resistance in gliomas represents a major therapeutic challenge in the management of these tumours. The mechanisms of drug resistance in these tumours are complex, multifarious and are the subject of ongoing clinical trials. In this chapter, we give an overview of the mechanisms of chemoresistance in the most common human gliomas which occur in the adult population. We also outline some targeted therapeutic agents currently under investigation in the management of these tumour types.

Keywords Chemoresistance • Ependymoma • Glioblastoma • Haemangioblastoma

Abbreviations

ABC	Adenosine triphosphate-binding cassette
EDGR	Epidermal growth factor receptor
IDH-1	Isocitrate dehydrogenase 1
L1CAM	L1 cell adhesion molecule
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MGMT	O6-methylguanine-DNA methyltransferase
MVP	Major human vault protein
NGAL	Neutrophil gelatinase-associated lipocalin
PCDC4	Programmed cell death 4

S.M. Crilly
National Neurosurgery Centre, Beaumont Hospital, Dublin 9, Ireland

P.J. O'Halloran (✉)
National Neurosurgery Centre, Beaumont Hospital, Dublin 9, Ireland

Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland
e-mail: phiohalloran@rcsi.ie

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_1

PDGFR-A	Platelet-derived growth factor receptor A
TMZ	Temozolomide
WT-1	Wilms' tumour 1
ZEB1	Zinc finger E-box-binding homeobox 1

1.1 Glioblastoma (IDH wild type ICD-O 9440/3, IDH Mutant ICD-O 9445/3 WHO Grade IV)

1.1.1 Introduction

Glioblastoma (GBM) is the most common primary brain tumour. Reported incidence of GBM in the human population is 3/100,000. The average age at diagnosis is 64 years. GBM is one of the most aggressive forms of cancer in humans; the current median survival with best available therapy is 14 months [1].

Current treatment involves maximum safe surgical resection followed by concurrent radiotherapy and temozolomide (TMZ) chemotherapy [2]. A variety of molecular markers have been identified as having different prognostic significance in GBM. These include the methylation status of the gene promoter for O6-methylguanine-DNA methyltransferase (MGMT), isocitrate dehydrogenase enzyme 1/2 (IDH-1/2) mutation, TP53 mutation and epidermal growth factor receptor (EDGF) overexpression and amplification [3, 4] .

Molecular and genetic characterisation of GBM is an active area of research. GBM is understood not to represent one single disease entity, but a disease with many variants dependent upon the presence or absence of distinct molecular markers. The presence or absence of different biological markers is a known factor in tumour sensitivity to chemotherapy/radiotherapy. There exist at least three distinct biological subtypes of GBM. Proteomic pathways and mechanisms of signal transduction differ among these subtypes [5].

Classical/proliferative subtype: exemplified by frequent mutations or amplifications in the gene encoding epidermal growth factor receptor (EGFR).

Proneural subtype: exemplified by mutations in TP53, isocitrate dehydrogenase 1 (IDH-1) and platelet-derived growth factor receptor A (PDGFR-A).

Mesenchymal subtype: exemplified by mutations in the neurofibromatosis type 1 (NF-1) gene.

These subtypes differ in their natural history and their response to targeted therapeutics. In this chapter, we give an overview of the mechanisms of chemoresistance in GBM and the targeted therapies that may be used in order to overcome these therapeutic challenges.

1.1.2 Mechanisms of Chemoresistance in GBM

1.1.2.1 CD 133 + Stem Cell-Associated Resistance/NOTCH Pathway

CD 133 is a novel cell membrane protein that has been identified as a marker of GBM stem-like cells in the central nervous system. It is known that such cells drive tumour progression, that is, cancer stem cells (CSCs). The work of Singh et al., in Toronto, has elucidated the role of CD133+ in human brain tumour clones. Specifically, that in nonobese diabetic, severe combined immunodeficient (NOD-SCID) mice, these clonal cell populations (derived from human tumours) can initiate tumorigenesis in vivo [6]. CD133+ CSCs can escape lethal damage by activation of DNA damage repair checkpoints, including checkpoint proteins Chk1 and Chk2 [7]. The NOTCH pathway is involved in the proliferation/cell survival of stem-like cells in embryonal cell lines [8]; therefore, inhibition of the NOTCH pathway using gamma-secretase inhibitors reduces stem cell marker CD133 in GBM nanospheres. The use of gamma-secretase inhibitors is one potential therapeutic adjunct in GBM therapy. Gilbert et al. describe the enhancement of temozolomide treatment in GBM with the addition of gamma-secretase inhibitors, through the inhibition of the NOTCH pathway in an experimental model of GBM. Specifically, ex vivo TMZ and gamma-secretase inhibitor treatment of glioma xenografts in immunocompromised mice extended tumour latency and survival and in vivo blocked tumour progression in 50 % of mice with pre-existing tumours [9].

1.1.2.2 Downregulation of Lipocalin 2

Lipocalin 2 is a member of the lipocalin family, involved in binding/transporting lipids/hydrophobic molecules. Lipocalin 2 is involved in a wide range of physiological processes including regulation of the innate immune response, iron sequestration and modulation of the functioning of matrix metalloproteinases [10, 11]. Increased lipocalin 2 expression has been discovered in a variety of human neoplasms including breast, lung, colon, thyroid and pancreatic carcinomas [12, 13]. Neutrophil gelatinase-associated lipocalin (NGAL) has been shown to be highly upregulated in non-small cell lung carcinoma (NSCLC) cells, specifically those with acquired erlotinib resistance, and is known to mediate apoptosis resistance in this tumour type [14]. The role of lipocalin 2 in glioma tumorigenesis has yet to be fully elucidated. Lipocalin 2 is known to be actively secreted by microglia and astrocytic cells and is known to play a role in apoptosis in these cell types. It has also been proven to be involved in reactive astrocytosis in response to cellular injury in the central nervous system [15, 16]. In a study published in 2009, Zheng et al. proved that downregulation of lipocalin 2 contributes to chemoresistance in GBM

cells. Specifically, they elucidated one of the putative mechanisms behind BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) chemotherapy resistance using a variant of C6 rat glioma cells, that is, decreased expression of lipocalin 2 in BCNU-resistant C6R cells. They showed, however, no downregulation of lipocalin 2 receptors in these cells with lipocalin 2 downregulation. From a therapeutic viewpoint, the addition of recombinant lipocalin 2 or the use of lipocalin 2 copy DNA (cDNA) improved the sensitivity of C6 cells and human glioma cells to BCNU, while the knockdown of lipocalin 2 by antisense cDNA transfection decreased tumour chemosensitivity. Mechanistically, lipocalin 2 enhanced BCNU-induced Akt dephosphorylation, thereby inducing apoptosis. Therefore, lipocalin 2 may be a putative molecular target to inducing chemosensitivity in GBM cells [17].

1.1.2.3 Decreased PDCD4 Expression and Tumour Progression

Programmed cell death 4 (PDCD4) is a tumour suppressor protein which has been studied primarily in breast cancer tumorigenesis, which is known to be unregulated in apoptosis [18] and downregulated in several forms of cancer [19]. In human lung carcinoma, the loss of PDCD4 expression correlated with tumour progression and poor prognosis [20]. Gaur et al. demonstrated that oncogenic miR-21 microRNA is expressed at higher levels in GBM-derived cell lines and that downregulation of miR-21 in GBM-derived cell lines leads to increased PDCD4 expression which inhibits tumour formation. They reported that GBM-derived cell lines transfected with anti-miR-21 and short interfering RNA (siRNA) to PDCD4 showed tumour growth, therefore, indicating that upregulation of PDCD4 is a potential chemotherapeutic target in GBM therapy [21].

1.1.2.4 Wilms' Tumour 1 Transcription Factor Silencing

Wilms' tumour 1 (WT-1) is a transcription factor involved in multiple biological processes, is known to be constitutively overexpressed in GBM cell lines and is essentially absent in the normal human brain [22]. The function of WT-1 in GBM is unknown in humans. WT-1 silencing causes IGF-1R overexpression. IGF-1R is regarded as a proliferative factor and anti-apoptotic; Chen et al. demonstrated that WT-1 functions as a survival factor for GBMs, possibly through the inhibition of IGF-1R expression [23].

1.1.2.5 ZEB1 Pathway

Zinc finger E-box-binding homeobox 1 (ZEB1) has been shown to influence invasion, chemoresistance and tumorigenesis in GBM. It has also been shown that ZEB1 expression in GBM patients is predictive of shorter survival and poorer response to temozolomide (TMZ). ZEB1 has been shown to interact with miR-200 on

downstream effectors ROBO1, OLIG2, CD133 and MGMT and considered as potential therapeutic targets in GBM therapy [24].

1.1.2.6 Adenosine Triphosphate-Binding Cassette (ABC) Superfamily

Multidrug resistance in paediatric GBM is known to be in part mediated by the adenosine triphosphate-binding cassette (ABC) transporters. The expression of various multidrug-resistance genes has been described for various childhood neuroepithelial tumours. Valera et al. have described differential expression of multidrug-resistance genes in a cell line of paediatric GBM following exposure of the cell line to vinblastine [25]. Drug resistance has been associated with the presence of the ABC efflux transporter which excludes drugs from the intracellular compartment. It has been hypothesised that a specific subset of GBM cells named initiating cells are more adept at self-renewal/proliferation. These cells have been shown to express adenosine triphosphate-binding cassette at very low levels, and this is thought to be associated with differentiation. Rama et al. have described this overexertion of ABC transporters in differentiated GBMs versus initiating cells. Therefore, the blockade of ABC transport proteins may be possible targets for reducing chemoresistance in GBM [26].

1.1.2.7 P38 MAP Kinase Signalling

Mitogen-activated protein kinase (MAPK) kinase 3 (MKK3) has been identified as an important activator of p38 MAPK in glioma. These members of the MAPK family are associated with tumour invasion, progression and patient survival. Demote et al. have shown that inhibition of MKK3 or p38 leads to reduced glioma invasiveness in vitro. Therefore, treatment with a chemotherapeutic agent to interfere with MKK3/p38 signalling in addition to temozolomide increases chemosensitivity in gliomas [27]. The use of beta-elemene, which is an extract of the traditional Chinese herb *Curcuma wenyujin*, has a demonstrated anti-proliferative effect in human and mouse tumour cells in vivo and in vitro [28, 29]. Mechanistically, beta-elemene inhibits growth of GBM cells through a p38 MAPK-dependent pathway. Yao et al. found that beta-elemene inhibited GBM cell proliferation causing G0/G1 phase arrest in C6 and U251 GBM cell lines through the upregulation and phosphorylation of p38 MAPK [30].

1.1.2.8 Neural Adhesion Molecule L1CAM

Neural adhesion molecule L1 cell adhesion molecule (L1CAM) is an axonal glycoprotein belonging to the immunoglobulin supergene family. This cellular adhesion molecule plays an important role in the development of the central nervous system including roles in neuronal migration and differentiation. GBM tissues exhibit

elevated expression of the adhesion molecule L1CAM. Held-Feint et al. investigated the mechanism of L1CAM in GBM cells and the role of this molecule in tumour chemoresistance. Specifically, that L1CAM and tumour growth factor-beta 1 (TGF-beta 1) activity are interrelated in GBM cell cultures. Also, that L1CAM expression in GBM cells reduced the degree of apoptosis subsequent to treatment with temozolomide (TMZ) chemotherapy. They also demonstrated that knockdown of L1CAM by siRNA increased GBM stem-like cell chemosensitivity and that overexpression of L1CAM diminished the apoptotic response in these cells. They postulated this to be related to caspase-8 expression in GBM and glioma stem-like cells. That is, downregulation of caspase-8 by TGF-beta 1 and upregulation of L1CAM lead to apoptosis resistance in GBM, thereby, highlighting L1CAM downregulation as a potential target for GBM resistance [31].

1.1.2.9 MicroRNA-Induced Chemo-/Radiosensitivity

MicroRNAs are a newly discovered family of genes encoding small RNA molecule which bind through partial sequence homology to the 3′ untranslated regions (3′ UTRs) of target genes which are involved in gene expression [32]. They are known to play an important role in tumorigenesis, cell survival and apoptosis. MicroRNA-21 is significantly elevated in GBM and is known to play a role in tumorigenesis and invasiveness. Ren et al. proved that the addition of a molecular inhibitor of microRNA-21 to taxol chemotherapy increased the chemosensitivity of PTEN mutant/PTEN-wild-type GBM cells to taxol chemotherapy [33]. Similarly, Wong et al. also proved that microRNA-21 inhibition in addition to temozolomide chemotherapy results in higher apoptotic levels in an in vitro GBM model [34].

Han et al. discovered that beta-catenin, a cellular pathway which is dysregulated in many cancers, regulates miR-21 in human glioma cells. It does so in a signal transducer and activator of transcription 3 (STAT-3)-dependent manner. In essence, they demonstrated that in GBM cells and LN229 glioma xenografts, the beta-catenin/STAT3/miR-21 pathway regulates tumour growth, proliferation and invasiveness by controlling the RECK protein, a membrane-anchored matrix metalloproteinase (MMP) inhibitor whose absence has been associated with several tumour types including human gliomas [35, 36].

MicroRNA-211 (miR-211) is another putative mRNA known to be involved in tumour invasiveness in melanoma, important in regulating apoptosis and tumour progression [37, 38]. Asuthkar et al. found that miR-211 is involved in the regulation of MMP-9 and plays a functional role in GBM and that miR-211 inhibits glioma cell invasiveness and migration. They speculated that upregulating miR-211 and downregulation of MMP-9 may have applications for GBM chemotherapeutics in the future [39].

Moller et al. reviewed the literature on microRNA in GBM in a systematic review published in 2013. In their excellent review on this subject, they found 163 papers, identifying 253 unregulated, 95 downregulated and 17 disputed microRNAs with respect to levels of expression; the vast majority (85 %) of the miRNAs had not been

meaningfully functionally characterised with respect to their role in gliomas. They focus on 26 miRNAs that have been proven to be involved in the mesenchymal mode of invasiveness and migration [40]. They outline the fact that there are 365 miRNAs which have been associated with gliomas, but, only 15 have been widely studied (miR-7, miR-10b, miR-15b, miR-17, miR-21, miR-23a, miR-25, miR-124, miR-128a, miR128b, miR-132, miR137, miR-195, miR-221 and miR222) and accounting for 62 of the 102 papers they reviewed [40].

1.1.2.10 Vascular Endothelial Growth Factor (VEGF) Inhibition in Recurrent GBM

Vascular endothelial growth factor is a potent mediator of angiogenesis in GBM. Rubenstein et al. demonstrated that pharmacological blockade of VEGF activity inhibited intracranial growth of GBM [41]. Subsequently, Kreisl et al. published the results of a phase II trial in 2009 demonstrating that single agent bevacizumab had significant biological activity in those with recurrent GBM. They found that 71 % of patients underwent a radiographic response as per the Levin criteria when treated with bevacizumab and that progression-free survival was 16 weeks (95 % CI, 12–26 weeks) [42, 43]. Although anti-angiogenic therapy appears to improve the survival in patients with recurrent GBM, it is thought to eventually increase tumour invasiveness in the case of relapse [44]. De Groot et al. describe this effect in their 2010 paper where they describe an apparent phenotypic shift to a predominantly infiltrative pattern of tumour progression following treatment with bevacizumab [45].

Two trials published in 2014 (RTOG 0825, and the AVAglio trial) demonstrate no overall increase in survival in newly diagnosed GBM patients who undergo treatment with bevacizumab [46, 47]. Specifically, the work of Gilbert et al., published in the NEJM in February 2014, demonstrated no increase in overall survival, with modest increase in progression-free survival of the disease. There was a modest increase in rates of side effects associated with bevacizumab including hypertension, thromboembolic events, intestinal perforation and neutropenia [46]. Similarly, the AVAglio trial showed that the addition of bevacizumab to temozolomide chemoradiotherapy conferred no survival advantage in GBM, with an increase in associated morbidity relative to treatment with placebo [47].

1.1.2.11 c-MET Pathway in GBM Resistance to Bevacizumab

c-MET hyperactivation has a known role in increasing tumorigenicity and tumour resistance to DNA-damaging agents. Li et al. have previously demonstrated in 2010 that c-MET is activated in GBM neurospheres and that c-MET expression correlates with stem cell marker expression, supporting embryonic stem cells and the induction of formation of pluripotent stem cells [48]. Jahangiri et al. conducted a microarray analysis of bevacizumab-resistant GBM samples. Specifically, they found that c-MET was unregulated in the samples that were pretreated with bevacizumab. These

samples showed greater intra-tumour hypoxia also. They then treated these bevacizumab-resistant GBM cells with a c-MET inhibitor (XL184), which had the effect of downregulating c-MET tyrosine kinase phosphorylation, reducing tumour volume by a factor of 3 and increasing overall survival in a murine model of bevacizumab-resistant GBM. They then further proved the interrelatedness of c-MET and bevacizumab chemoresistance in GBM, by engineering the resistant GBM subclone to express three short hairpin RNAs (shRNA) which had the effect of downregulating c-MET protein expression. They found that these cells in which c-MET was knocked down when cultured in hypoxic conditions grew faster relative to a non-c-MET knock-down subtype cultured in hypoxia but cumulatively exhibited less cells after a 48-hour culture period. Their data demonstrated that c-MET could be targeted alongside VEGF blockade to reduce resistance to anti-angiogenic therapy [49].

1.1.2.12 EGFR/EGFRvIII Pathways

The epidermal growth factor receptor (EGFR), which is a receptor tyrosine kinase, is frequently upregulated or mutated in GBM. It has been shown that a certain subset of human GBM patients benefits from the EGFR kinase inhibitors erlotinib and gefitinib [50, 51]. It has also been demonstrated that GBMs constitutively express EGFRvIII, which is known to have a significant role in activating the phosphatidylinositol 3-kinase (PI3K) signalling pathway, which has been shown to be involved in cell proliferation, survival and invasiveness in human cancer [52, 53]. EGFRs with mutations in their tyrosine kinase domains dysregulate anti-apoptotic signalling via PI3K-Akt. Akt is a cellular kinase involved in apoptosis [52].

The phosphatase and tensin homolog (PTEN) gene on chromosome 10 is a tumour suppressor protein, which is a known inhibitor of the PI3K signalling pathway and is commonly mutated or absent in human GBM cell lines [54]. Mellinghoff et al. demonstrated that the co-expression of EGFRvIII and PTEN in GBM cells was significantly associated with clinical/biological response to EGFR kinase inhibitors (erlotinib, gefitinib). Conversely, they demonstrated that a loss or underexpression of PTEN led to a significantly diminished response to EGFR kinase inhibitors [55].

1.1.2.13 mTOR/PI3K/Akt Pathway

The mTOR/PI3K/Akt pathway is known to be heavily involved in cell survival, apoptosis and invasiveness in human GBM and other forms of cancer. PTEN inactivation in concert with this is a known driver of tumorigenesis [56]. The PI3K/Akt pathway has been shown to interact with the nuclear factor kappa beta (NFkB) pathway and the receptor-interacting protein 1 (RIP1). RIP1 has been shown to inhibit the tumour suppressor protein p53 in human GBM, and overexpression of the protein in GBM has been shown to convey a worse prognosis [57]. Cheng et al.

investigated whether the dual inhibition of the Akt pathway and EGFR pathways in human glioma samples had an effect on apoptosis, cell survival or other proliferative cellular properties of GBM cells. The reasoning behind this study was that the sensitivity to specific pathway inhibitors of EGFR in other cancers has been previously demonstrated to be attenuated by modulation of the Akt pathway [58]. Specifically, they combined the novel allosteric Akt inhibitor, MK-2206, with the EDGR inhibitor gefitinib and showed pharmacological synergism and enhanced antitumour activity in a mouse glioma model [59].

Temisirolimus is an ester analogue of sirolimus and has been shown to interact with mammalian target of rapamycin (mTOR). It does this in a mechanism whereby it binds to immunophilin FKBP-12 which forms a complex that interacts with mammalian target of rapamycin kinase to reduce the downstream effect of the mTOR pathway which is a downstream mediator of the PI3K/Akt pathway in GBM. It has undergone clinical trials for recurrent GBM and has been shown to modestly increase time to tumour progression in some patients 5.4 months versus 1.9 months in those who responded to the drug [60].

1.1.2.14 PDGFR Pathways

GBMs are among the most vascular tumours and by definition undergo potent angiogenesis in relation to their growth and proliferation. They have been shown to have increased expression of vascular endothelial growth factor (VEGF), which has been shown to be involved in angiogenesis and endothelial cell migration [61]. Platelet-derived growth factor (PDGF) and platelet-derived growth factor receptor (PDGFR) have been shown to be constitutively overexpressed in human GBM cell lines. It has been shown to be involved with the recruitment of pericytes in angiogenesis and the maturation of blood vessel formation [62]. The North American Brain Tumor Consortium Study 06-02 tested the use of panzopanib, multi-targeted tyrosine kinase inhibitor, against vascular endothelial growth factor receptors (VEGFR)-1, -2, and -3, platelet-derived growth factor receptors-α and -β and c-Kit in a phase II clinical trial for recurrent GBM. They showed that this agent did not significantly increase progression-free survival in patients with recurrent GBM; however, it was shown to diminish oedema and mass effect in a small proportion of patients [63].

1.1.2.15 Integrins

Integrins are transmembrane receptors that bind multiple extracellular ligands via an arginine-glycine-aspartic acid (RGD) peptide. Ligand binding activates integrins to regulate invasion, migration, proliferation, survival and angiogenesis. Integrins are widely expressed by both the GBM cells and the tumour vasculature [64]. Phase II studies of the effectiveness of integrin inhibitors in recurrent GBM have been published. Cilengitide (competitively binds αvβ3 and αvβ5 integrin receptors)

therapy was shown to have a modest benefit in terms of progression-free survival (the 6-month progression-free survival with 2000 mg oral cilengitide was shown to be similar to that of temozolomide for patients with recurrent GBM [65]).

1.1.2.16 Hyperoxia Increases Chemosensitivity to TMZ in GBM

Intra-tumoural hypoxia in GBM is common and has been associated with the development of resistance to temozolomide chemotherapy [66, 67]. GBM is histologically characterised by poorly organised areas of increased vascularity, hypoxia and necrosis. Hyperoxia has been previously associated with increased sensitivity to chemotherapy in other cancer subtypes. The mechanism by which hyperoxia attributes its therapeutic benefit in GBM is not completely understood. Putative mechanisms include prevention of blood-brain barrier disruption, reduction of peri-tumour oedema and reduction of neutrophil-endothelial cell adhesion. Also at a cellular level, hyperoxia is thought to alter cytokine expression, the expression of growth factors and transcription factors involved in the apoptotic pathway [68, 69]. Sun et al. investigated whether there was a dose-dependent relationship between temozolomide resistance in D54-R and U87-R temozolomide-resistant GBM subclones, cells which had previously been treated with sublethal doses of temozolomide in order to develop chemoresistance. In effect, what they demonstrated was, at a specific-defined cell line and well-defined optimum oxygen concentration, that the resistant cells had the same chemotherapeutic response to temozolomide as their parent temozolomide chemosensitive cells, that is, that hyperoxia resensitised a chemoresistant GBM subclone of cells to become chemosensitive to temozolomide therapy [70].

1.1.2.17 miR-218-RTK-HIF Pathway in Mesenchymal GBM

The mesenchymal GBM subtype microRNA-218 (miR-218) is decreased significantly in highly necrotic mesenchymal GBM. Reduced miR-218 is known to confer GBM resistance to chemotherapy. Low miR-218 levels are known to be associated with increased levels of miR-210, which has been associated with hypoxia and necrosis in GBM [71]. Mathew et al. demonstrated that low miR-218 levels exhibited increased expression of a hypoxia-inducible factor (HIF) metagene, that is, specifically in highly aggressive mesenchymal GBM and promotes tumorigenesis in this tumour type. They postulate that the use of a combination of synthetic miR-218 with chemotherapy particularly in mesenchymal GBM could be of therapeutic utility in terms of increasing chemosensitivity [72].

1.1.2.18 Galectin-1

Galectins are a structurally related family of animal lectins defined by two properties: (1) they have an affinity for beta-galactoside sugars, and (2) they have a sequence homology [73, 74]. In 1997 a group led by Dr. Avraham Raz at Wayne

State University published a paper detailing the correlation between galectin expression and the grade of gliomas in the central nervous system [75]. Specifically, they looked at galectin-3 and showed that its expression was highly correlated with the glioma grade. Numerous other studies have implicated galectin expression in astrocytomas [76–78]. Three specific galectins (galectin-1, galectin-3 and galectin-8) have been proven to have important roles in various components of glioma tumorigenesis, cell proliferation, angiogenesis and invasiveness.

The first of these galectin molecules is galectin-1 (Gal1). This is likely the most important of these molecules in terms of GBM chemoresistance because it is involved in a diverse range of cellular processes which mediate glioma cell migration, angiogenesis and chemosensitivity. Le Mercier et al. studied the effect of reducing galectin-1 expression in GBM cells by the use of siRNA. They found that this treatment increased their sensitivity to pro-apoptotic/pro-autophagy chemotherapeutic agents. What they found was by reducing Gal1 expression in a particular clone of GBM cells, the effectiveness of various chemotherapeutic agents increased, specifically temozolomide, both in vivo and in vitro [79]. Interestingly, downregulation of Gal1 was found not to act in a pro-apoptotic/pro-autophagic manner, but it was found to modulate p53 transcriptional activity and decrease the activity of several genes targeted by p53, specifically *DDIT3/GADD153/CHOP*, *DUSP5 ATF3* and *GADD45A*. Significantly, they also found that this had the effect of decreasing the expression of seven genes known to be implicated in GBM chemoresistance (*ORP150*, *HERP*, *GRP78/Bip*, *TRA1*, *BNIP3L*, *GADD45B* and *CYR61*), some of which are located in the endoplasmic reticulum and are known to be involved in the regulation of the endoplasmic stress response pathway, which is involved in the cellular response to hypoxia, a known tumour activator of galectin-1 activity [77, 80, 81] These data demonstrate the possible utility of galectin-1 downregulation as a chemotherapeutic target to enhance the therapeutic effectiveness of temozolomide in GBM.

1.1.2.19 MDR-Related Genes

An article published in 1994 by Hagesawa et al. was one of the first implicating the possible role of multidrug-resistance (MDR) genes in GBM chemoresistance. The multidrug-resistance phenotype in human tumours is partly associated with overexpression of the 170 kDa P-glycoprotein encoded by the *multidrug-resistance-1 (MDR1)* gene. What they found was that the GBM cell lines they studied, which had elevated multidrug-resistance-associated protein (MRP) mRNA levels, showed the highest resistance to multiple anticancer agents such as etoposide, vincristine and adriamycin and decreased intracellular accumulation of etoposide [82]. Subsequent to this study, a paper by Walther et al. studied the effect of MDR1 expression had on GBM chemotherapy resistance. They transduced the human *TNFα* (*hTNF*) gene carrying retroviral vector pN2tk-hTNF into U373MG human GBM cells. This resulted in the expression and secretion of biologically active hTNF which had the downstream effect of reducing P-glycoprotein expression with enhanced rhodamine-123 uptake and

potentiation of cytotoxicity of the MDR-relevant drugs vincristine and doxorubicin. Abe et al. further explored this subject in 1998 [83]. In summary, what they found was that the proportion of the ATP-binding membrane glycoprotein MRP and P-gp-positive cells in a culture of GBM cells increased after chemotherapy treatment. They speculated that MRP as well as P-gp may be involved in acquired or intrinsic drug resistance in human glioma. Given the experiments of Walther et al. and others, it may be possible to potentiate the cytotoxicity of MDR-related drugs by reducing P-glycoprotein expression via modulating TNF. This could work, in turn, to reduce chemoresistance to vincristine and doxorubicin in GBM.

1.1.2.20 Major Vault Protein

Major human vault protein (MVP) has been implicated in multidrug resistance in GBM. The major vault protein is a molecular homolog of lung resistance protein (LRP) which is expressed in lung cancer cells and is known to convey chemoresistance in this tumour type. Vaults are 13 kDa ribonucleoproteins discovered by Nancy Kedersha and Leonard Rome at UCLA in 1986 which are expressed heavily in the gastrointestinal tract and macrophages [84, 85]. The major vault protein has been implicated in multidrug resistance in astrocytic tumours. Berger et al. demonstrated that the MVP expression level correlated with chemosensitivity against several antineoplastic drugs in glioma including anthracyclines, CDDP and VP-16. They also demonstrated, using gradient centrifugations, that all MVP in glioma cells are assembled in particles behaving like intact vaults [86]. The mechanism by which vaults convey chemoresistance in GBM was unknown, but, they were thought to be involved in nucleocytoplasmic transport. More recently, Lötsch et al. demonstrated that vaults have a tumour-promoting potential by stabilising EGFR-/PI3K-mediated migration and survival pathways in human GBM and that MVP/vaults significantly support migratory and invasive competence as well as starvation resistance of glioma cells [87, 88]. Vaults remain an enigmatic target of chemoresistance in GBM therapy.

1.1.2.21 Anti-apoptotic Protein (Bcl-2)

The majority of human malignant glioma cells express Fas/Apo-1 and are susceptible to Fas/Apo-1 antibody-mediated apoptosis. Expression of the anti-apoptotic porto-oncogene Bcl-2 is inversely proportional to the sensitivity of glioma cells to antibody-mediated Fas/Apo-1-mediated apoptosis [89]. Several clinical trials are underway targeting Bcl-2 family proteins in human GBM [90] Tagscherer et al. describe the use of a Bcl-2 small molecule inhibitor ABT-737 in GBM [91]. ABT-737 shows high affinity to Bcl-2, Bcl-XL and Bcl-w and exhibits potent antitumour activity. They demonstrate that ABT-737 potently induces apoptosis in GBM cells and that ABT-737 sensitises the cells to anticancer drugs and to the death ligand TRAIL.

1.2 Oligodendroglioma (IDH Mutant and 1p/19q Co-deleted ICD-O 9450/3, WHO Grade II)/Anaplastic Oligodendroglioma (IDH Mutant and 1p/19q Co-deleted ICD-O 9451/3, WHO Grade III)

Oligodendrogliomas are considered as WHO grade II tumours in adults and accounts for 4–15 % of all intracranial tumours [92, 93]. They are increasingly recognised due to the analysis of the distinct molecular signatures these tumours possess. They are more common in males usually presenting in the second to fourth decades of life. The goals of treatment are symptom management, prevention and/or delaying tumour progression and increasing time to tumour malignant transformation. Oligodendrogliomas are most often supra-tentorial, commonly in the frontal lobes and calcified, non-enhancing lesions on CT imaging.

Kraus described shared allelic loss of chromosome 1p and 19q in both oligodendrogliomas and oligoastrocytomas [94]. In a paper published in 1998, Cairncross showed that in anaplastic oligodendrogliomas, loss of chromosome 1p is a predictor of chemosensitivity, and combined loss involving chromosomes 1p and 19q is associated with both chemosensitivity and longer recurrence-free survival after chemotherapy (alkylating agents) [95]. The European Organisation for Research and Treatment of Cancer (EORTC) has been instrumental in demonstrating that the extent of surgical resection in oligodendroglioma/oligoastrocytoma is indicative of outcome [96, 97].

To date, the treatment of low-grade gliomas including oligodendrogliomas and oligoastrocytomas has been the subject of four randomised trials [98–101]. Median response to PCV chemotherapy was 60–80 %, with a median response duration of 12–18 months. Melphalan chemotherapy showed some promise at a 55 % response rate at 6 months. van den Bent published the results of a trial in the Lancet in 2005 which demonstrated that median response to temozolomide chemotherapy was 40 % which was sustained for 6–7 months [102].

In terms of radiotherapy treatment, it has been demonstrated that there is no significant difference in the EORTC and RTOG trials between early radiotherapy versus follow-up late radiotherapy in terms of survival. However, progression-free survival was increased in the group who had early radiotherapy [103]. Conventionally, radiotherapy is given at a dose of 50–54Gy which offers the best balance of treatment response vs. neurotoxicity. A study published by the Southwestern Oncology Group in 1993 demonstrated that adjuvant chemotherapy (CCNU) has not been shown to confer a survival benefit in low-grade glioma versus radiotherapy alone [98].

The anaplastic oligodendroglial tumours representing WHO grade III lesions are diagnosed most commonly in males, typically in the fifth and sixth decades of life. The treatment of anaplastic oligodendroglial tumours (anaplastic oligodendrogliomas/anaplastic oligoastrocytomas) has been the subject of two randomised clinical trials. These data show an improvement in time to tumour progression when treated with adjuvant PCV and radiotherapy when compared to radiotherapy alone [99, 104–106]. On the occasion where oligodendroglial tumours recur following PCV chemotherapy, cisplatin has some evidence of utility [89].

More recently, there have been reports of the use of imatinib mesylate, a novel tyrosine kinase inhibitor (used in the treatment of gastrointestinal stromal tumour) in the treatment of grade III astrocytomas, including anaplastic oligodendroglioma [107]. Desjardins et al. describe the use of imatinib and hydroxyurea for recurrent WHO III glioma (following temozolomide chemoradiotherapy) with moderate therapeutic effect in some patients [108].

Based on the importance of genetic tumour typing in oligodendroglial tumours, it is recommended that all patients undergo determination of the 1p/19q status [109]. It is now accepted that all patients with anaplastic oligodendrogliomas that are 1p/19q co-deleted should undergo combination of PCV chemotherapy and radiotherapy as demonstrated by the EORTC 26951 and RTOG 9402 trials [110, 111].

It is likely that in the near future, further molecular characterisation of these tumour types will take place, with the development of novel treatment paradigms improving overall survival.

1.3 Ependymoma (ICD-O 9391/3, WHO Grade II)/ Anaplastic Ependymoma (ICD-O 9392/3, WHO Grade III)

Ependymomas are thought to arise from the periventricular ependymal lining and comprise the third most common paediatric brain tumour. There is a lack of molecular phenotyping of this disease due to the relative rarity of this glioma type.

1.3.1 Treatment Options for Ependymomas

1.3.1.1 Lapatinib

Molecular studies of ependymomas show that most tumours have ErbB2 overexpression and unmethylated MGMT promoter status. Lapatinib is a molecular target of ErbB2. Gilbert et al. published the results of a phase II study of lapatinib and dose-dependent temozolomide treatment in adults with recurrent ependymoma, which was the first prospective therapeutics trial in adult ependymoma [112]. They showed that lapatinib and dose-dependent temozolomide were well tolerated by patients and that the combination of the two had activity against the ependymoma spectrum dependent on location and tumour grade [94].

1.3.1.2 Lonafarnib

As part of a phase I trial, the Pediatric Brain Tumor Consortium Study studied the safety and profile of the farnesyltransferase inhibitor lonafarnib in advanced CNS cancer in paediatric patients and has established safe therapeutic dose targets for

use in children. Farnesyltransferase inhibitors are small molecules that reversibly bind to the farnesyltransferase CAAX-binding site [113], leading to inhibition of progerin farnesylation, which is a crucial post-translational processing step for protein intercalation into the inner nuclear membrane. This drug has also been used with utility in the treatment of Hutchinson-Gilford progeria syndrome in terms of improving neurological outcome of these patients from cerebrovascular disease [114, 115].

1.3.1.3 Actinomycin D Treatment in High-Risk Ependymomas

It has been shown that abnormal p53 expression in ependymoma confers a worse prognosis, occurring in approximately 22 % of intracranial ependymomas. Tazaridis et al. showed that a majority of high-risk ependymomas also had a homozygous CDKN2A deletion. Their experiments showed that the loss of function of p53 was in a biological manner leading to overexpression of MDM. They showed that low-dose treatment of ependymomas with actinomycin D reactivated p53 and was of potential therapeutic benefit in a subset of patients with p53 mutations [116].

1.3.1.4 Histone Deacetylase Inhibitors

Given their periventricular location, complete resection of ependymomas is not always possible. The intensive chemoradiotherapy cure is rare following partial resection. The experiments of Milde et al. introduce a novel chemotherapeutic approach to dealing with this issue. Specifically, they obtained tumour cells from a patient with supra-tentorial metastatic ependymoma (cytogenetic group 3/molecular subgroup C DKFZ-EP1NS cells) which were shown to recapitulate the original tumour in a niche-dependent manner. These cells are shown to be chemoresistant to temozolomide, vincristine and cisplatin. However, when treated with the histone deacetylase inhibitor vorinostat, they expressed neuronal-specific markers and lost stem cell-specific properties [117]. Vorinostat shows some promise for treatment of partially resected ependymomas following recurrence after conventional chemoradiotherapy.

1.4 Haemangioblastoma (ICD-O 9161/1, WHO Grade I)

Haemangioblastoma is a benign, capillary vessel-rich neoplasm located in the cerebellum or spine of 13–72 % of patients with von Hippel-Lindau (VHL) disease [118]. To date no systemic therapy has been approved for the use against haemangioblastomas in von Hippel-Lindau disease.

1.4.1 Treatment Option for Haemangioblastoma

1.4.1.1 Semaxanib/SU 5416

Semaxanib is a multi-target tyrosine kinase inhibitor with downstream anti-angiogenic effects. In 2004, Madhusudan et al. discuss their success in treating haemangioblastoma with semaxanib. They noted two of the six patients treated underwent stabilisation of their disease; however, this compound is no longer clinically available [119].

1.4.1.2 Bevacizumab

A clinical trial involving oral treatment of haemangioblastomas (#NCT01015300) with bevacizumab was terminated early due to inability to recruit sufficient patient numbers. However, intra-vitreal anti-VEGF therapy has shown promise in the treatment of retinal haemangioblastomas. Hrisomalos et al. report improved visual acuity/optical coherence tomography thickness following bevacizumab therapy in patients with retinal haemangioblastomas due to VHL [120].

1.4.1.3 Vatalanib

Vatalanib is an oral anti-VEGF inhibitor which targets all known VEGF receptor tyrosine kinases. It is currently under investigation in a phase II clinical trial in the treatment of haemangioblastomas in von Hippel-Lindau patients (NCI trial #NCT00052013).

1.4.1.4 Vorinostat

Vorinostat is a histone deacetylase inhibitor which is currently undergoing a clinical trial in the treatment of haemangioblastoma in VHL (NCT02108002). Several other targeted therapies have been tried in VHL for the treatment of haemangioblastoma including panzopanib, ranibizumab and sunitinib, all with limited or anecdotal success [121].

1.5 Conclusion

In this chapter, we have given a brief overview of the most common adult primary brain tumours, the mechanisms by which they evade conventional chemotherapeutic drugs and ongoing investigations into therapeutic options which might induce chemosensitivity in these tumours.

Firstly, we discussed GBM, the most common primary brain tumour in adults. This is a formidable tumour associated with high mortality and significant chemoresistance. The biological mechanisms whereby this tumour evades conventional chemotherapy are protean. The most common pathways are discussed briefly in this chapter and will be discussed in greater detail in the following chapters. Further molecular characterisation of this tumour is increasing our understanding of the different phenotypes of this tumour and helping to explain the differences in biological behaviour exhibited among different tumour subtypes. Increasing understanding of the complex biological pathways involved in this tumour surely represents the best mechanism by which to combat drug resistance in this tumour type.

We then discussed the oligodendroglial tumours and the importance of the allelic loss of chromosome 1p and 19q in determining prognosis in these tumour types. Again, analogous to GBM, further characterisation of the biological pathways involved in tumorigenesis and invasiveness in this tumour type will yield new therapeutic options targeted against this tumour type.

The ependymomas are a rare tumour type in adults thought to arise from the periventricular lining of the ventricular system within the brain. Molecular characterisation of the pathways which promote tumorigenesis, invasiveness and chemoresistance in this tumour is sparse relative to the astrocytic/oligodendroglial counterparts. The management of these tumours is complex due to their periventricular location which makes achieving a complete resection difficult. This obviates the importance of developing appropriate chemotherapeutic agents targeted against this tumour type.

Lastly, we briefly discussed haemangioblastoma, a capillary-rich neoplasm often presenting in the spine or cerebellum, particular in patients with the von Hippel-Lindau disease. The mainstay of trails against this tumour type is monoclonal antibodies targeted against vascular endothelial growth factor receptor (VEGF) many of which are still ongoing. We await the results of these data to further elucidate the role of anti-angiogenic therapy in this tumour type.

The following chapters, will further elucidate what is known about these mechanisms and discuss possible therapeutic targets to these resistance mechanisms.

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Chapter 2
Targeting Chemotherapy Resistance in Glioblastoma Through Modulation of ABC Transporters

Amanda Tivnan

Abstract Glioblastoma (GBM) is a highly aggressive Grade IV solid central nervous system neoplasm with an incidence rate of 3–4 per 100,000 people worldwide and the average 5-year survival rate of GBM patients is less than 5 %, leading to the fact that GBM is the most lethal form of brain tumor. The presence of several adenosine triphosphate-binding cassette (ABC) transporters is thought to contribute to the sustained progression of GBM tumours, inhibiting and rapidly removing anticancer drugs from GBM tumour cells. ABC transporters are transmembrane pumps which use ATP hydrolysis to facilitate translocation of substrates across cellular membranes. Overexpression of ABC transporters including P-gp (ABCB1), ABCCs, or MRPs and breast cancer-resistance protein (BCRP, ABCG2) on the GBM cells themselves is thought to instill chemoresistance and active drug extrusion at the tumor site rendering the temporal effect of successfully administered drugs negligible, if at all. In this regard, the role of individual ABC transporters and their contribution to chemoresistance and potential as targeted therapies of GBM chemosensitization will be discussed in this chapter.

Keywords Adenosine triphosphate-binding cassette (ABC) transporters • Chemoresistance • Glioblastoma

Abbreviations

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
B-BB	Blood-brain barrier

A. Tivnan, PhD (✉)
Royal College of Surgeons in Ireland, 123 St Stephen's Green, Dublin 2, Ireland
e-mail: amandativnan@rcsi.ie

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_2

B-CFB	Blood-cerebrospinal fluid barrier
B-TB	Blood-tumor barrier
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
EGF	Epithelial growth factor
Gy	Gray (unit of ionizing radiation)
PD-1	Programmed cell death protein 1
VEGF	Vascular endothelial growth factor

2.1 Introduction

Glioblastoma (GBM) is a highly aggressive Grade IV solid central nervous system neoplasm with an incidence rate of 3–4 per 100,000 people worldwide [1], and an average 5-year survival rate of less than 5 %, leading to the fact that GBM is the most lethal form of brain tumor (http://www.braintumourresearch.org/our-reports). GBM represents approximately 15 % of all primary brain tumors diagnosed annually in the USA, increasing in frequency with age and showing more prevalence in men than women (http://globocan.iarc.fr/). Although the incidence has decreased in the context of primary and CNS tumors when determined histologically since 1995, it has remained the highest recorded type of glioma, accounting for 55.1 % diagnosed with respect to all other histological glioma subtypes between 2008 and 2012 [13]. Although the incidence rates for brain and nervous system tumors have been collated across Europe [14], the incidence rates for specific brain tumor types such as GBM have yet to be compiled.

Despite decades of ongoing clinical research, the median survival rate for GBM patients beyond 12 months has not changed significantly. Initially, standard clinical care involved extensive surgical resection followed by adjuvant radiation therapy (RT), which included 45–50 Gy of deep RT to the tumor site, given daily over a period of 4 to 5 weeks, resulting in a doubling in median survival time from 4–6 to 10–11 months [15]. Whole-brain RT became the standard of care, as non-specific targeting of uncontrollably growing cells proved to be efficacious in GBM treatment. Understandably, the brain contains many neural structures which are very sensitive to RT, limiting the amount of tolerated RT which could be used in patients; however, alternative RT regimen did not alter survival rates. Up until the mid-1990s, nitrosoureas, alkylating agents used in chemotherapy, showed benefit to patient survival by approximately 2 months [16]. Systemically administered bis-chloroethylnitrosourea (BCNU) forms interstrand cross-links in DNA, preventing replication and transcription. BCNU administration was the standard of care for adjuvant chemotherapy at that time and was administered at the time of surgery to the resection cavity. The BCNU studies span over four decades, until Stupp and colleagues established a new system of chemotherapy, the current standard of care. The Stupp protocol involves tumor resection followed by RT in

combination with adjunct and concomitant temozolomide (TMZ) chemotherapy, increasing the median survival rates from 12.1 months with RT alone to 14.6 months for TMZ/RT treatment; however, little improvement has been made since this time. Despite treatment, GBM recurrence at distal sites is typically 6.9 months [17], and in instances where repeat resection is not a viable option, adjunct chemotherapy is ineffective at stopping tumor progression and morbidity, with several studies attributing such treatment resistance to increased expression of multidrug resistance proteins including members of the ATP-binding cassette (ABC) family [6, 18–20].

2.2 Adenosine Triphosphate-Binding Cassette (ABC) Superfamily

Adenosine triphosphate-binding cassette (ABC) transporters are transmembrane pumps which consist of multiple subunits of transmembrane and membrane-associated ATPases, the latter of which uses ATP hydrolysis to facilitate translocation of substrates across cellular membranes. Substrates of these transporters can be both organic and inorganic, ranging from amino acids, lipids, and sterols to primary and secondary metabolites and drugs [21]. ABC genes, of which 48 have been identified in humans, are essential for human processes [22] with mutations being linked to a myriad of diseases such as cystic fibrosis [23, 24], Stargardt's disease [25–27], and Dubin-Johnson syndrome [28, 29] and contributing to multiple drug resistance through transporter protein overexpression in several cancers [30–33].

2.2.1 Structure

The primary sequence of the ABC transporter family is highly conserved throughout evolution with four subunits or domains, two nucleotide-binding domains (NBDs) which are ATP-binding domains and two transmembrane domains (TMD1 and TMD2). The NBD domains contain Walker A (or P-loop), Walker B motif, a Q-loop, and H motif, and an α-helical signature (C) domain "LSGGQ" [21]. The Walker A and the second Walker B domains are found in all ATP-binding proteins; however, the C motif is specific to ABC transporters. The TMDs which consist of between six and ten transmembrane α-helices, depending on the transporter class [21], form a transmembrane "pore" which can be classified as inward (open to the cytoplasm) or outward (open to the exterior) facing, with no known sequence homology resulting in the diversity of substrate binding. Genes encoding these transporters are organized as either full proteins (*2 NBF and 2 TMDs*) or half transporters which are later assembled as homo- or heterodimers.

2.2.2 *Mechanism of Transport*

ABC transporters typically have to pump substrates against a chemical gradient, requiring energy to fuel this process, provided as a result of ATP hydrolysis. In brief, ABC transporters undergo a catalytic cycle from a ground to activated state whereby direct binding of a specific substrate to the TMDs occurs in conjunction with two ATP molecules binding to the NBDs. TMDs change conformation from either inward to outward facing or vice versa; ATP is hydrolyzed with the result of ATP and phosphate; and reduced TMD substrate affinity leads to solute release. At this point, NBDs dissociate and return to the inactivated/ground state. This widely accepted theory of ABC transporter function is known as the "alternating access model" [34].

More specifically, the structure of NBDs in ABC transporters is an ongoing area of debate, with theories speculating that the resting state is composed of monomer NBDs requiring the binding of free ATP prior to dimerization or, alternatively, the maintenance of a ground inactivated dimer state with ATP preloaded requiring stimulation through substrate binding to the TMD before functional dimerization and transport activity. The mechanism through which TMDs change conformation after substrate binding is thought to involve coupling helices called intercellular loops (ICLs) which are found in close contact with the helical domains, in a groove between the two lobes of the NBD [35]. A Q-loop extends up from the TMD and overlaps a structurally diverse region (SDR) of 30 nucleotides with a downstream X-loop from the NBD [36]. Once bound, the substrate stimulates a change in the ICLs, causing the Q-loop to move. This movement can then, in theory, increase the affinity of the NBDs for unloaded ATP leading to dimerization of the NBD itself through ATP hydrolysis or promote dimerization of the inactive-dimer-ATP-bound ground state.

Although this process varies among ABC transporter classes, the basic steps of ATP-dependent NBD dimerization and TMD conformational switching are shared constitutively leading to translocation of a particular substrate across a membrane against a chemical gradient.

The ABC superfamily is the largest family of transmembrane proteins with seven subfamilies designated A–G based on sequence homology [37]. ABCA proteins are predominantly expressed in the central nervous system (CNS) and the hematopoietic system involved in lipid transport and homeostasis with 12 members being identified [37–40]. The *ABCB* gene is primarily expressed in the blood-brain barrier (B-BB) and liver involved in toxin extrusion; however, overexpression results in multiple drug resistance [22]. The most extensively studied is ABCB1, also known as P-glycoprotein (P-gp) or multidrug resistance protein 1 (MDR1), which is discussed in detail in Sect. 2.3 in the context of its contribution to GBM drug resistance. The ABCC subfamily contains 13 members, nine of which are also referred to as multidrug resistance proteins (MRPs) involved in transport, toxin excretion, and signal transduction [41], the role of which in chemotherapy-resistant GBM is outlined in Sect. 2.4 of this chapter. The ABCD subfamily is exclusively expressed in the peroxisome and the role of ABCD1 in fatty acid metabolism has been linked to adrenoleukodystrophy (ALD), a neurodegeneration and adrenal deficiency disease [42–44].

ABCE and *ABCF* genes contain ATP-binding domains; however, these genes do not encode transmembrane regions. The ABCG subfamily has an orientation opposing all other ABC genes with an ATP-binding domain in the N terminus and transmembrane at the C terminus. ABCG2 is also known as the breast cancer-resistance protein (BCRP), and although its native function is not known, chromosomal translocation resulting in ABCG2 amplification causes drug resistance to common anticancer drugs such as topotecan, mitoxantrone, and doxorubicin [45–47]. The role of BCRP in GBM drug resistance is further discussed in Sect. 2.5.

With respect to glioblastoma, tumor sustainability may be due to the inability of several anticancer drugs to cross the B-BB, blood-cerebrospinal fluid barrier (B-CFB), and blood-tumor barrier (B-TB) due to the presence of several ABC transporters [2–5]. In addition, overexpression of ABC transporters including P-gp (ABCB1), ABCCs or MRPs, and breast cancer-resistance protein (BCRP, ABCG2) on the GBM cells themselves is thought to instill chemoresistance and active drug extrusion at the tumor site rendering the temporal effect of successfully administered drugs negligible, if at all [6–12]. In this regard, the role of individual ABC transporters and their contribution to chemoresistance and potential as targeted therapies of GBM chemosensitization will be discussed in this chapter.

2.3 P-Glycoprotein (P-gp/Pgp/ABCB1/MDR1/CD243)

P-Glycoprotein is encoded by two multidrug resistance 1 genes (*MDR1 and MDR3*). The predominant protein isoform is the multidrug resistance protein 1 (MDR1), also known as ATP-binding cassette subfamily B member 1 (ABCB1) or cluster of differentiation 243 (CD243). The MDR3 product is not known to confer substrate resistance in humans [48]. P-gp was the first identified ABC transporter and is, therefore, the most extensively studied, showing a very broad range of substrate specificity (Table 2.1).

Although the presence of the B-BB acts as a major impediment to the therapeutic effect of several drugs on brain cancers, the active efflux of anticancer drugs by ABC transporters further reduces any effect which may be noted by such chemotherapeutics. In 2005, the FDA approved the concomitant use of temozolomide (TMZ) with radiotherapy for the treatment of newly diagnosed GBM, a standard of care which is maintained today, known as the Stupp protocol [49]. As TMZ is the current first-line treatment option for GBM, identification of mechanisms of TMZ resistance is an important avenue of research, holding the potential to improve clinically used chemotherapeutic agents. TMZ resistance, in many cancers, has been proven to be multifactorial, including changes in cell cycle in response to treatment, increased mismatch repair genes, and increased expression of O-6-methylguanine transferase (MGMT) expression [50, 51]. Although TMZ has been the only chemotherapy which has proven to increase survival rates, an achievement attributed to the drugs' ability to traverse the B-BB with ease, the presence of P-gp and several other ABC transporters whose substrate specificity extends to TMZ lends to one of the mechanisms which contribute to

Table 2.1 MRP transporter substrate and inhibitor lists

Transporter name	Distribution	Substrate	Inhibitors
P-Glycoprotein (P-gp/Pgp/ABCB1/CD243/MDR1)	• Intestinal epithelium • Liver cells • Proximal tubule of the kidney • Capillary endothelial cells (B-BB and blood-testis barrier) • Cancer cells	Aldosterone Amprenavir Bilirubin Cimetidine Colchicine Cortisol CPT-11 Cyclosporine Doxorubicin Dexamethasone Digoxin Diltiazem Domperidone Etoposide Estradiol-17B-D-glucuronide Erythromycin Fexofenadine Quinidine Tacrolimus Temozolomide Vinblastine Immunosuppressive agents HIV type I antiretroviral therapies Lipids Xenobiotics	Amiodarone Azithromycin Atorvastatin, bromocriptine, carvedilol, cyclosporine, captopril Clarithromycin Erythromycin, GF120918, itraconazole, ketoconazole, LY335979, meperidine Methadone Nelfinavir, pentazocine Progesterone Piperine Quercetin Quinidine Quinine Reserpine Reversan Ritonavir Tariquidar Verapamil

ABCC1 (MRP1)	• Ubiquitous Highest in • Intestine • Testes • Kidney • Malignant cancer cells of the brain • Capillary endothelial cells (B-BB and blood-testis barrier)	Adefovir Indinavir Saquinavir Ritonavir Methotrexate, edatrexate ZD1694 Doxorubicin Daunorubicin, epirubicin Idarubicin Etoposide, vincristine, vinblastine, paclitaxel Irinotecan Hydroxyflutamide, FR901228 NSC-630176 Difloxacin, grepafloxacin N-Ethylmaleimide, thiotepa, cyclophosphamide, melphalan, chlorambucil, ethacrynic acid, metolachlor Atrazine, sulforaphane, aflatoxin Arsenic NNAL SN-38 E3040S	Reversan MK571, indomethacin, benzbromarone, euchrestaflavanone, sophoraflavanone, cyclosporine A, quercetin

(continued)

Table 2.1 (continued)

Transporter name	Distribution	Substrate	Inhibitors
ABCC2 (MRP2)	• Intestine • Liver • Kidney • Brain	Indinavir Cisplatin Vinblastine Doxorubicin Methotrexate Etoposide, mitoxantrone, valsartan Olmesartan, glucuronidated SN-38	Cyclosporine Delavirdine, efavirenz, emtricitabine, benzbromarone
ABCC3 (MRP3)	• Kidney • Intestines • Adrenal gland • Liver • Pancreas • Capillary endothelial cells (B-BB and blood-testis barrier)	Etoposide Methotrexate Teniposide Fexofenadine, methotrexate, vincristine, Teniposide, acetaminophen, glucuronide	Delavirdine, efavirenz, nevirapine, emtricitabine, lamivudine, tenofovir, indomethacin, furosemide, probenecid, MK751
ABCC4 (MRP4)	• Bladder • Lung • Muscle • Pancreas • Prostate • Gonads • Capillary endothelial cells (B-BB and blood-testis barrier)	6-Mercaptopurine 6-Thioguanine and metabolites Methotrexate Acyclovir Ritonavir Tenofovir Topotecan PMEA Furosemide, ceftizoxime Cefazolin	MK571 Indomethacin, diclofenac, celecoxib, sulfinpyrazone, quercetin

ABCC5 (MRP5)	• Ubiquitous • Highest in • B-BB • Heart • Liver • Lung • Urethra • Placenta • Skeletal muscles	6-Mercaptopurine 6-Thioguanine and metabolites Methotrexate PMEA 5-Fluorouracil, rosuvastatin, atorvastatin	Sulfinpyrazone, benzbromarone Trequinsin Dipyridamole Zaprinast NPPB
ABCC6 (MRP6)	• Kidney • Liver • Low expression in the lung, intestines, retina, skin, and vessel walls	Cisplatin Daunorubicin Etoposide Anthracyclines Epipodophyllotoxins Leukotriene C4	Benzbromarone Indomethacin Probenecid
ABCC7 (MRP10)	• Ubiquitous expression at a low level • Highest expression in • Pancreas • Prostate cancer, hepatocellular cell lines • Breast cancer	Taxanes Epothilone B Vinca alkaloids Cytarabine Tamoxifen	Cepharanthine Lapatinib Erlotinib Nilotinib Imatinib Sildenafil Vardenafil

(continued)

Table 2.1 (continued)

Transporter name	Distribution	Substrate	Inhibitors
Breast cancer resistance protein (BCRP/ ABCG2)	• Intestine • Liver • Breast • Placenta • Brain	Daunorubicin Doxorubicin Topotecan Rosuvastatin Sulfasalazine Mitoxantrone Irinotecan Imatinib Methotrexate Anthracyclines SN-38 Nucleoside analogues, prazosin, pantoprazole Statins	Elacridar (GF120918) Abacavir Amprenavir Aripiprazole Atazanavir Atorvastatin Cerivastatin Daunomycin Dehydroepiandrosterone sulfate Delavirdine Efavirenz Erlotinib Fluvastatin Ko143 Lopinavir Nelfinavir Nilotinib Pitavastatin Prenylflavonoid Rosuvastatin Saquinavir Simvastatin SN-38 Sulfasalazine

the acquired resistance of TMZ transport into the brain. ABC transporters on the luminal surface of the B-BB, such as P-gp, transport drugs from the endothelial cells of the B-BB back into blood circulation, thereby reducing their bioavailability, reducing the drugs' capacity to elicit DNA damage, and having a profound effect on TMZ-induced tumor death. In addition, increased expression of P-gp on the tumor itself has a direct correlation to the therapeutic effect of the P-gp substrate anticancer drug [8, 52–54].

It has also been shown that treatment of GBM cells with TMZ led to increased expression of P-gp itself through epidermal growth factor (EGF) regulation of the *MDR1* gene [55]. This increased P-gp expression leads to increased drug extrusion and increased resistance. The diverse localization of P-gp has been found to contribute to multidrug resistance in many different forms of cancer [56–59]. The contribution of P-gp to GBM leads to rapid and prolonged resistance resulting in short progression-free survival for patients [55]. From a clinical perspective, P-gp-positive cells from surgical specimens increase with respect to malignancy grade, i.e., from low-grade glioma to high grade or GBM [60]. In addition to P-gp overexpression, genetic alterations such as MDR1 polymorphisms play an important role with respect to GBM patient's response to chemotherapeutic regimes.

In principle, P-gp-induced multidrug resistance may be overcome using treatment combinations of chemotherapy and P-gp inhibitors or, alternatively, non-P-gp substrate anticancer agents. Researchers have evaluated the potential of P-gp inhibition, which is used in several other forms of cancer [61–65], with respect to increasing therapeutic transport into the brain [12, 66–69]. The two most commonly used P-gp inhibitors, verapamil and cyclosporine A, are first-generation P-gp inhibitors, which have been assessed with respect to their ability to inhibit P-gp-mediated transport and drug resistance in glioblastoma models [66, 70–74]. Second- and third-generation P-gp reversal agents have also been developed and evaluated in cancers other than glioma: valspodar, dexverapamil and tariquidar, biricodar, elacridar, OC144-093, and R101933 [75–81]. Additional approaches include P-gp monoclonal antibodies, an immunotherapy-based approach [82–85]; however, this has yet to be applied to glioblastoma. There are a very limited number of glioma clinical trials which involve P-gp and are mainly focused on the assessment of P-gp expression or polymorphism contribution to novel drug resistance (Table 2.2). A considerable disadvantage to the use of such inhibition, specifically for brain cancer, is the fact that increased permeability of the B-BB to such a broad range of substrates may lead to drug neurotoxicity and restricted dosage limitations.

Although the role of the tumor microenvironment and hypoxia to apoptosis resistance has been readily studied [120–123], recent studies have linked hypoxia to chemodrug resistance through the *ABCB1* gene. The P-gp-encoded gene contains a hypoxia-responsive element in the promoter region and can, therefore, be regulated by hypoxic microenvironment conditions via HIF1α [124]. Increased hypoxia or tumor cycling hypoxia which occurs as the tumor establishes a reliable blood flow to facilitate tumor expansion can lead to increased P-gp expression and increased drug resistance. This pathway is particularly applicable to GBM drug resistance pathways as these highly heterogeneous tumors quite often have a necrotic and hypoxic core which, in addition to radiotherapy resistance [125], may also be multidrug resistant due to increased P-gp expression [124].

Table 2.2 Clinical trial involving ABC transporters

Link to ABC transporters	NCT number	Cancer type	Status	Research-relevant and trial result references
P-gp imaging	NCT01281982	Glioma	Terminated December 2015	[86–88]
P-gp expression correlation to prognosis	NCT02197637	Glioma	Open. Due to finish March 2017	[89, 90]
P-gp antagonist PSC 833	NCT00001302	Breast, kidney, neoplasm, lymphoma, metastasis, ovarian	Completed in 2002	[76, 78–81, 91–100]
P-gp as a stratification factor	NCT01459484	Osteosarcoma	Estimated completion January 2020	[101–103]
ABCC1 polymorphisms	NCT00898456	Myeloid leukemia	Estimated completion January 2020	[104, 105]
Multidrug resistance genes	NCT00898404	Acute lymphoblastic leukemia	Estimated completion January 2020	[106–108]
Genetic variations of ABC transporters ABCC1, P-gp	NCT01282658	Colorectal cancer	Completed May 2013	[109–111]
Genetic variations of ABC transporters ABCC1, P-gp	NCT01280448	Lung cancer	Completed September 2013	[112, 113]
Prediction response to chemotherapy. MRP1 P-gp	NCT00551798	High-grade lymphoma Hodgkin's disease	Completed January 2011	[114, 115]
Correlation to drug resistance (P-gp, MRP1, BCRP, and MDR-3)	NCT00753207	Breast	Completed January 2014	[116–119]

2.4 Multidrug Resistance Proteins

This family of ABC transporters contains six members, MRP1–6 (ABCC1–6) which have progressively been identified since 1992 [126–132] and are known to contribute to ATP-dependent decrease in anticancer drug efflux and, therefore, multidrug resistance. In this chapter, the author has focused on multidrug resistance proteins 1 (ABCC1, MRP1), 3 (ABCC3, MRP3), and 5 (ABCC5, MRP5) with regard to their contribution to glioblastoma drug resistance.

2.4.1 Multidrug Resistance Protein 1 (MRP1, ABCC1, MRP)

ABCC1 was first identified by Cole et al. in 1992 [126] as a glutathione-conjugated toxic compound unidirectional transporter. Further to its role in multidrug resistance, the ABCC1 transporter is thought to contribute to the avoidance of xenobiotic accumulation and toxicity [133, 134], aid in the transport of inflammatory mediators such as LTC4 [135], and protect against oxidative stress [136–138]. The expression in nonmalignant tissue is ubiquitous, with the highest mRNA noted in the basolateral cellular surface of tissues such as the lungs, kidneys, skeletal muscle, and testes (Table 2.2); however, there have been no definitive studies which attribute the expression of MRP1 in these tissues to drug elimination or absorption, rather than tissue distribution and toxicity avoidance. Notably, it is the contribution which MRP1 plays to multidrug resistance in cancer cells which is of most interest.

The role of MRP1 and polymorphic variants of ABCC1 with respect to patient response to several novel or clinical chemotherapeutic agents has been completed and is currently underway in many clinical trials for a myriad of cancers (Table 2.2). Many of these studies assess the expression of both P-gp and MRP1 in comparison to clinical prognosis due to the broad substrate overlap which is noted between these two transporters.

With regard to brain cancer, Abe et al. carried out several studies into the correlation of MRP1 and P-gp expression and glioma grading [6, 139] with confirmation in 1998 [140] that the increased expressions of ABCC1 and P-gp between pre- and post-chemotherapy suggest a role of these transporters in acquired and intrinsic drug resistance in glioma. MRP1 has been shown to only be highly expressed in high-grade gliomas (HGGs), especially GBM evident in over 55 % of samples in a study by Pinto de Faria et al. [141]. Since then, the role of MRP1 in glioblastoma drug resistance has been evaluated [8, 10, 128, 142] with recent findings of MRP1 expression on tumor-associated microvessel endothelial cells [11] providing support to the role of MRP1 in GBM intrinsic multidrug resistance. In 2015, the author identified the role of MRP1 in sensitization to two clinically relevant chemotherapeutic agents, vincristine and etoposide, in both primary and recurrent patient-derived GBM cell lines [12]. Additionally, the author assessed two nonspecific MRP1 small-molecule inhibitors, reversan and MK571, whose role in alternative cancers, such as neuroblastoma, has been assessed with promising results [37, 143, 144]. The findings suggest that specific MRP1 inhibition by targeted short interfering (si)RNA molecules in both primary and recurrent patient-derived GBM lines leads to chemosensitization to vincristine and etoposide treatment; however, temozolomide-induced cell death can only be achieved by additional inhibition of P-gp and/or breast cancer resistance protein (BCRP/ABCG2) supporting the fact that temozolomide is not a substrate for MRP1 [142].

2.4.2 Multidrug Resistance Protein 2 (MRP2, ABCC2, cMOAT, cMRP)

ABCC2 is also known as the canalicular multispecific organic anion transporter (cMOAT) due to its expression in the canalicular, or apical, section of the hepatocyte, being responsible for biliary transport in the liver [145–147]. MRP2 mutations have been proven to contribute to Dubin-Johnson syndrome, a typically asymptomatic autosomal recessive condition whereby the patient has an increase in conjugated bilirubin devoid of liver enzyme elevation [148–150]. Additional expression in the endothelial cells of the proximal renal tube highlights the role of MRP2 in small organic anion transport [151, 152], and clinically MRP2 transport inhibition may lead to iatrogenic Fanconi syndrome, an inhibition of mitochondrial DNA synthesis as a result of increased organic anion buildup in the kidneys [153].

Although the role of MRP2 in the native function of the human B-BB has been readily studied [154–157], the implication of MRP2 expression in glioma [10, 158] is the identification of topoisomerase II inhibitors as a substrate for this ABC transporter. Topoisomerase inhibitors are drugs which interfere with the action of the topoisomerase enzyme, responsible for controlling DNA structural formation during cell cycle events. These inhibitors include etoposide and teniposide, which are used clinically for recurrent GBM treatment, as well as several other epipodophyllotoxins, aminoacridine, and mitoxantrone. Increased MRP2 expression correlates to increased resistance to topoisomerase inhibitors in patient-derived GBM cells [159]. Although specific MRP2 inhibition in GBM has not been studied to date, successful pan inhibition of MRP1, 2, and 3 in HIVE-viral therapy studies in canine kidney cell lines has been developed [160], a technique which may be applicable for GBM cell chemosensitization.

2.4.3 Multidrug Resistance Protein 3 (MRP3, ABCC3, MOAT-D, cMOAT-2, MLP-2)

ABCC3 was first identified in the liver [161]; however, its noted expression in many tissues including the adrenal glands, kidney, small intestine, colon, pancreas, placenta, gallbladder, lungs, spleen, stomach, brain, and tonsils signifies its importance in xenobiotic and drug efflux. ABCC3 polymorphisms have been associated with negative clinical outcome in several cancer forms and arthritis [162–168]. Although it has been suggested that the contribution which MRP3 plays to drug resistance is minor compared to its nearest homologue MRP1 (ABCC1) [8], increased MRP3 expression in GBM biopsies correlated with a higher risk of mortality [169], and researchers have postulated the use of this multidrug resistance protein for targeted antibody therapy of malignant gliomas such as glioblastoma [169, 170]. Although the concept of MRP3 inhibitors may seem favorable with respect to improved drug response in high-grade

malignant glioma, the role of MRP3 in transporting bilirubin glucuronides into the blood under conditions of impaired biliary bilirubin excretion requires an air of caution for systemic administration of such inhibitor-based therapeutics.

2.4.4 *Multidrug Resistance Protein 4 (MRP4, ABCC4, MOAT-B)*

ABCC4 is the smallest ABC transporter and is a known mediator of secondary messenger signaling through cAMP translocation in several different cell and tissue types [171–176]. Dysregulation of MRP4 expression has been connected to multidrug resistance through modulated transport of anticancer drug substrates [37, 177–179] with various levels of MRP4 expression noted in glioblastoma, retinoblastoma, neuroblastoma, and prostate, lung, ovarian, and pancreatic tumor cell lines [180–182]. With respect to glioblastoma, very few studies have focused on MRP4 expression. Studies by Rama et al. showed that GBM-initiating cells express little or no ABC transporters [183]; however, drug-resistant cancer stem cells from differentiated malignant patient tumors were noted to express increased levels of MRP4, in addition to P-gp, MRP2, and BRCP [184] supporting a role for ABC transport inhibition in drug effect enhancement. The role of MRP4 in cancers such as neuroblastoma has been found to be a prognostic indicator of progression-free survival independent of drug efflux potential [37, 144, 179, 182], a role which has yet to be investigated in additional cancers including glioblastoma.

2.4.5 *Multidrug Resistance Protein 5 (MRP5, ABCC5, MOAT-C, Pabc-1)*

Multidrug resistance protein 5 (MRP5) is a 160 kDa protein with a broad range of substrate specificity, overlapping with several other members of the ABC subfamily of transporters (Table 2.1). Highest expression patterns in naïve tissues include the heart, urethra, astrocytes, and pyramidal neurons of the brain and the B-BB. Although very few studies have been carried out on the role of MRP5 in glioblastoma, as noted by Alexiou et al., MRP5 expression in GBM tumor specimen was noted in <45 % of patients, with increased expression correlating to reduced survival. This correlation was identified to be an independent prognostic indicator for GBM survival [185], making MRP5 protein or ABCC5 transcript inhibition a viable target in an attempt to reduce GBM chemoresistance.

2.4.6 Multidrug Resistance Protein 6 (MRP6, ABCC6, MLP-1) and Multidrug Resistance Protein 7 (MRP7, ABCC10, EST182763, SIMRP7)

The final ABCC family members discussed in this chapter are MRP6 (ABCC6) and a newly included member MRP7 (ABCC10) in 2001 [186]. Although the roles of MRP6 [187–190] and MRP7 [186, 191–197] have been investigated in many forms of cancer, their role in adult brain cancer has yet to be elucidated. Clinically, mutations of the ABCC6 gene lead to the accumulation of calcium and mineralization of the elastic fibers in the connective tissue of the body [198], while ABCC10 mutations have been noted in patients with kidney tubular dysfunction [199]; however, their expression profiles with respect to glioma have not yet been elucidated.

2.5 Breast Cancer Resistance Protein (BCRP, ABCG2, Cdw338)

All genes which are members of the ABC family encode for transporter proteins responsible for transporting solutes, drugs, and xenobiotics across cell membranes. The ABC gene family is divided into seven distinct subfamilies named ABC1, ABCB or MDR/TAP, ABCC or MRPs, ABCDs or ALD, ABCE (OABP), ABCF (GCN20), and ABCG or White genes, of which the breast cancer resistance protein (BRCP) is a member of the White subfamily [200]. BCRP was initially discovered in multidrug-resistant breast cancer cell lines conferring resistance to a variety of chemotherapeutic agents including most topoisomerase I and II inhibitors such as topotecan, irinotecan, and doxorubicin [201]. The BCRP transporter plays a significant role in barrier function, being highly expressed in the intestine, B-BB, placenta, and liver, preventing drug transport into tissues such as the brain, gut, and also tumors. Similar to MRP2 (ABCC2), BRCP is also involved in biliary and renal excretion of drugs [202–207].

In brain cancer cells, as discussed by Bleu et al. [208], increased ABCG2 expression can occur through several mechanisms including activation of the PI3K/Akt pathway, PTEN deletion, and hypoxia. In glioma, amplification of growth factor receptors can lead to activation of the PI3K/Akt pathway which facilitates translocation of the ABCG2 transporter from the cytoplasm to the cell membrane. Similarly, PTEN deletion, as is noted in several cancer forms [209–213], leads to the increased expression of ABCG2 through notch activation, translocation into the nucleus, and activation of ABCG2 transcription. Translocation of hypoxia-induced HIF1 and HIF-2α to the promoter region of the ABCG2 gene leads to increased transcription and expression. In this vein, BCRP was found to be localized to the nuclear membranes of both glioblastoma cells and patient biopsy samples [214], and microvessel endothelium of human brain and glioma cells [215, 216], highlighting its contribu-

tion to chemodrug resistance. In addition, BCRP expression has been associated with increasing glioma grading suggesting a role of BCRP as a prognostic marker for progressive astrocytoma [217].

2.6 Targeting Chemoresistance

As discussed in this chapter, the ability of many members of the ABC transporter family to confer drug resistance in various cancer forms has been well established in vitro, and although correlations between expression levels and clinical outcome in glioma patient samples have been verified, a clinically relevant role for ABC transporters in GBM treatment has yet to be definitively confirmed. In this section, we will discuss the use of ABC-targeted therapies, including RNA interference (RNAi)-based studies, microRNA (miRNA)-based therapeutics, small-molecule inhibitors, and immunotherapy-based approaches to inhibiting ABC transporters and increasing anticancer bioavailability in GBM cells in vivo and clinically.

In vivo studies by Parrish et al. [218] proved that although P-gp and BCRP inhibition using elacridar improved palbociclib (PD-0332991, a cyclin-dependent kinase 4/6 inhibitor) distribution in the brain, it was ineffective at improving orthotopic tumor burden in a patient-derived GBM model. This was also the case for the chemotherapy sunitinib where elacridar improved brain distribution but did not alter the efficacy of sunitinib to hinder tumor progression [219] and likewise for imatinib mesylate (Gleevec) [220]. Although the P-gp inhibitor PSC833 has been assessed in several forms of cancer including breast, kidney, and ovarian cancer and lymphoma (Table 2.2), its role in targeting chemoresistance in GBM has, to date, only been assessed in vitro or in vivo with respect to brain distribution studies and has yet to progress to tumor burden impact studies [73, 221–223].

Specific targeting of ABC transporters using RNA interference (RNAi)- or microRNA (miRNA)-based therapeutics has been extensively studied in vitro [12, 142, 224–228], and although progression to in vivo studies holds several challenges with regard to tumor delivery including biodistribution and limited delivery of effective intratumoral doses, many researchers have shown extremely promising results [229–237]. Researchers are attempting to overcome such delivery issues through direct RNAi administration to either the tumor itself or the cranial cavity post-tumor excision in surgical resection orthotopic models of glioblastoma [238]. Notably, nanoparticle-mediated delivery of short interfering (si) RNA molecules in combination with chemotherapeutic agents in models of glioblastoma [239–247] holds great promise, with encouraging preliminary results from nanoparticle-delivered ABCC-specific siRNA [248]. Of particular interest is the use of noninvasive intranasal delivery of RNAi molecules for glioblastoma in an orthotopic murine model of GBM [249]. Such techniques would facilitate researchers to circumvent the drug efflux effects of ABC transporter expression

at the B-BB and allow direct targeting of the tumor itself. Notably, however, nanoparticle encapsulation and surface modification may also assist in overcoming intrinsic ABC transporter expression which would still pose a challenge with regard to intratumoral uptake of native drugs.

Immunotherapy is, by definition, the use of a patient's immune system to treat and prevent malignant tumor growth and progression. This approach to inhibiting tumor growth in glioblastoma is readily underway in Phase II/III clinical trials for targets such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and immune checkpoint inhibitors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), along with adoptive cell therapy and peptide vaccine assessment for this aggressive brain tumor [250–257]. To date, however, there have been no studies to evaluate ABC-based immunotherapies in GBM. P-gp monoclonal antibodies (mAbs) have been shown to induce chemosensitization in lymphoma [83, 84], and MRP1 mAbs for therapeutic use have been successfully developed and characterized [258, 259] providing potential for further research of these molecules in GBM.

2.7 Conclusion

This chapter is a concise review of the functional role of ABC transporters in the aggressive brain cancer, glioblastoma, and the chemoresistant implication of the expression of these proteins at both the blood-brain barrier and also within the tumor cells themselves. This expression provides intrinsic chemoresistance to several clinically suitable chemotherapeutic agents, and their increased modulation in response to drug exposure, hypoxia, tumor progression, and genetic mutation results in highly chemoresistant recurrent tumors which are devoid of treatment response. This chapter provides a detailed list of all known ABC transporter substrates and inhibitors (Table 2.1) in addition to currently active and completed clinical trials involving ABC transporters in various cancer types (Table 2.2).

Latest developments in drug delivery into the brain using nanoparticle technology have provided an opportunity for researchers to evaluate the effects of glioma-targeted delivery of several novel and clinically relevant drugs in vivo in higher dosages devoid of off-target neurotoxic effects. In this regard, it would be of great interest to evaluate the role of ABC inhibition through noninvasive intranasal ABC-targeted drug delivery in combination with systemic chemotherapy administration.

The aggressive nature of this form of cancer, and the dismal prognosis currently available for these patients, requires novel approaches to drug delivery methodologies with ABC transporter modulation holding an extremely promising avenue for treatment progression.

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Chapter 3
Resistance of Glioblastomas to Radiation Therapy

Han Shen and Eric Hau

Abstract Glioblastoma is the most lethal primary brain tumour with a median survival of 12–14 months because of resistance to radiotherapy and other chemotherapies. Ionising radiation represents the most effective treatment for glioblastoma, but radiotherapy remains only palliative due to radioresistance. The mechanism of radioresistance in glioblastomas is a complex phenomenon and has been extensively studied in the last decade, and effective radiosensitisers have always been sought experimentally. The radiosensitivity of tumour cells is regulated by a series of internal factors, such as cell cycle arrest, cell apoptosis and DNA damage. In addition, the existence of glioma stem cells and their growing microenvironment also play an important role in radioresistance. In this chapter, we will summarise the proposed mechanisms of radioresistance in glioma cells and also review how the therapeutic strategies can be developed to target these mechanisms for an improved radiosensitisation of these aggressive brain tumours.

Keywords Glioblastoma • Radiotherapy • Resistance

Abbreviations

2-DG	2-Deoxy-D-glucose
3-BP	3-Bromopyruvate
3D	Three dimensional
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
DCA	Dichloroacetate

H. Shen, BMed (✉)
Targeted Therapies Research Program, Children's Cancer Institute Australia, Lowy Cancer Research Centre, University of New South Wales, Randwick, NSW 2031, Australia
e-mail: hshen@ccia.unsw.edu.au

E. Hau
Cancer Care Centre, St George Hospital, Kogarah, NSW 2217, Australia

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_3

DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
GSH	Glutathione
GSSH	Oxidised glutathione
Gy	Grey
H2AX	Phosphorylated histone
HIF	Hypoxia-inducible factor
HK	Hexokinase
NADP	Nicotinamide adenine dinucleotide phosphate
PARP	Poly(ADP-ribose)polymerase
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

3.1 Introduction

Glioblastoma (GBM) is the most aggressive malignant primary brain tumour in adults (WHO grade IV) [1]. It has an annual incidence of 2–3 cases per 100,000 populations in Europe and North America and accounts for 52% of all primary brain tumours. Significant survival benefits have been achieved with postoperative radiation therapy to doses of 50–60 Gy, but dose-escalation attempts beyond 60 Gy have led to increased toxicity without additional survival benefit. To improve local control and lessen toxicity with these infiltrating tumours, innovative imaging techniques are actively being developed to better delineate tumour extent and related radiation therapy treatment fields. Currently, the standard of care for patients with newly diagnosed GBMs includes maximal safe resection of the tumour, followed by 6-week course of radiation therapy (typical dose is around 60 Gy) plus concomitant and adjuvant chemotherapy with temozolomide [2].

Despite aggressive multimodality strategy, long-term control of such malignancy is rarely achieved and it ultimately recurs. Most recurrences appear within 2 cm of the resection margin and within the irradiated volume in nearly all patients [3]. It was proposed that escalating the dose of radiation would improve local control, but recent clinical trials failed to support this hypothesis [4], suggesting the radioresistance of high-grade gliomas remains one of the main reasons for failure of treatment. Brachytherapy and stereotactic radiosurgery are effective therapies for recurrent GBMs but tend to be associated with notable toxicity [5]. More recently, re-irradiation strategies employ concurrent use of bevacizumab to limit treatment-related injury while still permitting delivery of meaningful doses [6, 7]. These clinical trials are ongoing and merits of these strategies are not yet clear but appear promising.

3.2 The Intrinsic Radioresistance of Glioma Stem Cells

GBMs are characterised by diffuse and infiltrative growth into surrounding normal brain tissue. However, they frequently relapse after radiation therapy as focal masses, indicating that only a fraction of tumour cells is responsible for recurrence. There is increasing evidence that human GBMs possess a small portion of stem-like cells, referred to as glioma stem cells, that is not only capable of initiating a tumour but also resistant to radiation therapy. These glioma stem cells were first isolated from tumour specimens of GBMs and were characterised as being able to form colonies under cell culture conditions (clonogenic). Additionally, these glioma stem cells could also be induced to differentiate along astrocytic and neural lineages [8]. Following the first report, another group of researchers using an orthotopic model demonstrated the ability of these glioma stem cells to induce tumours at a very high frequency. Specifically, by using stereotactic device, intracranial inoculation of as few as 100 glioma stem cells into immune-compromised mice was adequate for inducing tumour formation. Moreover, these tumour xenografts phenotypically resemble the patient's original tumour and could be serially transplanted. By contrast, implantation of up to 105 non-glioma stem cells could not induce tumour formation [9]. In light of these findings, several studies subsequently analysed the behaviour of patient-derived GBM cells sorted by expression of candidate stem cell markers or by different culturing methods (neural sphere culture or adherent monolayer culture) in serum-free medium supplemented with multiple exogenous growth factors. These glioma stem cells were characterised in terms of in vitro self-renewal (defined by neurosphere formation), differentiation potential and in vivo tumorigenicity (defined by tumour initiation in orthotopic xenograft models).

This aforementioned evidence has led to the hypothesis that the nearly inevitable relapse of GBM is primarily attributed to the persistence of these glioma stem cells after multimodality treatment. Several studies, therefore, analysed the prognostic values of putative stem cell markers and related cellular features in tumour specimens. Consistently, they all observed significant correlation of stem cell markers, e.g. CD133 or nestin expression, with shorter overall survival [10–13]. Notably, one of the studies above further indicated the correlation was independent of patient age, symptom duration, extent of glioma resection, MGMT methylation and p53 status [11]. Confirmation for a role of glioma stem cells in determining the response to radiotherapy also comes from a recent study that analysed the expression of CD133 in tumour specimens from ten glioma patients who had gone through surgical resection before and after radiotherapy delivered by stereotactic radiosurgery followed by external beam radiation [14]. The percentage of CD133-positive cells was significantly increased in the post-irradiated tumour samples compared to that in the original specimens. These data are keeping in line with the hypothesis that glioma stem cells are able to survive high doses of radiation, although there was no causative relationship established based on these findings. Attempts were also made by preclinical studies to unravel the hypothesis that glioma stem cells are responsible for the very low cure rates observed after radiotherapy. Hitherto, only two studies compared the

radiation responses of glioma stem cells with their differentiated counterparts. Specifically, both studies compared the clonogenicity of glioma stem cells with that of differentiated cells using a clonogenic survival assay, the gold standard endpoint for testing the intrinsic radiosensitivity of tumour cells. Bao et al. conducted clonogenic survival assays on both CD133-positive and CD133-negative cells derived from the same specimen and observed that CD133-positive cells were more radioresistant than those with CD133-negative after treatment with 5-Gy irradiation [15]. These findings were also confirmed by another recent study showing the clonogenic surviving fraction of CD133-positive derived from patient-derived GBM cells was significantly higher than that of the CD133-negative after being treated with irradiation [16]. Furthermore, Bao et al. also noticed that the percentage of CD133-positive cells was elevated in gliomas after radiotherapy. To identify possible mechanisms underlying an intrinsic radioresistance of CD133-positive cells, further mechanistic experiments were performed to analyse proteins involved in apoptosis and early DNA damage checkpoint responses. Western blot for apoptotic markers, poly(ADP-ribose) polymerase (PARP) cleavage and annexin V demonstrated lower rates of apoptosis in CD133-positive cells as opposed to CD133-negative cells. Moreover, the expressions of cell cycle checkpoint-related proteins (phosphorylated ATM, Rad17 and Chk1/2) were also upregulated in CD133-positive cells compared to CD133-negative cells. Quantification of phosphorylated histone (H2AX) before and after irradiation also confirms a potential mechanism involving faster repair of DNA double-strand breaks in CD133-positive than in CD133-negative cells [15]. Following these landmark studies, there are several studies investigating different responses of glioma stem cells and non-stem cells to radiation therapy. Most of the findings are consistent with previous reports and further suggest that these intrinsic cellular features of glioma stem cells can be targeted to modify the sensitivity of tumour cells to radiation therapy. For instance, it has been suggested that the radiosensitivity of glioma stem cells can be enhanced by targeting SirT1 (a member of the mammalian sirtuin family, deacetylates various transcription factors to trigger cell defence and survival in response to stresses and DNA damage) [17], Notch signalling (an essential pathway involved in the maintenance of a variety of adult stem cells through promoting self-renewal and repressing differentiation) [18], Brclin and ATG5 (autophagy-related proteins) and Chk1/2 (cell cycle checkpoint kinases) [15]. Radiobiological modelling of treatment outcomes using a due component linear quadratic model has also been able to predict the remarkable radiation resistance displayed in GBMs to both conventional and hypofractionated radiotherapy and suggest this may arise from accelerated regrowth of tumour from the stem cell compartments [19].

3.3 Microenvironment, the Radioresistant Entity in GBMs

Currently, the majority of existing assays examining radiation sensitivity do not take an important factor into account: the microenvironment where both glioma stem and non-stem cells reside. It has been proposed that radioresistance is more likely to be a

property of the microenvironment unit, a functional entity in which glioma stem cells are capable of maintaining or enhancing their intrinsic cellular features that contribute to radioresistance [20]. Indeed, it is rational to believe that the in vivo microenvironment creates a more favourable niche for tumour cells that would survive higher doses of radiation compared to those growing in a petri dish with culture medium. Indirect evidence for this proposal that microenvironment determines radiosensitivity of glioma cells comes from a preclinical study comparing the induction and repair of irradiation-induced DNA double-strand breaks [21]. The authors compared the level of H2AX foci in CD133-positive cells grown in vitro and in vivo after irradiation and observed that the number of H2AX foci decreased much faster in the in vivo setting, suggesting a much more efficient DNA repair ability of these cells residing in the microenvironment niche. Additionally, novel in vitro culture techniques have also been utilised to study the impact of microenvironment on radiosensitivity. Hovinga et al. employed a three-dimensional (3D) organotypic explant system of surgical GBM specimens to test the efficacy of radiotherapy and demonstrated that radiation alone was less effective on the clonogenicity of tumour cells in the explants than in neurosphere cultures [22], although the latter is more complex than the conventional cell culture method with attached cells growing as a monolayer.

It has been well documented that there is a reciprocal interaction between endothelial cells and glioma cells. On one hand, tumour cells have been proven to exert a pro-angiogenic action that is mediated by stimulation of endothelial cells via secreting vascular endothelial growth factor (VEGF). On the other hand, endothelial cells are known to play an important role in the maintenance of glioma cells through emanating several factors that positively regulate signalling pathways. To unravel how the microenvironment influences the radiation response of glioma stem cells, researchers first investigated the distribution of cells expressing different stem cell markers within glioma specimens. Calabrese et al. first reported that nestin-positive cells resided much closer to endothelial cells (CD34-positive) compared to nestin-negative cells [23]. This finding was also confirmed by another study showing a close relationship between CD133-positive cells and vascular structures in paraffin-embedded sections from GBM specimens [24]. In light of these findings based on clinical specimens, another interesting study analysing the association between endothelial cells and glioma cells with 3D coculture system demonstrated that the vascular networks formed by both tumour cells and endothelial cells were more stable than that formed by endothelial cells alone [25]. More importantly, this study further showed a survival advantage of coculture (glioma and endothelial cells) in mosaic vasculature over the vascular network formed by endothelial cells alone after irradiation. Consistent with these findings are the results of a study comparing radiation-induced apoptosis in endothelial cells. The number of apoptotic cells was quantified either in monoculture or in coculture with glioma cells [26], and the results demonstrated that there were significantly lower levels of apoptotic cell death in the coculture system after irradiation. Although the evidence of mutually radioprotective effect between glioma cells and endothelial cells is accumulating, the relationship between the radiosensitivity of glioma cells and the endpoint assay used in these studies, e.g. the regression of vascular structure and the apoptotic cell

death of endothelial cells, remains unclear. In this regard, the gold standard clonogenic survival assays should be incorporated into further investigations to confirm the existence of mutual benefits between glioma cells and endothelial cells that might modulate the radiosensitivity of gliomas, even if the underlying mechanisms of such a relationship will require more robust models to be further elucidated.

Aside from cell-cell interactions summarised above, cell-extracellular matrix (ECM) contact is also thought to have a great impact on cellular mechanisms leading to increased cell survival upon exposure to ionising radiation. First of all, ECM has been proposed to serve as a deposit for proteins which modulate radiation responses. The direct evidence for this proposal was from several recent studies showing that basic fibroblast growth factor (bFGF) was able to not only stimulate the growth of glioma stem cells [27] but also lower their radiosensitivity by inhibiting radiation-induced apoptosis [15]. bFGF belongs to a family of potent mitogens that induce proliferation and differentiation of mesenchymal, neuroectodermal and epithelial cells [28]. More evidence from studies of other tumour types indicates that bFGF serves as an important inducer of radiation damage repair [29]. This radioprotective effect is probably not due to the preferential repair of DNA breaks induced by irradiation but rather to an inhibition of interphase apoptosis involving protein kinase C [30]. Secondly, ECM has been thought to serve as a substratum for triggering integrin-mediated signalling cascades in tumour cells after radiation. The evidence supporting this proposal came from the findings that elevated radioresistance of glioma cells was linked with the expression and activation of beta 1 [31], alpha v beta 3 and alpha v beta 5 integrins [32]. Given the underlying mechanisms of action of integrins in modulating radiosensitivity has been primarily studied in other cancer types, such as breast cancer [33], nasopharyngeal carcinoma [34] and prostate cancer [35], characterisation of glioma cells attaching to various ECM components is also needed to clarify whether any of these factors play a similar role in determining the fate of glioma cells after irradiation. Last but not least, ECM has been proposed to create a more favourable environment for repopulation of tumour cells that survive radiotherapy. This proposed mechanism is supported by several studies investigating tenascin C, an ECM protein that is highly expressed during embryogenesis and tissue repair and in pathological conditions such as chronic inflammation and cancer [36]. It has been demonstrated that the endogenous pool of tenascin C isoforms in gliomas promotes the proliferation, invasion and migration of tumour cells [37, 38]. Recent studies of human gliomas not only demonstrated a strong correlation of tenascin C with tumour blood vessels [39] but also identified tenascin C as a novel candidate marker for glioma stem cells by using tissue microarrays [40]. Moreover, a post-irradiation increase in tenascin C has also been observed in glioma patients, suggesting it may have a radioprotective role [41]. As a molecular target, monoclonal antibodies against tenascin (anti-tenascin mAb) have been developed, and preliminary but promising results have been reported in small pilot clinical studies [42, 43]. Taken together, these findings highlight the importance of studies analysing the complexity of ECM and the tumour cells when assessing the response to irradiation. It is worth noting that although these specific

ECM components influence the survival of glioma cells post-irradiation, the heterogeneity observed from GBMs suggests the individual mechanisms may not exert these actions universally across all the glioma cells.

3.4 The Role of Hypoxia, HIF-1 and Tumour Glucose Metabolism in Radioresistance of GBMs

Radiation is an important treatment modality that kills tumour cells through induction of oxidative stress. When ionising radiation passes the living tissue, the ionisation of H_2O leads to the production of reactive oxygen species (ROS) that contain chemically active oxygen molecules leading to oxidative stress and DNA damage. Oxygen molecules (O_2) can stabilise the chemical composition of the DNA damage by reacting with the free radicals, such that O_2 chemically "fixes" DNA damage. Unlike the balance achieved in normal tissues, the consumption of O_2 by tumour tissue is much higher than the O_2 supply from the surrounding blood vessels. Malignant solid tumours with inadequate blood supply and inconsistent perfusion therefore contain large portions of hypoxic cells which exhibit a high degree of resistance to radiotherapy due, in part, to an increase of hypoxia-inducible factor-1α (HIF-1α) and expression of other cellular survival molecules [44]. In GBMs, hypercellular zones called pseudopalisades typically surrounding necrotic foci are constantly exposed to moderate levels of hypoxia ($pO_2 = 2.5–5\%$) [45]. Radiation itself has also been shown to stabilise the activity of HIF1α, which in turn regulates a plethora of genes involved in angiogenesis, invasion, metabolism and protection against oxidative stress [46, 47]. The residual tumour cells surviving after radiotherapy eventually proliferate and lead to cancer relapse.

Normal cells under aerobic conditions generate energy by catabolising glucose via inefficient glycolysis followed by a more efficient mitochondrial oxidative phosphorylation. Because hypoxia decreases the rate of mitochondrial oxidation, tumour cells switch to glycolysis for energy production under hypoxic conditions [48]. This process in which pyruvate, lactate and hydrogen ions are produced is called anaerobic glycolysis or Pasteur effect [49]. However, a hallmark of cancer cells, defined as reprogramming energy metabolism, is that they catabolise available glucose to lactate irrespective of the availability of oxygen (aerobic glycolysis), referred to as the Warburg effect [50]. Given the inefficient energy production by glycolysis compared with oxidative phosphorylation oxidation via the citric acid cycle in mitochondria, this glycolytic switch may seem counterintuitive. However, the glycolytic intermediate glucose-6-phosphate can also be utilised in the pentose phosphate pathway in which precursors of nucleotides and amino acids are synthesised. These macromolecules also serve as building blocks that are required for rapid tumour cell growth and proliferation. Therefore, using glycolysis provides a growth advantage for tumour cells and leads to malignant progression [51]. GBMs, like most malignant solid tumours, are highly glycolytic, producing large amounts of lactate as a metabolic byproduct [52]. It has been validated that tumours with high levels of glycolysis are less responsive to

radiotherapy and behave more aggressively [53]. More recent reports have also identified the Warburg effect to be implicated in resistance to cytotoxic stress induced by radiotherapy [54]. The underlying mechanisms of the radioresistance resulted from hypoxia, and high glycolytic states have been explored by increasing studies. Briefly, hypoxic tumour cells are less sensitive to radiotherapy as a consequence of the interference of hypoxia with the fixation of free radical-induced DNA damage [55]. On the flip side, tumour cells counter the direct effect of radiotherapy, radiation-induced oxidative stress, by increasing their endogenous antioxidant potential through constantly accumulating pyruvate, lactate, GSH/GSSG and NAD(P)H/NAD(P)+[56]. These molecules generated from the glycolytic pathway establish an intracellular redox buffer system that inevitably scavenges free radicals and ROS [57].

From the therapeutic point of view, tumour glucose metabolism can be targeted at several levels, either indirectly by targeting HIF-1 or directly via inhibiting enzymes involved in the glycolytic pathway. HIF-1 inhibition results in metabolic changes with a decreased rate of glucose uptake and lactate production [58] and an increase in oxygen consumption, reflecting enhanced mitochondrial oxidation [59]. As ROS are produced during mitochondrial oxidation, these metabolic alterations could enhance the therapeutic efficacy of radiotherapy [60]. The efficacy of many novel anticancer agents that target signal transduction pathways may be attributed partly to their indirect activity of HIF-1 inhibition. Although a large number of novel compounds have been shown to inhibit HIF-1, no compound has been demonstrated to directly and specifically inhibit HIF-1 activity so far. BAY87-2243, an inhibitor of HIF-1 activity and a stabiliser of HIF-1α, is now being tested in a phase I clinical trial (ClinicalTrials.gov identifier: NCT01297530) and could be the first compound of the class. Another novel antisense oligonucleotide EZN-2968 targeting HIF-1α is currently being evaluated in a phase I trial [61]. Given HIF-1 not only regulates the glycolytic pathway but also modulates the transcription of many genes involved in critical aspects of cancer biology, including immortalisation, maintenance of cell stemness, genetic instability, vascularisation, metabolic reprogramming and invasion/metastasis [62], a small molecule of HIF-1 inhibitor would be a very promising therapeutic agent to synergistically target malignancies with radiotherapy.

A structural analogue of glucose, 2-deoxy-D-glucose (2-DG), inhibited glycolysis via competitive inhibition of hexokinase 2 (HK2) that controls the first rate-limiting step of glycolysis [63–65]. Treatment with 2-DG is cytotoxic and radiosensitises human glioma cells [66]. This cytotoxic and radiosensitising effect of 2-DG is mediated by disruptions in GSH metabolism and decreased NADPH content [67], which reflects the close connection between tumour glucose metabolism and an aberrant cellular redox status. Phase I/II clinical trials examined the effect of the combination of 2-DG with hypofractionated radiotherapy in patients with glioma and showed this combined treatment was well tolerated without severe acute toxicity [68]. This regimen resulted in a moderate increase in median survival with a significantly improved quality of life [69]. Lonidamine, an orally administered small molecule that inhibits glycolysis by inactivating the mitochondria-bounded HK, has been described since the early 1980s [70]. Lonidamine was tested in a randomised study for GBMs in combination with radiotherapy, but failed to

show therapeutic benefit in terms of time to progression and overall survival [71]. Another leading compound 3-bromopyruvate (3-BP), a pyruvate analogue, is both an alkylating agent and an inhibitor of HK2. 3-BP was shown to inhibit tumour growth in a dose-dependent fashion in vivo [72]; however, the inability of 3-BP to cross the blood-brain barrier prevented its further application in gliomas [73].

Another target of tumour glucose metabolism is pyruvate dehydrogenase kinase 1 (PDK1), which controls the amount of pyruvate entering the citric acid cycle by negatively regulating pyruvate dehydrogenase (PDH) activity. This results in decreased pyruvate oxidation in mitochondria [74]. It has been validated that inhibition of PDK1 alters the glucose metabolism and increases oxygen consumption of tumour cells, which in turn resensitises the tumour cells to radiotherapy. Dichloroacetate (DCA), a small molecule PDK inhibitor that has the potential for such metabolic targeting, has been shown to reverse the Warburg effect by shifting glucose metabolism from glycolysis to mitochondrial oxidation and to inhibit tumour cell growth [75, 76]. By combining with irradiation, DCA has been demonstrated to enhance the radiosensitivity of several tumour types in vitro [77–79] and GBMs in vivo [47]. DCA has been used as an orphan drug for various acquired and congenital disorders of mitochondrial metabolism for decades and has recently been demonstrated to be feasible and well tolerated in patients with recurrent malignant gliomas in a recent phase I clinical trial [80]. In addition, a recent study has tested the efficacy of DCA in a small cohort of GBM patients, suggesting metabolic modulation through PDK inhibition as a novel therapeutic strategy for the treatment of this devastating brain tumour [81]. Interestingly, this study also observed that apoptosis was further increased in the glioma stem cells treated with DCA plus temozolomide, indicating DCA may potentiate the effect of standard chemotherapy in the current clinical setting. Taken together, all these data warrant further evaluation of the triple combination (DCA, temozolomide and radiotherapy) in clinical trials for newly diagnosed GBM patients.

In summary, inhibiting tumour glucose metabolism at several levels has been shown to decrease the amount of antioxidant molecules and to radiosensitise GBM cells in preclinical studies. The combination of the glycolysis inhibitor 2-DG with hypofractionated radiotherapy shows promising results in the phase I/II clinical trials with no severe toxicity. However, daily use of anti-glycolytic treatment in combination with daily-fractionated radiotherapy for several weeks may lead to more severe adverse effects, given the brain is highly dependent on glucose for its energy metabolism. In this regard, DCA may be superior to other glycolytic inhibitors as it reduces glycolysis by shunting pyruvate into the citric acid cycle where ATP should still be generated to maintain the energy supply for normal brain.

3.5 Conclusion

Although substantial research efforts have been made to develop novel therapeutic strategies, multimodal treatment still fails to cure most solid cancers, irrespective of the stage and spread of the diseases. Radiation therapy is still the cornerstone in the

management of patients suffering from malignant tumours, but it eventually fails against recurrent tumours due to radioresistance. Cancer stem cells offer novel targets to enhance the efficacy of radiation therapy, thus future targeted therapy should have this aim incorporated. In addition, the tumour microenvironment has been a main focus and the therapeutic target in the field of radiation biology in terms of hypoxia. The aberrant glucose metabolism of cancer cells is also closely linked with the hypoxic niches where they reside. Given targeting HIF-1 and glucose metabolism has been extensively explored and proven to be a radiosensitising strategy in preclinical studies, future studies should examine whether HIF-1 and glucose metabolism inhibitors are effective to conquer the radioresistance of malignant tumours in clinical practice.

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Chapter 4
The Blood-Brain Barrier in Glioblastoma: Pathology and Therapeutic Implications

John Kealy and Matthew Campbell

Abstract Glioblastoma (GBM) is a highly malignant form of brain tumour for which the prognosis is generally very poor and treatment options are limited. GBM is associated with rapid and aggressive tumour growth with associated cerebral oedema. Central to the difficulty associated with treating GBM is the challenge of getting chemotherapeutic drugs to cross the blood-brain barrier (BBB). Although vasculature within and around a GBM becomes more permeable due to pathological changes in the BBB, large areas of the tumour remain resistant to systemically administered agents. Here, we will introduce the concept of the BBB and its normal role in the healthy brain before describing how it becomes compromised in cases of GBM. This will cover physiological, genetic and functional aspects of BBB function and dysfunction. Finally, the therapeutic implications of modulating BBB permeability and receptor-mediated transport will be discussed with a focus on chemotherapeutic drug delivery.

Keywords Blood-brain barrier • Brain • Glioblastoma • Tumour

Abbreviations

BBB	Blood-brain barrier
GBM	Glioblastoma
NVU	Neurovascular unit
TJ	Tight junction

J. Kealy • M. Campbell (✉)
Smurfit Institute of Genetics, Lincoln Place Gate, Trinity College Dublin, Dublin 2, Ireland
e-mail: matthew.campbell@tcd.ie

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_4

4.1 Introduction

In order for the central nervous system to work effectively and efficiently, it requires a tightly regulated means of supplying neurons with nutrients and removing unwanted substrates from the cerebrospinal fluid (CSF). This is achieved by dense vascularisation of the cerebral parenchyma [1] where controlled trafficking of molecular species between the central nervous system and the periphery can occur. Access to the central nervous system is restricted by the blood-brain barrier (BBB), a complex interface between the blood stream and the cerebral parenchyma [2]. The presence of tight junctions and transporter proteins allows the BBB to selectively regulate the passage of molecules to and from the brain [3–5].

Glioblastoma (GBM) is a particularly malignant form of brain tumour for which the prognosis is generally poor and treatment options are limited [6]. GBM is associated with rapid and aggressive tumour growth with an associated cerebral oedema, and prognosis is poor for patients diagnosed with the condition [7]. Part of the difficulty in treating in GBM is the challenge of getting chemotherapeutic drugs to cross the BBB, even though the BBB in GBM becomes more permeable due to pathological changes in the BBB [8–11]. However, dynamic changes in BBB permeability can be achieved by targeting these tight junctions and transporters with suitable treatments [12]. This can be used to increase the efficacy of drug delivery [13] or removal of pathological material from the cerebral parenchyma, for example, the removal of amyloid beta in Alzheimer's disease [14].

The aim of this chapter will be to introduce readers to the BBB and its normal role in the healthy brain before describing how it becomes compromised in cases of GBM. This will cover physiological, genetic and functional aspects of BBB function and dysfunction. Finally, the therapeutic implications of modulating BBB permeability and receptor-mediated transport will be discussed with a focus on chemotherapeutic drug delivery.

4.2 The BBB in Normal and Pathological Conditions

Normal brain function requires rapid and controlled access to metabolic resources and molecular products from the periphery. At the same time, removal of unwanted material from the brain to the periphery is also vital. As such, the brain has evolved a pervasive and efficient vascular system that permeates through it; it is estimated that a neuron is never further than 20 μm from a capillary. Recent 3D imaging of the mouse brain shows that penetrating vessels with a diameter of approximately 23 μm access the tissue before branching into microcapillaries as small as 3 μm in diameter, ensuring a dense vascularisation of the brain [1]. The issue of supply of necessary metabolites and removal of waste is well catered for by this extensive microcapillary system, and access to the cerebral parenchyma is restricted by the BBB so that dangerous biochemical species cannot cause damage to delicate neuronal tissue.

The BBB itself is composed of a layer of endothelial cells lining the lumen of the capillary in order to create an interface between peripheral circulation and the cerebral parenchyma where substances can then enter the cerebrospinal fluid (CSF). Endothelial cells control transcellular transport from the blood into the CSF, expressing transporters for molecules that are essential for normal metabolism and homoeostasis such as glucose, insulin and amino acids [3, 5]. The endothelial cells form adherens junctions and tight junctions between each other in order to limit paracellular transport between the vascular and cerebral compartments [12]. Interendothelial adherens junctions are mainly composed of members of the cadherin family such as vascular endothelial (VE)-cadherin and N-cadherin [15]. Tight junctions are composed of about 30 proteins including occludin, tricellulin and members of the claudins and junction adhesion molecule families [16, 17]. Tight junction proteins are anchored to the intracellular cytoskeleton of the endothelial cells by transmembrane proteins such as zonula occludens-1 (ZO-1) [18]. Tight junctions restrict molecules with a size greater than 400–450 Da from passing between endothelial cells [19, 20]. Furthermore, movement of small ions across the BBB is also restricted given its electrical resistance, on average around 1870 omega (Ω) cm^2 [21]. Taken together, the endothelial cell layer can regulate access to and from the central nervous system to a high degree, with the result that approximately 98 % of drugs developed to target neurological disorders being unable to cross the BBB [22].

The microcapillaries that supply the brain are surrounded by a group of different cell types that form the neurovascular unit (NVU), including neurons, astrocytes, microglia and pericytes. The non-neuronal cells of the NVU act both as scaffolding and as mediators of molecular transport into and out of the brain. Within the NVU, contractile pericytes form layers on the abluminal surface of the microcapillaries, regulating permeability in the mature BBB [23]. Pericytes are covered by a macromolecular layer known as the basal lamina which is in turn enclosed by perivascular endfeet from astrocytes to create a supportive sheath (the glia limitans) around the microcapillaries that supply the brain [24–26]. Microglia then form a line of protection on the brain side of the BBB, able to mount an immune response should an unwanted substance make it through the BBB [27].

Although the cerebral endothelial cell layer is the main workhorse of the BBB, the pericytic and glial support structure surrounding the microvasculature is necessary to the development and maintenance of BBB integrity and functionality. Development of the BBB is achieved by radial glia first via the production of retinoic acid to induce BBB formation and secondly by stabilisation of developing vasculature through Wnt signalling [28]. At the same time, interactions between pericytes and endothelial cells functionally regulate BBB integrity [29] via signalling involving transforming growth factor β (TGFβ) [30], platelet-derived growth factor B [31] and the forkhead transcription factor Foxf2 [32]. Once the BBB is mature, astrocytes form endfoot projections that can modulate permeability—possibly through TGFβ [33] and angiotensin signalling [34]. In the NVU, astrocytes act as a go-between for neurons and vasculature, synchronising cerebral blood flow, maintaining homoeostasis and regulating water content in the cerebral parenchyma [25]. Astrocytic endfeet are known to regulate transport of Na^+ and Cl^- across the endothelial cell layer [35] through intercellular Ca^{2+}

signalling between astrocytes and endothelial cells [36]. Astrocytic endfeet also regulate neurovascular coupling via a nitric oxide-dependent intracellular Ca^{2+} signalling cascade [37], ensuring that metabolic supply is maintained during neuronal activation. Pericytes also play a role in controlling the environment within the NVU through the regulation of cerebral blood flow [38] and by coordinating astrocytic endfoot position on the walls of the cerebral vasculature [39].

It is important to note that the BBB is not homogenous; different regions show variations in permeability. For example, the circumventricular organs entirely lack a layer of endothelial cells and have almost free access to peripheral blood flow [40, 41]. Even where an endothelial cell layer is well established, the permeability of the BBB is not a static value as BBB permeability is a dynamic process with up- and down-regulation of tight junction proteins occurring constantly [12]. Diurnal and seasonal effects on BBB permeability have been described [42, 43], and recent work from our group shows that concentrations of molecular regulators of BBB permeability follow a circadian rhythm (unpublished data). This reinforces the idea that the BBB is a constantly changing entity under normal conditions and this dynamism requires careful consideration in addressing questions about pathological processes and therapeutic interventions.

4.3 The Compromised BBB in GBM

GBM is a malignant class of Grade IV tumours that tends to form in the brain or spinal cord, mainly in adults aged 50–60 [44]. The prognosis for GBM is poor in many cases given the aggressive growth of the tumour and difficulties in treating GBM with standard oncological treatments [45, 46]. This means that the five-year survival of patients diagnosed with GBM is only 1.9 % in patients undergoing radiotherapy alone [7]. Typically, abnormal differentiation of brain tissue results in a mass of cancerous tissue though the exact source of the initial insult is under debate. It has been suggested that GBMs develop from aberrant glial cells but more recently the idea that cancer stem cells (CSCs) are responsible for the condition. These CSCs develop from a suitable progenitor cell line where the normal developmental cascade has been altered leading to unregulated growth [44]. Given the fact that GBMs tend to form numerous cell types during their growth, it is likely that multipotent progenitor cells are at fault in this condition [47–50] and the location of GBM development within the brain may further influence the fate of these CSCs [51, 52]. In particular, co-activation of the Ras and Akt pathways has been identified as being necessary for GBM induction [53, 54]. The role of Ras is confirmed by the presence of altered Notch signalling in GBM cell lines [55]. Ras has also been implicated in the maintenance of GBM with suppression of Kras expression resulting in GBM apoptosis and regression in a mouse model of GBM [56].

Tumour growth and maintenance are facilitated by newly formed blood vessels that give the cancerous cells access to the peripheral blood supply; a well-established hallmark of higher-grade brain tumours is an extensive network of microcapillaries

within the cancerous region [57–59]. Out of all brain cancers, GBM shows a relatively high level of biomarkers relating to proliferation and angiogenesis [60], which is unsurprising given the aggressive nature of GBM. There seems to be a reciprocal relationship between the developing tumour and the vasculature of the brain; there are high levels of perivascular nestin-positive CSCs in the early stages of GBM growth [61], nestin being a marker of angiogenesis that is upregulated in cancerous cells [62]. Calabrese and colleagues (2007) also describe direct interaction between cultured CSCs and endothelial cells and, most importantly, that endothelial cells promote GBM development in vivo. This is a subversion of the regulatory role of endothelial cells on normal neural stem cell development [63].

A number of molecular pathways regulating angiogenesis in GBMs have now been identified. Vascular endothelial growth factor (VEGF) in particular stands out as an important regulator of angiogenesis [64]. CSCs actively promote VEGF signalling, directly acting on local endothelial cells to promote angiogenesis [65]. Normally, endothelial cells do not express the tyrosine kinase receptor for VEGF, but it is expressed on endothelial cells associated with tumour formation [64]. Inhibition of VEGF signalling following transfection of human GBM cells into the brains of nude mice was subsequently shown to inhibit GBM growth and decrease the rate of angiogenesis in vivo [66]. Specific targeting of the VEGF tyrosine kinase receptor using a diphtheria toxin conjugated to tumour-specific isoforms of the VEGF receptor has been also shown to prevent tumour-associated angiogenesis and inhibit GBM growth in vivo [67–69]. Translation of anti-VEGF treatments to human clinical cases of glioma has shown therapeutic promise; however GBM still remains resistant to treatment even with these new therapies [70–72]. Part of this resistance may be due to the issue with invasive cells that migrate away from the GBM core where vascularisation is at its most dense [73], a process that is itself dependent on VEGF signalling [74].

These new blood vessels develop a BBB but one that shows marked differences compared to that in normal tissue [4, 75] with alterations of both adherens [10, 11] and tight junction proteins [8, 9]. Additionally, epigenetic modulation of GBM development [76] and GBM's susceptibility to radiation therapy [77] are associated with changes in the expression of markers for tight and adherens junctions, indicating an intimate relationship between endothelial integrity and GBM growth. It is well established that switching of cadherin expression in the adherens junction is involved in GBM development [78, 79] and these alterations have knock-on effects on tight junction stability [80, 81].

At the level of the tight junction, there is almost complete loss of claudin-1 [9] and reduced levels of claudin-3 [82], claudin-5 and occludin [9] associated with GBM. At the same time, decreased levels of claudin-1 and claudin-5 in human GBM samples are accompanied by significant increases in the expression of the adherens junction protein β-catenin [83]. Liebner and colleagues (2000) also describe alterations in plakoglobin and beta-catenin, further suggesting abnormally formed tight junctions in this pathological state. In clinical cases of GBM, these regions of abnormal tight junctions can be identified through contrast magnetic resonance imaging (MRI); a contrast agent (gadolinium) is injected into the patient

and areas of high BBB permeability show a hyperintensive signal in T1-weighted scans [84, 85]. Using this method, regions of increased BBB permeability have been identified in patients [86], fitting with the molecular data from preclinical studies outlined above. This loss of tight junction integrity can be reversed in vitro using an anti-TGFβ antibody [4]. Interestingly, there appears to be a connection between TGFβ effect on endothelial cells and angiogenesis; in vitro analysis shows that TGFβ upregulates VEGF and inhibition of TGFβ signalling leads to an increase in claudin-5 levels [87], and human neuroimaging suggests that the extent of BBB "leakiness" is an indicator of patient survival [85].

Abnormal tight junctions in GBMs are associated with changes in basal lamina composition, namely, decreases in agrin, a basal lamina protein associated with BBB function, and increases in tenascin, which is normally absent in the basal lamina [88]. Data from an in vitro model of BBB using cultures of rat endothelial cells suggests that there may be a transient decrease in BBB permeability given that there is a fibroblast growth factor-2-dependent increase in occludin and ZO-1 protein levels following initial exposure to human GBM cells accompanied by an increase in transendothelial electrical resistance (TEER) [89]. However, this may be a transient increase in tight junction efficiency that is lost as the GBM becomes established in the tissue.

The role of the BBB in GBM is of further clinical significance when considering how disruption in fluid clearance can lead to serious cerebral oedema [90]. Under normal conditions, the BBB is responsible for regulating osmotic processes via the aquaporin family of proteins [91, 92]. In particular, aquaporin-4 has a clear role in water transport; it is the most abundant water channel in the central nervous system and is found throughout the glia limitans in the astrocytic endfeet lining the BBB [93]. Aquaporin-4 is implicated in multiple regulatory processes including, but not limited to, regulation of extracellular space volume, circulation of CSF, waste clearance and cell migration [94]. Importantly, directly disrupting aquaporin-4 function using aquaporin-4-immunoglobulin G causes a significant increase in BBB breakdown [95].

As noted above, the BBB becomes disturbed within the GBM and cerebral oedema has been identified in regions neighbouring the GBM [86]. Changes in aquaporin expression have been described during brain tumour development, namely, increases in aquaporin-1 [96] and aquaporin-4 [97]. Aquaporin-4 has been shown to be responsible both for the induction of cerebral oedema and for its resolution in a number of pathological states [98, 99]. Nevertheless, even though aquaporin-4 expression increases along with levels of cerebral oedema [100], aquaporin-4 levels are not predictive of patient survival and may follow other processes involved in tumour growth rather than oedema itself [101]. This is supported by the association between increased aquaporin-4 expression in tumours and simultaneous increases in VEGF and hypoxia-inducible factor-1α [102], suggesting a link with angiogenesis during tumour development.

Increases in BBB permeability during GBM may also be linked to aquaporin expression; in GBM the expression of aquaporin-4 moves from its polarised configuration in the astrocytic endfeet [91] and instead covers the cell bodies of the cancerous cells leading to dysregulation of the BBB [103]. Complicating the matter,

aquaporin-4 seems to play a direct role in oedema during therapy against GBM; treatment with radiotherapy and chemotherapy modulates perivascular levels of aquaporin-4 leading to a reduction in cerebral oedema associated with GBM [104]. However, until recently there has been little work performed on targeting the aquaporin system directly in combating cerebral oedema in neuropathological disorders [99]. Other symptoms of GBM have been addressed by targeting aquaporins; successful attempts at modulating the aquaporin system have been made in order to attenuate angiogenesis, cell migration and growth in aquaporin-1-null mice [105] and following knockdown of aquaporin-4 expression in human cell cultures and in nude mice in vivo [106].

4.4 Therapeutic Implications of the BBB in Treating GBM

It must be considered that even though the BBB within the GBM is compromised, it is still largely functional and will continue to perform its job in excluding large molecules from the cerebral parenchyma [20]. This creates the challenge of targeting the GBM effectively using chemotherapies as many commonly used chemotherapeutic drugs are in the size range of 450–850 Da so the BBB will still greatly limit access of many therapeutic compounds to the brain [20, 22]. The current standard for GBM treatment is surgical resection [107] followed by radiotherapy with an adjuvant chemotherapy [48]. Nonetheless, response to chemotherapy in GBM is poor, meaning that therapeutic success is still very low [7], so any attempts at increasing the efficiency of drug delivery to the brain are desirable. Therefore, techniques to increase BBB permeability using osmotic, genetic and physical interventions or via receptor-mediated transport have been developed in order to aid in delivering drugs that would be too large to cross the BBB under normal circumstances.

Osmotic modulation of BBB permeability can be accomplished using a variety of techniques. The most commonly used approach is the use of a concentrated solution of the sugar mannitol to increase BBB permeability across the entire brain for several minutes [108, 109] with Na^{+}/Ca^{2+} exchange governing the length of time that BBB permeability is increased [110, 111]. 20 % mannitol was shown early on as a way to improve chemotherapy survival times [112]. However, its use in treating brain cancers has been controversial as osmotic modulation tends to cause widespread opening of the BBB with debatable effects on the therapeutic index of co-administered chemotherapeutic agents [113] and mannitol can induce seizures in patients with epileptiform activity persisting for days [114]. Therefore, mannitol has fallen out of favour as methods for bypassing the BBB with fewer side effects have been introduced. These newer approaches favour selective opening of the BBB; for example, localised BBB opening has been achieved using a convection-enhanced delivery of ethylamine-human serum albumin (EA-HSA) in order to allow greater access of systemically administered methotrexate to the cerebral parenchyma, resulting in reduced tumour growth and increased survival in a rat model of glioma [115].

RNA interference is a method of knocking down gene expression by delivery of a tailored piece of RNA via a viral or non-viral vector that shows great promise across a range of neurological disorders [116]. It has been used to directly target GBM as many of these vectors are designed to pass through the BBB [117–120]. However, RNA interference can also be used to alter BBB permeability in order to allow other therapeutic compounds to access the cerebral parenchyma. Global or selective genetic modulation of the endothelial tight junction can be achieved systemically or locally using short hairpin (sh) or small interfering (si) RNAs. Modulation of BBB permeability using siRNAs has proven to be of therapeutic use in a mouse model of Alzheimer's disease, knocking down occludin and claudin-5 levels increases the permeability of the BBB enough to allow significant clearance of amyloid beta from the brain to the periphery [14]. For GBM, this type of approach could aid in delivering drugs as large as 1 kDa from the periphery across the BBB without inducing oedema [121, 122], allowing many standard chemotherapy drugs to enter the cerebral parenchyma. Furthermore, RNA interference can be utilised to enhance delivery of drugs that are already small enough to pass through the BBB [123], meaning that a smaller dose is needed to be systemically administered which may reduce side effects associated with a particular drug. In terms of GBM and other brain cancers, RNA interference has so far been successfully used preclinically to knock down aquaporin expression in vitro and in vivo. Knockdown of aquaporin-4 significantly reduces water mobility under normal conditions in vivo [124] and significantly reduces GBM migration and growth by disrupting pathways involved in cell invasion and adherence [106].

A third way of altering BBB permeability is the use of physical means such as focussed ultrasound which has recently been shown to be a promising non-invasive method to treat a number of cancers including prostate cancer [125] and liver carcinomas [126] as well as ablation of brain tumours in humans [127–130]. Ablation using this method is problematic at present due to side effects with overheating of nontarget brain tissue. However, at lower intensities it can also be used as a way to increase BBB permeability in vivo [131] though this has yet to be attempted in humans [132]. A 1 MHz sonication pulse can significantly increase BBB permeability in tumours in rats [133–136], and using MRI to guide focussed ultrasound application, it is possible to selectively increase BBB permeability in precise target regions of the rat brain in vivo [137]. This can then be used to allow greater access to the brain for a number of therapeutic compounds including drugs and genetic therapies: uptake of doxorubicin [138] and temozolomide [139] is significantly increased following focussed ultrasound exposure and oligonucleotides, and DNA plasmid delivery can be made even more effective when focussed ultrasound was combined with nanoparticle delivery [140, 141]. Enhanced localisation of focussed ultrasound combined with targeted drug delivery can be achieved using microbubbles preloaded with the desired drug [142, 143].

Finally, receptor-mediated transport is a method of crossing the BBB without altering its baseline level of permeability; instead drugs are bound to a ligand that can normally cross the BBB unimpeded [144]. A number of suitable endocytotic receptors have been identified including low-density lipoprotein receptor-related

protein-1 and protein-2 (LRP-1 and LRP-2), transferrin receptor, insulin receptor and insulin-like growth factor receptor [144]. LRP-1 is found on endothelial cells and is responsible for transcellular transportation for multiple ligands across the BBB [145], and as such, it has become a target for the development of drug carriers that co-opt LRP-1 to carry therapeutic compounds across the BBB into the cerebral parenchyma [146]. Delivery of the chemotherapeutic drug doxorubicin and paclitaxel to the brain via LRP-1 has been achieved by binding doxorubicin to p97 [147] and by binding paclitaxel to angiopep-2 [148]. This significantly increases the effectiveness of GBM uptake of Adriamycin and paclitaxel in mouse models of glioma. Researchers have also taken advantage of transferrin's ability to guide material across the BBB (discussed in greater detail below) with doxorubicin having been successfully delivered in this manner [149].

Nanoparticles have also been used for many years to aid the delivery of drugs across the BBB [150, 151] via receptor-mediated transport involving apolipoproteins B and E [152–154]. Doxorubicin has been successfully delivered to the rodent brain by binding it to polysorbate-coated nanoparticles [155, 156]. Not only does nanoparticle-bound doxorubicin show effectiveness in treating GBM in preclinical experiments [157], the data also suggests that binding doxorubicin to polysorbate-coated nanoparticles also reduces the drug's systemic toxicity [158]. Similarly, methotrexate can be delivered to the brain using the same type of polysorbate-coated nanoparticles [159]; there was a significant decrease in tumour size and a significant increase in the rates of apoptosis in a rat model of glioma using an alternative nanoparticle system (methotrexate was loaded into lipid core nanocapsules) [160].

Combining RNA interference and receptor-mediated transport may result in better therapies, and research involving the transferrin receptor has seen convergence of these techniques leading to increased efficacy in the treatment of GBM [161, 162]. It has been long known that transferrin receptor levels are greatly increased in GBM [163] and these levels are significantly increased following radiotherapy [164], making them an attractive option for delivering drugs to cancerous brain tissue. Early in vitro research using transferrin conjugated to toxins showed that targeting the transferrin receptor could be a way to selectively target GBMs in vivo [165, 166]. Furthermore, RNA interference and traditional receptor-mediated transport can be made more efficient by conjugating transferrin onto nanoparticles; for example, spherical nucleic acids can be conjugated onto gold [167], cationic solid lipid [168] and hyaluronan-grafted lipid-based nanoparticles [169], whereas transferrin can be conjugated onto poly(lactic-co-glycolic acid) [170], poly(ethylene glycol)-poly(l-lactic-co-glycolic acid) [171] and gold nanoparticles [172].

Using oligonucleotides against laminin-8 (a vascular basement membrane protein that is upregulated during GBM) conjugated to an antibody against the transferrin receptor, significant decreases in GBM microvasculature density and significant increases in survival were obtained in nude rats [173]. Polypropylenimine dendrimers can be used as a non-viral alternative to deliver DNA to target cells [174], and conjugating these with transferrin has been shown to be effective in delivering treatments directly to cancerous cells with little toxicity [175]. This has allowed direct delivery of siRNA to GBM cells without using a viral vector [176].

It has also been demonstrated that conjugating microRNA to transferrin and to a nanoparticle delivery system can result in higher levels of transport across the BBB than transferrin alone [177].

4.5 Conclusion

The BBB's tight control over access to and from the brain becomes disrupted in the presence of GBM as expression of tight junction proteins decreases, leading to an increase in BBB permeability. Increases in angiogenesis help to nurture the GBM, and alterations in aquaporin-4 levels contribute to cerebral oedema around the tumour. Despite the compromised nature of the BBB within the GBM, delivery of chemotherapeutic drugs remains problematic. BBB permeability can be further increased by osmotic modulation, RNA interference and focussed ultrasound treatment. Alternatively, receptor-mediated transport can be used to "piggyback" into the cerebral parenchyma using the transporters naturally expressed on endothelial cells. This in turn can be facilitated by the use of nanoparticle conjugates.

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Chapter 5
Resistance of Brain Tumours to Small-Molecule-Targeted Therapies: Lessons from Various Cancer Types

Fiona O'Neill

Abstract Brain tumours are a heterogeneous group of central nervous system (CNS) neoplasms. The current treatment regime for brain tumours consists of surgical resection or sequential or concurrent chemoradiotherapy with chemotherapy such as temozolomide. Despite improvements in these treatments, survival rate is less than 10 % 5 years after diagnosis, and there are limited second-line treatments available. Although recent improvements in understanding the genomics of brain tumours such as glioblastomas (GBMs) have implicated several pathways, clinical success of targeted therapies has been limited. This is due in part to resistance (both intrinsic and acquired) to the therapies but also in part due to the intratumoural heterogeneity, limited vasculature, lack of CNS penetration and the blood-brain barrier limiting the efficacy of the agent. The use of targeted therapies has come to the forefront of oncology in the past decade. Monoclonal antibodies and small-molecule-targeted therapies such as tyrosine kinase inhibitors are currently being used for the treatment of a number of receptor and pathway alteration-driven forms of cancer. Resistance to targeted therapies has been the Achilles heel of the successful application of these emerging agents. By investigating the response to these therapies in many cancer types and examining any mechanisms of resistance, they may be utilised in the treatment of brain tumours.

Keywords Targeted therapies • Brain • Resistance

F. O'Neill (✉)
National Institute for Cellular Biotechnology, DCU, Collins Avenue, Dublin 9, Ireland
e-mail: Fiona.oneill4@mail.dcu.ie

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_5

Abbreviations

ADC	Antibody drug conjugate
CML	Chronic myeloid leukaemia
CNS	Central nervous system
EGFR	Epidermal growth factor
EIADS	Enzyme-inducing antiepileptic drugs
ERK/MAPK	Extracellular-signal-regulated kinase/mitogen-activated protein kinase
HER2	Human epidermal receptor 2
NSCLC	Non-small cell lung cancer
OS	Overall survival
PDGFR	Platelet-derived growth factor receptor
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinases
RCC	Renal cell carcinoma
RTK	Receptor tyrosine kinase
TKI	Tyrosine kinase inhibitor
TMZ	Temozolomide
TRAIL	TNF-related apoptosis-inducing ligand
VEGFR	Vascular growth factor receptor
WHO	World Health Organization

5.1 Introduction

Brain tumours are a heterogeneous group of central nervous system (CNS) neoplasms. Classification of these tumours is dictated by their histology and locations. The most common primary brain tumour is gliomas which account for approximately 80 % of malignant adult brain tumours [1]. Gliomas are formed from the glial component of the nervous system. These are the most common primary brain tumours. Gliomas are very heterogeneous with an infiltrative growth pattern with the majority being resistant to both radiotherapy and chemotherapy [2]. Brain tumours have been classified by the World Health Organization (WHO) according to their cell type and malignancy. Gliomas can comprise of astrocytomas, oligodendrogliomas, oligoastrocytomas and ependymomas. Astrocytomas are the most frequent intracranial neoplasm and can be responsible for more than 60 % of all primary tumours [3]. Table 5.1 describes the WHO classification for astrocytomas based on histological criteria and molecular characteristics.

The current treatment regime for brain tumours is comprised of surgical resection or sequential or concurrent chemoradiotherapy with chemotherapy such as temozolomide [4]. Despite improvements in these treatments, survival rate is less than 10 % 5 years after diagnosis and there are limited second-line treatment available [5].

Table 5.1 WHO classification of diffuse astrocytomas [3]

WHO grade	WHO designation	Histology criteria	Molecular characteristic
II	Astrocytoma	Zero criterion nuclear atypia	P53 mutations Overexpression of PDGFR
III	Anaplastic astrocytoma	Nuclear atypia and mitotic activity	LOH on chromosome 19
IV	Primary glioblastoma	Nuclear atypia, mitoses, endothelial proliferation and/or necrosis	EGFR overexpression LOH on chromosome 19/MMAC1/PTEN Loss of DCC
IV	Secondary glioblastoma	Nuclear atypia, mitoses, endothelial proliferation and/or necrosis	P53 mutations LOH on chromosome 19/MMAC1/PTEN Loss of DCC

Advances in understanding the genomic makeup of these tumours have implicated a number of different pathways and receptors [6]. Receptor tyrosine kinases (RTK) such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular growth factor receptor (VEGFR) and c-Kit have been found to be amplified in patient tumours, which contribute to tumour growth and possibly to the transformation process to a more malignant phenotype [7, 8] (Fig. 5.1). Due to the expression of these receptors, one new approach in the treatment of brain tumours has been the use of small-molecule-targeted therapies. Although recent improvements in understanding the genomics of GBMs have implicated several pathways, clinical success of targeted therapies has been limited. This is due in part to resistance (both intrinsic and acquired) to the therapies but also in part due to the intratumoural heterogeneity, limited vasculature, lack of CNS penetration and the blood-brain barrier limiting the efficacy of the agent [6, 9, 10].

The use of targeted therapies has come to the forefront of oncology in the past decade. Monoclonal antibodies and small-molecule-targeted therapies such as tyrosine kinase inhibitors are currently being used for the treatment of a number of receptor and pathway alteration-driven forms of cancer. Resistance to targeted therapies has been the Achilles heel of the successful application of these emerging agents. The resistance can be as a result of pre-existing mutations/alterations in the drug target (intrinsic) or induced following drug treatment (acquired) [11], and mechanisms by which this resistance occurs have not yet been fully characterised.

These targeted therapies have been used successfully in the treatment of other cancers including chronic myeloid leukaemia, human epidermal receptor 2 (HER2) breast cancer and non-small cell lung cancer. Resistance mechanisms have been investigated for these, and it is possible that lessons may be learned in how to overcome or prevent resistance of these therapies when treating brain tumours.

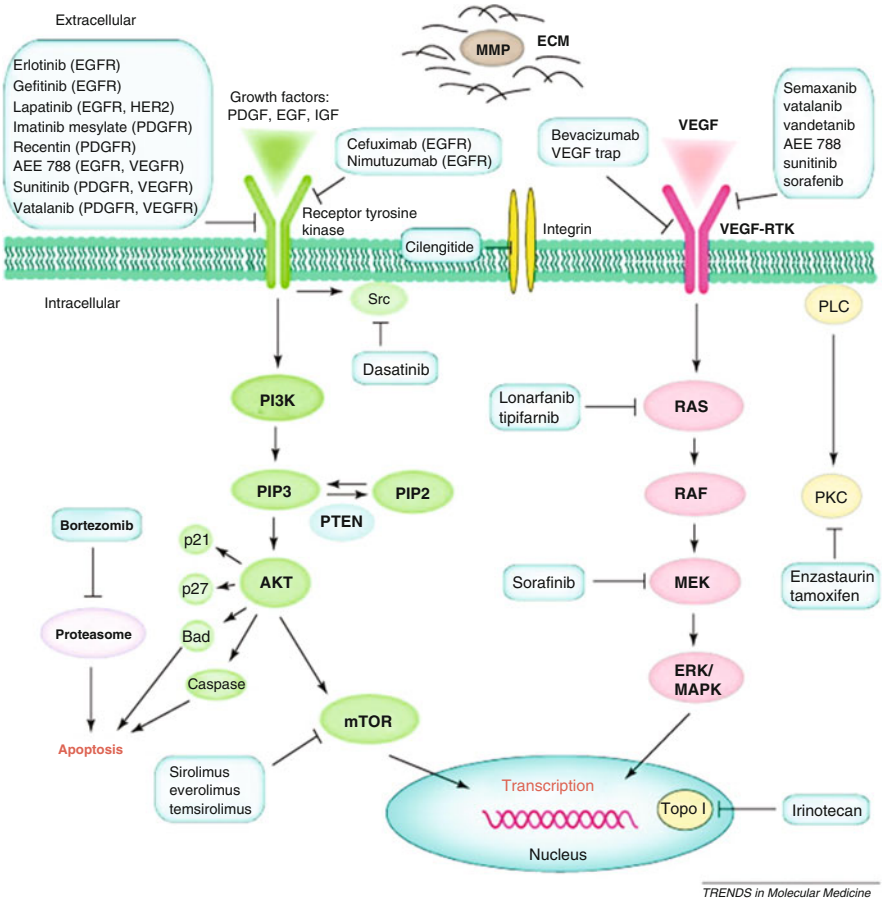

Fig. 5.1 Overview of current molecular-targeted therapies for malignant gliomas [6]

5.2 Receptor Tyrosine Kinases

5.2.1 EGFR Inhibitors

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase which plays an important role in normal tissue development and carcinogenesis and has been found to be activated or overexpressed in 70 % of solid tumours. Activation of this receptor has been associated with increase proliferation, motility and invasion and inhibition of apoptosis and an increase in angiogenesis [12]. Due to this overexpression, EGFR has been extensively investigated as a targeted therapy candidate, which has resulted in the development of a substantial arsenal of inhibitors (Table 5.2).

Table 5.2 Small-molecule inhibitors for EGFR

Drug name	Drug company	Target	Disease(s) studied
Erlotinib	Roche	EGFR	NSCLC [13], pancreatic [14], glioblastoma [15]
Lapatinib	GlaxoSmithKline	EGFR and HER2	Breast cancer [16], colorectal [17], gastric [18] glioma [19]
Afatinib	Boehringer Ingelheim	EGFR and HER2	NSCLC [20], breast cancer [21]
Gefitinib	AstraZeneca	EGFR	NSCLC [22], oesophageal [23] glioblastoma [24]
AC80	Ambit Biosciences	Pan HER	Head and neck [25, 26]

Erlotinib is an EGFR-specific tyrosine kinase inhibitor (TKI). It inhibits autophosphorylation of EGFR, resulting in the inhibition of EGFR-dependent cell proliferation. In combination with temozolomide, it has shown antitumour activity, in a small group of patients with glioblastoma, resulting in an increased survival time [15]. Erlotinib was initially approved, in 2004, for the treatment of locally advanced or metastatic non-small cell lung cancer (NSCLC) after failures of other regimes, and in 2005, approval was given for the treatment of pancreatic cancer in combination with gemcitabine in patients with locally advanced, unresectable or metastatic pancreatic carcinoma.

Lapatinib, a dual kinase inhibitor developed by GlaxoSmithKline, targets both HER2 and EGFR receptors [27]. By inhibiting the tyrosine kinase domains of the receptors, lapatinib prevents activation of important pro-cancer pathways such as ERK/MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinases) which have vital roles in cell proliferation and survival [27, 28].

Afatinib is also a tyrosine kinase dual inhibitor of HER2 and EGFR [29]. This agent, developed by Boehringer Ingelheim, has been shown to irreversibly inhibit the HER2 and EGFR receptors [30]. Due to inhibition of the EGFR receptor, the inhibitor has been proven to be effective in the treatment of non-small cell lung cancer, and a number of phase 2 and 3 clinical trials have been undertaken studying these malignancies. The results of these trials have indicated that in patients who have developed resistance to a number of first-line treatments such as gefitinib and erlotinib, treatment with afatinib may be beneficial [31, 32].

Gefitinib is a specific inhibitor of EGFR; it inhibits the intracellular phosphorylation of Akt and PI3K. This results in inhibition of proliferation, angiogenesis and induction of apoptosis. Patients with phosphorylated Akt have been shown to be better responders to gefitinib, suggesting patients with Akt activation may be more sensitive to gefitinib [33].

In addition to these established EGFR inhibitors, new molecules are being developed to target members of the EGFR family. AC480 is a potent pan-HER inhibitor that has been tested in a number of different cancer types. Recent studies have indicated that it can enhance radiosensitivity and response of head and neck squamous cell carcinoma cell lines, both in in vitro and in vivo [25, 26].

As these inhibitors are being utilised in more cancer types, including brain cancer, the issue of resistance (either intrinsic or acquired) is cultivating a lot of interest from researchers. In many instances, development of resistance to these molecules is inevitable, the mechanisms of which have not yet been fully characterised but a large number of studies have provided potential hypotheses.

Mechanisms of resistance for lapatinib have been many and varied. Activation of AXL [34], involvement of the oestrogen receptor [35] and mutations in the PI3K pathways have all been investigated [36, 37]. The primary causation of resistance to erlotinib is the mutation of the EGFR target, specifically a secondary mutation of the target called T790M [38]. Gefitinib and afatinib are also susceptible to T790M mutation as a mechanism of acquired resistance [39–42]. It has been determined that as many as 50 % of patients that acquire resistance to these inhibitors do so through the expression of the T790M mutation. The remaining 50 % have a number of varying mechanisms that include, but is not limited to, activation of alternative tyrosine kinase receptors, independent activation of molecular effectors downstream to the target protein, activation of tumour-induced angiogenesis, c-MET amplification and enhanced interference with aerobic glycolysis [41, 43, 44].

Due to the overexpression of EGFR in a number of GBM patients, a number of clinical trials have been undertaken to evaluate these targeted therapies in the treatment of the disease. Unfortunately the results were not as compelling as they have been in the treatment of other neoplasms. There has also been some research into the use of a model of targeted therapies called antibody-drug conjugates (ADCs). ADCs are complex molecules composed of an antibody linked to a biologically active cytotoxic (anticancer) payload or drug [45]. A number of clinical trials have sought to evaluate the addition of EGFR-targeting ADCs that can improve progression-free (PFS) and/or overall survival (OS) [46, 47].

Initial studies completed investigating the response of gefitinib in recurrent GBM reported no tumour response [48]. Responsiveness to erlotinib in patients with glioblastomas appears to be dependent on the co-expression of mutated EGFR (EGFRvIII) and the tumour suppressor gene PTEN [49]. EGFRvIII is the most common extracellular domain mutation [50]. However, a more recent phase II study indicated that monotherapy in patients with co-expression mutated EGFR (EGFRvIII) and the tumour suppressor gene PTEN showed minimal efficacy [51]. In recurrent patients, a recent phase I study has indicated that afatinib has a limited activity as a single agent in patients [52]. The combination of lapatinib and temozolomide (TMZ) was also investigated in recurrent high-grade gliomas. Although limited numbers participated, it was felt that the results warranted a phase II study to be undertaken [53].

5.2.2 VEGFR Inhibitors

In addition to EGFR, it has been determined that the expression of additional tyrosine kinase receptors such as platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) and c-Kit can also be targeted using small-molecule therapies for the treatment of a number of cancer types including brain tumours (Table 5.3).

Table 5.3 Small-molecule inhibitors for VEGFR

Drug name	Drug company	Target	Disease(s) studied
Cediranib	AstraZeneca	VEGF, PDGF, c-Kit	Cervical [54], glioma [55], breast [56]
Pazopanib	GlaxoSmithKline	VEGF, PDGF, c-Kit	Renal [57], glioma [19, 58], glioblastoma [58]
Sorafenib	Bayer Schering	VEGF, PDGF, c-Kit	Hepatocellular [59], pancreas [60], glioblastomas [61]
Sunitinib	Pfizer	VEGF, PDGF, c-Kit	Renal cell, recurrent anaplastic [62], glioblastoma [63]

In order for tumours to proliferate, it is vital that they are capable of developing a new blood supply from pre-existing vessels. Researchers found that by inhibiting this process known as angiogenesis through inhibiting the expression of VEGFR, an important factor involved in the process, that it had the potential of to be anti-cancerous, and as such an array of molecules were generated. One such molecule is the monoclonal antibody bevacizumab [64]. First approved in 2004 for the treatment of metastatic colon cancer, bevacizumab in combination with chemotherapy showed significant benefit to patients with solid tumours. Due to the expression of VEGFR and the necessity for improvement in the treatment of recurrent glioblastomas and gliomas, there was a strong biological rationale for the use of bevacizumab in the treatment of these tumours [2].

Initial studies completed utilised the combination of bevacizumab with irinotecan and bevacizumab alone, which was tolerated well by patients and resulted in increases in PFS and OS [65, 66]. While this treatment has been widely used in the treatment of brain tumours, resistance to the therapy is still an issue [67]. As a result, research has turned to additional targeted therapies that inhibit the VEGFR, many of which have been used successfully in other cancer types.

A potent small-molecule inhibitor of VEGFR, cediranib, has also shown that it can target PDGFR and c-Kit [68, 69]. It has been investigated in the treatment of an array of different cancer types including cervical cancer [54], glioma [70] and breast cancer[56]. Cediranib has been tested in as many as 10 clinical trials for its effects in gliomas. Results from these trials have been varied. Initial data indicated the potential for this inhibitor in the treatment of gliomas [71]; however, later studies have shown that cediranib when compared to cediranib in combination with lomustine or lomustine alone did not prolong progression-free survival [55].

RTKs such as pazopanib, sorafenib and sunitinib have also been utilised in the treatment of a number of neoplasms. All three of these small molecules have the ability to target VEGFR as well as PDGFR and c-Kit. They have been utilised successfully in the treatment of renal cell cancer (RCC) and have been investigated in a large number of other cancer types [57, 72, 73]. Unfortunately, investigations of these therapies in brain tumours have not produced compelling results. Pazopanib and sunitinib were investigated as single agents in recurrent glioblastomas and showed a very poor response rate much less than 10 % [58, 74]. Studies investigating sorafenib in combination with chemotherapy also resulted in modest response rates of approximately 12 % [75]. As with RTKs that target EGFR, molecules that target VDGFR are also susceptible to the development of resistance.

Resistance to pazopanib, sorafenib and sunitinib has been associated with a number of different hypotheses. Resistance to sunitinib has in part been associated with an escape from the anti-angiogenesis mediated by IL-8 [76]. IFNγ-related angiostatic chemokines have also been shown to be responsible for recurrence of angiogenesis in tumours undergoing treatment with sunitinib [77]. Recent research has suggested that cross-resistance between VEGFR/PDGFR inhibitors can occur. This cross-resistance has been attributed to increased intracellular drug accumulation accompanied by increased lysosomal storage, reduced drug levels in plasma and also alternative signalling pathways being recruited [78, 79].

5.2.3 PDGFR and C-Kit Inhibitors

Platelet-derived growth factor receptors (PDGF-R) are cell surface tyrosine kinase receptors for members of the platelet-derived growth factor (PDGF) family that are important in the regulation of cell proliferation, cellular differentiation, cell growth and development and progression of disease types such as cancer. The proto-oncogene, c-Kit, encodes CD117, a transmembrane tyrosine kinase which is expressed during normal growth and development in a variety of tissues [80]. Expression of this tyrosine kinase has been associated with a number of different cancer types including NSCLC, melanoma and GISTs [81]. In addition to inhibition of VEGFR, inhibition of PDGFR and c-Kit in cancer cells has been exploited as a potential anticancer strategy (Table 5.4).

Imatinib was developed for the treatment of chronic myeloid leukaemia (CML), a small molecule that belongs to the group of phenylaminopyrimidines. It has been shown to interact with and inhibit tyrosine kinases and specifically target the Bcr-Abl tyrosine kinase [88] which the abnormal protein generates and prevents cell proliferation. The introduction of imatinib into the portfolio of drugs employed for the treatment of chronic myeloid leukaemia has been extremely successful, with one study indicating that, since its introduction, imatinib has improved the 8-year survival rate to 87 % from a 20 % pre-imatinib rate [82]. In addition to Bcr-Abl, imatinib also inhibits PDGFR, stem cell factor C-Kit and C-Abl, causing cell cycle arrest and/or apoptosis [89, 90]. It is due to the ability of this molecule to inhibit these additional targets that it was investigated for the treatment of gliomas [91–93].

Table 5.4 Small-molecule inhibitors for PDGFR and c-Kit

Drug name	Drug company	Target	Disease(s) studied
Imatinib	Novartis	PDGFR, c-Kit, Bcr-Abl,	CML [82], ovarian [83], glioma [84]
Dasatinib	Bristol-Myers Squibb	PDGFR, c-Kit, Bcr-Abl, Src	CML [85], prostate [86], recurrent glioblastoma [87]

Investigations into the use of imatinib as a single agent in the treatment of gliomas provided disappointing results, despite the strong biological rationale. A phase I–II study indicated that the 6-month PFS was 3 % for GBM and 10 % for patients with anaplastic glioma. This study was further complicated by the observation that patients taking enzyme-inducing antiepileptic drugs (EIAEDs) had a lower plasma exposure of the drug in comparison to those who were not taking the drug. As a result of this observation, a number of patients are excluded for additional stages of the trial [94]. Imatinib in combination with hydroxyurea, a ribonucleotide reductase inhibitor, was well tolerated; however, the response rate in malignant glioma patients was not very compelling. [84].

Dasatinib, a thiazole-based ATP-competitive, dual Src/Abl kinase inhibitor, was approved for the treatment of imatinib-resistant and imatinib-intolerant patients across all phases of chronic myelogenous leukaemia (CML) [95]. Dasatinib is effective in treating imatinib-resistant CML as dasatinib is insensitive to the activation state of Bcr-Abl. Imatinib is effective in sustaining the Bcr-Abl protein in its inactive conformation; however, mutations at 17 amino acid positions render imatinib ineffective by "locking" Bcr-Abl in its active conformation. Dasatinib is less selective in its binding requirements and binds to the active form of the Abl kinase thus overcoming 14 imatinib-resistant mutations [96]. Preclinical data have suggested that SRC may be activated in glioblastoma tumours. Due to this observation, studies have been undertaken utilising dasatinib in combination with other therapies and as a single agent. A phase I/II trial in combination with CCNU resulted in significant toxicities and led to suboptimal level of drug exposure [97]. More disappointingly, a phase II trial which looked to enrol recurrent GBM patients on a number of different criteria, which included activation or expression of at least two dasatinib targets, indicated that dasatinib was not effective in this setting [87].

Unfortunately, as has been observed in many other targeted therapies, resistance can occur in approximately one third of patients following treatment with imatinib [98, 99]. Extensive research has been undertaken to elucidate the mechanisms of imatinib resistance, which include amplification of BCR-ABL1, overexpression of the multidrug-resistant P-glycoprotein (MDR-1), alpha acid glycoprotein binding, BCR-ABL1 and the emergence of mutations in the ABL-kinase domain as well as the development of BCR-ABL1-independent pathways of signal transduction [100–104]. While dasatinib has been utilised as a second-line treatment for imatinib-resistant CML, research has suggested that a second mutation on the *BCR-abl* gene could contribute to the resistance mechanism [105].

5.3 TRAIL

TNF-related apoptosis-inducing ligand (TRAIL) is a protein that acts as a ligand to induce apoptosis in cells [106]. Since its discovery, there has been significant interest in the use of this protein as an anticancer therapy [107]. One such molecule, TIC10, has been found to activate the gene for the TRAIL protein. In addition to activation of the TRAIL protein, TIC10 inactivates the cell proliferation—and cell

survival—promoting kinases Akt and ERK [108]. Although there have only been a limited number of clinical trials, at present there are no more than eight trials ongoing (www.clincialtrials.gov). In vitro and in vivo data have indicated that there may be promising results. The trials have spanned a number of different cancer types including colorectal (CRC), NSCLC, triple negative breast cancer, lymphoma and glioblastoma [109–111]. Preclinical evaluation of the drug in colorectal cancer indicated that the TIC10 molecule had the ability to inhibit self-renewal of colorectal stem-like cells and as such held promise as a treatment for chemotherapy-resistant CRC [112]. There have also been some preclinical and clinical evaluations for the use of this drug in the treatment of both advanced and recurrent glioblastomas [110, 113]. In vivo and in vitro data have indicated that the combination therapy of TIC10 and Bcl-2/Bcl-xL inhibition shows promise as an approach for the treatment of glioblastomas [114].

As with more established small-molecule inhibitors, there have been reports of resistance to these TRAIL molecules. In some cases, there has been an intrinsic resistance to them. One approach to overcoming intrinsic resistance has been sensitisation of the cells using chemotherapy or radiation in combination with the TRAIL molecule such as TIC10. These combinations need to be approached with caution so as to limit toxicity to normal cells [115]. Acquired resistance has also been reported. As of yet there is not a complete understanding of the mechanisms which drive this resistance; however, there have been a number of different theories investigated. It has been suggested that multiple redundant pathways may play a role in the resistance mechanism. cFLIP, anti-apoptotic B-cell lymphoma 2 protein and X-linked inhibitor of protein inhibitor, along with mutations in the death receptors DR4 and DR5, have all been implicated [116–118].

5.4 Conclusion

Uses of targeted therapies and small-molecule inhibitors have been successful in the treatment of solid tumours since their introduction. While there has been a strong biological rationale to utilise these therapies in the treatment of brain tumour and an increase in the knowledge relating to the genomic makeup of these tumours, the clinical results delivered to date have been disappointing. This is due, in part, to the complex nature of the tumours, the lack of CNS penetration of the drugs and also resistance to the therapies themselves. Lessons can be learned from how this resistance can be overcome from other tumour types; however, the remaining complexities associated with brain tumours still need to be overcome.

Another issue that needs to be tackled is the lack of suitable biomarkers for these therapies. It will be vital to be able to determine which patients will benefit most from these treatments. Development of predictive biomarkers may help to alleviate the risk of acquired resistance as they may have the power to stratify patients, ensuring that only those who will benefit from the selected treatment will receive it. The use

of targeted therapies for the treatment of brain tumours, while complex, does still hold promise for the improved prognosis of this disease.

Acknowledgement The author wishes to acknowledge and thank Dr. Patrick Morris for his assistance.

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Chapter 6
Drug Repurposing to Circumvent Chemotherapy Resistance in Brain Tumours

Richard Hill, Samantha A. Murray, Zaynah Maherally, Samantha C. Higgins, and Geoffrey J. Pilkington

Abstract The incidence of primary brain tumours in the UK is steadily rising; prognosis for this devastating disease is dismal and not significantly improving. The relative failure for effective therapy may be attributed to heterogeneity with over 120 histological entities. Additionally, the biology is incredibly complex with numerous pathways and cascades working simultaneously or in harmonisation. Moreover, we find that as the tumour evolves, it commandeers alternative pathways for its survival. At its most aggressive form, glioblastoma produces guerrilla cells that trek into the normal brain parenchyma where they are protected by the intact blood-brain barrier (B-BB). The majority of drugs cannot cross the intact B-BB; the dosage required to cross the damaged area around the tumour would be toxic systemically. Effective therapy, therefore, has a number of hurdles against chemotherapy resistance. In this chapter we look at the tumour biology and conventional therapeutic resistance; we then look at the potential of using drugs originally used to treat other diseases. Indeed, repurposed drugs are drawing increasingly more interest, especially as after approximately ten years of development; the new drug failure rate of 25,000:1 costs the industry an astonishing fortune of around $2.6 billion per year. Here, we refer to UK regulations and doctor trepidation for prescribing outside of the drug's licence—'off-label'—and give examples of some of the repurposed agents which have gained attention within the laboratory and within our current legislation require additional clinical trials for marketing authorisation and prescription without fear of potential litigation.

Keywords Blood-brain barrier • Glioblastoma • Repositioning • Repurposing

R. Hill • S.A. Murray • Z. Maherally • S.C. Higgins • G.J. Pilkington (✉)
Cellular and Molecular Neuro-oncology, Institute of Biomedical & Biomolecular Sciences, School of Pharmacy & Biomedical Sciences, University of Portsmouth, Portsmouth PO1 2DT, UK
e-mail: Geoff.Pilkington@port.ac.uk

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*, Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_6

Abbreviations

ALDH	Aldehyde dehydrogenase
CED	Convection-enhanced delivery
ECM	Extracellular matrix
EGF	Epidermal growth factor
GBM	Glioblastoma
JAMs	Junctional adhesion molecules
MDS	Methylation-resistant DNA synthesis
MGMT	Methylguanine-DNA methyltransferase
MMPs	Matrix metalloproteinase
MMR	Mismatch repair
mTORC2	Rapamycin complex 2
PDGF	Platelet-derived growth factor
PDK1	3-phosphoinositide-dependent kinase
P_e	Transendothelial permeability coefficient
PTEN	Phosphatase and tensin homolog
TEER	Transendothelial electrical resistance
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
ZO-1, ZO-2 and ZO-3	Zonula occludens proteins 1, 2 and 3

6.1 Introduction

In 2013 alone there were 10,624 new cases of 'brain, other CNS and intracranial tumours' accounting for 3 % of all new cases in the UK [1]; the 5-year survival remains at a dismal figure of around 19 % [2] (2010–2011) with the average number of years lost amongst all cancer patients—the highest for brain tumour patients, at just over 20 years [3]. However, the number of tumours reported increases significantly when metastatic brain tumours are considered. Brain metastases typically cause profound neurological symptoms and dramatically impact patient quality of life [4]. Accurate statistics for metastatic spread to the brain are not available, and this has been attributed to reporting and diagnostic methods as well as life expectancy where the metastases may not present clinically in the patient's lifetime or the extent of illness prevents diagnosis; at publication of this chapter, the current best estimate for metastases to the brain from all primary cancer sites combined is 6 % [5]. However, the primary site of the cancer influences the statistical likelihood of metastasis to the brain; in adult lung cancer patients, 40–50 % present with brain metastasis, and for breast cancer patients, approximately 15–25 % metastasis to the brain is reported [6], while in melanoma patients, almost 60 % will develop brain metastases [7]. Irrespective of the source (primary or secondary), all are potentially life threatening. The brain is encased and protected by the skull, thus, it is not possible for the brain to expand to accommodate the tumour

mass. Consequently as the tumour grows, there is the compression and displacement of normal brain tissue. This expansion can cause blockage of the cerebrospinal fluid flow as well as elevation of the intracranial pressure within the brain and enlargement of the brain ventricles which may result in hydrocephalus [8]. Furthermore, a large number of brain tumours give rise to peritumoural oedema which in combination with hydrocephalus and normal tissue displacement creates a 'perfect storm' of conditions that result in the plethora of symptoms presented by brain tumour patients.

In contrast to the vast majority of all other cancers, brain tumours are unique in that there are over 120 different types [9] which is likely to increase in number due to the planned update of the 'World Health Organization (WHO) classification' in 2016 [10]. The consideration of the therapeutic treatment(s), both conventional and novel, for each of these different types of brain tumour is far beyond the scope of this chapter, and, therefore, focus will be given to the most common types of brain tumour/the most severe types and types where pioneering treatment has been applied.

Of all of the primary brain tumours diagnosed, approximately 80 % are gliomas [11, 12] including astrocytomas, oligodendrogliomas and ependymomas. The incidence of glioblastoma and giant cell glioblastoma alone, across all ages in England for 2013, was reported as 2,424, an average year-on-year increase of approximately 8 % since 1983 [13]. The World Health Organization (WHO) malignancy grading system places glioma into four subgroups, with increasing malignancy I–IV, based on key features including nuclear atypia, mitotic activity, microvascular proliferation and necrotic regions [9]. The most severe of these in adults are the grade III/IV tumours including glioblastoma (GBM), a grade IV tumour that accounts for over 50 % of all malignant gliomas, and although they can arise at any age, the mean age at diagnosis is 64 years [14]. The clinical presentation of GBM (as well as most other types of brain tumour) is diverse and is likely to directly relate to the region(s) of the brain where the tumour is located. For example, a tumour mass within the frontal lobe can present with emotional changes and disinhibition, memory loss, paralysis on one side of the body, vision loss and seizures. In contrast, a parietal lobe tumour can trigger spatial disorders or impaired speech. An occipital lobe tumour can result in the loss of vision, while a brainstem tumour can present with hearing loss, facial muscle weakness, vomiting or double vision. While these can offer the clinician a region that could harbour a tumour mass, this is far from definitive, and there are multiple conserved symptoms irrespective of tumour location including headaches, visual problems and increased intracranial pressure inducing nausea, vomiting, drowsiness and delayed responses [15].

Unless linked to rare genetic diseases such as neurofibromatosis type 1 and type 2, tuberous sclerosis, Li-Fraumeni syndrome [16] and von Hippel-Lindau syndrome [17] or due to the iatrogenic effect of ionising radiation treatment, especially in children [18], the aetiology for brain tumours is unknown. A critical component of glioma is the transition to malignant glioma by the acquisition of genetic mutations and the deregulation of growth factor signalling pathways. As a result of this, glioma pathogenesis to malignant glioma is commonly mediated by vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and the loss of phosphotensin analogue (PTEN). Due to this aberrant activation (or the loss of repression), the downstream cascades of proliferative pathways (including PI3K/AKT) are triggered [19].

6.2 Biology

When one considers GBM, there are two major clinical forms, primary (de novo) and secondary. Most GBMs arise rapidly de novo, with no previous lesions, and are termed primary GBM. GBMs that arise from diffuse astrocytoma (grade II) or anaplastic astrocytomas (grade III) are termed secondary GBMs. Thus, GBM, which is the most common astrocytic glioma, can arise with no prior neoplastic disease [20] or can progress from lower-grade gliomas [21]. Moreover, the time to progression from diffuse astrocytoma (grade II) to GBM varies considerably from less than a year to more than 10 years [9]. The dismal prognosis for glioblastoma patients is due to the invasive nature of the tumour which results in complete resection by surgery, and, despite progress in radio-/chemotherapy, less than half of patients survive more than a year. Primary GBMs are genetically characterised by loss of heterozygosity on chromosome 10q, EFGR amplification, p16INK4A deletion associated with the retinoblastoma tumour suppressor gene (*RB1*) [22] which controls progression through G1 to S phase of the cell cycle and finally PTEN mutations [23]. Despite the short duration of symptoms in many cases of GBM, the lesions are often surprisingly large at the time of presentation. The lesion is usually unilateral but those in the brain stem and corpus callosum can be bilaterally symmetrical. Infiltrative spread is a hallmark feature of all diffuse astrocytic tumours; however, GBMs are well known for rapid invasion of neighbouring brain structures. A very common feature is extension of the tumour through the corpus callosum into the contralateral hemisphere, creating the image of a bilateral, symmetrical lesion referred to as the 'butterfly glioma' [24]. As the term glioblastoma 'multiforme' suggests, the histopathology of this tumour varies greatly. While some lesions show a high degree of cellular and nuclear polymorphism with numerous multinucleate giant cells, others are highly cellular [9].

As already noted, brain tumours may be primary (intrinsic) or secondary (extrinsic), when cells leave a primary somatic cancer, intravasate into the bloodstream, arrest at a distant organ and eventually develop into gross lesions at the secondary site including the CNS [25]. The brain is, in fact, a major site for growth of secondary cancers, with 25% of malignant tumours showing involvement in the brain [26]. Secondary lesions such as these are generally well circumscribed, whereas primary brain tumours show marked dissemination at the brain/tumour interface with poor definition of the tumour edge. This is due to neoplastic cells migrating several millimetres and sometimes even centimetres away from the main tumour mass [27, 28]. There is a preferential invasion of glioma cells along white matter tracts (the most permissive substrates for CNS invasion, which offer the least resistance and function as a 'railway track' for tumour cell spread). Indeed, some gliomas remain confined to the white matter, stopping abruptly at the grey-white matter junction. Other migratory patterns include preferential growth around neurons in the grey matter, perivascular growth and subpial spread [29]. These typical behaviours imply that glioma cells have either a tropism for particular sites or a restricted ability to invade in the potential regions between specific cell combinations [24]. These motile cells termed 'guerilla'

cells give rise to recurrent tumours, following surgical and adjuvant chemo- and radiotherapeutic intervention [22]. The ability of glioma cells to invade the surrounding brain tissue prevents gliomas from complete surgical resection and subsequently successful therapy. It has been demonstrated that during this invasive procedure, cells transiently arrest from the cell cycle [29], therefore leaving them refractory to radiotherapy, while their position within areas of intact B-BB protects them from a number of chemotherapeutic agents which can pass into the major tumour mass by virtue of a disrupted B-BB [27, 28]. The mechanisms of invasion, which include complex, interacting processes between cell adhesion molecules, remodelling of the extracellular matrix (ECM), matrix metalloproteinases (MMPs), cytoskeleton elements [30], gangliosides and growth factors [31], which were first described by Liotta [32] are still in use to design current models of invasion. Other components which have been associated to invasion include tenascin C, integrins, cadherins, NCAM and connexin 43 [33].

Drug delivery to the brain is hampered by a major obstacle in the B-BB, and many new approaches to circumvent this have been suggested. These approaches either serve to increase drug delivery of intravascularly administered drugs by manipulating either the drugs or the capillary permeability or to increase drug delivery by local administration [34]. Further, as stipulated by the 'go-or-grow hypothesis' [23], that is, cells either move (invade) or divide and not both, so this transient arrest from the cell cycle means that radiotherapy (and some cell cycle-specific antitumour drugs) will not affect migratory cell populations.

The invasive phenotype of low- and high-grade tumours is acquired early on in tumourigenesis [24]. Despite the migratory behaviour and invasiveness of primary brain tumours, they rarely metastasise to distant organs [35]. Pilkington [27] explained the above statement by suggesting that:

1. Time—gliomas are fatal even before metastases become detectable.
2. Immunity detection and eradication by patient's own immune system.
3. Seed-soil hypothesis—whereby glioma cells are unable to grow in a different microenvironment.
4. The B-BB may prevent neoplastic glia from leaving the brain and entering the circulatory system.
5. Neoplastic glia failing to express the required cell adhesion molecules to adhere to other organ endothelium; the fifth hypothesis is the most likely candidate [23].

6.3 Heterogeneity

Clinical management of gliomas currently relies heavily on accurate histopathological diagnosis of grade and subtype for provision of appropriate therapy to individual patients [36].

Genotypically, gliomas and cultured glioma cells have been demonstrated to differ within and amongst individuals, according to chromosome number, marker chromosomes and DNA content. Phenotypically, cultured glioma cells differ in morphology,

growth properties, antigenic expression and tumourigenicity in nude mice and in response to both radiotherapy and chemotherapeutic agents. The existence of this inherent complex antigenic heterogeneity requires the design of multimodality therapeutic regimes for each individual patient and tumour [37]. Cultured glioma cells, at least during the early passage, retain the cellular heterogeneity seen in histological sections taken at the time of surgery, but this is generally lost with overpassaging [38] which emphasises the need to use low-passage primary glioma cultures in research strategies.

Anaplastic changes in gliomas can be diffuse, or it may be possible to distinguish areas with low- and high-grade histological features that may be indicative of progression in some parts of the tumour to a higher grade. Many gliomas show mixed histology with features of more than one histological subtype present within the same tumour sample or in a sample obtained from the same patient obtained at different times [36]. Tumour heterogeneity has important morphological, molecular and clinical implications. The knowledge of oncogenic alterations involved in each tumour can be important to correlate the morphological features, the genetic background, the prognosis and the clinical response to therapy with anticancer agents. Based on the molecular background of the tumour, there are new cancer gene therapy protocols such as inhibitors of tyrosine kinase for the platelet-derived growth factor (PDGF) receptor in gliomas [39].

Angiogenesis, which is the generation of new capillary blood vessels, is a characteristic of GBM and is an essential component of many physiological and pathological conditions including growth and metastasis of primary tumours. Solid tumours require angiogenesis to spread more than a few cubic millimetres, and GBMs are known to be one of the most highly vascularised of all human tumours [26]. It has, however, been demonstrated that the rapid growth of GBM results in focal ischaemia and hypoxia, which in turn results in angiogenesis mediated by vascular endothelial growth factor (VEGF) [40]. It has been shown that VEGF and PDGF are greatly overexpressed by tumour cells in human GBM [41, 42]. Tumours are also able to target and use pre-existing host vessels by induction of angiopoietin-2 expression by host endothelial cells, thus resulting in the regression of pre-existing vessels, tumour necrosis and subsequent onset of hypoxia-induced VEGF-mediated angiogenesis [43].

6.4 Role of the Blood-Brain Barrier (B-BB) in Hampering Treatment

The first report of a barrier to the brain was observed in 1885 by a German scientist Paul Ehrlich who noted that after a parenteral injection of a variety of vital dyes into adult animals, almost all organs were stained except for the brain and spinal cord [44] (Ehrlich, 1885). The nature of the B-BB was debated well into the twentieth century when it was shown that the principal reason why certain dyes do not penetrate from the blood into the brain was because they bound to plasma proteins, mainly albumin. The B-BB is now known to be a selective diffusion barrier which is highly restrictive in the transport of substances between the blood and the central nervous system (CNS) [45, 46] as well as cancer cells from other organs [47]. The

B-BB acts as a protection for neural tissue as it depends on the partition from the systemic circulation to maintain a specific neural tissue environment and a barrier from toxic materials in the bloodstream [48–50]. Regulation of water permeability, ion concentrations, delivery of amino acids and sugars and prevention of exposure to circulating immune cells and antibodies are all contributory factors of the B-BB [50]. The B-BB is therefore essential for the normal function of the CNS [46].

Cellular transport of material across the barrier can occur via two pathways:

1. Transcellular flux, transport across/through the endothelial cells
2. Paracellular flux, transport between the endothelial cells

Transcellular flux is low in cerebral microvascular endothelial cells and may be due to passive diffusion or by active transport mechanisms [51–53]. Small lipophilic molecules such as carbon dioxide, oxygen and ethanol can liberally diffuse across the lipid membranes of the endothelium, unlike small polar solutes which must be transported by specific carriers, such as the glucose transporter, GLUT-1, needed for brain function [54]. In addition to specific carriers for influx, efflux-specific carriers are critical for the removal of potentially toxic metabolites such as glutamate [54].

6.5 Cellular Components of B-BB

The B-BB is a complex system composed of different cellular components including brain endothelial cells lining the cerebral vasculature [54], capillary basement membrane, pericytes embedded within the basement membrane [55] and astrocyte end feet ensheathing the vessels [45]. These components form what is termed as the functional ‘neurovascular unit’ (Fig. 6.1) [54, 56].

6.5.1 Cerebral Microvascular Endothelial Cells

Cerebral microvascular endothelial cells differ from endothelial cells found in the rest of the body by virtue of increased mitochondrial content [57], sparse pinocytic vesicular transport [58, 59], extensive tight junctions [46, 60, 61] and a lack of additional fenestrations [58], all of which contribute to their ability to maintain a tight barrier.

6.5.2 Tight Junctions

The tight junction network of the B-BB is a striking complexity of transmembrane proteins including junctional adhesion molecules (JAMs) and cytoplasmic proteins associated with tight junctions [62, 63]. The transmembrane proteins comprise three integral membrane proteins: occludin and claudins, proteins which anchor plasma

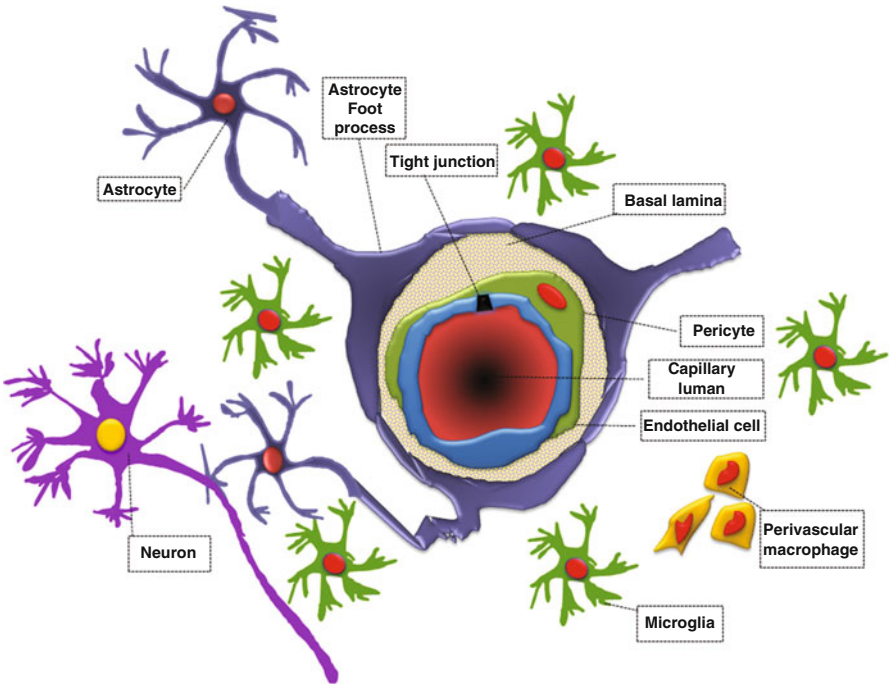

Fig. 6.1 The neurovascular unit. The B-BB composed of cerebral microvascular endothelial cells grasped by pericytes is embedded within the basement membrane that is surrounded by astrocytes and microglia. Tight junctions present between the cerebral endothelial cells form a diffusion barrier, which severely restricts penetration of water-soluble compounds, including polar drugs, into the brain

membranes of adjacent cells [64], and JAMs which are thought to play a significant role in early-phase close cell-cell contact and in tight junction formation [65]. These transmembrane proteins are linked to cytoplasmic proteins including zonula occludens proteins 1, 2 and 3 (ZO-1, ZO-2 and ZO-3) by the carboxy termini of the transmembrane proteins [65]. Catenins and E-cadherin are also involved in tight junctions. Ultimately, these tight junctions play a critical role in the B-BB. Occludin expression correlates with the barrier properties of the endothelial cells and the extent of permeability [66, 67] and is downregulated in various brain disorders, accompanied by tight junction disruption [68] implicating the level of expression of occludin in being important for tight junction maintenance of the mature B-BB [69]. Complex tight junctions found in the brain capillaries cause a tightness of around 50–100 times higher than that found in peripheral microvessels [54]. Tight junctions are responsible for the polarisation of the cell and the separation of an apical from a basal domain as well as the restriction of the paracellular pathway [70].

The transendothelial electrical resistance (TEER) and transendothelial permeability coefficient (Pe) for small-soluble inert tracers are methods to evaluate tight junction function and the B-BB paracellular transport, two parameters of the quality of the B-BB models [71]. TEER is the measure of the electric (ionic) conductance

of the monolayer. As the cell monolayer on the apical membrane becomes confluent, the TEER detects an increase in electrical resistance. It can, therefore, quantitatively measure the 'tightness' of the monolayer and, in turn, cell health by evaluating the resistance to ions across the B-BB [72]. The TEER value in vivo has been measured at around 1500 Ωcm2 [73, 74], whereas in vitro endothelial monolayers range between 20 and 1400 Ωcm2 [75] with the highest in vitro bovine TEER system measuring around 2000 Ωcm2 [76].

6.5.3 Astrocytes

Astrocytes have long, radial outgoing cell protrusions, a shape which contributes to sustaining the CNS, supporting neuronal metabolism, envelopment and isolation of synapses and maintenance of the blood-brain barrier [77, 78]. With their end feet, wrapped around capillaries, they interact with microvascular endothelial cells [54, 79] and work as mediators between the microvessels and synapses. This involves the regulation of local blood flow and oxygen and glucose transport [80]. Astrocytes are known for participating in the formation of the blood-brain barrier, modulating the expression and polarisation of transporters, enhancing tight junction formation and promoting specialised enzyme systems [54, 79, 81].

6.5.4 Pericytes

Pericytes are less well characterised compared to the other cellular components of the B-BB [82]. Pericytes are biochemically, physiologically and morphologically heterogeneous and are thought to regulate proliferation and differentiation of cerebral microvascular endothelial cells and give structural stability for the barrier [83]. With an estimated ratio of 1:3, pericytes surround brain vascular endothelial cells [84], separated by a thin layer of basement membrane. Pericytes are contractile, α-smooth muscle actin expressing cells [85] with direct peg-and-socket contacts to endothelial cells, initiating multiple signalling pathways [86]. By means of their contractility, they contribute to the regulation of the local blood flow by controlling capillary diameter [85, 87, 88]. A loss of pericytes leads to locally reduced cerebral blood flow and a breakdown of the blood-brain barrier [89, 90]. Pericytes also release structural constituents of the basement membrane and ECM [84].

6.5.5 Basal Lamina

Basement membranes are dynamic thin sheetlike structures of extracellular matrix that provide a supporting structure on which endothelial cells encompass a number of molecules [91]. Extracellular matrix (ECM) plays an important role in maintaining the health

and function of vascular endothelial cells. It resides between the endothelial cell and astrocyte end feet and is composed of highly complex arrays of approximately 50 glycoproteins which induce fibronectin, type IV collagen, nidogens, laminin and heparan sulphate proteoglycans (agrin and perlecan) [92]. The microvascular basement membrane comprises of three-dimensional networks made by laminin and collagen IV. The interconnections and integrity of these networks are upheld by nidogens and perlecan. Heparan sulphate proteoglycans cross-link the laminin and collagen IV networks and bind soluble factors; fibulins bind nidogens and laminins [92]. Therefore, ECM proteins are vital for the formation and preservation of the basement membrane [93] and play important roles in cell adhesion, migration, differentiation and growth [94].

6.6 Examples of Methods for Therapeutic Approaches Overcoming the Obstacle of the Blood-Brain Barrier

(a) *Nanomedicine/nanoparticle carriers*: Paracellular transport across the blood-brain barrier is controlled by tight junctions, efflux pumps and pinocytic vesicles, denying access to 98 % of small-molecule drugs and 100 % of large-molecule drugs, allowing passage only to highly lipophilic molecules with less than eight hydrogen bonds and a molecular weight less than 400 Da [95, 96]. Although glioma progression, and if undertaken neurosurgery, compromises the integrity of the local B-BB, only tumour cells in the vicinity of the breach may be exposed to chemotherapy, leaving tumour cells in the surrounding parenchyma protected by the intact B-BB [97]. Other than altering the B-BB (see review by Karim [98]), a method that is increasingly gaining research interest is that of nanomedicine- or nanoparticle-mediated delivery, which allows anticancer agents that would otherwise be prohibited from traversing the blood-brain barrier admittance to the brain when encapsulated in lipid or other polymeric nanocarrier. Nanoparticle design is tailored using discrete molecular forces such as chemical bonding, electrostatics, steric interactions and physical adsorption, thereby enabling nanoscale assemblies of specific size, hydrophobicity and surface charge nanocarriers [99]. There are currently two active clinical trials listed on the worldwide database ClinicalTrials.gov that include the search term ‘nano’; these are a Phase II Study of Combined Temozolomide and SGT-53 for Treatment of Recurrent Glioblastoma (NCT02340156) and a Phase I Study of Convection-Enhanced Delivery of Liposomal-Irinotecan Using Real-Time Imaging with Gadolinium in Patients with Recurrent High-Grade Glioma (NCT02022644).

(b) *Drug depot polymer*: Used as a surgical adjunct to total (>90 %) resection for both the first and recurrent GBM; a biodegradable polymer (composed of polyanhydride poly[1,3-bis(carboxyphenoxy)propane-co-sebacic-acid]), impregnated with only one type of chemotherapeutic drug, the alkylating agent, BCNU (carmustine) [100–102], is placed into the surgical cavity where a sustained release of the active drug is proportional to biodegradation of the polymer. Early animal pharmacokinetic studies measured the reach of exposure to be within a

perimeter of around 5 cm and found present up to the end of a 30-day trial [103]. The National Institute for Health and Care Excellence (NICE) guidelines for brain cancers recommends the use of the drug depot polymer Gliadel® which has been shown to have some modest but significantly prolonged median survival for patients with recurrent GBM (31 versus 23 weeks) [100]. Phase III studies in newly diagnosed GBM patients also showed significant longer median survival compared with placebo controls (13.9 vs 11.6 months) [104]. With exception to three-year post-marketing surveillance (observation) which is due to complete in March 2017, only one clinical study of Gliadel® is presently recruiting, a phase II trial (safety/efficacy study) sponsored by the University of California, of NovoTTF-100A (tumour-treating fields that interfere with multiplication of the GBM cells), Gliadel® and Bevacizumab in the first relapse (NCT02348255); this is due to complete in December 2017.

(c) *Convection-enhanced delivery*: A major criticism of drug depot polymers is that they rely on diffusion to deliver the active agent into the surrounding brain parenchyma. One alternative is to increase the pressure gradient; this is the aim of convection-enhanced delivery (CED). CED has been under development since the early 1990s [105] with clinical trials since the early 2000s [106]. CED is performed by stereotactically placing microcatheters intratumourally [106] or in oedematous brain parenchyma surrounding a resection cavity [107]. The pressure gradient is established very precisely with a motor-driven pumping device. Clinical trials have thus far been largely disappointing with problems of infusate reflux or 'backflow' and subtherapeutic drug concentrations in the target area [108]. Advances in the technology required focus on catheter diameter (addressed by decreasing the size of the catheter and introducing a recessed step [109]), tissue trauma on catheter implantation and the speed of catheter insertion [110]. These issues are being addressed with stereotactic robotic technology at the Bristol Frenchay Hospital in collaboration with Renishaw engineers who have developed neuromate® and stereotactic planning software neuroinspire™. The hospital is now planning to use this equipment in a paediatric phase I clinical trial.

6.7 Conventional Therapy

The standard treatment modality following a diagnosis of GBM involves a combinatorial approach. Where possible, there is maximal surgical resection of the tumour mass (based on MRI analysis) and radiotherapy. Radiotherapy alone can significantly increase median survival although the most common radiological response is to stabilise the disease, but ultimately disease progression follows. The mechanism(s) for resistance to radiotherapy remains poorly understood, although widespread hypoxia within grade III/IV tumours is considered to be an important factor. Current post-surgical treatment involves adjuvant temozolomide chemotherapy with concomitant radiotherapy. Temozolomide treatment provides a modest increase in median patient survival although this is not as striking as increases seen following chemotherapeutic

intervention for other types of cancer [111]. Subgroup analysis of GBM patients has demonstrated that a discernible clinical response to temozolomide was principally limited to those tumours containing a specific epigenetic alteration, specifically promoter methylation of the O^6-methylguanine-DNA methyltransferase (*MGMT*) gene [112]. Critically, while these patients show an improvement (compared to surgical resection and radiotherapy alone), almost all demonstrate eventual progressive disease in the absence of *MGMT* promoter methylation [113]. Based on these data, it is not surprising to conclude that there are alternate pathways utilised within GBMs to resist alkylating agent chemotherapy. With this in mind, significant attention has been directed towards understanding and preventing GBM resistance to chemotherapy investigating key pathways and proteins, particularly those involved in the mismatch repair (MMR) pathway. A recent study has demonstrated that the c-Myc-dependent downregulation of the micro-RNA (mIR)29c generates a glioma that is highly resistant to temozolomide [114]. This was characterised further and shown to be mediated by an increase in *REV3L* (a DNA repair polymerase) expression and a concomitant increase in DNA repair activity [114]. The clinical importance of these findings was noted when GBM patients were evaluated for miR-29c expression where the higher expression of this micro-RNA was associated with a significantly improved survival rate. Similarly it has been shown that resistant GBM cell lines exhibit methylation-resistant DNA synthesis (MDS), defective cell cycle checkpoints and an impaired DNA damage response network, suggesting that, in temozolomide-resistant GBMs, this signalling is isolated from the ongoing DNA alkylation damage introduced by temozolomide that significantly contributes to temozolomide resistance [115].

Beyond mismatch and DNA repair networks, it has been suggested that GBM biology may be enabled by the unfolded protein response (UPR), which can both support secretory pathway function and promote stress resistance via altered metabolism [116, 117]. The UPR or elements of it (e.g. BiP/GRP78) have been associated with reduced responses to cancer chemotherapy [118–120]. Furthermore, chemotherapy resistance correlates with both hypoxic signalling and elevated aerobic glycolysis (the 'Warburg effect') in order to maintain sufficient intracellular ATP levels [121]. Hypoxia also correlates with UPR activation (reviewed in Iurlaro [122]) where protein markers of each overlap (e.g. tribbles homolog 3 [123]). Thus, there is an intersection of tumour stress, chemotherapy resistance and metabolic upregulation in the UPR. Unfortunately, with all current front-line therapeutics, recurrence is common, and thus overall the clinical prognosis for GBM patients is poor.

6.8 Targeted Therapy

As previously alluded to, it is known that the loss of PTEN expression (by mutation approximately 40 %) or loss of heterozygosity (approximately 80 %) [124] and PI3K/AKT/mTOR signalling is increased in roughly 90 % of all GBMs; the therapeutic inhibition of this network (summarised in Fig. 6.2) has been extensively investigated.

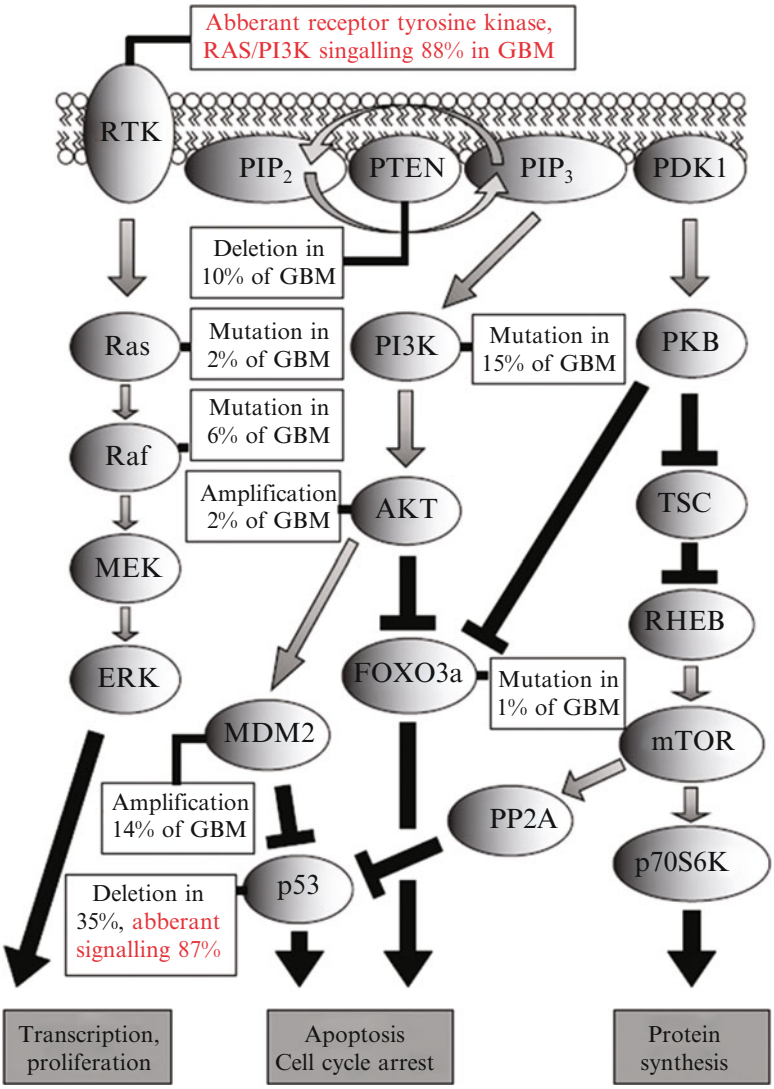

Fig. 6.2 Overview of the PI3/AKT signalling network. Each box highlights the general final percentage of glioblastomas with alterations in at least one known component gene within these networks. The general cellular response is also indicated following the activation/inactivation of these pathways (such as apoptosis or protein synthesis)

The phosphatidylinositol 3-kinase (PI3K) family of intracellular lipid kinases directs a plethora of diverse signalling networks that regulate proliferation, differentiation, migration, metabolism and survival [125, 126]. Upstream receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR), activate class IA PI3Ks. There are additional PI3K classes (I–III) that are grouped

according to their substrate preference although these are beyond the scope of this chapter. The binding of the p85 regulatory subunit of PI3K to phosphotyrosine residues on activated RTKs mediates a conformational change in p85. This change abolishes the inhibition of each PI3K p110 isoform (α, β and δ) which allows PI3K localisation at the plasma membrane and catalyses the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) through the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2). PIP3 is a critical activator of the serine/threonine kinase AKT/protein kinase B. Following the binding of PIP3 to AKT, there is the membrane recruitment of AKT and phosphorylation by 3-phosphoinositide-dependent kinase (PDK1) and the mammalian target of rapamycin complex 2 (mTORC2). Once recruited to the cell membrane, AKT phosphorylates, activates or inhibits numerous target proteins.

The PI3K network is negatively regulated by a number of proteins with a key regulator being the phosphatase and tensin homolog (PTEN). This protein suppresses PI3K activity by mediating the dephosphorylation of PIP3 to the biologically inactive PIP2. Furthermore, activation of the mTOR complex 1 (mTORC1) and p70S6 kinase modulates PI3K activation through negative feedback inhibition [126]. Considering the critical cellular processes that are regulated by this network, it is not surprising that loss of function or gain of function mutations are frequently observed in the proteins within the PI3K signalling pathway in almost all types of cancer. This includes p85, p110, PTEN and AKT [127–129]. Indeed, the amplification of EGFR has been reported in approximately 45 % of GBM patients [130]. The amplification (or activating mutations) of *PIK3CA* (which encodes the p110α subunit of PI3K) or *PIK3R1* (which encodes the p85 regulatory subunit of PI3K) has also been reported in approximately 15 % of all GBM patients [127, 128, 131]. Further to the gain of function mutations, loss of function mutations, chromosomal deletions and/or the epigenetic gene silencing of PTEN has been reported in almost half of all GBM patients [132] and, importantly, correlates with a significantly worse prognosis [133]. As a result of this constitutive activation and deregulation of the PI3K network, the inhibition of this pathway has attracted significant attention within the pharmaceutical industry for the development of anticancer therapeutics (reviewed in detail in Wang [134]).

A number of novel PI3K inhibitors have been tested in the clinic to treat GBM. In particular, rapamycin analogues (that inhibit mTORC1) have been tested however in each trial; little significant impact on patient survival and overall outcome was observed [135, 136]. BKM120 (Novartis) is a pan-class I PI3K inhibitor without mTOR and Vps34 activity. It inhibits wild-type PI3Kα, β, γ and δ and inhibits the in vitro growth of classic GBM cell lines independently of PTEN and/or EGFR [137, 138] although this is p53 dependent [139]. Critically, for clinical applications against GBMs, this compound does cross the blood-brain barrier and in pilot studies significantly improved the survival of *NOD/SCID* mice harbouring intracerebral U87 tumour xenografts [139]. Currently in GBM, there is an ongoing phase II clinical trial with this agent in patients with first or second GBM recurrence (clinical trial NCT01339052 being coordinated by the Dana-Farber/Brigham and Women's Cancer Center, USA). Prior to this, PX-886 (Oncothyreon) was developed and is a

derivative of wortmannin that irreversibly inhibits PI3K. In glioma cells, this agent inhibits cell proliferation with the strongest effect reported in PTEN-negative cell lines [140]. Based on promising studies, this agent has been tested in a now completed phase II clinical trial (NCT01259869 competed in February 2015) although the results have not been released at the time of writing.

Many PI3K/AKT mutations also activate the mTOR pathway, and it has been suggested that the dual inhibition of both the PI3K and mTOR pathway represents a more effective therapeutic strategy. Of these, the most widely studied is the compound BEZ235 (Novartis), a dual PI3K/mTORC1/2 inhibitor [141]. BEZ235 has been used in a wide range of in vitro studies, and in glioma cells, BEZ235 treatment induced G1 cell cycle arrest and autophagy and reduced VEGF expression. Furthermore, BEZ235 significantly increased the survival of tumour-bearing mice [142]. There are many other examples of specifically targeting these networks [143–145].

A therapeutic that has been extensively tested is the anti-vascular endothelial growth factor (anti-VEGF) monoclonal antibody bevacizumab (Avastin®) approved by the FDA for the treatment of GBM [146]. Bevacizumab treatment was reported to significantly improve tumour outcome and progression-free survival when compared to standard front-line therapeutics [147, 148]. While initially encouraging, there are highly significant caveats to the therapeutic targeting of angiogenesis. First of all, glioma cells can invade the brain diffusely over long distances without necessarily requiring angiogenesis. This was demonstrated in 2003 within an in vivo model where after the treatment of intracerebral xenografted human glioblastoma cells with an anti-VEGFR-2 antibody, there was a striking increase in the number and total area of small satellite tumours clustered around the primary tumour mass. Critically the authors concluded that GBM invasion was tightly associated with pre-existent blood vessels and hypothesised that an increased co-option of the host vasculature was a compensatory mechanism that is selected for after anti-angiogenesis treatment [149]. At the time this was a controversial report, and there were publications that questioned these findings and suggested that the risk of distant or diffuse recurrence at the time of failure of bevacizumab treatment was not higher than with anti-VEGF-free treatment regimens, suggesting that there is not a specific GBM response to bevacizumab that promotes distant tumour growth at recurrence [150]. In the same month, an independent group revealed by MRI that bevacizumab treatment caused a strong decrease in contrast enhancement while having only a marginal effect on tumour growth. This, the authors argue, reveals that the vascular remodelling induced by anti-VEGF treatment leads to a more hypoxic tumour microenvironment. As a consequence, there is a metabolic change in the intratumour microenvironment towards glycolysis that the authors suggest leads to enhanced tumour cell invasion into the normal brain [151]. More recently it has been suggested that GBM-originated neovascularisation (including tumour-derived endothelial cell-induced angiogenesis) and vasculogenic mimicry are critical factors in the resistance to anti-VEGF therapy [152]. A published 2014 Cochrane systematic review reported insufficient evidence for anti-angiogenic therapy to prolong life in patients with high-grade malignant tumours [153].

At their core, the application of these (and other targeted therapies) requires identifying which patients could benefit from these inhibitors (e.g. *PTEN* loss as opposed to a gain of function *PIK3R1*mutation) and the optimisation of these PI3K pathway inhibitors in combination with other therapies, for example, the administration of MAP kinase inhibitors alongside conventional treatments such as radiation therapy, chemotherapy and anti-angiogenic therapies.

6.9 Repurposed and Reformulated Therapeutics

While there are a vast number of new drugs and targeted therapeutics being developed, there is an estimated failure rate of 25,000:1 for drugs reaching the marketing authorisation stage. This factor underlies the often exorbitant price associated with new drugs, as pharma seeks to recover costs for a pyramid of historic failures. A recent estimate of the costs involved in bringing a new FDA-approved drug to market in 2013 is a staggering $2.6 billion over a developmental period of at least ten years [154]; it is no wonder then that an area which has caught significant recent attention is the repurposing/repositioning of drugs. These are drugs that have been through safety and pharmacokinetic trials in humans and either have been shelved—as inferior against existing alternatives—or are currently licensed for an alternative use. Candidate drugs may have been discovered either in a different therapeutic field with the same molecular pathway target, therefore of medium novelty; in a different therapeutic field with a different molecular pathway, therefore of high novelty; less often, in the same therapeutic field, the same molecular target, which makes it a licence extension (not repurposing); or off target in the same therapeutic field but a different molecular pathway (but this is rare). Further incentives for marketing repurposed drugs are to be found in the new EU Regulation No. 536/2014 of the European Parliament and of the Council on clinical trials on medicinal products for human use, repealing Directive 2001/20/EC. The main aim of the new Clinical Trials Regulation is to streamline applications in the EU with a single entry point via an EU portal and database and to create a single authorisation procedure with a single assessment outcome. However, this regulation also introduces a proportionate approach to trial supervision and conduct; of interest here is the definition for a low-intervention clinical trial, where a trial drug (excluding a placebo) is either covered by its marketing authorisation or used with evidence-based data and supported by published scientific evidence on the safety and efficacy of that product and where the intervention poses only very limited additional risk to the subject compared to normal clinical practice. These 'low-intervention clinical trials' will be subject to less stringent rules, as regards monitoring, requirements for the contents of the master file and traceability of investigational medicinal products [155].

Drugs are granted marketing authorisation by a medicines regulator; for UK-only use, licences are granted by the Medicines and Healthcare Products Regulatory Agency (MHRA), or for EU use, the European Medicines Agency (EMA). Licences are granted for specific indications and patient populations, with a specific formulation

(e.g. liquid, tablet or route of administration) in recommended doses. Prescribing by healthcare professionals outside of a drug's marketing licence or 'off-label' is permitted in the UK through the 'specials' regime (Article 5 of Directive 2001/83/EC), when no other suitable alternative is available, so long as risks and benefits have been weighed against current medical/scientific evidence, and the patient has given explicit consent and accurate records are kept. However, it is up to the discretion of the prescribing professional whether they are willing to prescribe off-label due to the current legal position in terms of liability action which could be brought against them by a patient who has suffered an adverse reaction. In the UK, an injured patient can take liability action against the licence holder or manufacturer of a drug that was prescribed within its licence under Product Liability Directive (EEC/85/374). However, should the drug be prescribed off-label, resulting in patient injury that is not due to product defect and the patient can prove that the physician was negligent then, under 'strict liability', a case can be brought for civil liability (negligence) or even criminal liability and disciplinary sanctions [156]. Due to this position, we find in the UK that there may be hesitation to prescribe off-label; we have certainly witnessed this first hand when we were working with the antidepressant clomipramine for GBM. At the time of writing this chapter, the Off-Patent Drugs Bill (which would have required the 'Secretary of State to take steps to secure a licence for off-patent drugs in new indications and to require the National Institute for Health and Care Excellence to conduct technology appraisals for off-patent drugs in new indications and for connected purposes') did not get government backing and was withdrawn. However, revisions were made to the Access to Medical Treatments (Innovation) Bill (also known as the Saatchi Bill, after Lord Saatchi who introduced the Bill after his wife's death) which aims to make off-label prescribing more consistent with the implementation of a nationwide database of innovative (off-label) treatments conducted by physicians (Figs. 6.3, 6.4 and 6.5).

6.9.1 All-Trans Retinoic Acid (ATRA)/Tretinoin

One repurposed drug that has been widely investigated is all-trans retinoic acid (ATRA), a derivative of retinoid (chemically related to vitamin A), used in the treatment of acne or more recently to treat acute promyelocytic leukaemia (APL). ATRA/ has been shown to be capable of differentiating a variety of stem cells including normal neural progenitor cells. Importantly it was shown that proliferation and self-renewal of neurospheres were reduced following exposure to all-trans retinoic acid [158]. It was also demonstrated that alterations in the extracellular signal-regulated kinases (ERK1/2) were associated with regulation of differentiation, proliferation and apoptosis. It was also suggested that all-trans retinoic acid may have therapeutic potential by differentiating GBM cancer stem cells and rendering them sensitive to other targeted (or conventional) therapy [158].

This agent was investigated further, and it was recently reported that within GBMs there are multiple mutations within the retinoic acid synthesis process and, consequently, resistance to treatment with all-trans retinoic acid [159]. Many enzymes

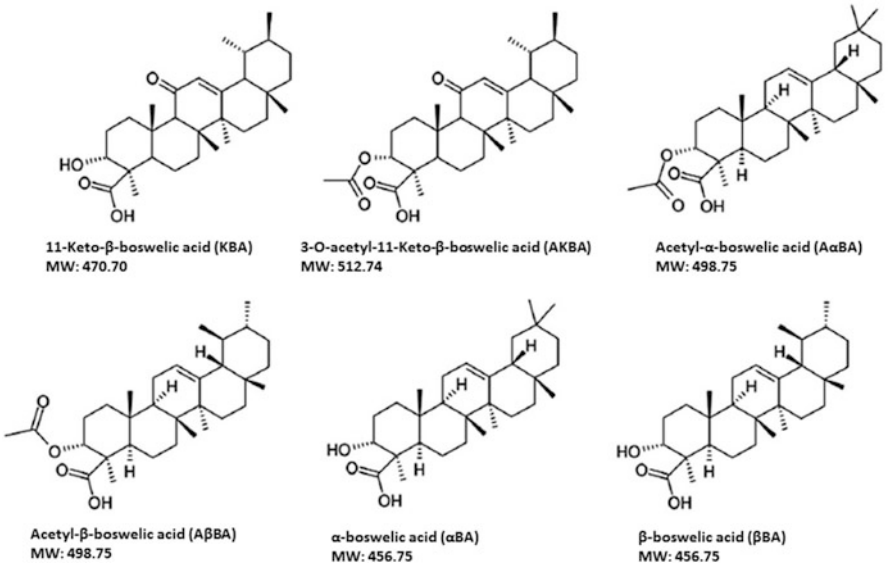

Fig. 6.3 Structures of major triterpenoid metabolites [157]

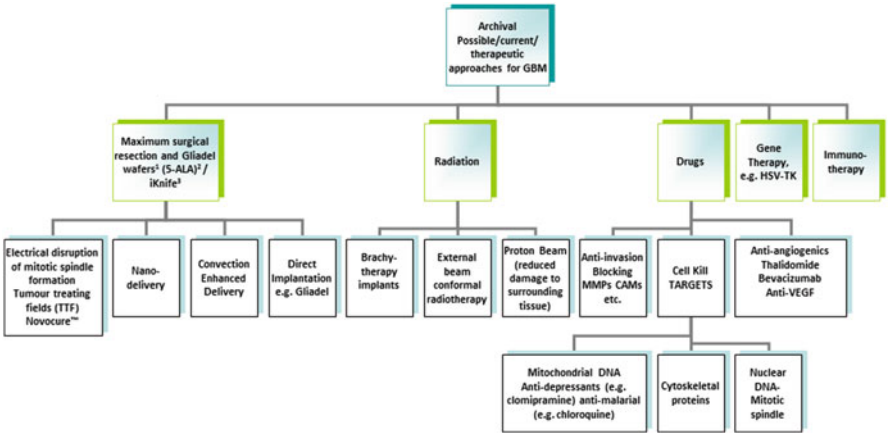

Fig. 6.4 Possible current therapeutic approaches for GBM. (1) Gliadel® wafer (carmustine implant) is a local chemotherapeutic agent that is applied locally during surgery. (2) 5-Aminolevulinic acid (5ALA) enables visualisation of the infiltrative zone of the tumours by UV-defined fluorescence. (3) Raman spectroscopy and intelligent knife (iKnife) approach enable the removal of tumour along with sampling and analysis of chemical composition of the tumour with the use of an electrosurgical knife that uses an electrical current to rapidly heat and vaporise the tissue; this creates smoke that is drawn into the extraction system for analysis by Raman spectroscopy which differentiates between tumour and normal brain

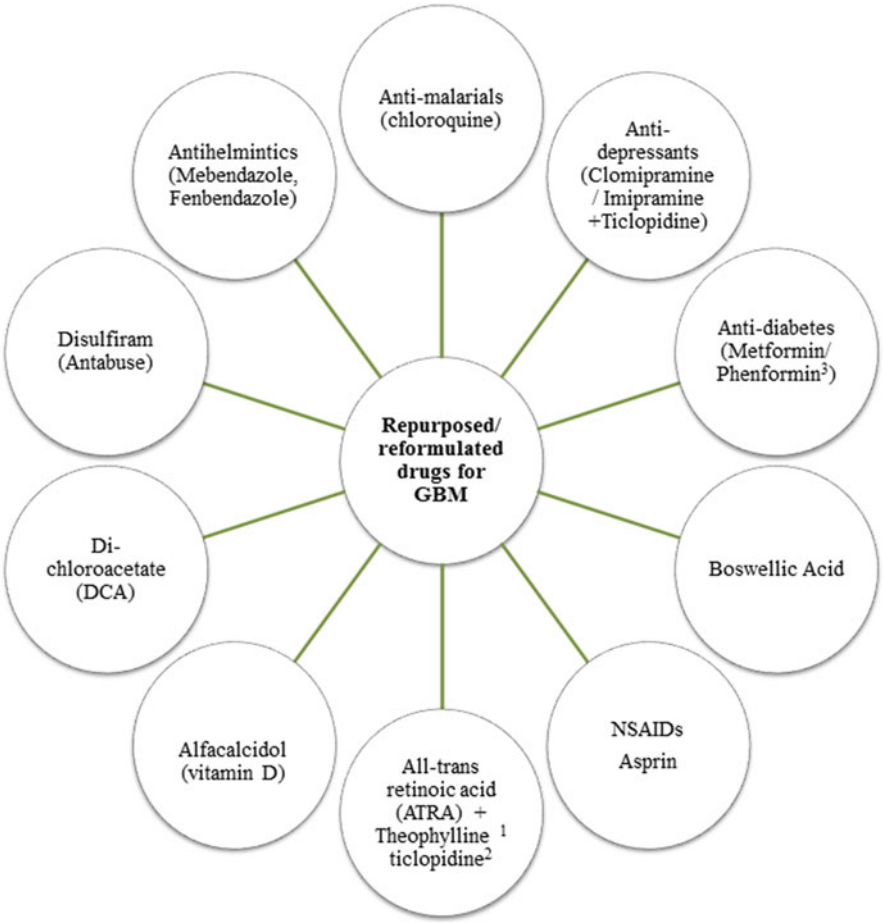

Fig. 6.5 Promising repurposed/reformulated drugs for GBM. A large range of repurposed drugs, used either in their original form or following reformulation, have been used in the quest to find new ways of treating GBM. (1) Theophylline maximises cellular cAMP (increased cAMP levels in cells results in enhanced apoptotic response to certain repurposed agents. This can be achieved using the methylxanthine drug, theophylline, used for respiratory disease which prevents cellular cAMP breakdown to 5′AMP, thus maximising cellular cAMP. (2) Ticlopidine—potentiator of tricyclic response in GBM. (3) Phenformin plus DCA or inhibitors of lactic acidosis

involved in retinoic acid synthesis were downregulated in a large number of GBM samples (compared to normal brain samples), and in almost 70 % of all GBM samples tested, retinoic acid was undetectable [159]. Interestingly, aberrant retinoic acid receptor α (RARA) localisation was observed in GBM cell lines. In normal astrocytes, this was detected in the cell nucleus, cytoplasm and cell membrane. Upon retinoic acid exposure, RARA translocated completely to the nucleus. Based on these studies, it can be hypothesised that therapy with retinoic acid might be effective only for those GBM patients with RARA localisation in the cell nucleus [159]. If this hypothesis is correct,

it suggests that the 10 % of recurrent GBM patients who responded to treatment with isotretinoin (13-cis retinoic acid marked as Accutane) in a phase II clinical trial [160] may have been in this small subgroup with normal retinoic acid receptor α localisation. When used at a therapeutic dose, some significant toxicity was observed, particularly neutropaenia or leukopenia. Furthermore, in vitro studies demonstrated that some GBM cells are inhibited by treatment with retinoids, while others are stimulated [161]. Consequently, molecular biomarkers/ are needed to guide which patients could respond to retinoid treatments such as 13-cis retinoic acid and all-trans retinoic acid.

6.9.2 Alfacalcidol (Vitamin D)

Another interesting repurposed drug is alfacalcidol which is a synthetic vitamin D analogue that is used to treat ailments related to vitamin D deficiency such as rickets. It was reported in 2001 that alfacalcidol is able to redifferentiate neoplastic cells in vitro and binds to nuclear receptors in GBMs. This binding regulates mitotic activity and is therapeutically useful. Strikingly, this group reported that in a phase I trial, the agent was safe and induced, in some patients, synergy with classical surgery-radiotherapy-chemotherapy treatments, yielding progressive and durable regression of the tumour [162]. What is striking in this study is that the median survival reported for patients in this trial was 21 months; greater than the 14-month median survival typically observed in GBM patients. However, a larger phase II trial was never instigated following this study.

6.9.3 Chloroquine

Chloroquine was introduced in the United States in the 1940s to treat malaria, and a derivative of this, hydroxychloroquine, has also been used for years as an antimalarial. More specifically, chloroquine is a lysosomotropic agent that prevents endosomal acidification [163]. It accumulates inside the acidic parts of the cell, including endosomes and lysosomes, resulting in the inhibition of lysosomal enzymes that require an acidic pH, and prevents fusion of endosomes and lysosomes. Chloroquine has been shown to inhibit autophagy as it raises the lysosomal pH, which leads to the inhibition of both fusion of autophagosomes with lysosomes and lysosomal protein degradation [164]. Initial clinical trials revealed a benefit of adding chloroquine to conventional anti-GBM therapy [165]. These initial results were not, however, reproduced in a recent phase I/II clinical trial of hydroxychloroquine in combination with conventional radiation therapy and temozolomide in newly diagnosed GBM patients. This study established that autophagy inhibition is achievable with hydroxychloroquine; however, dose-limiting toxicity prevented escalation to higher doses. At 600 mg/d hydroxychloroquine, autophagy inhibition was not consistently achieved in patients, and no significant improvement in overall survival was observed [166]. The authors of this study conclude that a definitive test of the role of autophagy inhibition in the

adjuvant setting for glioma patients requires the development of lower-toxicity compounds that could achieve more consistent inhibition of autophagy than hydroxychloroquine. An important caveat to this study was that *MGMT* promoter methylation status was not evaluated and this may have impacted on the hydroxychloroquine combinational therapy when administered with temozolomide. The use of this agent however is still the subject of significant attention. In particular, it has been reported in other clinical trials that patients with newly diagnosed GBM benefited from chloroquine in combination with conventional therapy (surgical resection, temozolomide and radiation therapy) [167, 168]. This combinational approach has also been examined at the molecular level. Specifically, combination in vitro treatment of U87 cell (cells which contain wild-type p53) with temozolomide and chloroquine synergistically reduced cell proliferation and enhanced apoptosis, with increased sub-G1 hypodiploid cells and caspase activation. Interestingly, this dual treatment also upregulated total p53 and phosphorylated p53 levels, whereas p53 knockdown abolished the combination effect [169]. It is hypothesised that combination treatment with temozolomide and chloroquine in GBM is via differential autophagy-associated mechanisms that are dependent on p53 status [169]. It has also been demonstrated that epidermal growth factor receptor (EGFR)-overexpressing U373 glioblastomas, when subcutaneously implanted in mice, were sensitive to intraperitoneal chloroquine injections and, in particular, in vitro U373 cells engineered to overexpress EGFR were more sensitive to chloroquine treatment than control U373 glioblastoma cells [170]. Thus, it is believed that chloroquine could be markedly effective against EGFR-driven cancers such as GBM.

6.9.4 Dichloroacetate

The drug dichloroacetate (DCA), unlicensed but widely available and prescribed in the NHS under the 'special' regime, has been used since the 1980s to treat congenital disorders of mitochondrial function, typically presenting with lactic acidosis, in patients with severe malaria and homozygous familial hypercholesterolemia, and for severe brain injury. Caution is required with this drug, however, as adverse effects include polyneuropathy on prolonged use, abnormal oxalate metabolism and metabolic acidosis [171]. DCA acts by inhibiting pyruvate dehydrogenase kinases (PDK), activating pyruvate dehydrogenase (PDH) and diverting pyruvate away from glycolysis and towards oxidative respiration in the mitochondria [172]. Thus, this repurposed drug acts against the 'Warburg effect' targeting the signature metabolic remodelling of cancer cells, decreasing glycolysis and increasing normal aerobic respiration [173]. The initial study that was conducted demonstrated no haematological, hepatic, renal or cardiac toxicity, while there were serum concentrations of DCA sufficient to inhibit pyruvate dehydrogenase kinase (which was highly expressed in all GBMs tested in this study). It is argued, therefore, that DCA is a viable therapeutic approach in the treatment of GBM. However, the number of patients in this initial study was low and a larger study is required [172].

6.9.5 *Imipramine (Tofranil™/Melipramine) and Ticlopidine*

The role of catabolic recycling of cellular components by autophagy in malignant progression and clinical response to therapy in GBM patients has become the subject of significant investigation. One particular subset of repurposed drugs are tricyclic antidepressants based on the observation that patients who are prescribed these drugs are associated with a significantly decreased incidence of glioma [174]. Imipramine (marketed as Tofranil and also known as melipramine) is mainly used in the treatment of major depression and enuresis (inability to control urination). In vivo studies demonstrated that imipramine-treated animals with intracranial tumours displayed a lower histopathological grade tumour and had significantly lower proliferative index [175]. It was further demonstrated that imipramine could synergise with a second repurposed drug, ticlopidine (an anti-platelet drug in the thienopyridine family that is an adenosine diphosphate (ADP) receptor inhibitor). Haematological adverse reactions with respect to ticlopidine listed in the summary of product characteristics include life-threatening neutropaenia/agranulocytosis, thrombotic thrombocytopenic purpura (TTP) and aplastic anaemia. Importantly, it was shown that imipramine and ticlopidine dual treatment targeted autophagy and thus inhibited GBM growth and increase survival [175]. This large in vivo study also revealed that this dual regime significantly altered GBM histopathology generating large areas of tumour necrosis and significantly reduced cellularity compared to matched control tumours [175]. From this study, it is argued that while imipramine monotherapy yielded a modest increase in survival of GBM-bearing mice, in combination with ticlopidine, there was a synergistic increase, dramatically increasing GBM survival. Ticlopidine targets the purinergic receptor P2RY12 which is not expressed in high levels in normal tissues (excluding platelets), but is upregulated in a range of tumour types [176]. Critically although P2RY12 alone is ineffective as a monotherapy, it could synergise with various tricyclic antidepressants.

6.9.6 *Clomipramine (Anafranil®)*

Agents that act on the mitochondria to induce tumour cell death are few and far between, but the tricyclic antidepressant, clomipramine (Anafranil®), in common use since its development in the 1960s [177], is one such agent. In more recent years, clomipramine has also been shown to function as an antineoplastic agent. Studies, albeit, in vitro, in cancers such as leukaemia [178, 179], melanoma [180], glioma and neuroblastoma [181–183] as well as extensive studies in our laboratories on glioblastoma and low-grade astrocytoma [184–188] have shown that clomipramine results in tumour cell-specific, mitochondrially mediated apoptosis (as opposed to many anticancer therapeutics that have their effect by targeting the nucleus/DNA) [186, 189]. More recent in vitro studies have addressed the use of clomipramine in combination with other agents against glioma with high success [187, 190].

The mitochondrial membrane potentials in cancer cells, particularly GBMs, are frequently reduced in comparison with those of non-neoplastic cells which allows a win-

dow of opportunity for small-molecule agents to enter the tumour cell mitochondria and reduce oxygen consumption with subsequent release of cytochrome c and activation of a caspase pathway to apoptosis that is cancer cell specific. Clomipramine has also been used in combination with Gleevec in vitro, where it was reported that combination treatment resulted in the inhibition of cell growth and enhanced apoptotic cell death. There was also the reported inhibition of DNA synthesis and cAMP [190]. In addition to this, there was also a significant synergistic induction of autophagy by the dual treatment combination, suggesting the potential clinical application of this combination in the treatment of drug-resistant GBM [190]. While clomipramine has been used in these (and other) studies, commercial development in cancer therapy has not been forthcoming, and clinical use in GBM has been confined to vast numbers of anecdotal cases, many of which reported superior outcome when measured against mean survival times.

An epidemiological case-controlled study using the General Practice Research Database compared previous tricyclic antidepressant (TCA) usage of 31,953 cancer patients (773 of which were glioma patients) against case-controlled patients matched for age, gender and GP practice (at a ratio of 2:1). This study reported that the chances of being diagnosed with a glioma were reduced by 40 % for patients taking TCAs and further reduced in patients who were either taking a high-dose TCA (50 %) or for an extended period of time (64 %) (determined from the British National Formulary); all results were statistically significant. The only other statistically significant group of cancers in which TCAs had an effect were in colorectal cancer [174]. This research is especially important as it points to a margin of specificity for TCAs in cancer, albeit in a protective/preventative capacity.

6.9.7 Metformin

Metformin has been used for over 40 years as a first-line defence drug against type 2 diabetes [191] (Witters 2001); the compound is an analogue derived from galegine, isolated from the plant *Galega officinalis*, also known as French lilac, goat's rue and professor weed [192]. Typically prescribed to treat hyperglycaemia in type 2 diabetes where the site of action for this drug is on the cell mitochondria, it disrupts cell respiration, specifically by inhibiting respiratory complex I [193]. Metformin belongs to a group of compounds known as the biguanides that also includes phenformin and buformin, the latter two have enhanced glucose-lowering properties compared to metformin, but have an increased risk of causing lactic acidosis. It has been reported that Type 2 diabetic patients on long-term metformin treatment have reduced incidences of many types of cancer, including breast, liver, lung and pancreatic cancer [194–196]. Metformin has also shown improved overall improved survival of diabetic patients with breast, colorectal and head and neck cancer [197–199].

The principal mechanism of action of this drug is by suppression of the process of gluconeogenesis used in the manufacture of new glucose by the liver. Metformin has been shown to enable normal but not T-cell-deficient SCID mice to reject solid tumours [200]. Despite this, it is generally believed that the anticancer action of metformin may be mostly due to lowering glucose and insulin.

Metformin enters cells via the plasma membrane monoamine transporter (PMAT) and organic cation transporter 1 (OCT1). It has multifaceted mechanisms of action including activation of adenosine monophosphate-activated protein kinase (AMPK) [201–205]; this AMPK-dependent pathway leads to inhibition of a number of downstream pathways: the phosphatidylinositol 3-kinase (PI3 K) pathway, the mammalian target of rapamycin (mTOR) and the extracellular signal-regulated kinases 1 and 2 (ERK1/2) [206]. Cell regulator cyclin D1 is also inhibited; this reduction in cyclin D1 levels results in cell cycle arrest in G0/G1 phase [207]. Metformin directly impairs mitochondrial function resulting in reduced mitochondrial respiration by inhibition of complex I of the electron transport chain, often referred to as the AMPK-independent pathway [208]. This inhibition of complex I is likely to be the reason for the lactic acidosis side effect; however, it has been reported that antidiabetic biguanides do not induce lactic acidosis in nondiabetic individuals [209–211]. In vitro metformin decreases glioma proliferation, induces autophagy and apoptosis and causes cell cycle arrest through activation of AMPK and inhibition of the mTOR pathway [212]. Sesen et al. also demonstrated that metformin treatment in combination with temozolomide and/or irradiation induces a synergistic antitumour response in glioma cell lines [212]. Cell migration is also reduced by metformin in cells that lack expression of the PTEN suppressor gene [213]. Moreover, metformin has been shown to promote differentiation of cancer 'stem-like' glioma initiating cells and reduce self-renewing properties [214].

Although, metformin is the most widely studied of the biguanides, phenformin is more lipophilic and has reduced IC50 values [215], indicative of increased cytotoxicity. Due to the potential issue of lactic acidosis, phenformin has been discontinued for use in many countries. To address this issue, combination studies with glycolytic inhibitors have been reported with sodium oxamate and 2-deoxyglucose with increased efficacy against glioma and other cancer cells [216, 217].

At the time of writing, there are two phase I clinical trials that include metformin. The first of these trials for patients with a recurrent brain tumour combines radiation therapy with low-carbohydrate diet and metformin is active and recruiting. The study aims to selectively starve tumour cells, making them vulnerable to ionising radiation (NCT02149459). The second trial is ongoing, but no longer recruiting; this is a factorial trial of temozolomide, memantine, mefloquine and metformin for post-radiation therapy glioblastoma multiforme which aims to find the highest tolerable dose of temozolomide in combination with the other agents and/or metformin. No study results have been posted as yet (NCT01430351).

6.9.8 Disulfiram (Antabuse)

The drug disulfiram (DS) (marketed as Antabuse) has been in clinical use since the 1940s to combat alcohol abuse by inhibiting the enzyme acetaldehyde dehydrogenase. The repurposing of this agent has received significant attention due to a diverse range of inhibition against key GBM components that include inhibition of the 'cancer stem cell marker' aldehyde dehydrogenase (ALDH) [218], MGMT inhibition [219], polo-like kinase inhibition [220] and inhibition of two matrix

metalloproteinases, MMP-2 and MMP-9 [221]—critical for GBM invasion. In vitro DS requires the use of the essential trace element copper (or other metal ions), for redox reaction that leads to selective apoptosis in the cancer cells [222]; various levels of copper are found in serum supplemented cultures [223]; this is because cancer cells, in comparison to normal cells, have higher levels of reactive oxygen species (ROS) activity which can cause deleterious effects. Further exposure, therefore, by ROS-generating agents will deplete the cellular antioxidant capacity preferentially causing apoptosis in the cancer cells [224]. For this reason, copper supplementation is being used in clinical trials with DS. Three clinical trials are registered on the database ClinicalTrials.gov:

The oldest (NCT01907165), a phase I study, registered by Washington University School of Medicine to start in October 2013 is currently recruiting histologically confirmed glioblastoma patients to be assigned to TMZ plus DS or TMZ plus DS plus copper gluconate.

The second, a Greek, phase II study, registered by the Olympic Medical Centre (NCT01777919) will also be studying the effect of DS/copper as an adjuvant and concurrent chemotherapy in newly diagnosed GBM, but is yet to start recruiting.

Finally, a phase II study sponsored by Sahlgrenska University Hospital in Sweden (NCT02678975) has been registered to randomise patients either to standard therapy of alkylating agents plus chemotherapy or to alkylating chemotherapy plus DS plus copper; this study is due to start in September 2016.

To further strengthen the case of DS against GBM, a recent in vitro study demonstrated that brain tumours with high aldehyde dehydrogenase (ALDH) activity have a number of characteristics which are similar to those of brain tumour-initiating cells [225]; the therapeutic potential, therefore, is that the main cellular target of DS, ALDH, is particularly prevalent in 'classical'-type glioblastomas expressing EGFR; cautiously, however, a restriction regarding this statement is that the brain tumour type tested was atypical teratoid/rhabdoid brain tumours [225] (malignant paediatric brain tumour rather than a GBM).

6.9.9 Mebendazole

Mebendazole, now generic, for the treatment of a broad range of worm infestation (including pin-, round- and hookworm) mediates an effect by binding to the tubulin subunits in the gut of the worm. It has been reported that fenbendazole, a benzimidazole antihelminthic, inhibited brain tumour engraftment, and following more extensive studies, it was shown that mebendazole was a more promising drug for GBM therapy, significantly extending mean survival up to 63 % in syngeneic and xenograft orthotopic mouse glioma models [226]. The authors of this study suggest that this repurposed agent could be further tested in clinical trials and a phase I trial has been initiated for newly diagnosed high-grade GBM in combination with temozolomide and conducted at the Sidney Kimmel Comprehensive Cancer Centre (NCT01729260).

6.9.10 *Boswellia serrata*

Boswellia serrata is an active compound isolated from Indian frankincense; it is a pentacyclic terpenoid commonly used in Ayurvedic medicine to treat a number of inflammatory illnesses such as asthma, Crohn's disease and arthritis [227]. The therapeutic potential of boswellic acid derivatives has been widely reported, and several studies have recently reported to possess anti-angiogenic, anti-inflammatory, anti-invasive and pro-apoptotic potential against cancers such as hepatocellular carcinoma [228], prostate [229–231], pancreatic [232], colon [233–236], melanoma [237], meningioma [238], glioma [239, 240], myeloma [241], AML [242–244] and leukaemia [240]. *Boswellia serrata* (*Salai/Salai guggul*) (family, Burseraceae; genus, *Boswellia*) is a large branching tree found in India, Northern Africa and the Middle East; the gum resin extracted from the tree is known as Indian frankincense, Indian olibanum, *dhup* and *salai guggul*. The constituents of the oleo-gum resins contain 30–60 % resin and 5–10 % essential oils, which are soluble in the organic solvents, and the rest is made up of polysaccharides (~65 % arabinose, galactose and xylose) which are soluble in water. The resin contains monoterpenes (α-thujene), diterpenes (macrocyclic diterpenoids, such as incensole, incensole oxide, isoincensole oxide), triterpenes (such as α- and β-amyrins), tetracyclic triterpenic acids (tirucall-8,24-dien,21-oic acids) and the much researched pharmacologically active metabolites pentacyclic triterpenic acids—collectively known as boswellic acids (BAs) of which 75 active isomers have thus far been detected [245], 6 of which are focused on by researchers: 11-keto-β-boswellic acid (KBA), acetyl-11-keto-β-boswellic acid (AKBA), β-boswellic acid (βBA), acetyl-β-boswellic acid (AβBA), α-boswellic acid (αBA) and acetyl- α-boswellic acid (AαBA) [157].

Boswellia serrata resin dry extract (BSE) was given Orphan Designation in 2002 by the European Commission (EU/3/02/117) for the 'treatment of peritumoral oedema derived from brain tumours'. BSE is generally not a standardised formulation, although manufactured preparations are available, H15 being the most widely used standardised formula. Current proprietary formulations of BSE differ by the concentration/inclusion of various boswellic acid isomers; the starting resin material is processed by various methods which remove the majority of the essential oils and polysaccharides, the remaining fraction containing mainly organic acids. In vitro anti-inflammatory studies in the 1990s found that 5-lipoxygenase (5-LO) and human leukocyte elastase (HLE) were inhibited by 11-keto-β-boswellic acid (KBA) and acetyl-11-keto-β-boswellic acid (AKBA) [246–249]; based on these results, it was believed that these particular isomers were pharmacologically the most important active components of BSE, resulting in a focus of experimentation on these isomers. However, plasma studies contradicted the importance of these particular isomers when following oral administration—levels of KBA and AKBA were found to be too low or undetectable [157, 250, 251], with more recent studies highlighting the importance of four other BAs (as per Fig. 6.1). As boswellic acid and clomipramine may manipulate many of the same mitochondrial effector pathways [252] in order to exploit their antineoplastic effects, we hypothesise that, when administered

in combination, the synergistic effects may be sufficient to overcome therapeutic resistance in glioblastoma. Boswellic acid is a small, lipid-soluble molecule capable of crossing the blood-brain barrier; oral administration in a suitable carrier compound—currently under development with a Portsmouth Brain Tumour Research Centre collaborator (patent pending)—will result in reduced metabolism of the drug and subsequently more efficient uptake into the bloodstream. However, our pilot serum measurement taken in collaboration with the Eschborn team using their published method [253] showed that taken over 7 days (1 g three times a day), it resulted in >30 μg/ml BAs in serum [unpublished data], predicting that 60 % of the serum concentration would reach the brain [254]. This would adequately meet the concentration required for glioma cell kill. Using the purest isomer combination (under development), this formulation, method of administration and treatment course should ensure steady-state levels in patient populations.

6.10 Conclusion

The repurposing of approved drugs offers significant opportunity and hope for the clinician to combat GBM, a cancer that unlike many others still presents a very poor clinical prognosis. The current standard front-line treatments (surgical resection, adjuvant radiotherapy and temozolomide) offer only marginal patient improvement compared to surgical resection alone. The application of repurposed drugs offers a substantial benefit in terms of accrued knowledge regarding safety and toxicity alone, while many of the repurposed drugs themselves could be prescribed to GBM patients on the basis of their original therapeutic use (e.g. clomipramine as an antidepressant). There are clearly many obstacles faced within the neuro-oncology field; however, the alternative uses for a number of widely prescribed drugs (and understanding the molecular mechanisms that these agents exploit within GBM) could open up new treatment options for a disease that, to date, desperately requires them.

Acknowledgement The authors would like to thank Dr. Aditi Nadkarni for her advice and editing suggestions on this chapter.

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Chapter 7
Small-Molecule Inhibitors in Glioblastoma: Key Pathways and Resistance Mechanisms

Jenny L. Pokorny, Gaspar J. Kitange, and Daniel J. Ma

Abstract Glioblastoma, the most common and aggressive form of primary adult brain tumor, is a devastating disease with a dismal two-year survival. Attempts to improve patient survival include a variety of treatment options, from monoclonal antibodies, vaccines, and microbubbles to exosomes and small-molecule inhibitors, all of which are in various stages of preclinical and clinical development. The most frequently tested type of novel therapeutics are the small-molecule inhibitors targeting key signaling pathways dysregulated in GBM, including TP53, retinoblastoma, and the receptor tyrosine kinase-driven EGF, PDGF, and c-MET pathways. This chapter will compare preclinical and clinical results for a subset of inhibitors targeting the receptor tyrosine kinase families EGF, VEGF, and PDGF along with the PI3K/Akt/mTor pathway and cell cycle inhibitors. In the discussion, potential resistance mechanisms which continue to pose significant barriers to effective small-molecule inhibition treatment of GBM will be discussed along with possible improvements.

Keywords Glioblastoma • Small-molecule inhibitor

Abbreviations

AG	Anaplastic glioma
CDK	Cyclin-dependent kinase
DSBs	Double-stranded breaks
EGF	Epidermal growth factor
ErbB	Erythroblastic leukemia viral oncogene homolog
mTor	Mammalian target of rapamycin

J.L. Pokorny (✉)
Department of Neurosurgery, Stanford University,
1201 Welch Rd, MSLS Building, Stanford, CA 94305, USA
e-mail: jpokorny@stanford.edu

G.J. Kitange • D.J. Ma
Department of Radiation Oncology, Mayo Clinic,
1st St SW, Guggenheim Building, Rochester, MN 55905, USA

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_7

mTorc1-2	Mammalian target of rapamycin complex 1 and 2
PDGF	Platelet-derived growth factor
PFS	Progression-free survival
PTEN	Phosphatase and tensin homolog
RT	Radiation therapy
RTK	Receptor tyrosine kinase
TSC2	Tuberous sclerosis 2

7.1 Introduction: Current Glioblastoma Therapy Options

The current standard of care for newly diagnosed patients with glioblastoma (GBM) includes surgery, radiation therapy (RT), and chemotherapy with the DNA methylating agent temozolomide (TMZ) [1]. Both TMZ and RT kill GBM cells by inducing DNA double-strand breaks (DSBs), which in turn activates apoptotic cell death [2]. Cells surviving the initial therapy rapidly proliferate leading to recurrent disease, for which known effective therapeutic options are limited [1]. FDA-approved treatments for recurrent GBM are limited to the antiangiogenic agent Avastin (bevacizumab) and the DNA alkylating drugs lomustine (CCNU) and carmustine (BCNU), while surgery done at recurrence has yielded equivocal results for patients [1]. The growth of both primary and recurrent tumors is primarily driven by vast signaling pathways activated or dysregulated in GBM. System-wide analysis conducted by The Cancer Genome Atlas in over 200 GBM patient samples reveals many commonly mutated and dysregulated pathways in GBM [3]. For instance, as shown in Fig. 7.1, 88 % of patients analyzed had altered signaling in the RTK/RAS/PI(3)K network with 45 % of patients showing either mutation or amplification of EGFR alone, while mutation and homozygous deletion of PTEN was found in 36 % of patients. Alterations in both the p53 and RB signaling pathways are also common (87 % and 78 %, respectively), with homozygous deletion of CDKN2A (ARF/P16/INK4A) and CDKN2B represented in approximately half of patient samples. The majority of the signaling pathways noted in this study provide a survival advantage either by decreasing apoptosis or increasing cell proliferation and angiogenesis, suggesting that key member proteins could provide novel targets for signaling modulation [4].

Similar system-wide genomic analysis performed in other cancer types has uncovered novel targets and pathways which could be used to provide direct clinical benefit [5, 6]. Although targeted chemotherapeutics created to modulate specific pathways identified in GBM patients could be similarly identified and targeted, no small-molecule inhibitor has been FDA approved for treatment of GBM patients. This is, at least in part, because of a lack of significant therapeutic benefit and/or toxicity that was observed in the initial preclinical and clinical studies [7, 8]. Significant complications continue to plague the development and testing of novel therapeutics in GBM, a few of which will be covered toward the end of this chapter in Sect. 7.3, Resistance Mechanisms to Small-Molecule Inhibitors.

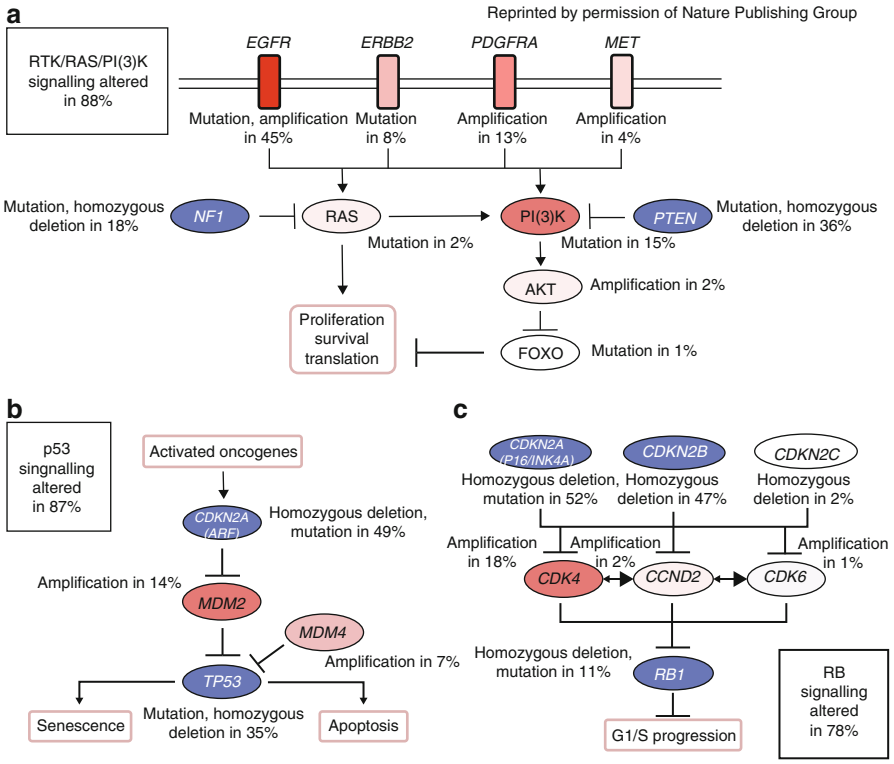

Fig. 7.1 Commonly dysregulated pathways and pathway components in GBM patients. Sequencing of 206 glioblastoma patient tissue samples was performed to uncover the most commonly dysregulated pathways and pathway components present in GBM patient tissues. Reprinted by permission from Macmillan Publishers Ltd: Molecules [3], copyright (2009)

7.2 Small-Molecule Novel Therapeutics

7.2.1 Receptor Tyrosine Kinase Inhibitors

The receptor tyrosine kinases (RTKs) are frequently mutated and overexpressed in GBM, with a high frequency of alterations reported in the PDGF, EGF, and c-MET families, among others [9]. Mutational events in RTKs are often concurrent with other activating and silencing mutations, such as loss of the tumor suppressor genes phosphatase and tensin homolog (PTEN) and tumor protein (p53) [3]. Alterations in RTKs affect a wide range of downstream cellular pathways and processes including apoptosis evasion, growth, survival, and focal adhesion, as noted in Fig. 7.2. Figure 7.3 presents a simplified schematic of RTK/PI3K/Akt signaling and many of the chemotherapeutics directed against specific members of these pathways, a few of which will be discussed throughout this chapter. The following section will provide a detailed account of key inhibitors targeting the RTKs and pinpoint the observed clinical benefit, if any, in GBM.

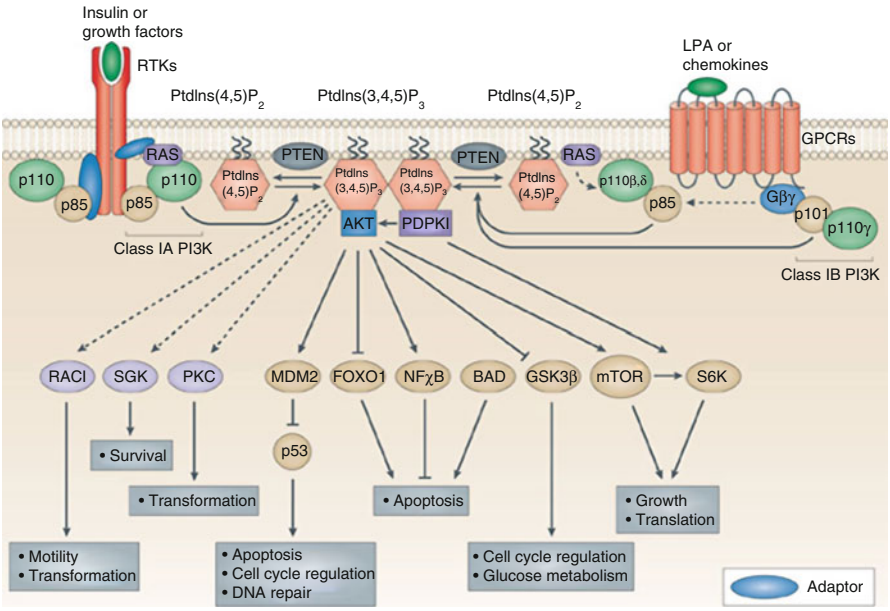

Fig. 7.2 Receptor tyrosine kinase pathway proteins and downstream signaling effects. The activation of receptor tyrosine kinases (RTKs), in addition to G-protein-coupled receptors (GPCRs), drives a multitude of downstream targets including Akt, mTor, and PI3K and their respective pathways, many of which are known to be dysregulated in various forms of cancer including GBM. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery [56], copyright (2009)

7.2.1.1 Epidermal Growth Factor Inhibitors

One of the most extensively studied and frequently targeted of the RTKs across all cancer types is the erythroblastic leukemia viral oncogene homolog (ErbB) or epidermal growth factor (EGF) family of receptors [10, 11]. The EGF family consists of four members: EGFR/ErbB1/Her1, ErbB2/Her2, ErbB3/Her3, and ErbB4/Her4 [10]. Though only EGFR, Her3 and Her4 have known ligand-induced kinase activity, dimerization and oligimerization of all EGF family members allows for a wide variety of signaling pathway options [10, 11]. A key regulator of downstream RTK activity, PTEN was implicated as a possible negative regulator of the EGFR inhibitor response [12]. However, preclinical and clinical studies from other groups have repeatedly failed to confirm the importance of PTEN in receptor tyrosine kinase inhibitor (RTKi) efficacy [13, 14]. Thus, the relevance of PTEN status to EGFR inhibitor efficacy remains unknown.

EGFR targeting is a particularly attractive therapeutic strategy for GBM patients as approximately 34–63 % will have an amplification of EGFR and, of those, 25–64 % will also have an excision of exons 2–7, called EGFRvIII, which creates a constitutively activated protein kinase [15]. Deletion of exons 2–7 has been shown to drive EGFR addiction in cells; thus EGFR inhibition should be an effective therapeutic

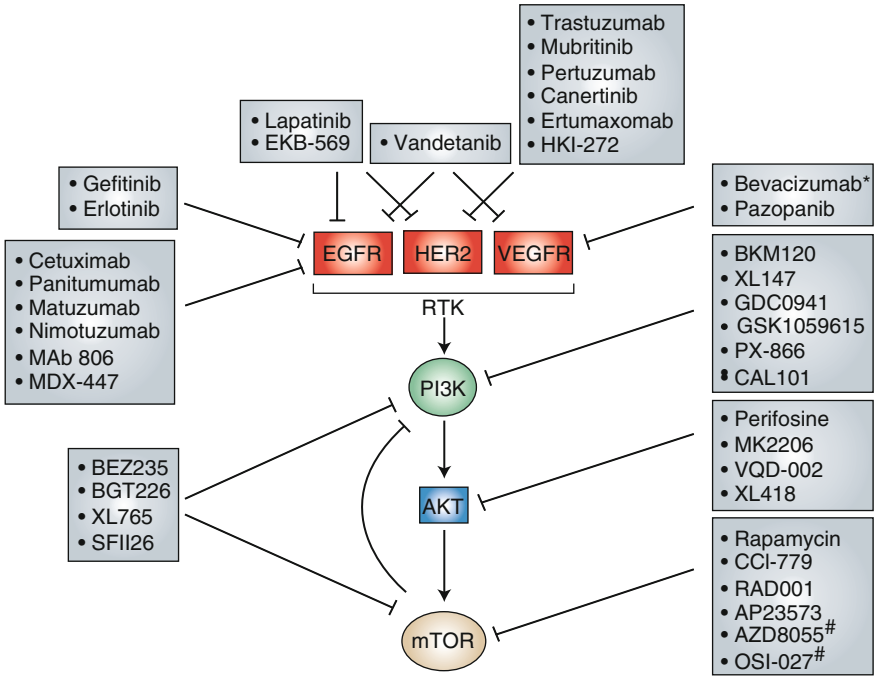

Fig. 7.3 Small-molecule inhibitors and their targets. Several small-molecule inhibitors targeting RTK, PI3K, and mTor have been tested preclinically and clinically for GBM and other cancer types. Only a select few examples from this extensive list are discussed in this chapter. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery [56], copyright (2009)

strategy for GBM patients [11, 16]. Activation of EGFR drives downstream signaling through a wide variety of pathways including JAK/STAT, RAF/MEK/ERK, and PI3K/AKT/mTor; thus uncontrolled or amplified EGFR activity is likely a critical tumorigenic event [11, 17]. Not surprisingly, there are a large number of EGF-directed chemotherapeutics which are in various stages of testing for GBM, as illustrated in Fig. 7.3. Three of the inhibitors included in Fig. 7.3, erlotinib, lapatinib, and gefitinib, have been extensively evaluated both preclinically and clinically for therapy in GBM, and the results of those studies are discussed below.

A. Erlotinib (Tarceva, Astellas Pharma)

Erlotinib is a selective and reversible, ATP-competitive intracellular kinase domain inhibitor of EGFR (ErbB1), FDA approved for treatment of non-small cell lung cancer patients (NSCLC) [16, 18]. Similar to GBM, a large number of NSCLC patients will have activating mutations in EGFR (approximately 40–80 %), with about 90 % of those mutations occurring in the kinase domain [19, 20].

Preclinical Evaluation

Xenograft studies in patient-derived orthotopic lines treated with erlotinib showed that EGFRvIII status is likely not a reliable predictor of single agent response [21]. One of only two responsive lines from the panel of 11 tested was an EGFR WT tumor. Although PTEN status did appear to predict for favorable response, since only two lines were responsive, the total number of lines tested were too few to make predictive statements on the role of PTEN status based on these data alone.

Clinical Evaluation

Erlotinib has been tested in five separate Phase II clinical trials (a sixth trial will be discussed in detail in the section for rapamycin). The first trial tested erlotinib in a nonrandomized and open-label study as a single agent in patients who had completed TMZ and RT treatments and were on their first relapse [22]. Forty-eight patients for the two-stage study were accrued, but low response rates (one complete response and three partial responses) in stage 1 led to the study being ended before the start of stage 2. Assessment of EGFR amplification did not indicate any significant survival difference between EGFR-amplified and non-amplified patients (progression-free survival (PFS) at 6 months 21.7 % vs 18.3 %, amplified and non-amplified, respectively). In the second trial, 96 patients were divided into two groups (group 1, 53 patients with recurrent GBM, oligodendroglioma, or anaplastic astrocytoma, and group 2, 43 GBM patients who were nonprogressive (NP) after RT) and treated with single agent erlotinib [8]. Group 1 patients were allowed no more than two prior relapses and two prior therapies. Group 2 patients were not allowed to have received any prior chemotherapy, including TMZ. Erlotinib treatment yielded a PFS at 6 months in recurrent GBM patients of only 3 % (27 % for recurrent AG patients), while estimated 1-year PFS was 9 % in the NP group. Pharmacokinetic analysis conducted in a subset of patients indicated that erlotinib penetration into tumors was insufficient to effectively inhibit EGFR phosphorylation [8, 23]. In the third trial, 97 newly diagnosed patients received erlotinib in combination with TMZ and RT and then continued erlotinib treatment with adjuvant TMZ [13]. No difference in median OS was seen in patients treated with erlotinib when compared to EORTC 26981/22981-NCIC historical controls (15.3 vs 15 months), and response had no correlation with PTEN or EGFR status. In the fourth study, 27 newly diagnosed patients were treated concurrently with TMZ, RT, and erlotinib, within 28 days of biopsy or resection [7]. PFS at 6 months was only 30 % for this study. The study authors also noted that the combination of RT, TMZ, and erlotinib had "unacceptable toxicity." Finally in the fifth trial, 110 recurrent GBM patients were randomly assigned to receive either erlotinib (54 patients) or a control compound (either TMZ or BCNU (27 or 29 patients, respectively), depending upon whether the patient had been treated with TMZ previously) [24]. At 6 months, patients receiving erlotinib had a PFS of 11.4 %, while patients in the control arm had a PFS of 24.1 %. Collectively, the poor results

from xenograft studies and clinical trials illustrate how little is known about the complexity of the EGFR signaling pathway and which factors are truly predictive of response to EGFR inhibition.

B. Lapatinib (Tykerb, Novartis)

A second EGFR inhibitor, lapatinib, also inhibits ErbB2 (Her2) [25]. Although Her2-driven pathways have not been implicated in GBM as they are in breast cancer, heterodimerization and oligimerization of all four EGF receptors are known signaling mechanisms; thus, dual inhibition of EGFR and Her2 as opposed to inhibition of only EGFR is a potential method to target and inhibit interactions that both receptors play roles in [10, 26]. Further, some studies have found that primary (de novo) GBM tumors have Her2 overexpression, though GBMs arising from lower-grade tumors (secondary GBM) do not appear to exhibit the same phenotype [27].

Preclinical Evaluation

Only one in vivo study of lapatinib treatment of GBM exists currently, and although five patient-derived xenografts were used in the study, only one xenograft line, GBM6, is noted by the authors as being responsive to in vivo treatment with lapatinib (placebo treatment vs lapatinib, $p<0.05$) [25]. Interestingly, GBM6 was also included in the abovementioned in vivo study with erlotinib, and in that study, treatment of GBM6 with erlotinib did not produce a statistically significant survival benefit ($p=0.536$) [21]. Thus, if the lapatinib results in GBM6 are reproducible, they may indicate that GBM6 is uniquely sensitive to Her2/neu inhibition or to the combination of EGFR and Her2/neu inhibition. Further, these results may hint at a certain subset of GBM tumors that are sensitive to dual inhibition of EGFR and Her2/neu.

Clinical Evaluation

Lapatinib has been tested in two Phase I/II trials, both of which were conducted in recurrent GBM patients. In the first Phase II trial, the best response was stable disease in four patients, while the remaining 13 patients had early progression; thus the study was ended prematurely [28]. Neither EGFRvIII nor PTEN status was predictive of outcome. The second trial tested the combination of pazopanib and lapatinib in 41 GBM patients at first or second recurrence. Patients were split into two groups: 1. EGFRvIII/PTEN positive and 2. EGFRvIII/PTEN negative [29]. This study also failed to meet primary endpoints with a PFS at 6 months of 0 % in the EGFRvIII/PTEN positive and 15 % in the EGFRvIII/PTEN negative groups. Although pharmacokinetic evaluation of pazopanib levels indicated effective concentrations reached, lapatinib doses achieved were subtherapeutic. Neither PTEN nor EGFRvIII status was predictive of outcome in this study. Although the preclinical results for lapatinib indicate a potential, select group of GBM patients that may respond to lapatinib, clinical studies have yet to effectively identify and benefit that specific patient population.

C. Gefitinib (Iressa, Astra-Zeneca, and Teva)

Gefitinib, similar to erlotinib, is an ATP-competitive selective inhibitor of the EGFR kinase domain [18]. Although there are slight differences between the two compounds (molecular weight and increased likelihood for adverse events (with erlotinib), etc), both retrospective and prospective studies conducted in NSCLC patients have failed to find a significant therapeutic difference [19, 30–32]. Similar comparative studies of erlotinib and gefitinib have not been conducted for GBM, either preclinically or clinically, so it is unclear if treatment with gefitinib and erlotinib in GBM patients will prove to yield essentially analogous results as well.

Preclinical Evaluation

Joshi et al. reported mixed results from in vivo studies performed in 9L rat gliosarcoma and the human GBM cell line 020913 with gefitinib [14]. Animals injected intracranially (IC) with 020913 and treated with gefitinib had a statistically significant survival benefit over placebo treated animals ($p=0.0001$). However, rats implanted with 9L cells did not yield a similar benefit ($p=0.13$). In their analysis of these results, the authors speculate that the 020913 growth conditions (stem cell media supplemented with EGF and FGF) may select for EGF dependence, whereas 9L cells, which are grown in complete media, are not similarly selected. Growth conditions have in fact been found to directly affect expression of EGFR [15]. Although the EGFR status of 9L cells is a point of contention, with some groups stating that 9L cells do not express EGFR, while others have noted that 9L cells do overexpress EGFR [33, 34], the relevance of EGFR status for inhibitor efficacy is unknown. Further, according to in vitro data noted by the authors, 9L cells treated with gefitinib were more sensitive than 020913 cells. Thus again, though differential effects with EGFR inhibition have been noted, no clear explanation has yet been found for these differences.

Clinical Evaluation

Gefitinib was tested in three Phase II trials, two conducted in recurrent and one in newly diagnosed GBM patients. The first study accrued 28 patients with mixed high-grade gliomas (grades III and IV) in which there was no appreciable efficacy [35]. PFS at 6 months for all patients was 14.3 % (12.5 % in the GBM patient subgroup). Five patients had stable disease and none had partial response. Neither EGFR expression nor gene status nor p-Akt level (a downstream target of EGFR signaling, discussed in greater detail in Sect. 7.2.2) predicted for outcome. In the second trial of gefitinib in recurrent patients, PFS at 6 months was 13 % with no objective tumor responses noted [36]. In the third trial, 96 newly diagnosed patients first underwent RT and then were treated with single agent gefitinib [37]. Patient results, when compared to historical controls, showed no significant difference in survival with gefitinib treatment (PFS at 12 months, post-RT vs historical controls 16.7 % and 30.3 %, respectively). Although there are slight differences between erlotinib and gefitinib, neither drug appears to provide significant benefit to GBM patients clinically.

7.2.1.2 Vascular Endothelial Growth Factor and Platelet-Derived Growth Factor Kinase Inhibitors

The vascular endothelial growth factor (VEGF) family consists of five members, VEGFA, B, C, D, and placental growth factor (though the best characterized is VEGFA) along with the three receptors, VEGFR1, 2, and 3 [38]. Initially identified as an endothelial cell mitogen, the VEGF family actually has functions in a wide variety of cell types and cellular functions, such as cancer stem cell function, tumorigenesis, and stimulation of epithelial to mesenchymal transition [38]. The platelet-derived growth factor (PDGF) family also has multiple components with five isoforms (PDGF-AA, -BB, -CC, -DD, and -AB). Binding of these factors leads to homo- or heterodimeric complexing of PDGFRα and PDGFRβ which ultimately drives downstream effects such as migration, cell survival, and growth [39]. Similar to EGF, both VEGF and PDGF expression have been found to be upregulated in GBM [3, 9]. PDGF and PDGFR are overexpressed in approximately 16% of GBM, while VEGF-driven angiogenesis is considered a key factor in tumor growth and survival [40, 41]. Several inhibitors with activity against VEGF and PDGF are currently undergoing investigation for treatment of GBM, the results for two of which will be discussed in detail below.

A. Sorafenib (Nexavar, Bayer)

Sorafenib is a multi-targeted kinase inhibitor with activity against both the VEGF (VEGFR2 and 3) and PDGF (PDGFβ and KIT) families [42]. Sorafenib also inhibits both C-Raf and B-Raf along with MEK and ERK phosphorylation [42] and has been shown to induce apoptosis in a variety of cell lines through its downregulation of the pro-survival factor Mcl-1 [43].

Preclinical Evaluation

Available preclinical in vivo studies of sorafenib are limited, with only one study conducted in mice with orthotopically implanted U87 cells. Single agent sorafenib treatment in this study had a significant survival benefit over vehicle treatment ($p < 0.05$) [44].

Clinical Evaluation

Sorafenib has been tested in five clinical trials in GBM patients, the first of which, a Phase I/II, combined sorafenib with temsirolimus. However, the study was ended before the start of Phase II as no patients from Phase I made PFS at 6 months [45]. Median PFS in this study was 8 weeks. A separate Phase II trial with sorafenib in combination with bevacizumab conducted in recurrent GBM patients showed no benefit of the combination over bevacizumab historical controls (PFS at 6 months was 20.4% for sorafenib and bevacizumab combination vs 16–24% for bevacizumab historical controls) [46]. The third and fourth Phase II studies both tested the

combination of sorafenib with low-dose TMZ in recurrent GBM patients, yet each had markedly different results. In both trials, patients received 800 mg/day of sorafenib and similar doses of TMZ (40 mg/m^2/day vs 50 mg/m^2/day). However the PFS at 6 months for each study is strikingly different. In the first study, the PFS at 6 months was only 9.4 %, while the second trial had a PFS at 6 months of 26 % [47, 48]. It is unclear why similar study designs yielded such dissimilar results. However, the results from both groups indicate that the combination of TMZ and sorafenib is of limited benefit to recurrent GBM patients. In the fifth study, 47 newly diagnosed patients who first underwent combined treatment with TMZ and RT were then treated with sorafenib and TMZ as maintenance therapy. Unfortunately, only about 60 % of patients successfully finished concurrent TMZ and RT and continued on to adjuvant TMZ and sorafenib. Though the PFS at 6 months for the entire treatment group was 50 %, the addition of sorafenib provided no survival benefit over historical controls [49]. Apparently, clinical sorafenib treatment does not have the same efficacy as what was found preclinically, especially when sorafenib efficacy is compared to historical controls.

B. Sunitinib (Sutent, Pfizer)

Sunitinib malate is a multi-targeted receptor tyrosine kinase inhibitor which inhibits PDGFRα and -β, VEGFR1 and 2, as well as RET, c-KIT, CSF-1R, and FLT3 [50]. Although multi-targeted kinases offer a wide range of inhibitor activity and thus provide more opportunities for efficacy, it becomes challenging, if not impossible, to thoroughly understand the mechanisms of action of a multi-targeted therapeutic. Differential efficacy can potentially be attributed to any or all of the known targets or the further downstream effects, making determining biomarkers and selecting patient populations extremely difficult.

Preclinical Evaluation

A 2007 study demonstrated a significant survival benefit in mice with U87 IC implanted tumors and treated with sunitinib at 80 mg/kg over placebo-treated mice ($p<0.0001$) [50]. A second study by Joshi et al. reported that animals implanted with either 9L rat glioma or 020913 human GBM cells and treated with single agent sunitinib at 15 mg/kg did not have any survival benefit ($p=0.13$ compared to placebo-treated mice) [14]. Although treatment with sunitinib in the first study showed survival benefit, the dose used is significantly higher than that used in patients in the clinical trials discussed below (37.5 mg), thus calling into question the reliability of those survival data.

Clinical Evaluation

Sunitinib has been tested in two Phase II trials, the first of which was conducted in newly diagnosed patients with unresectable GBM [51]. Patients were treated with sunitinib pre-RT and then concurrent with and post-RT treatment. Out of the 12

patients accrued for the study, only one (8.3 %) exhibited stable disease, while 11 (91.3 %) had disease progression on treatment. The lack of response in this patient population led to the study being terminated early. In a second Phase II study, patients with recurrent GBM or recurrent gliosarcoma were stratified by their previous exposure to bevacizumab into bevacizumab-resistant and bevacizumab-naïve groups [52]. Only 3/29 of the bevacizumab-naïve patients achieved radiographic response (10 %), while 0/29 of the bevacizumab-resistant patients did. Single agent sunitinib treatment provided no improvement in median time-to-progression or overall survival (1.6 and 3.8 months, respectively). Although the inhibition of multiple targets potentially provides more opportunities for target inhibition and treatment efficacy, neither sorafenib nor sunitinib appears to provide any significant benefit in GBM patients.

7.2.2 PI3K/mTor/Akt Pathway Inhibitors

Many of the downstream targets and pathways of the RTKs have been implicated as essential drivers in gliomagenesis, apoptosis evasion, and cell growth. One of the most targeted and dysregulated of those downstream pathways is the phosphoinositide 3-kinase/ mechanistic target of rapamycin (PI3K/mTor) pathway [53]. The PI3 kinases are subdivided into three different classes, though this chapter will only focus on the activity of the Class I PI3 kinases. Activation of a target receptor (either an RTK or G-protein-coupled receptor) induces interaction of PI3K, either directly or via a mediator, thus driving generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) [53, 54]. Figure 7.2 gives a simplified view of the signaling components involved in this pathway. Once generated, PIP3 interacts with Akt, causing conformational changes which expose the two activating phosphorylation sites, T308 and S473 [53]. Upon activation, Akt can then phosphorylate a wide range of proteins (as noted in Fig. 7.2), including tuberous sclerosis 2 (TSC2), a negative regulator of the mammalian target of rapamycin complex 1, or mTorc1 [53].

A component of both mTorc1 and mTorc2, mTor is a serine/threonine kinase with broad activity in cell proliferation, growth, and survival [55]. Although mTor was initially named after its role in the cellular response to rapamycin, acute rapamycin treatment only affects mTor when complexed as mTorc1, a master regulator of protein synthesis (via 4EBP1 and S6K) and cellular nutrient response [55, 56]. mTorc2, on the other hand, is structurally affected only by chronic rapamycin treatment, although not all cell types respond uniformly [57]. mTorc2, unlike mTorc1, is insensitive to nutrient-driven signaling and instead plays a role in cell survival and cytoskeletal organization [55, 57].

7.2.2.1 A. Rapamycin (Sirolimus, Pfizer)

Rapamycin is a macrolide antibiotic with known activity as an antifungal, immunosuppressive, and antineoplastic [58]. All of these effects are due to rapamycin's interactions with immunophilin FKBP12, the complexing of which inhibits

substrate recruitment and catalytic accessibility to mTor, though only when mTor is complexed as mTorc1 [58]. Rapamycin does have some marginal activity against mTorc2 as well [57].

Preclinical Evaluation

Fischer rats implanted IC with the RG2 cell line and treated with rapamycin had modest though statistically significant survival benefit (19.5 vs 24 days, $p<0.01$) [59]. In a second in vivo study, CD1 nude mice implanted with U87MG cells also had a significant survival benefit with rapamycin treatment ($p<0.0001$) [60]. Interestingly, mice implanted with patient-derived glioma lines and treated with single-agent rapamycin in studies from Zhuang et al. and Mendiburu-Eliçabe et al. failed to recapitulate the survival benefit noted in the U87 and RG2 studies, though Zhuang et al. did find that rapamycin may be a radiosensitizer as the combination of rapamycin and RT provided a significant survival benefit over RT alone ($p<0.005$, RT vs rapamycin + RT) [61, 62].

Clinical Evaluation

An initial Phase I study in GBM patients selected for PTEN loss, which included specimen sampling to track drug concentration in the tumor, found that although tumor levels of rapamycin were sufficient to inhibit mTor, the magnitude of mTor inhibition achieved varied widely across all patients. Further, although Ki-67 expression (a marker of cell proliferation) was found to decrease in 7 of the 14 patients after treatment with rapamycin for 1 week, in direct correlation to the amount of mTor inhibition ($p=0.0047$), the level of Ki-67 downregulation was not associated with intratumoral concentration of the drug [63]. A Phase II open-label study in 32 unselected, recurrent, heavily pretreated GBM patients treated with erlotinib and rapamycin showed no improvement in PFS (estimated PFS at 6 months was 3.1 %) or OS with the combination, and patient response was not correlated with PTEN expression [64]. Although response was correlated with p-Akt levels, the statistics were barely significant ($p=0.045$). The comparison of preclinical and clinical rapamycin results indicates that the survival data from U87 and RG2 may not be as reliable as those data from the patient-derived lines.

7.2.2.2 B. Rad001 (Everolimus, Novartis)

Rad001 is a rapamycin ester analog (rapalog) which was designed to overcome rapamycin's instability and insolubility. Similar to rapamycin, Rad001 is an immunosuppressant and an effective inhibitor of mTor activity, specifically when mTor is complexed as mTorc1 [59, 65]. In preclinical models Rad001 is well tolerated even at very high doses [66].

Preclinical Evaluation

In a panel of 17 orthotopically implanted, patient-derived lines, which included seven lines deficient in PTEN, only one line, GBM10, had a significant survival benefit with single-agent Rad001 treatment [67]. The only other line found to respond to Rad001 was a PTEN WT line (GBM22). Molecular analysis to determine specific mechanisms indicated that PTEN status was an insufficient biomarker for response of tumors to single agent Rad001. Although a second group found that single agent Rad001 treatment of animals with U87MG tumors provided significant survival benefit, the clinical relevance of immortalized lines to the patient experience is likely limited [68, 69].

Clinical Evaluation

Rad001 has been tested in one Phase II clinical trial, N057K, which added Rad001 to the combination of TMZ and RT along with adjuvant TMZ. The authors found that the inclusion of Rad001 in this newly diagnosed patient population provided no significant benefit when compared to historical controls (PFS at 6 months was 52 %) [70] (personal communication). Kreisl et al. in a Phase I study testing the combination of Rad001 and gefitinib in 22 recurrent patients found that, though the combination of the two drugs provided some stable disease (8) and partial responses (2), these responses were not durable (PFS at 6 months was 4.5 %). Although Rad001 is supposed to be an improved version of rapamycin, Rad001 fails to provide any more patient benefit than rapamycin does.

7.2.2.3 C. XL765 (Voxtalisib, Exelis)

XL765 is an ATP-competitive, selective, dual pan-Class I PI3K and mTor inhibitor undergoing testing in a wide variety of cancer types [71]. Although many mTor inhibitors, including the rapalogs, have been found to provide treatment benefit in some cancer types, selective inhibition of mTor often leads to compensatory upregulation of Akt, a consequence that is mitigated with usage of a dual PI3K/mTor inhibitor [54, 58, 72, 73].

Preclinical Evaluation

In vitro testing of XL765 conducted in five patient-derived xenograft lines treated with single agent XL765 showed excellent activity against all lines tested, while the combination of XL765 and TMZ showed evidence of synergistic activity in four out of the five lines tested [72]. Although in vivo testing of XL765 and TMZ in one line, GBM39, provided survival benefit over control (XL765 +TMZ vs control, 117 vs 55 days, $p<0.001$), the combination of XL765 and TMZ failed to provide statistically significant benefit over the single agent TMZ arm (117 vs 83 days, $p=0.09$).

Clinical Evaluation

XL765 has currently only been tested in one Phase I study in AGand GBM patients. Fifty-four patients who were either already receiving TMZ treatment (group 1) or were newly diagnosed (group 2) received XL765 either in combination with adjuvant TMZ (group 1) or along with RT and TMZ combined therapy dosing (group 2). Of the 47 evaluable patients, XL765 produced partial response in only two patients (overall response rate of 4 %) and stable disease in 32 patients (68 %) [71]. Inhibition of both PI3K and mTor does not appear to be a useful sensitization strategy, though further studies are necessary to completely rule out dual PI3K/mTor inhibitors as a possible treatment for GBM patients.

7.2.3 Inhibitors of Cell Cycle Progression

The cell cycle and DNA replication are tightly regulated by proteins that either inhibit or potentiate progression of cell division [74]. Retinoblastoma (pRb) and p53 are among the key proteins guiding cell cycle progression, with cell cycle dysregulation, either via the pRb or p53 pathway, a common occurrence in GBM and considered to be a critical step in gliomagenesis (Fig. 7.1) [3]. The high prevalence of cell cycle dysregulation in GBM has generated interest in creating pharmacological agents which can disturb various components of this complex system, though currently very few clinical studies have been conducted with cell cycle inhibitors in GBM.

7.2.3.1 G1/S Inhibitors

The retinoblastoma family consists of three members: Rb (pRb)/p105, p107, and Rb2/p130, all of which are cell cycle regulators via their inhibitory activity against the E2F family of transcription factors [75]. pRb, when hypophosphorylated, binds and inhibits the transcription factor E2F [75]. pRb inhibition is relieved by activated cyclin-dependent kinase 4/6 (CDK4/6) complexed with cyclin D1, which phosphorylates pRb, releasing E2F and ultimately allowing for progression through G1 into S-phase and finally cell division [75]. Inactivation or loss of pRb, which occurs in approximately 25 % of GBM, allows for persistent activation of the E2F family of transcripts and uninhibited cell division [75, 76]. In GBM with intact pRb, deletion of p16 and p14, negative regulators of the complexing of CDK4/6-cyclin D, along with amplification of CDK4/6 has been shown to mediate persistent hyperphosphorylation of pRb [3, 77]. Inhibition of G1/S components is an attractive sensitization strategy in GBM due to the high prevalence of cell cycle dysregulation found in these tumors. Several G1/S inhibitors, including roscovitine, palbociclib, abemaciclib, flavopiridol, and many others, have been tested in a variety of cancers, though only one, palbociclib, has been FDA approved for use in breast cancers.

A. Flavopiridol (Alvocidib, Tolero Pharmaceuticals)

Flavopiridol is a multi-targeted phosphokinase inhibitor with activity against several cyclin-dependent kinases (CDK1, 2, 4, 6, and 7) as well as some receptor tyrosine kinases and signal transducing kinases (i.e., Erk-1) [78]. Flavopiridol exhibits cytotoxicity against both actively dividing cells and resting cells [78]. Though the drug is currently not FDA approved for treatment of any neoplasm, it was designated as an orphan drug for acute myeloid leukemia by the FDA in 2014.

Preclinical Evaluation

Only one in vivo study in the murine GL261 line has been conducted with flavopiridol [79]. In this study, animals with IC GL261 tumors were treated days 7–11 after injection and then harvested on days 14, 21, and 28 for assessment of tumor volume, microvessel density, and apoptosis (by TUNEL). Though there was a significant decrease in tumor volume in mice treated with flavopiridol on day 14 post-injection ($N=6$, $p<0.02$, in comparison to control treated mice), by days 21 and 28, there was no longer any significant difference in tumor volume. Although these data indicate, as the authors note, that flavopiridol is capable of reaching the tumor and penetrating the brain, further long-term survival studies in primary human lines are necessary to accurately represent flavopiridol efficacy preclinically.

Clinical Evaluation

Currently, no clinical trials for GBM have been conducted with flavopiridol.

B. Palbociclib (PD0332991, Pfizer)

Palbociclib is an inhibitor of both CDK4 and CDK6-cyclin D1 kinase activity, with equal inhibition achieved for each kinase [80]. Tumors without pRb expression have been found to be resistant to palbociclib, while those that express pRb are potentially responsive to treatment [80].

Preclinical Evaluation

Single agent administration of palbociclib to mice with either orthotopically implanted U87 or GBM39 xenografts yielded significant benefit in comparison to placebo treatment ($p<0.001$) [81]. In the same study, the combination of radiation and palbociclib in U87 implanted mice further improved survival, though only when RT was administered after palbociclib ($p=0.01$ for single agent RT and palbociclib compared to the combination of palbociclib pretreatment and RT posttreatment). However, the combination of TMZ and palbociclib in a separate cohort of mice did not provide added benefit over the cyclical dosing of TMZ alone (78.1 vs 81.4 days, $p=0.970$). A second study conducted in the patient-derived GBM6 line

also showed some slight though statistically significant ($p < 0.01$) benefit with single agent treatment of palbociclib [82]. Data from these patient-derived lines indicate that palbociclib may have modest efficacy in GBM patients.

Clinical Evaluation

Only one clinical trial in recurrent GBM or gliosarcoma which is Rb positive is currently ongoing (ClinicalTrials.gov Identifier: NCT01227434). At this time no results are available.

7.2.3.2 G2/M Inhibitors

p53 is a key regulator of the cell cycle and a known tumor suppressor. Genotoxic and cytotoxic stress drive p53 to stall cell cycle progression, mainly at the G1/S checkpoint, or induce apoptosis [83]. Figure 7.4 is a simplified illustration of a few of the proteins responsible for DNA damage sensing and cell cycle progression, including p53. Mutation or deletion of p53 is found in approximately 35 % of GBM, though p53 pathway alterations are detected in 70 % of samples tested [3]. MDM2, a negative regulator of p53 function, keeps these cell cycle and proapoptotic pathways in check, and in the case of GBMs with intact p53, MDM2 amplification (found in approximately 11 % of GBMs) inhibits p53 activity [3]. p53-null tumors thus rely heavily on the G2/M checkpoint and the activity of the checkpoint proteins Wee1 and Myt1 to maintain genomic integrity [84–86].

Wee1 and Myt1 are tyrosine kinases in the serine-threonine family of protein kinases that are key regulators of mitotic entry [85]. Wee1's kinase function is specifically directed at phosphorylation of CDK1 at tyrosine 15, an inhibitory phosphorylation that halts cell cycle progression at G2/M, as illustrated in Fig. 7.4 [87]. Once the G2/M checkpoint is successfully completed, Cdc25 reactivates CDK1/Cyclin B by removal of the tyrosine 15 phosphorylation. CDK1/Cyclin B in turn targets Wee1 for hyperphosphorylation at threonine 293, marking it for translocation outside of the nucleus and degradation [87, 88].

In p53-null tumors, pharmacological inhibition of Wee1 may be particularly effective due to synthetic lethality [85]. The idea of synthetic lethality posits that loss or disruption of one gene, "A," can be compensated for by a second gene "B." However loss or disruption of both genes leads to cell death [89]. Thus p53-null tumors which are incapable of maintaining genomic integrity by arresting at the G1/S checkpoint along with pharmacological inhibition of Wee1 (leading to loss of a functional G2/M checkpoint) could potentially cause synthetic lethality. Additional cytotoxic damage introduced after loss of the G1 and G2 checkpoints could potentially allow for propagation of highly lethal adducts, such as those induced by TMZ and other cytotoxic agents. Thus, the combination of a cytotoxic agent like TMZ and a Wee1 inhibitor poses an attractive sensitization strategy to increase efficacy in GBM patients.

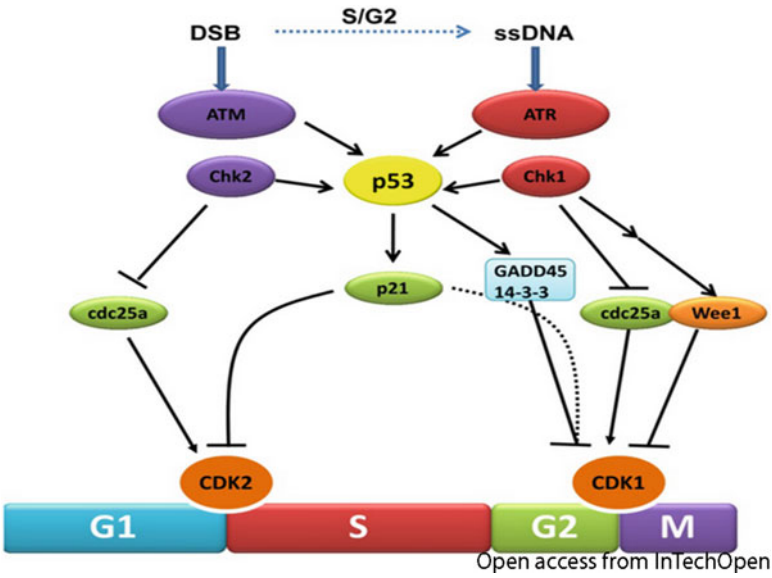

Fig. 7.4 The cell cycle and checkpoint proteins. Induction of DNA damage in the form of a DSB or ssDNA activates DNA damage repair proteins causing arrest at one of the cell cycle checkpoints via p53. Cells lacking p53 activity rely heavily on the cell cycle arrest at G2 driven by Wee1 to maintain genomic integrity [124]. Open access from InTechOpen

A. MK-1775 (AZD-1775, Astra-Zeneca)

MK-1775 is an ATP-competitive, selective inhibitor of Wee1 with single agent in vitro activity in a wide range of tumor cell lines [90], while the combination of MK-1775 and cytotoxic agents such as TMZ and gemcitabine further increases efficacy [91, 92]. Though results from Guertin et al. and Pokorny et al. indicate that p53 status did not predict for single agent or combination efficacy with TMZ, Rajeshkumar et al. did find that p53 status was relevant for the combination of gemcitabine and MK-1775 in pancreatic cancer lines.

Preclinical Evaluation

MK-1775 treatment of the clinically relevant xenografts (GBM22 and GBM12) implanted orthotopically failed to provide any survival benefit over placebo (36 vs 34 days $p=0.15$) [91]. MALDI-MSI data from animals with either IC or flank tumors treated with a single dose of 200 mg/kg MK-1775 indicated that exposure levels in the brain were heterogeneous. Pharmacokinetic data further indicated that drug levels achieved in the brain (maximum 5 %) were likely insufficient to provide therapeutic benefit, even in the highly sensitive GBM22 line. Administration of MK-1775 to mice bearing GBM22 heterotopic tumors provided survival benefit

over placebo (median survival 38 vs 30 days, $p=0.01$), especially when combined with TMZ (median survival with TMZ of 91 days vs 240 days with the combination of TMZ and MK-1775 treated with a protracted dosing schedule, $p=0.02$).

Clinical Evaluation

Although there are no Wee1 inhibitors FDA approved for treatment of any malignancy, over 18 clinical trials (one of which is being conducted in recurrent and newly diagnosed GBM patients) in a wide range of cancer types are listed on clinicaltrials.gov. Preclinical studies of Wee1 inhibitors as a single agent and in combination with FDA-approved cytotoxic agents have shown promising results; thus Wee1 inhibitors may very well be approved for clinical use in the near future [92, 93]. Understanding the Wee1 specific pathways and potential biomarkers as well as the reasons for differential response and lack of brain efficacy as noted from the study above will be essential for successful clinical application of Wee1 inhibitors in the future.

7.3 Resistance Mechanisms to Small-Molecule Inhibitors

Although several different compounds targeting a wide variety of pathways considered to be essential for GBM proliferation and survival have been tested both preclinically and clinically, none have provided benefit significant enough to warrant FDA approval. There are at least six main reasons for the lack of clinical efficacy seen among the drugs noted here. The first is the issue of pathway redundancy, while the second is the generation of secondary mutations with small-molecule treatment. The third highlights the difficulty of targeting extremely complex and incompletely understood pathways. The fourth point will focus on specific differences in EGFR mutations between NSCLC and GBM and why inhibitors, such as erlotinib and gefitinib, may be unrealistic options for GBM treatment. The fifth point covers the heterogeneous nature of GBM tumors, and the sixth will consider the unique environment presented by the brain and the obstacles that must be overcome when attempting to introduce novel therapeutics.

First, pathway redundancy is a key impediment to development of maximally effective drugs. Although novel therapeutics may target proteins and pathways considered key for cell survival, compensatory upregulation of untargeted pathways provides cells the ability to utilize alternatives which are still available. For instance, many NSCLC patients undergoing treatment with EGFR inhibitors regularly develop resistance when cells upregulate other pathways including c-MET, IGFR, VEGFR, and PDGFR [17]. Systems-level studies of the ErbB family and other key mitogenic factors commonly found to be upregulated in GBM have shown that these factors have “modularity” and “show redundancy of regulatory circuits” [94]. In fact, neither high brain accumulation of targeted drugs nor effective downregulation of drug target can guarantee patient efficacy [94]. Results from a Phase II trial

definitively show that gefitinib crosses into the brain in very high concentrations (brain concentration 22 times higher than plasma) and is capable of efficiently reducing EGFR phosphorylation [94]. Yet EGFR perturbation did not lead to modulation of any downstream signal transducers, and in fact, EGFR phosphorylation was not even found to be a factor for "overall activation of the pathway" [94]. Similar issues have also been noted in hepatocellular carcinoma patient resistance to sorafenib, in prostate cancer patient resistance to Rad001, and in breast cancer patient resistance to lapatinib, in which cross talk and upregulation of untargeted or compensatory pathways are recognized as key mechanisms of acquired resistance [73, 95, 96]. If further analysis of the EGF pathway concludes that there is redundancy and modularity, those findings will significantly affect future attempts to target EGFR with single agents such as gefitinib and erlotinib.

A second mechanism of resistance is the generation of secondary mutations after initial tumor treatment. Along with upregulation of compensatory pathways, approximately 50 % of NSCLC patients treated with EGFR inhibitors will become resistant by emergence of a T790M mutation, which replaces the threonine for a bulkier methionine, preventing binding of the inhibitor, while maintaining catalytic activity [17, 97]. Similarly TMZ resistance in GBM patients has been linked to mutation of the mismatch repair gene, MSH6, while sunitinib resistance in gastrointestinal stromal tumors has been correlated with secondary mutation of KIT [98, 99]. Since regular biopsy of patient tumors is unrealistic in GBM, assessment of tumors at recurrence, when possible, along with generation of patient-derived xenografts with resistant phenotypes will allow for a better understanding of key mutations leading to therapeutic resistance.

A third mechanism of resistance is the complexity of the pathways targeted and the difficulty of correctly implicating biomarkers. In 2005, Mellinghoff et al. reported that PTEN status (WT or deleted) was a predictive marker for EGFR inhibitor efficacy. However in all of the EGFR and PI3K inhibitor trials described above in which PTEN status was noted, PTEN status failed to definitively correspond with inhibitor efficacy. Other groups have more recently implicated phosphorylation of PTEN at tyrosine 240 [100] and upregulation of EGFRvIII and PI3Kp110δ [101] as possible drivers of RTKi resistance as opposed to the more binary PTEN status. These findings indicate that our current level of pathway understanding, particularly for EGFR, is insufficient. In many of these failed clinical trials, a better understanding of molecular markers of response and the pathways targeted could lead to improved patient selection and study results.

A fourth resistance mechanism focuses specifically on the unique EGFR mutations found in GBM in comparison to those found in NSCLC tumors. Although erlotinib and gefitinib have both been FDA approved for treatment of NSCLC, a tumor with a high rate of EGFR activity, similar to GBM, efficacy achieved with these same inhibitors in GBM remains disappointing [19, 102, 103]. In-depth analysis of clinical lung cancer samples indicates that these tumors often have a high percentage of EGFR mutations, similar to GBM. However, the majority of EGFR mutations found in lung cancers are kinase domain (KD) mutations, as opposed to the extracellular domain (EC) mutations more commonly found in GBM [16].

Interestingly, a comparison of lung cancers that had either KD or EC mutations found that lines with EC mutations, when treated with gefitinib or erlotinib, were significantly more resistant to both erlotinib and gefitinib, in comparison to lines with KD mutations [16]. Although the idea of oncogene addiction posits that GBM cells that are EGFRvIII are addicted to the EGFR signaling pathway and EGFR inhibition should provide benefit, erlotinib and other similar inhibitors have not delivered [16]. The unique mutations found specifically in GBM could play an essential role in understanding this lack of efficacy.

Fifth, GBM is, by definition, a highly heterogeneous tumor [3, 4, 104]. Although whole genome sequencing and histopathology allow clinicians to categorize each tumor by its specific genetic abnormalities, sequencing and pathology results are limited by the samples taken [105, 106]. Unrepresented or underrepresented subpopulations of cells with different expression profiles which do not respond to the targeted treatment are a source for tumor resistance [107]. Although newer techniques such as single cell RNAseq allow a deeper understanding of tumor clonality and the possibility of improved chemotherapeutic targeting, significant cost and the issue of sampling bias mean that RNAseq is not yet ready for regular clinical use [105]. Further, all methods for categorizing tumors provide only a snapshot of the tumor as it is in the instant that the tissue samples are taken. Some of the studies noted above analyzed treatment efficacy based upon patient samples that were taken at initial surgery [29, 63, 64]. However a patient who presents at recurrence and is treated with a targeted therapy based upon limited and outdated pathology or sequencing data may not respond to treatment because the tumor has changed and the collected samples are not actually representative of the current tumor. Regular biopsy of GBMs is an unrealistic option; thus the development of assays that can be used to regularly monitor tumor expression patterns, via tumor-specific circulating DNA, for instance, is necessary to improve real-time molecular characteristics [108]. In essence, though targeted therapeutics are potentially promising options for GBM patients, limitations on histopathology and sequencing sample acquisition mean actual benefits are still quite limited.

The sixth and arguably one of the most important mechanisms of resistance is the unique environment of the brain and the blood-brain barrier (BBB) as significant impediments to effective drug delivery. Although the BBB provides essential protection to the normal brain against potential neurotoxins and harmful cells, it also acts as a safe haven for GBM tumors, keeping out commonly used chemotherapeutics [109]. Transcellular and paracellular passage into the brain parenchyma is regulated by two main mechanisms: the ATP binding cassette (ABC) transporters, located luminally on the endothelial cells, and tight junctions and adherens junctions present between the endothelial cells which constitute the brain vasculature, along with pericytes, astrocytes, and perivascular macrophages (illustrated in Fig. 7.5) [109]. Brain access is thus limited to diffusion of “very small or gaseous molecules (e.g., water, carbon dioxide)”; passive diffusion of larger solutes, which is limited by lipid solubility, electrical charge, and molecular weight; and active transport via specific solute carriers [109].

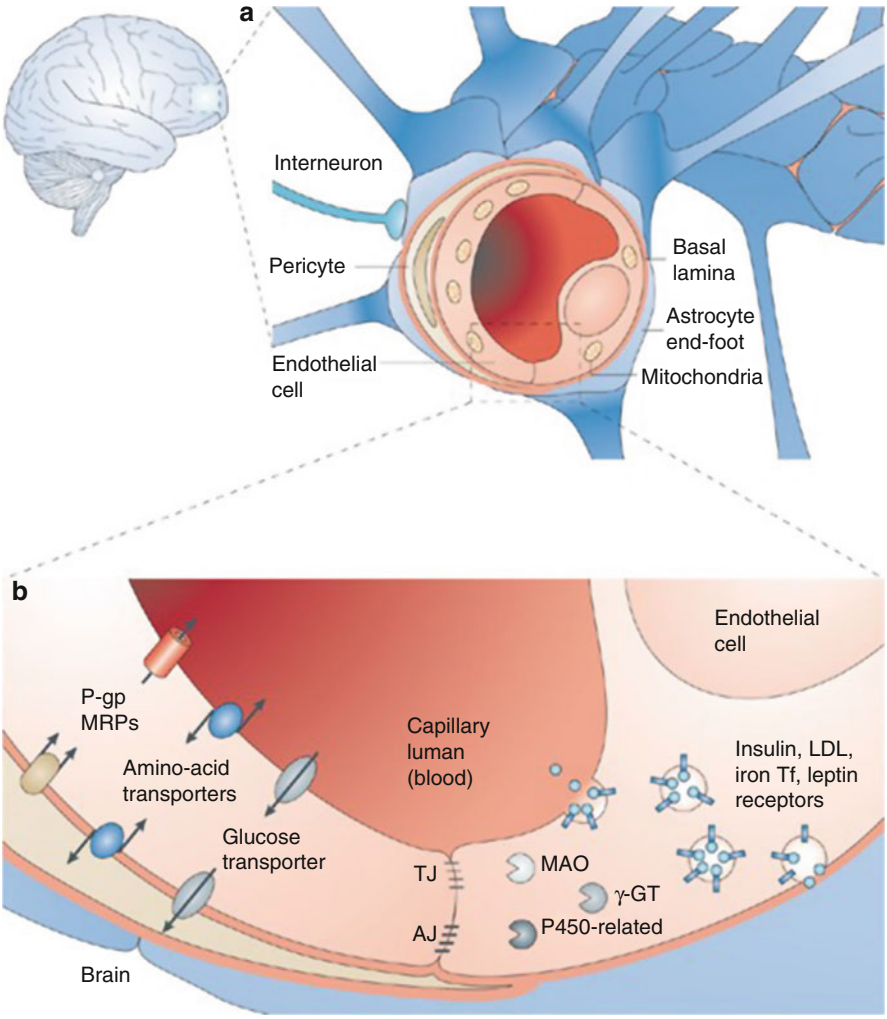

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Fig. 7.5 A diagram of various components of the blood-brain barrier. (**a**) Schematic of various cell types which line the blood vessels and constitute the blood-brain barrier, pericytes, endothelial cells, and astrocytes. (**b**) A detailed schematic of the luminal location of the drug pumps (p-gp and MRPs) along with the tight junctions (TJ) and adherens junctions (AJ) at the endothelial cell junctions which line the blood vessels. Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery [125], copyright (2007)

The ABC transporters p-glycoprotein (p-gp or multidrug resistance protein 1 (MDR1), also called permeability glycoprotein/ABCB1) and breast cancer resistance protein 1 (BCRP1/ABCG2) interact with and limit transcellular permeability of therapeutics present in the vasculature and prevent access to the brain by actively pumping out substrates. All but two (XL765 and MK-1775) of the small-molecule

Table 7.1 Summary of p-gp and BCRP1 substrate binding results for all compounds discussed in the chapter

	p-gp substrate	BCRP1 substrate
Erlotinib	X	X
Lapatinib	X	X
Gefitinib	X	X
Sorafenib	X	X
Sunitinib	X	X
Rapamycin	X	
Rad001	X	X
XL765	U	U
Flavopiridol	X	X
Palbociclib	X	X
MK-1775	U	U

All sources are referenced in the chapter text
U = data are unavailable

inhibitors discussed above have been shown to be a substrate for at least one of the drug pumps found in the brain (data summarized in Table 7.1) [66, 91, 102, 103, 110–116]. Inhibitors that are drug pump substrates may provide excellent therapeutic benefit in vitro (as noted for the Wee1 inhibitor MK-1775, [91]). However, substrates simply cannot accumulate in sufficient levels to have a meaningful therapeutic effect in the brain (hence the low brain accumulation of 5 % noted in PK results with MK-1775) [91]. Most GBM cells express P-gp at the level of the normal brain, with some evidence indicating that a subgroup of glioma cells express CD133, a proposed stem cell marker, along with increased expression of BCRP1 [107, 117]. Thus there may be a population of stem cells in the tumor which are able to evade drug effects by over-expression of drug pumps, ensuring propagation of the tumor even after chemotherapeutic treatment. Studies which take into consideration the basal expression of drug pumps in patients, as well as whether individual compounds bind to drug pumps, may be necessary for improved efficacy in the future.

The second component of the BBB, the tight junctions, also poses a significant barrier to effective chemotherapy delivery [109]. The tight junctions consist of claudin-5, occludin, and junctional adhesion molecules (JAMs) along with the intracellular adaptor proteins such as ZO-1, which link the transmembrane tight junction components with the actin cytoskeleton. The adherens junctions, of which VE-cadherin is one of the most important components, in combination with the tight junctions, connect the endothelial cells together and provide a relatively impermeable barrier in the normal brain [109, 118]. Although a compromised (“leaky”) BBB is considered to be a hallmark of GBM, careful studies of the brain environment actually indicate that patient tumors present with a heterogeneous distribution of BBB openness, with tumor cells found in areas of open and closed BBB [109]. Along with differential BBB integrity, Ortensi et al. also found that tumors often have increased expression of pro-invasive and stem cell markers in the tumor rim, the outer edge of tumor cells, in comparison to the more open and central tumor core [119]. It is possible that invading GBM cells are capable of adapting to the brain environment surrounding them and upregulating factors that improve their likelihood of survival [119]. It is likely these cells in the rim that prove to be difficult to reach and a significant barrier to effective small-molecule inhibitor treatment.

7.4 Future Perspectives

Considering the lack of promising results achieved with just the few chemotherapeutics described here, it is clear that new methods of drug delivery, discovery, and BBB penetration need to be created, along with a better understanding of the signaling pathways and factors found specifically in GBM tumors. To that end, a variety of modalities for improving brain access are being considered from microbubble injections targeted with focused ultrasound causing vibrations that can temporarily open the BBB [120] and bradykinin receptor agonists [121] to the drug pump inhibitors elacridar and tariquidar, which directly interact with and inhibit the substrate binding abilities of both p-gp and BCRP1 [122]. Although none of these modalities have yet been found to effectively overcome the BBB and allow for improved drug delivery, preclinical and clinical studies are still ongoing. Another promising option is to design inhibitors specifically for use in the brain with characteristics that allow for improved brain access and efficacy [123]. Regardless of the methodology, unless cancer researchers, clinicians, and drug developers can start to rethink the approach to GBM treatment, promising therapeutics will continue to fail at the clinical level.

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Chapter 8
Imaging Targeted Therapy Response and Resistance in Glioblastoma

Kate Connor, Monika A. Jarzabek, Kieron White, Andreas H. Jacobs, and Annette T. Byrne

Abstract Glioblastoma (GBM) is the most common and most malignant tumour of the central nervous system. Despite recent advances in understanding the biology of GBM, the disease still remains in desperate need of effective treatment options resulting in long-term improvements in overall patient survival. Molecularly targeted therapies, anti-angiogenic therapy and immunotherapy are promising avenues under investigation as future therapeutic options. Molecular imaging (MI) is an essential tool in the development of these targeted treatments, both preclinically and clinically. MI facilitates the preclinical study and interrogation of potential therapies. MI further supports non-invasive, longitudinal monitoring of therapy response and allows the study of emergence of treatment resistance via an imaging-guided therapeutic approach.

Keywords Glioblastoma • Targeted therapy • Resistance • Molecular imaging

Abbreviations

ADC	Apparent diffusion coefficient
BBB	Blood–brain barrier
BOLD-fMRI	Blood-oxygen-level-dependent contrast fMRI
Cho	Choline
Cr	Creatine/phosphocreatine
CT	Computed tomography

K. Connor • M.A. Jarzabek • K. White • A.T. Byrne (✉)
Centre for Systems Medicine, Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland
e-mail: annettebyrne@rcsi.ie

A.H. Jacobs
European Institute for Molecular Imaging (EIMI), Westfalian Wilhelms University (WWU) Münster, Münster, Germany

Department of Geriatrics and Neurology, Johanniter Hospital, Bonn, Germany

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*, Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_8

DKI	Diffusion kurtosis imaging
DSC-MR	Dynamic-susceptibility weighted contrast MR
DTI	Diffusion tensor imaging
EGFR	Epidermal growth factor receptor
FA	Fractional anisotropy
FLI	Fluorescence imaging
GLUTs	Glucose transporters
Lac	Lactate
LGG	Low grade glioma
Lip	Lipids
mI	Myoinositol
MI	Molecular imaging
MRI	Magnetic resonance imaging
MRS	MR spectroscopy
NAA	N-acetyl aspartate
PET	Positron emission tomography
rCBF	Relative cerebral blood flow
rCBV	Relative cerebral blood volume
SPECT	Single photon emission computed tomography
TT	Transit time

8.1 Introduction

Glioblastoma (GBM) is the most commonly diagnosed primary malignancy of the central nervous system (CNS) in adults. Each year there are approximately 23,000 cases diagnosed in the USA leading to approximately 15,000 deaths. Despite the many improvements made in the current standard of care over the last decade, patients diagnosed with GBM have a devastatingly low median life expectancy of less than 2 years.

Glioma is a term used to describe any tumour arising from the glial cells of the brain or spine, and GBM tumours arise from astrocytes or oligodendrocytes. Primary GBM occurs spontaneously in patients around 50 years of age and are characterised by genetic alterations, such as overexpression of epidermal growth factor receptor (EGFR). Secondary GBM develops by a stepwise progression from low grade glioma (LGG) through an increasing accumulation of genetic alterations, such as p53 and pRB mutation. GBM tumours are defined as the highest grade of glioma (HGG, grade IV) under the World Health Organisation (WHO) grading system [1]. These HGG are highly vascularised, often necrotic and have a propensity to infiltrate the surrounding structures of the brain. They are also associated with inflammation and oedema [2]. GBM is marked for its molecular heterogeneity both inter- and intra-tumourally [3]. The standard of care for treatment of GBM consists of maximal neurosurgical tumour resection with concomitant chemotherapy and radiotherapy (RT), followed by adjuvant chemotherapy. While surgically incurable

due to its infiltrative margins, resection in an imaging-guided approach is a vital step in disease management [4]. Currently, the most widely implemented treatment regimen is referred to as the 'Stupp protocol'. Under this protocol, patients receive daily treatment with the alkylating cytostatic drug temozolamide (TMZ), parallel to radiotherapy, followed by six cycles of adjuvant TMZ. This approach has demonstrated significant benefits to overall patient survival [5].

Despite recent advances and the emergence of novel therapeutic strategies, treatment remains palliative and the median patient survival remains dramatically short. The unfavourable prognosis associated with GBM is due primarily to the propensity of the tumour to develop resistance to therapy and ultimately to recur.

Development of molecularly targeted therapeutics with anti-GBM activity is, therefore, a critical and challenging goal, which is vital in order to improve patient survival rates. Several approaches are currently under investigation; however, none have yet demonstrated clear effects on overall patient survival [6]. A key factor in the development and evolution of new anti-GBM targeted therapies is the ability to monitor in real time the physiological and biochemical effects of therapy using molecular imaging (MI) and certainly to assess treatment resistance both in preclinical and clinical studies. MI allows optimisation and acceleration of novel targeted therapies. Thus, the pipeline for new therapy development relies on a combination of several imaging techniques in a multimodal strategy in order to maximise the information gained [7, 8].

8.2 Molecular Imaging Techniques Applied in GBM

Traditionally imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI) and intra-operative ultrasound were routinely used to monitor the therapeutic effects of cancer interventions. There are now, however, many additional non-invasive imaging modalities available, each with unique advantages, disadvantages and applications. MI is a powerful, non-invasive tool in the diagnosis, assessment and monitoring of patients harbouring brain tumours, which when employed positively impacts patient management. MI can be broadly defined as the in vivo characterisation and measurement of biologic processes on both cellular and molecular levels.

Currently, several of the MI modalities are used to evaluate GBM in preclinical and/or clinical settings. These may be broadly categorised into magnetic resonance imaging (MRI) approaches (DCE-MRI, DSC-MRI, DWI, DTI, BOLD-fMRI, ^{1}H MRI), nuclear-radioisotope-based imaging such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) and optical imaging (BLI, FLI). Primarily, these technologies facilitate the diagnosis and grading of the primary tumour, to record the extent of infiltration into the surrounding brain parenchyma, assist in planning and navigation in surgery intra-operatively and to monitor and assess treatment response and patient prognosis. Information gained from the use of MI is vital not only in deciding the treatment a patient will receive, but also in longitudinally monitoring the response to these treatments, and

importantly allows the quantitative and qualitative studies of the response and resistance [9]. In addition to traditional imaging techniques, further availability of improved contrast agents [10, 11] and radioligands has expanded the accessibility of real time information regarding tumour response to therapy. Each MI modality and its respective applications are further discussed below (Table 8.1).

Table 8.1 Imaging modalities employed in GBM assessment

		Application
MRI	T1-weighted	• Assessment of tumour location and volume • Determination of necrotic regions • Assessment of mitotic activity (DCE-MR)
	T2-weighted	• Assessment of tumour cellularity • Assessment of oedema
	MRS	• Detection of metabolites: • Assessment of cell proliferation (Cho/Cr, NAA/Cho) • Detection of membrane turnover (choline) • Detection of necrosis (creatine, phosphocreatine, lipid levels)
	DWI	• Assessment of tumour cellularity • Determination of ADC
	DTI	• Assessment of tumour invasiveness • Quantification of FA
	DKI	• Aids in differentiation of tumour grade
	DSC	• Determination of angiogenesis • Assessment of rCBV
	BOLD-fMRI	• Determination of different brain regions
PET	^{18}F-FDG	• Glucose metabolism • Visualisation of tumour
	^{18}F-FLT	• Assessment of cell proliferation
	^{18}F-FMISO	• Assessment of hypoxia
	^{11}C-MET	• Assessment of tumour size • Visualisation amino acid transport • Determination of invasion
	^{11}C-CHO	• Visualisation of differentiation
	^{18}F-RGD	• Assessment of avb3 integrin expression
SPECT	^{123}IMT	• Identification of tumour changes in response to therapy
	^{99m}Tc	• Tumour grading • Cellular proliferation
Optical	BLI	• Tumour location • Presence of metastasis • Tumour response to therapy
	FLI	• Tumour location • Presence of metastasis • Tumour response to therapy

MRI magnetic resonance imaging, *MRS* magnetic resonance spectroscopy, *DWI* diffusion weighted imaging, *DTI* diffusion tensor imaging, *DKI* diffusion kurtosis imaging, *DSC* dynamic susceptibility contrast enhanced MR, *PET* positron emission tomography, *FDG* fluorine-2-deoxy-D-glucose, *FLT* fluorothymidine, *MET* methionine, *CHO* choline, *FMISO* fluoromisonidazole, *SPECT* single photon emission computed tomography, *IMT* alpha-methyl-tyrosine, *Tc* technicium, *BLI* bioluminescence imaging, *FLI* fluorescence imaging

8.2.1 Magnetic Resonance Imaging (MRI)

MRI is the gold standard for imaging of brain tumours and has traditionally been used in the assessment of a variety of CNS abnormalities including tumours, metastases, infections and vascular diseases. The primary role of MRI in the initial brain tumour evaluation includes determining the location of the lesion (intra-axial vs. extra-axial), establishing the specific tumour location within the brain for treatment/biopsy planning, evaluating mass effect on the brain and ventricular system, determining the architecture of tumour vasculature, and used along with physiologic MRI sequences may suggest a possible diagnosis.

Several advanced MR techniques facilitate the monitoring of changes in tumour characteristics such as tumour size, vasculature, or perfusion, and also facilitate the study of drug responses [12]. These advanced MR techniques produce high-resolution images which may be utilised in assessing a number of molecular tumour features including cellularity and invasiveness, mitotic activity, and also vascular permeability, blood flow, blood volume and angiogenesis [13].

MRI relies on both tissue density and tissue relaxation properties to produce images. Often MR imaging requires the use of a contrast agent which can be used to assess the blood–brain barrier (BBB) damage. As a result, specific contrast agents capable of crossing the BBB have been designed due to the low endogenous permeability of the BBB [14]. Notably, some contrast agents are unable to cross the intact BBB and result in images of poorer resolution (e.g. images of LGG which has minimal BBB degradation). It is important to note that metabolic imaging modalities such as PET and MRS are, therefore, also necessary to not only achieve differential diagnosis but also further understand the tumour characteristics.

8.2.2 MR Imaging of Cellularity

An important application of MR is the evaluation of tumour cellularity and tumour invasiveness and can be carried out using both T2-weighted MRI and diffusion-weighted imaging (DWI or DW-MRI). Knowledge of tumour cellularity is important as it can inform as to the density and (indirectly) grade of the tumour. T2-weighted MRI is one of the basic pulse sequences of MRI, useful in the detection of oedema, inflammation and visualisation of white matter tracts [15]. DWI studies the Brownian motion of water molecules in order to generate images with greater contrast and higher sensitivity. This technique allows quantification of the apparent diffusion coefficient (ADC, mm^2/s). ADC is a value assigned to a defined region of interest (ROI) which represents the impedance of water molecule diffusion. It is often possible to correlate ADC with tumour size [16] to facilitate tumour grading [17]. Furthermore, ADC is often inversely correlated with cellularity [18]. However, this is inconsistent as necrotic regions common in HGG often contribute to a higher ADC value [15, 16]. Additionally, ADC values do not accurately represent the cellular heterogeneity present in HGG, due to the use of only specific ROIS for analysis which may not embody the tumour as a whole.

A second important use of MR is in the assessment of tumour invasiveness which, in turn, informs on the aggressiveness of the tumour. Conventional MR alone struggles to accurately assess the degree of tumour invasion due to the overlapping regions of inflammation and oedema with tumour cells. Diffusion tensor imaging (DTI) may instead be used. DTI creates a three-dimensional image of diffusion and allows the quantification of fractional anisotropy (FA) which facilitates visualisation of the white matter tracts of the brain and any changes they may undergo upon tissue injury. DWI/DTI is highly sensitive and has been shown to display tissue injury more rapidly than T1-weighted or T2-weighted MRI [19]. Diffusion Kurtosis Imaging (DKI) is an extension of DTI which assumes ideal Gaussian distribution of water movement and, while further studies are needed, has been shown to aid in the discrimination between HGG and LGG [20, 21].

It has previously been demonstrated that oxygenated and deoxygenated blood have magnetic properties [22] and can reflect acute (perfusion-related) tissue hypoxia [23]. Blood-oxygen-level-dependent contrast fMRI (BOLD-fMRI) is a method applied to visualise tumour blood flow allowing identification of different regions of the brain. Sensitivity of this method however is dependent on the rate of flow of oxygenated blood to the region under investigation.

8.2.3 MR Imaging of Mitotic Activity

A number of MR techniques are also employed in the imaging of mitotic activity allowing a correlation with prognosis. T1-weighted DCE-MRI and DWI may be used in this context. Correlation with Ki67 status can further inform this to reflect mitotic activity.

8.2.4 MR: Imaging of Angiogenesis

Imaging of angiogenesis is an essential tool in the grading of gliomas and in the assessment of response to therapy and treatment resistance. Angiogenesis may be histologically measured via assessment of microvascular density (MVD) or microvascular area (MVA); however, this is an invasive process requiring biopsy [24, 25].

Dynamic-susceptibility weighted contrast MR (DSC-MR) is a method of perfusion-weighted MR and an essential non-invasive tool in angiogenic studies. With a model that assumes that a contrast agent is restricted to the intravascular compartment, assumptions of relative cerebral blood volume (rCBV), relative cerebral blood flow (rCBF) and mean blood transit time (TT) may be made. An issue which arises in imaging angiogenesis is the rapid extravasation of the contrast agent due to leaky vasculature, resulting in underestimation of rCBV. Capillary permeability is another feature of angiogenesis in HGG [26].

8.2.5 MR Imaging of Tumour Metabolites and Necrosis

Proton MR spectroscopy (MRS) is a technique used to non-invasively measure the levels of a variety of brain metabolites in vivo and is complementary to MRI [27]. NAA (N-acetyl aspartate), choline (Cho), creatine/phosphocreatine (Cr), lactate (Lac), Lipids (Lip), myoinositol (mI) and glutamine/glutamate are commonly used in clinical MRS studies [28]. Each metabolite displays a unique peak in the spectrum and appears at a known frequency. These resonance peaks are as follows: NAA 2.02 ppm; choline 3.2 ppm; creatine/phosphocreatine 3.0 ppm; lipids 0.9–1.5 ppm; lactate 1.33 ppm; myoinositol 3.56 ppm; glutamine and glutamate 2.2 and 2.4 ppm [29]. The identification and quantification of these metabolites allow a greater understanding of the physiological state of a tumour: NAA is indicative of axonal integrity and neuronal density; choline fluctuations are associated with cell division and membrane turnover; creatine and phosphocreatine are marks of brain energy metabolism and reductions indicate tissue death; lipid increases are indicative of necrosis; higher level of myoinositol is indicative of low grade malignancy; finally, glutamine and glutamate are indicative of accelerated cell proliferation. No tumour-specific metabolite has yet been labelled; however, the ratios of certain metabolites relative to each other have been studied. Cho/Cr and NAA/Cho ratios have been studied to identify cellular proliferation within particular tumour regions. The use of these metabolites with regards to response to therapy will be discussed in further detail below. It should be pointed out that in contrast to other advanced MRI techniques, MRS has a limited spatial resolution (>1 mm).

T1-weighted MRI is an effective tool in the identification of necrotic regions of tissue. Necrosis is caused by tumour hypoxia resulting from insufficient tissue perfusion; however, it may also be a result of radiation treatment [30]. It is essential that radiation necrosis can be distinguished from necrosis present in recurrent or HGG. The appearance of treatment-induced tissue necrosis on conventional imaging and its associated clinical symptoms are similar to brain tumour recurrence and differentiation is therefore difficult. As biopsy is the most efficient method of differentiation, a non-invasive method is desirable.

In T1-weighted MRI, necrotic regions are commonly less enhanced and are easily visualised when compared to normal tumour and normal tissues. MRS may also be used in the identification of necrotic regions, as the NAA/Cho ratios and the lactate and lipid peaks may be indicative of necrosis. Choline levels have been shown to fluctuate and finally decrease when necrosis begins to appear. Inversely, lactate and lipid levels have been documented to increase upon development of necrosis due to the tissue destruction and cell lysis which occurs in necrosis [31].

8.2.6 Radioisotope Imaging

Compared to the frequency of use of MR in GBM patients, radioisotope imaging such as PET and SPECT is mostly employed in specialised Centres as specific radiotracers have to be produced on-site and requires a powerful set-up including

cyclotron and radiochemistry assuring GMP-based radiotracer production. PET is an inherent molecular imaging technique originally developed to quantify glucose or oxygen consumption of the brain. Values are determined in μmol/100 g/min. Over the past decades PET has evolved to be a highly useful clinical tool for studying tumour progression and treatment effects [12]. PET requires the use of radiotracers which are composed of radionuclides incorporated into common biological molecules such as glucose, ammonia or water. These radionuclides have short half-lives which commonly do not occur naturally. Various tracers are used in PET imaging for gliomas including ^{18}F-FDG, ^{18}F-MET, ^{18}F-FET, ^{18}F-FLT, ^{18}F-FMISO, ^{18}F-Fluciclatide, ^{18}F-Galacto-RGD and ^{11}C-CHO. These radionuclides decay *via* positron emission. Upon administration, the radionuclide is taken up in the appropriate location/tissue where positron emission causes the production of two gamma rays upon interaction occurs with an electron. These gamma rays are then detected allowing images to be reconstructed in 3D using appropriate software. Areas of concentrated tracer accumulation can then be visualised.

Tumour cells tend to exhibit a high level of glucose metabolism in addition to high expression of glucose transporters (GLUTs) when compared to non-tumour cells. As a result ^{18}F-FDG can be used in HGG due to its high uptake level. The FDG molecule acts like glucose during initial enzymatic reactions within cells, but the altered (-deoxy-) structure prevents further metabolism with subsequent accumulation. Normal brain tissue displays high levels of endogenous glucose uptake, particularly in the cerebral cortex and basal ganglia. Therefore, the standard use of ^{18}F-FDG is not useful in identifying malignancies of the brain due to high physiological background signal [32]. Several alternative radiotracers with improved noise-to-signal ratio have therefore been developed which includes amino acid tracers, radiolabelled choline and thymidine.

Amino acid tracers such as MET and FET are being taken up by amino acid transporters of tumour vasculature [33]. They serve excellent tumour-to-background signals allowing delineation of infiltrative tumour parts, targeted stereotactic approaches and the differentiation of biological active tumour parts from radiation necrosis [34–37].

^{18}F-FLT has also shown promise as a PET tracer as it may be used to identify proliferating tumour cells in vivo. Preferential uptake of thymidine by proliferating cells results in a higher uptake of this tracer in tumour cells, when compared to non-tumour cells. Studies have validated distinct ^{18}F-FLT uptake in GBM patients [34] and intracranial GBM orthotopic tumours and not the surrounding tissue, which could be further correlated with tumour size [38]. However, the drawback of FLT is the limited ability to pass the intact BBB. Therefore, FLT-based glioma diagnosis is restricted to HGG with disturbed BBB [9, 37, 39–42]. Clinical studies implementing FLT as early read-out parameter for anti-proliferative HGG therapies are currently ongoing.

Additionally glioma differentiation can also be assessed using ^{11}C-CHO (C-Choline). This radiotracer probably behaves in a similar manner than MET and FET [43]. Furthermore, the recent development of ^{18}F-RGD based tracers will allow an improved assessment of integrin expression in the gliomas vasculature [44].

SPECT is a further imaging technology used to generate 3D images of brain tumours. SPECT imaging requires the use of a tracer which directly emits gamma radiation such as technicium-99m (^{99m}Tc), Iodine-123 (^{123}I), Gallium-67 (^{67}Ga) and Lutetium-177 (^{177}Lu). ^{123}I-alpha-methyl-L-tyrosine (IMT) is an amino acid analogue which has been investigated for its use as a tracer in SPECT. Uptake of IMT is not dependent on the presence of BBB damage, and has been studied in the context of metabolic activity and shows promise in the detection of LGG [45]. Results are comparable to MET- and FET-PET.

8.2.7 Optical Imaging

Optical imaging is an essential tool employed in the preclinical assessment of GBM. Bioluminescence imaging (BLI) has been employed in the study of many cancer models and notably is an important tool in GBM tumour models. BLI is a form of optical imaging which relies on light production as a result of an enzymatic reaction, namely, that of luciferase and its substrate luciferin (or coelenterazine). Firefly luciferase, in the presence of ATP and oxygen, is an oxidative enzyme which induces the oxidation of luciferin producing oxyluciferin. Oxyluciferin contains a peroxide linkage resulting in its unstable state. Upon the return of this excited intermediate to its more stable ground state emission of a photon allows detection using the appropriate imaging method. Luciferase is useful as a reporter of transcriptional activity of cells in vitro, as well as in vivo applications such as whole animal imaging, tumour and metastasis imaging and ex vivo imaging. Moreover BLI allows longitudinal monitoring of tumour growth in response to therapy and also development of metastasis, and allows imaging of multiple animals at once [46–48]. There are, however, disadvantages to BLI, including the prerequisite of a system which stably expresses luciferase, and therefore cells often must be manipulated in order to express this. Moreover, methods to correct for scatter and attenuation have not been implemented (yet) for BLI, preventing exact 3D signal localisation and quantification. BLI is therefore limited to use in preclinical studies; however, its importance must not be underestimated [49].

Fluorescence imaging (FLI) is a second form of optical imaging which uses fluorescent dyes and molecular labels in order to visualise cellular structures and dynamics. FLI allows a broad range of observations including the location and dynamics of gene expression, protein expression and molecular interactions in both cells and tissues. FLI relies on the release of a detectable level of light from the labelled cell or tissue. FLI is primarily used in vitro due to the issues arising from low light penetration of tissue when implemented in vivo.

In order to image GBM in vivo in small animal models a newer detection system has, therefore, been implemented; near-infrared fluorescence (NIRF). This technique uses wavelengths in the range of 700–900 nm in order to minimise the autofluorescence of tissues detected and lessens the absorbance and scattering of light by tissue [50].

The use of these imaging techniques in evaluating response to therapy will be discussed in further detail below.

It should be pointed out that ALA-based optical imaging intra-operatively in patients with gliomas is increasingly being used for the past decade as it has been demonstrated that it detects tumour tissue intra-operatively which serves for improved resection which is related to improved overall survival [4].

8.3 Targeted Therapy in GBM

Targeted therapies differ from chemotherapy in a number of ways including often being cytostatic rather than cytotoxic, and acting on a specific molecular target with the aim of causing minimal damage to normal cells. Many different molecularly targeted therapies are under development, with the objective to increase efficacy of the current standard of care when used in combination, or for use as adjuvant therapy. As pointed out above, HGG harbour an assortment of genetic alterations, including epigenetic modifications, point mutations, translocations, amplifications and deletions which modify gene function and deregulate the normal cell cycling and signalling patterns of these cells. These alterations, while increasing the understanding of the genetic complexity of the disease, also create the potential for a variety of targeted treatment options. Moreover, despite this overall genetic heterogeneity, there are several common alterations broadly identified in glial tumours, providing targets which may be widely applicable; including alterations certain growth factors or their respective receptors [e.g. epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cell cycle regulating proteins and several components of the phosphoinositide-3-kinase (PI3K) pathway]. In a large scale review carried out by Seystahl et al. the need for improved standard of care in GBM is greatly highlighted. This review focused on a large number of clinical trials relating to GBM treatment approaches, none of which have provided an improved stance on the established standard of care; bevacizumab or chemotherapy with an alkylating agent [51].

Resistance to therapy is a significant, ongoing issue in GBM patients with a population of GBM patients resistant to TMZ. This TMZ resistance is as a result of multiple factors, primarily the hypomethylation of O-6-methylguanine-DNA methyltransferase (MGMT); a DNA repair enzyme. Expression of MGMT results in a more efficient DNA repair process thus reducing the efficacy of TMZ [52]. In addition to this, several further molecular pathways have been implicated in resistance including the EGFR pathway [53] and the PI3K pathway which will be discussed in further detail below (Fig. 8.1).

Targeted therapies for GBM may therefore be broadly classed into several groups: agents targeting the PI3K pathway, agents which target EGFR, immunotherapies, and the most prominent, anti-angiogenic therapy [54] (Table 8.2).

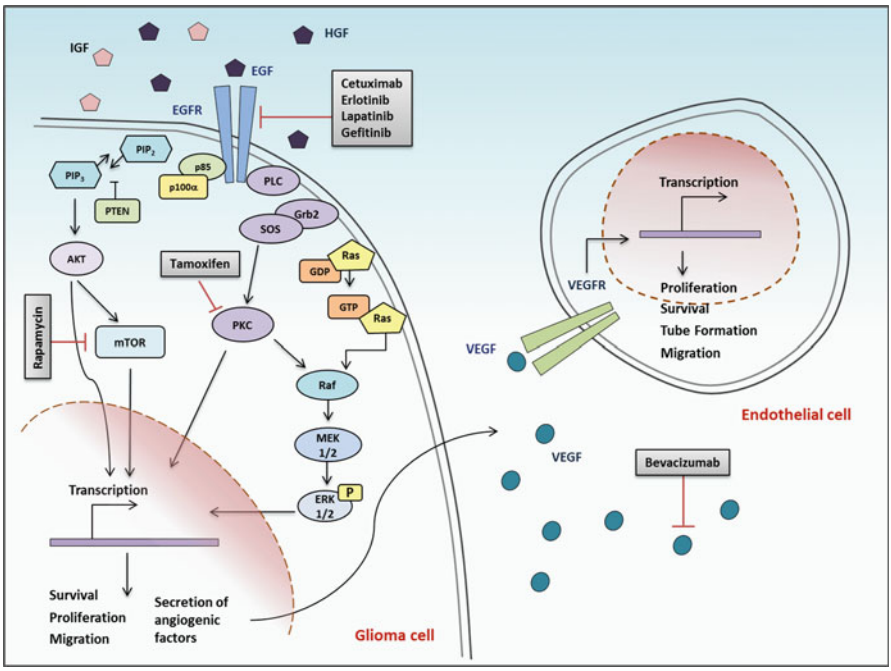

Fig. 8.1 Major pathways altered in GBM include the PI3K pathway and MAPK pathway. Dysregulated signalling through these pathways results in an oncogenic phenotype. Secretion of angiogenic factors such as VEGF by glioma cells induces proliferation, survival and migration in adjacent endothelial cells

Table 8.2 Targeted therapeutic approaches in GBM

Class	Drug	MI modality
Angiogenesis inhibitors	Bevacizumab	• PET • DSC-MRI • MRS
	Cediranib (ADZ2171)	
	Sunitinib (SU11248)	
	Imatinib (Glivec)	
PI3K pathway inhibitors	Rapamycin	• ^{18}F-FDG PET • ^{18}F-FLT PET • MRI
	BEZ235	• ^{18}F-FLT PET • ^{18}F-FET PET • DWI-MRI
EGFR inhibitors	Cetuximab	• PET • Optical imaging
	Carbozantinib	
	Gefitinib	
	Erlotinib	
Immune checkpoint inhibitors	Pembrolizumab	T1-weighted MRI
	Nivolumab	T2-weighted MRI

MRI magnetic resonance imaging, *MRS* magnetic resonance spectroscopy, *DSC* dynamic susceptibility contrast enhanced MR, *PET* positron emission tomography, *FDG* fluorine-2-deoxy-D-glucose, *FLT* fluorothymidine, *FET* fluoro-ethyl-tyrosine

8.3.1 Anti-Angiogenic Therapy

HGG are highly vascularised tumours in which metabolic needs of the tumour are only sustained via new blood vessel growth [54, 55]. Angiogenesis is driven by a number of complex pathways; however, vascular endothelial growth factor (VEGF/VEGF-A) is one of the key proangiogenic factors involved in this new vessel growth. Secretion of VEGF from glioma cells acts in a paracrine fashion upon nearby endocrine cells resulting in cell proliferation, survival and migration. Several novel anti-angiogenic therapies have been investigated in the treatment of GBM; however, trials have been disappointing in this area with minimal benefits to overall patient survival [5]. Rapid clinical improvement is often mediated by "normalisation" of the BBB with decreased peritumoural oedema (as depicted by MRI) while ongoing tumour activity can be depicted by FET-PET [56]. Initial anti-angiogenic therapies evaluated for anti-GBM activity include thalidomide, lenalidomide and carboxyamidotriazole; however, no patient benefits were observed. Bevacizumab, a monoclonal antibody against VEGF, remains the solitary angiogenesis inhibitor approved for use in GBM patients. Despite the success of bevacizumab in phase II trials, contradictory results in several patient cohorts have been documented and the clinical significance of bevacizumab in GBM patients remains debatable. Bevacizumab has however been demonstrated to reduce peritumoural oedema in a subsection of patients and reduce the need for corticosteroids [57]. In addition to bevacizumab, small molecule inhibitors cediranib (ADZ2171), sunitinib (SU11248) and imatinib (Glivec, INN) have also been investigated as treatment options for GBM patients [2].

Angiogenesis inhibitors are capable of inducing a normalisation of the tumour vasculature, allowing co-administered chemotherapeutics to act. Chronic inhibition of angiogenesis however can not only inhibit the tumour uptake of additional therapies, but may also activate compensatory pathways causing an adaptive tumour response whereby tumours become resistant and adapt a more invasive phenotype following treatment. This is particularly the case in chronic inhibition of VEGF with bevacizumab. Chronic bevacizumab therapy may result in a recurrence of the malignancy which is more aggressive and associated with oedema.

Defining angiogenesis inhibitor treatment response using imaging is challenging. Imaging the complex alterations of bevacizumab treatment in a rat model a HGG with multimodal PET (MET, FET) and multi-parametric MRI (T1w, T2w, DWI, ADC) have been nicely demonstrated by Viel et al. [58]. Reduction in tumour size is a basic indicator of response to anti-angiogenic therapy and may be evaluated using conventional MRI. This is however not always sensitive to minor effects or accurate due to the cytostatic activity of angiogenesis inhibitors. Furthermore blood vessel pruning by anti-angiogenic therapies however may influence the ability for contrast agent to reach the tumour and therefore sensitivity may be lost. As previously introduced, perfusion-weighted MRI may be used to visualise the effect of anti-angiogenic therapy, specifically through the use of DSC-MR and dynamic contrast enhanced MR (DCE-MR). Sorenson et al. have used DCE-MRI to measure volume transfer coefficient (K^{trans}), in patients treated with cediranib. By combining K^{trans}

values with particular biomarkers (microvessel volume and circulating collagen IV) a 'vascular normalisation index' was calculated. This value was predictive of overall survival (OS) and progression-free survival (PFS) following a single dose of cediranib. T1-weighted DCE-MR imaging can also be useful in measuring fractional volume of the extravascular extracellular space (v_e), and fractional blood plasma volume (v_p), which further informs tumour response to therapy. Furthermore, Sugahara et al. demonstrated that an enhanced lesion with a normalised rCBV ratio (tumour rCBV/contralateral tissue rCBV) higher than 2.6 suggests tumour recurrence while a normalised rCBV ratio lower than 0.6 implies pseudoprogression [59].

Another potential modality for imaging response to anti-angiogenic therapy is MRS (^{1}H MRSI). Several MRS studies have been carried out employing various metabolites as read-outs. Increased total Cho and decreased NAA levels are both indicative of brain tumour growth and NAA/tCho ratios are capable of distinguishing between normal brain tissue and tumour. In a study by Hamans et al. Bevacizumab treatment was observed to induce glycolysis, indicated by increases in lactate levels over time. This study also found that tumours are however heterogeneous in their lactate production and regions in the periphery of the tumour displayed no increases in lactate [29].

Several molecular factors have been indicated in resistance to anti-angiogenic therapy. Upregulation of c-MET signalling has been identified as a mediator of resistance in a population of GBM tumours and therefore may prove a useful therapeutic target [60]. Furthermore, targeting components of the HIF pathway may prove successful in reducing resistance due to the integral role this pathway plays in angiogenesis [61, 62].

8.3.2 PI3K Pathway Inhibition

The phosphoinositide 3-kinase (PI3K) pathway plays an important role in cell growth, cell survival, migration and cell cycle regulation. Upregulation in pathway activity is closely linked to tumourigenesis, and this pathway plays a major role not only in initial tumour development, but also in the tumour's potential response to treatment [63]. Many malignancies harbour a mutation in PI3K or one of its downstream effectors, PTEN, AKT and mTOR. Mutations in PIK3CA, the gene which codes for the catalytic subunit of PI3K, occur in approximately 25 % of GBM tumours. In addition to this, mutation or deletion of PTEN, a negative regulator of PI3K pathway signalling, occurs in approximately 50 % of GBM cases [64, 65]. Importantly, alterations resulting in overactivation of the pathway may be responsible for reducing the effect of TMZ treatment [66]. Combining this knowledge with the fact that GBM patients with an activated PI3K pathway have a worse prognosis than patients without, the PI3K pathway poses a potential target in GBM treatment. Mammalian target of rapamycin (mTOR) is a key regulator of PI3K pathway signalling and often presents upregulated activity in glioblastomas, resulting in increased cell survival, proliferation and migration. As a result mTOR inhibitors have been investigated at therapeutic targets in glioblastoma; however, results have been inconsistent.

Wei et al. proposed that ^{18}F-FDG- and FLT-PET could be used to monitor tumour response involving mTOR inhibitors. This study demonstrated the effect of rapamycin on tumour metabolism and growth in a subcutaneous GBM mouse model. Over a 72-h period, rapamycin decreased ^{18}F-FDG and ^{18}F-FLT uptake correlating with reduced tumour growth in U87 xenografts; however, no changes were observed in LN-229 xenografts [67].

In an in vitro study, Arcella et al. observed a strong anti-proliferative effect of the mTORC1 inhibitor Rapamycin in primary GBM cell cultures [68]. Furthermore, mTOR activity has also been demonstrated to play a role in GBM response to radiation therapy; Eshleman et al. observed that inhibition of mTOR via rapamycin treatment significantly improved the efficacy of radiation in U87 xenografts in nude mice [69, 70]. In a recent study by our group, response to therapy of mice orthotopically implanted with U87MG-luc2 cells was assessed following combination treatment with bevacizumab and the mTOR/PI3K inhibitor BEZ235. Response to therapy was monitored using DWI-MRI and multi-tracer PET (^{18}F-FLT, ^{18}F-FET). DWI-MRI identified significant reductions in tumour volume upon single agent treatment with bevacizumab; however, no additive effect was observed in combination treatment. Furthermore, no significant difference in uptake of either ^{18}F-FLT or ^{18}F-FET was observed between treatment groups (Figs. 8.1, 8.2 and 8.3) [71]. These

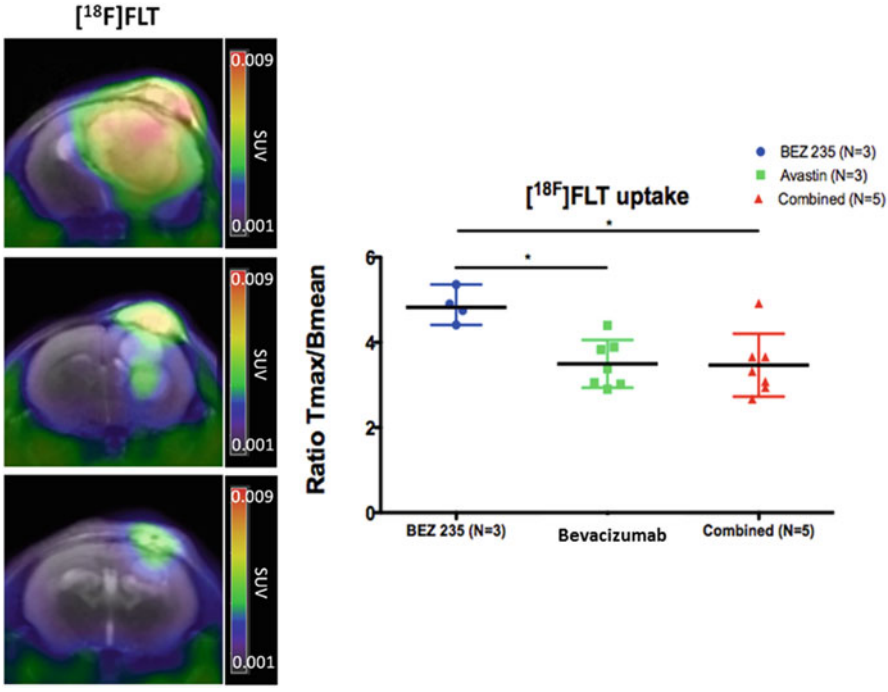

Fig. 8.2 PET images co-registered with MRI of effect of bevacizumab, BEZ235 and combination treatment on tumour uptake of ^{18}F-FLT indicating tumour proliferation. Figure adapted from O'Halloran et al. [71]

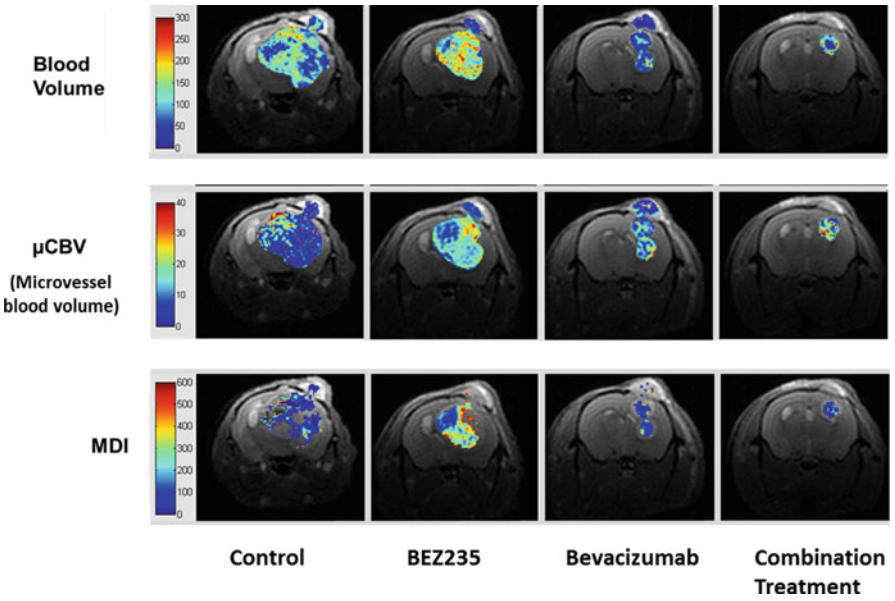

Fig. 8.3 MR images of effect of bevacizumab, BEZ235 and combination treatment on tumour blood volume, microvessel volume and vessel density (MDI). Figure adapted, with permission, from O'Halloran et al. [71]

mixed results suggest that despite the frequent activation of mTOR signalling in GBM, resistance to mTOR targeted therapy is a common issue highlighted in the minimal effect observed following mTOR inhibition. Several studies have attempted to understand the mechanisms of resistance employed by GBM tumours in response to PI3K pathway targeted therapy. In a study by Iwanami et al., the promyelocytic leukaemia (PML) gene was identified as an important factor in resistance to mTOR targeted therapies. PML plays a role in negatively regulating PI3K pathway signalling and is highly expressed in many GBM tumours. Following mTOR and EGFR inhibition, PML expression is observed to increase, preventing the induction of cell death. This exact mechanism of resistance is however unclear [72].

8.3.3 EGFR Inhibitors

The epidermal growth factor (EGFR) belongs to the HER family of receptors. Binding of its ligand EGF to its receptor initiates the activation of both the Ras/Raf/MEK pathway and the PI3K pathway, resulting in increased cellular proliferation and pro-survival signals. Aberrant EGFR signalling is common in cancer and EGFR amplifications and alterations are detected in approximately 40–50 % of primary GBM [27, 66]. In approximately 50 % of tumours with EGFR amplification, a specific EGFR mutant (EGFR vIII, EGFR type III, de2-7, ΔEGFR) can be detected

which results in ligand-independent activation of EGFR. Presence of this mutant results in dysregulated signalling and highly oncogenic phenotype [73]. EGFR overexpression has been associated with resistance to standard chemotherapy and radiation therapy (RT) [74]. EGFR is therefore an attractive target for therapy.

Many preclinical studies have observed promising results using inhibitors of EGFR. Gefitinib and Erlotinib, both EGFR inhibitors, have been considered for possible therapeutic use in GBM. Both have shown promising preclinical results, e.g. FLT-PET being capable of identifying treatment effect whereas FDG-PET does not [40]. In preclinical studies erlotinib was demonstrated to significantly inhibit the invasive phenotype of EGFR vIII overexpressing GBM [69] and reduce the viability of a panel of human glioblastoma cell lines. In clinical application, however, results varied and little effect was observed with erlotinib [75]. Similarly, gefitinib demonstrates promising results preclinically; however, this does not translate to patients. In a Phase II trial carried out in 100 patients with GBM, treatment with gefitinib displayed no significant increase on OS or PFS [76]. Similarly, in a Phase II trial carried out in 28 patients with HGG, limited activity was observed upon gefitinib treatment [77]. PTEN mutations which occur frequently in GBM are often responsible for resistance to EGFR inhibition; loss of PTEN results in constitutively active AKT signalling, thus eliminating the need for EGFR stimulation.

Due to the interest in EGFR inhibition in GBM there have been multiple imaging methods optimised for use in imaging specifically EGFR and its ligands. Specific EGFR-targeted PET and SPECT probes have been designed [78] for this purpose. Slobbe et al. carried out fluorine-18 labelling of Afatinib and demonstrated successful preliminary results [79]. Similarly, Wehrenberg-Klee et al. have demonstrated that the novel tracer ^{64}Cu-DOTA-cetuximab F(ab′)2 has promising new radiotracer for PET. This study demonstrated the use of this tracer in an intracranial mouse model and was successful in differentiating EGFR wild-type and EGFRvIII expressing GBMs. Furthermore this study successfully imaged EGFR expressing GBMs in a surgical resection model [78].

It is also possible to optically image EGFR-targeted therapy through the use of fluorescent conjugates of the EGFR ligand, EGF. Furthermore, anti-EGFR antibodies have been labelled with fluorophores and quantum dots to allow detection and visualisation of EGFR [73, 80].

In addition to anti-angiogenic therapy and therapies which target EGFR and mTOR, several studies have identified other possible candidates for inhibition. Jarzabek et al. have identified gossypol, a BH3 mimetic agent, as a potential therapeutic option. It was observed that gossypol acted synergistically in combination with TMZ. This manifested in a reduction of viability in HUVEC, U87-MG-luc2 and U343 cell lines. Furthermore, combination therapy of gossypol and TMZ inhibited cell proliferation and angiogenesis in a U87MG-luc2 xenograft mouse model, in addition to enhancing apoptosis in vivo [81]. A more recent study by Zakaria et al. investigated the effect of birinapant, an inhibitor-of-apoptosis-protein (IAP) antagonist, on TMZ response in a panel of GBM cells. A heterogeneous response was observed and divided into three categories; those which were sensitive to both TMZ and birinapant, those which were sensitive to birinapant but unaffected by TMZ and those which were resistant to both therapies [82].

8.3.4 Immune Checkpoint Inhibitors

Many malignancies result in the attenuation of the immune system. This is in part due to the secretion of immunosuppressive factors by tumour cells [56]. The principle purpose of immunotherapy is to enhance or reactivate a dampened immune response in order to allow the immune system to act against a tumour [83]. Immune checkpoints are molecules which are capable of upregulating and downregulating the activity of components of immune pathways; examples of which include PD-1, PD-L1, PD-L2 and CTLA-4. Blockade of these immune checkpoint molecules may be a promising approach in immunotherapy in cancer in general as well as in gliomas. PD-1 is a checkpoint protein located on T-cells which functions normally to downregulate the activity of T-cells. Many cancer cells protect themselves from the normal functions of the immune systems through an inhibition of T-cell signalling. PD-1 is a receptor which binds two endogenous ligands, PD-L1 and PD-L2. PD-L1, the primary ligand for PD-1, is expressed in macrophages, dendritic cells and also on glioma tumour cells [84]. CTLA4 is a receptor expressed by helper T-cells [85], which signals to downregulate the activity of the immune system, namely the T-cells. Checkpoint inhibitors may target the immunomodulatory effects of both PD-1 and CTLA-4 in order to restore the function of T-cells and enable their anti-tumour activity [86].

The therapeutic efficiency of PD-1 and PD-L1 antibodies has been investigated in a variety of cancer types [87] including GBM. Phase II trials of the PD-1 inhibitor pembrolizumab and the PD-L1 inhibitor MED14736 are currently ongoing [51, 88]. In a recent study by Bouffet et al., the efficacy of nivolumab in GBM cases with a heavy mutational burden (biallelic mismatch repair deficiency) was investigated. Nivolumab is an anti-PD-1 checkpoint inhibitor. Tumour response to therapy was monitored using T1w and T2w MRI to visualise tumour size and oedema. The initial and durable responses of recurrent GBM to immune checkpoint inhibition were shown [86]. Immune checkpoint inhibitors represent a promising strategy towards improved outcomes in GBM.

8.4 Theranostic Agents

A theranostic agent is one which combines both diagnostic and therapeutic capabilities in the interest of improving both disease management and treatment and individualising patient care. This combined approach can be used to accelerate drug development and aid precision of therapy for complex diseases such as cancer [89]. Theranostic agents which deliver therapeutic cargo but which also encompass contrast agents or dyes may be also used as non-invasive molecular imaging tools [90].

Many theranostics are nanoparticle (NP) based, making them suitable for the treatment of brain malignancies with targeted particles providing transport across the blood–brain barrier [91]. These theranostic agents may consist of gold nanoparticles, quantum dots, iron oxide nanoparticles, carbon nanotubes or be

silica based, therefore allowing a variety of surface chemistries [92]. The flexibility of this surface chemistry facilitates the functionalisation of these molecules, thus aiding tumour-specific delivery and payload release, and allowing for a variety of applications such as target cell surface receptor recognition and aided cellular internalisation [93]. Furthermore nanoparticles are particularly useful in theranostic design due to their propensity to accumulate in the vasculature of tumours as a result of the enhanced permeability and retention (EPR) effect [94].

For example the utility of such agents in GBM has been highlighted in a recent study by Ferrari et al. In this study Copper-64 has been shown to be a useful theranostic agent in a xenograft mouse model of glioblastoma. This study demonstrated that intravenously administered [^{64}Cu]$CuCl_2$ exhibits enhanced affinity for GBM tumour cells. The positron emission of ^{64}Cu allows PET imaging to be effectively carried out demonstrating a significant reduction in tumour volume and animal survival in [^{64}Cu]$CuCl_2$ treated animals [95]. In another recent study Yang et al. have developed a poly(aspartic acid) nanoparticle-based theranostic agent capable of delivering both iron oxide nanocrystals and doxorubicin to GBM tumours in vivo. This nanoparticle-doxorubicin compound demonstrated behaviour allowing application as a T_2 MR contrast agent while delivering doxorubicin to tumour cells [96].

Theranostic nanoparticle agents therefore represent a useful tool having a range of potential applications not only in glioblastoma detection and therapy but across oncology in general. Moreover there is significant scope to achieve a robust precision therapy strategy in complex oncology settings using “next-generation” smart nano-delivery theranostic agents.

8.5 Concluding Remarks

Resistance to therapy is a significant problem in GBM. Non-invasive MI-based technologies support the development of new experimental approaches as well as clinical efficiency read-outs by providing detailed molecular information relating to glioma activity and related vascular and immune cell components as well as mechanism of drug action, therapeutic efficacy and treatment resistance. Nevertheless, there is still a lack of effective imaging modalities which allow visualisation of conversion of a proliferative to an invasive glioma phenotype particularly after treatment with anti-angiogenic or anti-invasive drugs. Current efforts to define new treatment paradigms for GBM are primarily focused on novel targeted therapies. Moreover, it is becoming increasingly apparent that targeting a single cancer hallmark in GBM might be insufficient to affect a sustained response. It may, therefore, be necessary to employ a combined multi-targeted approach. Advanced multi-tracer PET and multi-parametric MRI are pivotal MI techniques to enable the interrogation of tumour behaviour in response to therapy, to provide information pertaining to tumour resistance and to allow efficient translation and reverse-translation of new experimental avenues and clinical studies to ultimately improve the patient outcome.

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Chapter 9
Drug Resistance in Malignant Meningiomas

Kyle A. Smith, Chris Miller, Domenico Gattozzi, and Roukoz B. Chamoun

Abstract Meningiomas are one of the most common intracranial tumors and arise from the arachnoid cap cell. Although the overwhelming majority of meningiomas are benign, approximately 5–15 % of meningiomas are non-benign. These tumors are histologically Grade II and III and have a propensity to be aggressive and occasionally metastasize. Multiple genetic abnormalities and epigenetic changes are involved in the formation and malignant transformation of meningioma. Given the aggressiveness of these tumors, the standard of care involves maximal surgical resection, when feasible and safe, followed by radiation. In cases of initial treatment failure or tumor recurrence, adjuvant therapy with chemotherapy or clinical trials becomes an option. Outcomes data for chemotherapy are rather scarce, but medical treatment options include mifepristone, hydroxyurea, somatostatin analogues, interferon-α, irinotecan, and temozolomide. Somatostatin analogues and interferon-α have shown promise, but prospective studies will be necessary to determine their effect on outcomes. Future development of medical treatments and chemotherapy depends upon the understanding of mitogenic and antiapoptotic pathways involved with malignant meningiomas. Multiple growth factors and receptors may serve as useful sites for therapy action. Multiple preclinical and clinical trials are underway for the disruption of these pathways. Future prospective, randomized clinical trials will be essential to evaluate the effect on tumor control, progression-free survival, and effect on overall survival.

Keywords Malignant • Atypical meningioma • Chemotherapy • Chemotherapy resistance

K.A. Smith • C. Miller • D. Gattozzi • R.B. Chamoun (✉)
Department of Neurosurgery, University of Kansas Medical Center, Kansas City, KS, USA
e-mail: rchamoun@kumc.edu

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_9

Abbreviations

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EORTC	European Organization for Research and Treatment of Cancer
GTR	Gross total resection
IGF	Insulin-like growth factor
NCCN	National Comprehensive Cancer Network
PDGF	Platelet-derived growth factor
PFS	Progression-free survival
PGDS	Prostaglandin D synthase
RT	Radiation therapy
RTOG	Radiation Therapy Oncology Group
SRS	Stereotactic radiosurgery
STR	Subtotal resection
TGF-α/-β	Transforming growth factor-α/-β
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

9.1 Introduction

Meningiomas are the most common intracranial tumors, comprising about 13–26 % of primary intracranial tumors [1–5]. Twenty percent of intracranial masses in males and 38 % of intracranial masses in females are meningiomas. The incidence of meningiomas is about 6 per 100,000, with a 2–7 per 100,000 incidence for females and 1–5 per 100,000 incidence for males [1, 3]. The incidence of meningioma increases with increasing age. Prevalence is around 97.5 per 100,000 [2]. Autopsies have demonstrated a 2.3 % rate of incidental meningioma occurrence [6]. In adults, meningiomas comprise 38 % of intradural spinal tumors, with only 3 % being malignant [7].

The progenitor cell for meningiomas has yet to be definitively elucidated; however, it is generally accepted that the arachnoid cap cell of the meninges is the cell of origin [8]. There is data showing that the progenitor cell is usually prostaglandin D synthase (PGDS) positive [9].

Symptoms of meningiomas usually arise from compression of adjacent brain structures leading to neurological deficit or seizure from cortical irritation [2]. Ninety percent of meningiomas are intracranial, and of these 90 % are supratentorial [4]. Intracranial meningiomas are noted to occur in a 2:1 female-to-male ratio, and in the spinal cord they occur in a 4:1 female-to-male ratio [10, 11]. The risk of meningioma formation increases with age [2, 8]. They are most commonly discovered in patients between 50 and 60 years old [1]. The risk of developing a meningioma is doubled with the presence of a first-degree relative with meningioma [8].

Table 9.1 Meningioma WHO grades and subtypes

Grade I (benign)	Grade II (atypical)	Grade III (malignant)
Angiomatous	Atypical	Anaplastic
Fibrous (fibroblastic)	Chordoid	Papillary
Lymphoplasmacyte rich	Clear cell	Rhabdoid
Meningothelial		
Metaplastic		
Microcystic		
Psammomatous		
Secretory		
Transitional (mixed)		

Meningiomas are graded histologically as Grade I, II, or III according to the 2007 World Health Organization (WHO) grading classifications. The majority of meningioma variants are WHO I. Subtypes of each WHO grade are listed in Table 9.1. Approximately 80–90 % of meningiomas are WHO Grade I, while atypical and malignant types of meningiomas encompass 5–15 % of meningiomas. However, unlike benign types, the more aggressive grades are frequently encountered in males [2, 5, 8, 9, 12–15]. Grade II and Grade III meningiomas are also more common in older patient populations and are more likely to be convexity versus skull base [1, 9]. Two percent of initially benign tumors progress to become malignant, and recurrent meningiomas (even from benign primaries) demonstrate a 28.5 % chance of being atypical or anaplastic, often with more complex genetic compositions [3, 16]. Malignant types of meningioma are more likely to recur, more likely to recur sooner, and more likely to lead to death within 2 years from diagnosis [4]. Meningiomas typically do not metastasize, but, when they do, common sites include the bone, liver, lungs, and pleura [17].

One in 33,000 people harbors a mutation of the *NF2* gene. This predisposes them to an increased incidence of spinal tumors, which includes meningiomas. There may be genetic differences between spinal and intracranial meningiomas, as well as their genetic bases, but larger studies need to be performed on the matter [7]. For a more detailed discussion on the genetics of meningioma, see the "Genetics" section below.

Aside from genetics there are other causes and associations linked with meningioma formation. Radiation is a well-known cause for meningioma formation, with a six- to tenfold increased incidence of meningioma with a history of radiation exposure [8]. There is a potential connection with head trauma and the formation of meningioma. One study noted that in males, head trauma severe enough to warrant medical treatment led to a fivefold increase in meningioma formation in the following 15–24 years compared to control subjects [18]. While no conclusive evidence has shown meningioma to be directly correlated to cellular phone and cordless phone use, the topic deserves mention as the current literature recommends caution given that existing data suggests a possible correlation. Larger studies with longer follow-up time intervals are needed to identify whether electromagnetic fields from

cordless and cellular phones are a driving force for meningioma formation [19]. Meningioma formation can also be associated with smoking. In males, personal history of smoking was noted to increase the chance of developing a meningioma; however, in women personal history of smoking was observed to decrease the risk of meningioma formation [20].

Given the increased incidence of meningiomas in the female population, several factors have been studied relating to female physiology in relation to development of meningioma. In a very large population study, estrogen-only hormone replacement therapy was noted to increase meningioma risk even after only 6 months of use, and use of hormone replacement therapy for 3 years increased the chance of intracranial meningioma by 1.4-fold. Spinal meningioma risk was similarly increased with estrogen hormone therapy, but not with a combination hormone replacement. The use of combined estrogen and progesterone hormone replacement therapy was not associated with increased risk of meningioma occurrence [11]. A similar large population study also demonstrated that any prior use of hormone therapy whatsoever increased meningioma risk compared to never having used postmenopausal hormone replacement therapy [21]. The number of pregnancies was noted to be inversely proportional to the risk of developing meningiomas. Menopause, regardless of cause, and breast cancer, however, were not related to meningioma development [22]. Breastfeeding was also noted to decrease risk of meningioma formation, especially if over 6 months of breastfeeding had been accumulated in a lifetime. Oral contraceptive use in women reportedly does not increase risk of meningioma formation, but higher body mass index does [23]. Compared to women with a normal body mass index, obese women have a 68 % increased risk of meningioma formation [24].

Current treatment strategies for meningiomas are discussed below, but the general standard is to attempt maximal surgical resection as this can be curative, especially for benign, low-grade lesions. Resection is limited by location, especially along the skull base and due to critical neurovascular structures. More innovative chemotherapies are discussed in more details in the “Treatment” section below. These are being studied mostly for the atypical and anaplastic types of meningiomas, which can be refractory to surgical therapy and radiation.

9.2 Standard Treatment

Meningioma treatment is tailored to the histological WHO grade. Standard treatment for atypical (WHO Grade II) and malignant (WHO Grade III) meningiomas begins similar to that of benign (WHO Grade I) meningiomas in that surgery is the primary treatment [3, 25]. Safe, maximal resection to obtain histological grade and tissue diagnosis is the goal of surgery. Complete surgical resection based on the Simpson grading system is generally attempted if the tumor is in an accessible location. Simpson Grades 1 and 2 are consistent with gross total resection (GTR) [3, 25, 26]. Oftentimes the complete resection is difficult or impossible for atypical and

malignant meningiomas due to their location and/or brain invasion. However, partially resected higher-grade meningiomas carry a high rate of recurrence and increased mortality [25–28]. Recurrence rates for high-grade meningiomas in general range from 29 to 94 % depending on the grade and subtype; furthermore these rates correlate with decreased overall survival [29]. Therefore, when complete resection cannot be obtained or the tumor histological grade is at least atypical (WHO Grade II), adjuvant therapies become part of the care regimen. Radiation therapy (RT) is typically delivered to residual tumor or to the resection margins in these cases [29].

Outcomes of radiation therapy are difficult to analyze due to the rarity of high-grade meningiomas, retrospective nature of studies, and variability of patient factors and tumor subtypes [3]. Furthermore, treatment modalities in RT and even the WHO grading scheme have undergone evolution over the time of these studies [29]. Nonetheless, there is a trend toward improved outcomes with postoperative RT in WHO Grades II and III meningiomas [29]. Adjuvant RT in particular is utilized in cases of WHO Grade III meningiomas with or without gross total resection and in cases of subtotal resection (STR) of atypical (WHO Grade II) meningiomas [4, 25, 29, 30]. This recommendation is congruent with the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology. The role of RT is less clear in cases of complete resection of atypical (WHO Grade II) meningiomas [4]. Several techniques of radiation therapy exist and are selected based on factors such as the remaining tumor size and location. These modalities are stereotactic radiosurgery (SRS), hypofractionated RT, and heavy particle irradiation [31–36]. Although RT protocols vary, fractionated radiation is typically delivered to a dose of 50–60 Gy [4]. SRS is typically utilized at a dose of 12–20 Gy [4].

Atypical (WHO Grade II) meningiomas tend to have an intermediate risk of recurrence and overall survival in comparison to benign and malignant meningiomas. Maximal resection, or Simpson Grades I–III, is associated with improved outcomes [37]. The estimated recurrence rate of atypical meningiomas after complete resection is 30–50 %; after incomplete resection recurrence rate is 60–100 % [38–42]. Furthermore, the 5-year progression-free survival (PFS) is estimated to be 59–90 % after complete resection and 30–70 % after subtotal resection [37]. RT is most frequently administered in Grade II tumors following incomplete resection or biopsy. RT following STR is generally recommended, and 5-year PFS outcomes range 43–91 % [25, 37]. SRS has been shown to demonstrate similar rates of PFS, but is more commonly used in smaller residual tumors [37]. In cases where complete resection is attained, the role of RT is less clear. Several studies report improved local control or a trend toward improved local control but failed to report PFS [37]. The risks of RT must be balanced with potential progression-free survival. The current literature is composed primarily of observational studies but suggests that adjuvant radiation improves PFS and local control without clear positive effect on overall survival [4, 40, 43, 44]. Complete resection and postoperative RT were found to be predictors of better outcome in multiple studies, primarily for local recurrence and progression-free survival [39–41, 45]. A summary of the treatment algorithm for atypical meningiomas is demonstrated in Fig. 9.1.

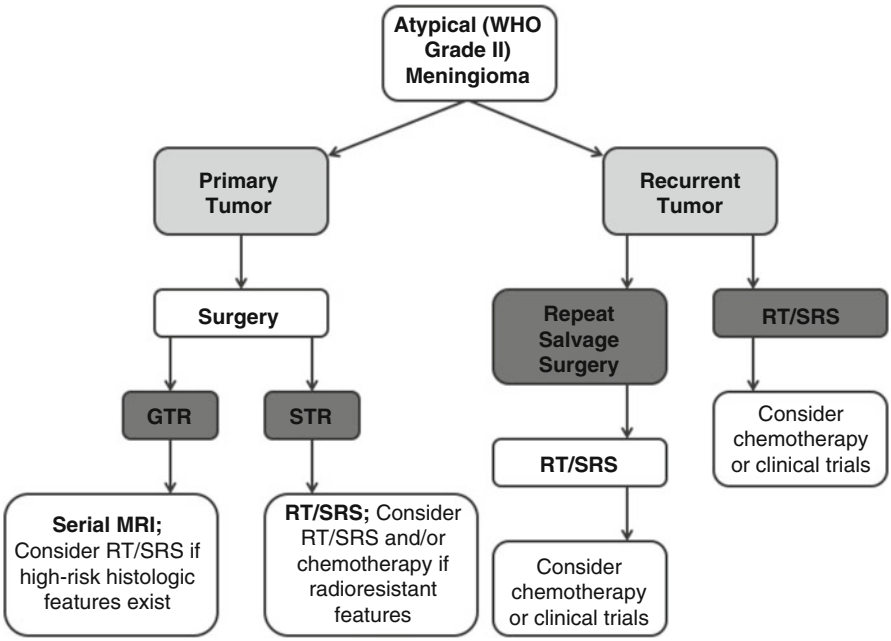

Fig. 9.1 Treatment algorithm for atypical (WHO Grade II) meningiomas. Treatment algorithm for atypical (WHO Grade II) meningiomas generally is divided by primary or recurrence of tumor. Recommendations for primary tumor generally include surgery followed by other treatment options depending on the extent of resection. Radiation therapy is generally recommended in cases of incomplete resection, but timing is determined by the practitioner following the patient with clinical imaging. The role for radiation therapy is less clear in cases of complete resection. In tumor recurrence, no single consensus exists and options include salvage surgery and/or radiation therapy

In the treatment of malignant (WHO Grade III) meningiomas, RT is a component of initial management due to the high rate of recurrence and progression even in the setting of complete resection. The majority of current literature for radiation comes from retrospective reviews in observational studies [5, 29, 46–50]. These data suggest that the 5-year overall survival is approximately 20–50 % with a recurrence rate of 60–90 % by 5 years. In cases of adjuvant RT, the recurrence rate is reduced by half, and survival is improved to greater than 50 %. The optimal dose of RT for malignant meningiomas is not known, but data suggest a total dose of 60 Gy to achieve local control of disease [31, 46, 47, 51]. A summary of the treatment algorithm for malignant meningiomas is demonstrated in Fig. 9.2.

Current prospective, nonrandomized trials are studying the role of postoperative RT for WHO Grades II and III meningiomas (RTOG 0539 and EORTC 22042). The Radiation Therapy Oncology Group (RTOG) 0539 trial has not published outcomes but studied intermediate-risk and high-risk disease treated with 6 weeks of RT following surgery. Doses were 54 Gy following gross total resection and 60 Gy following subtotal resection or in cases of recurrent Grade II meningiomas. The European Organization for Research and Treatment of Cancer (EORTC) 22042 trial used a

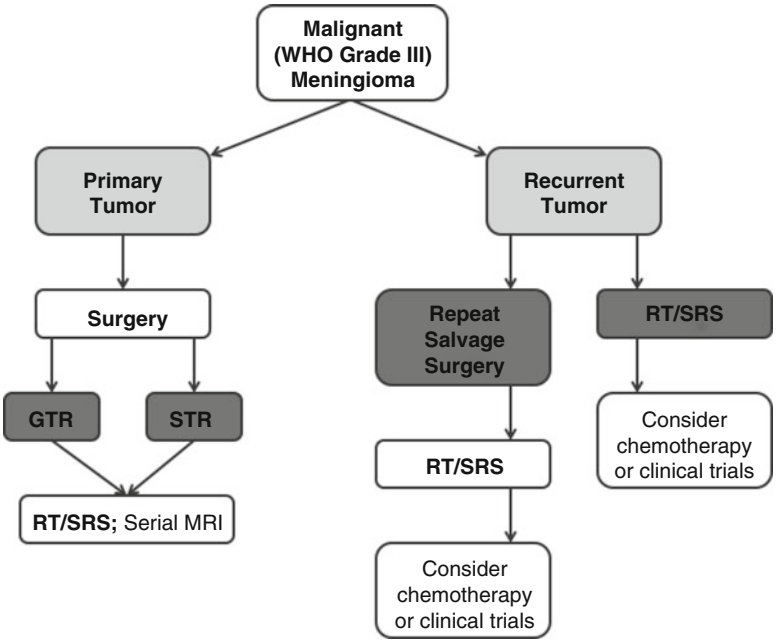

Fig. 9.2 Treatment algorithm for malignant (WHO Grade III) meningiomas. Treatment algorithm for malignant (WHO Grade III) meningiomas generally is divided by primary or recurrence of tumor. In cases of primary tumor, surgical resection is recommended followed by radiation therapy regardless of the extent of resection. Tumor recurrence of Grade III meningiomas is treated similar to Grade II meningiomas with salvage surgery and radiation therapy both being treatment options

similar RT protocol for atypical and malignant meningiomas following surgery. Outcome results of these studies are not yet available.

For all atypical and malignant meningiomas, close surveillance after treatment is necessary to monitor for recurrence. The National Comprehensive Cancer Network (NCCN) provides a baseline for minimum follow-up, but in general atypical meningiomas are imaged every 3–6 months for the first year and then every 6–12 months for several years. Malignant meningiomas are typically imaged in follow-up more frequently. Recurrence presents a slightly different view of treatment than newly diagnosed meningiomas, and decision-making is much more provider specific. Treatment options in recurrence are variable including repeat surgery, radiation, and clinical trials as demonstrated in Figs. 9.1 and 9.2.

9.3 Genetics of High-Grade Meningiomas

The genetic profiles of meningiomas remain a topic to be fully characterized, but to date a variety of associations of genetic aberrations have been linked to meningioma pathogenesis. The genetic abnormalities can be divided into two general categories: those that play a role in meningioma formation and those that play a role in

meningioma progression. It is reported that 20–57 % of meningiomas are diploid without chromosomal genetic abnormalities identified and that generally an increasing number and complexity of genetic aberrations parallel increasing tumor grade [52]. The main genetic alterations to consider are discussed below.

9.3.1 Mutations Associated with Meningioma Formation

9.3.1.1 Loss of Chromosome 22

Mutations on the long arm of chromosome 22 at the 22q12.2 locus have been implicated in meningioma formation. Individuals harboring a mutation of the neurofibromatosis type 2 gene develop multiple meningiomas [10]. In addition, over 50 %, and up to 80 % according to some sources, of sporadic low-grade meningiomas harbor this mutation [1, 9]. The gene involved in sporadic cases is merlin, also called schwannomin, and is a 4.1-type protein which normally functions to prevent cell growth by contact inhibition and maintains cell polarity, essentially functioning as a tumor suppressor [3, 10]. The locus for merlin lies between the myoglobin and c-sis proto-oncogene loci on this chromosome and is separate from the neurofibromatosis 2 gene present on chromosome 22 [3, 16]. Other genes on the long arm of chromosome 22 have been suggested as possible causative agents, including *BCR*, *Rgr* (an oncogene), and *ZCWCC1* (zinc finger protein-encoding gene) [30].

9.3.1.2 Loss of Chromosome 18

DAL-1 is differentially expressed in adenocarcinoma of the lung gene, a known tumor suppressor found to be strongly expressed in tissues of the lung and brain. DAL1/4.1B is a gene on chromosome 18, which encodes a 4.1 protein as its gene product, similar to the merlin gene product from chromosome 22 [53]. Studies noted a lack of DAL-1 expression in 60 % of sporadic meningiomas. Unlike merlin, which also functions as a tumor suppressor in schwannomas, DAL-1 is specific to meningiomas [53]. This gene also plays a role in tumor progression, as loss of this chromosome is found in increasing frequency paralleling increase in grade [54]. Loss of DAL-1 is considered an early part of meningioma progression. Its normal function entails disrupting cell motility through indirect effects on the actin cytoskeleton [53]. Abnormalities of chromosome 18 are uncommon in benign meningiomas [10].

9.3.1.3 Loss of Chromosome 7

Loss of the short arm of chromosome 7 is rarely encountered on cytogenetic analysis but deserves mention as monosomy of 7p is a common finding in meningiomas forming after radiation exposure [6]. This is of importance given that the dose of ionizing radiation per capita in the United States has increased by a factor of six

since the 1980s, with full dental X-rays, especially multiple sets and, at an early age, being the most common radiation exposure leading to increased meningioma formation in the US population [55]. Radiation-induced meningiomas also commonly undergo malignant transformation [16].

9.3.2 Mutations Associated with Meningioma Progression

9.3.2.1 Loss of Chromosome 1

Loss of 1p36.11 has also been associated with worse grade and prognosis with meningiomas, likely from loss of unknown tumor suppressor genes. These deletions are also more likely to be found in recurrent meningiomas [1]. Only 4.3 % of meningiomas recur with intact 1p, and those with 1p deletions are more likely to recur. Deletion of this region has been noted as the second most common deletion found in higher-grade meningiomas, after loss of chromosome 22 [14].

9.3.2.2 Loss of Chromosome 14

A potential gene target on the 14p arm is MEG3 (maternally expressed gene 3). While normally expressed in high amounts in normal brain and meningeal tissue, it was noted that both mRNAs from the MEG3 gene were expressed much lower in meningiomas. Not only did the amount of expression decrease with increasing meningioma grade, but the number of meningiomas expressing amounts comparable to normal meninges also decreased with increasing grade. In addition, the higher meningioma grades had higher probability of having a loss on chromosome 14, whereas more benign types of meningiomas were not noted to have this abnormality. It is thought this gene works in concert with the p53 pathway, but the details have yet to be elucidated [15]. Some authors point to this being more clinically relevant to males, evidenced by higher recurrence rates and shorter relapse-free time of survival [1].

9.3.2.3 Loss of Chromosome 9

Loss of the short arm of chromosome 9 at the 9p21 locus is associated with malignant progression of meningiomas. This has been noted to occur both as monosomy and as localized deletion. As the other cytogenetic changes involved in progression, this loss is found to occur more frequently in higher grades. One study showed that 17 % of benign meningiomas harbored this genetic change, compared to 52 % of atypical and 74 % of anaplastic. This deletion is noted to some extent in all meningiomas demonstrating brain invasion; however, all of those with the anaplastic histological grade harbored the deletion, compared to 45 % of invading tumors of benign histological grading. This abnormality has direct clinical relevance in regard to prognosis: 3-year

survival with anaplastic meningioma in the presence of 9p21 loss was 8.3 % compared to a 75 % 3-year survival for anaplastic meningiomas with chromosome 9 intact. Tumor recurrence was also more likely with this mutation [56]. The genes involved are *CDKN2a* (p16), *CDKN2B* (p15), and *p14ARF*. These three genes are tumor suppressors with possible effects on both the retinoblastoma and p53 pathways of cell regulation [56]. Some or all of the genes are all impaired to some extent from the loss on 9p, but in anaplastic meningiomas the likelihood of all three being homozygously deleted can be up to 46 %, compared to 3 % of atypical meningiomas. These genes are also subject to epigenetic silencing by methylation of gene regulator regions [57].

9.3.2.4 Gain of Chromosome 17

Amplification of chromosome 17q23 has been studied in meningiomas. There is evidence that this alteration is specifically related to transformation from a WHO II to a WHO III meningioma, as it characteristically only occurs with significant frequency in Grade III tumors. In one study, 48 % of Grade III tumors expressed this compared to less than 5 % of Grade II tumors, and it was not identified in any Grade I meningiomas. While a specific gene has not been discovered yet, it is important to note that the ERBB2 gene on this chromosome, which is implicated in breast cancer, was not amplified [13].

9.3.2.5 Loss of Chromosome 10

Loss of the PTEN gene (phosphatase and tensin homologue gene) has also been implicated in meningioma pathogenesis [1]. 10p12.31 has also been suggested as a target locus on this chromosome, with the MLLT10 gene being implicated, but as it is ubiquitously expressed in meningiomas, it is therefore of limited use as evidence for formation or progression [9]. Meningiomas with loss of heterozygosity at this locus are more likely to be meningothelial or transitional varieties and often express atypical or anaplastic characteristics [1].

9.3.2.6 Loss of Chromosome 18

The loss of chromosome 18 is also implicated with meningioma progression. This chromosomal loss is described above in the genetics of meningioma formation.

9.3.2.7 Gain of Chromosome 22

While monosomy of chromosome 22 is common among benign meningioma variants, multiple copies of this chromosome have been identified in more aggressive meningiomas. Trisomy and tetrasomy 22 have been identified in meningiomas

demonstrating more S-phase tumor cells. These aneuploidies also correlate with worse clinical outcome due to more aggressive tumor behavior [1].

9.3.3 Miscellaneous Mutations

Worth noting in this section on cytogenetic aberrations and progression of malignant meningioma is that some certain cytogenetic abnormalities found in higher grades are also present in lower grades, albeit less commonly. As mentioned earlier, monosomy of chromosome 22 is well accepted to be present in equal amounts in both low- and high-grade meningiomas; therefore, it is expected to play a role in initiation rather than progression. However, losses of chromosomes 1 and 14 were likewise found in all types of meningiomas, simply with higher frequency among higher grades [58].

Other mutations have been noted, most without identification of a target gene or gene product, and are therefore still being studied for clinical applicability. Such identified mutations include losses of chromosomes 1p, 6q, 10q, 14q, and 18q; gains on chromosomes 1q, 9q, 12q, 15q, 17q, and 20q; and other mutations on chromosomes 11 and 22 and the sex chromosomes [1, 3, 14, 17]. There is also report of unstable changes in the genome such as "rings, dicentrics, and telomeric associations."

9.3.4 Telomerase Activity

Telomerase activity is reported between 3 and 21 % for benign meningiomas, between 58 and 92 % of atypical meningiomas, and in 100 % of anaplastic meningiomas [10]. The hTERT reverse transcriptase and the hTR RNA component are implicated. The expression of hTERT is more sensitive to true telomerase activity and is a marker of tumor progression [1].

9.3.5 Epigenetic Changes

While deletion or mutation of tumor suppressor genes can lead to their inactivation, thereby promoting tumorigenesis, epigenetic changes have been recently studied in meningiomas and have been observed to produce the same effect. 5′ gene promoter regions carry CpG islands, which consist of CpG dinucleotides. These dinucleotides are found in high frequency in the 5′ gene promoter regions and, at baseline, are unmethylated. If these areas become hypermethylated, the tumor suppressor gene downstream becomes silenced without the need for mutation or deletion/loss of heterozygosity [12]. This could explain why some FISH studies fail to identify chromosomal abnormalities, whereas gene products of tumor suppressor genes are not

identified. These abnormalities were found in ten tumor suppressor gene promoters, including the abovementioned CDKN2A, CDKN2B, and p14ARF locations. Atypical and anaplastic meningiomas had much higher frequencies of hypermethylation noted, at 74 % and 69 %, respectively, compared to 6 % in benign meningiomas [12].

As described above, most abnormalities in the genetic profiles of meningiomas identified to date deal with an acquired state of aneuploidy. Not all the chromosomal abnormalities have resulted in discovery of a target gene or gene product, but the associations with meningioma type or behavior remain. Additionally, meningiomas remain graded based on histological appearance rather than on the identification of genetic abnormality. With the detection of more specific genetic alterations, therapeutic options may arise as targetable pathways or proteins are identified.

9.4 Chemotherapy Options

Despite meningiomas being dural based and not protected by the blood-brain barrier, chemotherapeutic options are limited for meningioma [37, 59–62]. Chemotherapy options and outcomes are listed in Table 9.2. Guidelines published by the NNCN place chemotherapeutic options as a last resort. Only with recurrent disease in which surgical removal is not possible and the patient is no longer eligible for radiation therapy is chemotherapy indicated [60]. These recommendations stem from the remarkably minimal literature on the use of chemotherapy for meningiomas [37, 60]. Currently the NCCN endorses three therapeutic agents for use in meningiomas: hydroxyurea, somatostatin, and interferon-α [60].

Table 9.2 Results of various meningioma chemotherapy trials

	HU [63]	HU malignant [64]	INF-alpha [65]	SS [66]	SS [67]	TMZ [68]	Irinotecan [69]	MP [70]	Placebo [70]
N	60	35	35	16	9	16	16	80	84
PFS 6 months (%)	10	3	54	44	44.4	0	6	–	–
PFS 12 months (%)	0	0	31	12.5	0	0	0	–	–
FFS 2 years	–	–	–	–	–	–	–	30	33
MTTP (months)	4	2	7	5	4.23	5	5	10[a]	11[a]
Cycles	192	88.5	242	92	–	29	31	–	–
Toxicity/cycle ≤3	26.04 %	41.81 %	26.03 %	3.26 %	–	93.10 %	96.77 %	39	29
Toxicity/cycle ≥4	0.00 %	0.00 %	0.83 %	0.00 %	–	3.45 %	9.68 %	8	6

PFS progression-free survival, *FFS* failure-free survival, *MTTP* median time to progression, *HU* hydroxyurea, *INF-alpha* interferon-alpha, *SS* somatostatin, *TMZ* temozolomide, *MP* mifepristone
[a]Median failure-free survival

Prior to reviewing the current understanding of chemotherapy for meningioma, it is important to note the benchmarks. With the exception of the mifepristone trial, there are uniformly no placebo arms in meningioma chemotherapeutic trials [70]. To compensate for this one study, chemotherapy effectiveness implies a progression-free survival at 6 months greater than 40 % for recurrent, radiation-refractory meningiomas [63–66, 68, 69]. However, there is no common consensus for what is an acceptable rate of progression-free survival.

Hydroxyurea was one of the first chemotherapeutics thought to be effective for meningioma. The mechanism of hydroxyurea is the inhibition of ribonucleotide reductase, thus preventing the formation of deoxyribonucleotides. Early studies demonstrated promise for hydroxyurea. The median time to progression ranged from 10 to 20 months. Rates of stable disease following treatment were 8–88 %. The issue with these initial studies is that much of their data includes patients that had not failed radiation therapy or had concurrent radiation therapy [63]. A later study by Chamberlin et al. using patients with radiation-refractory meningioma demonstrated less hopeful results. With WHO Grade I tumors that progressed following radiation therapy, progression-free survival was 10 % at 6 months and 0 % at 12 months with a median time to progression of 4 months [63]. For WHO Grades II and III subtypes refractory to radiation, the progression-free survival decreased to 3 % at 6 months and 0 % at 12 months [64]. The median time to progression was 2 months. The authors did, however, demonstrate that the therapy is well tolerated [63, 64].

Meningiomas have long been known to express hormone receptors. Frequency of hormone receptor expression in meningioma samples has been quoted as progesterone in 70 %, estrogen in 19 %, and growth hormone in nearly 100 % [59, 62, 71, 72]. These initially presented exciting targets for chemotherapy in resistant meningiomas. Mifepristone was most extensively studied after promising results were noted in a single-arm phase II clinical study in which 8 or 24 patients had minor responses with symptoms or radiographic findings [73]. This was followed by a phase III study that employed a placebo arm. Unfortunately, the results of this study proved negative as mifepristone demonstrated 30 % failure-free survival and placebo demonstrated 33 % failure-free survival at a 2-year follow-up [70].

Additionally, there has been preliminary research into somatostatin analogues to inhibit growth hormone receptors. Chamberlin et al. published a series of 16 patients treated with cycles of a long-acting somatostatin analogue, sandostatin [69]. In the patient population 14/16 patients demonstrated progression of recurrent meningioma prior to enrollment. All patients had demonstration of the presence of somatostatin receptors in the tumor. Prior treatment varied but included surgery, radiation therapy, and chemotherapy. Patients were monitored with imaging and neurologic status. The study demonstrated 44 % progression-free survival at 6 months and 12.5 % PFS at 12 months [69]. Median time to progression was 5 months. One other similar phase II study with nine patients demonstrated similar results with a 44.4 % progression-free survival at 6 months and 0 % PFS at 12 months [67]. Median time to progression was 4.23 months [67]. Somatostatin had the lowest reported toxicity of the NCCN endorsed therapies [60, 67, 69].

Chamberlin et al. have additionally studied interferon-α in WHO Grade I meningiomas recurrent after resection and refractory to radiation therapy. There are various antitumor effects that interferon-α evokes. Notably, interferon-α has shown inhibition of meningioma cells in culture [30, 59, 72]. Chamberlin et al. reported a progression-free survival of 54 % at 6 months and 31 % PFS at 12 months [65]. Median time to progression was 7 months [65]. The toxicity was similar to that of hydroxyurea, but notably higher than somatostatin and had the only Grade 4 toxicities.

Two more chemotherapeutics warrant mentioning, irinotecan and temozolomide. Irinotecan was initially studied in vivo and in vitro animal studies for effect on meningioma cells. The results showed a growth inhibitory effect with a more marked effect on malignant meningioma cells [74]. The drug has further been studied in a phase II trial in 16 patients with recurrent progressive meningioma following resection and radiotherapy. Unfortunately, these results were negative with a progression-free survival of 6 % at 6 months and 0 % at 12 months. Furthermore, there were 30 Grade 3 and three Grade 4 toxicities in 31 cycles of chemotherapy. Of note the human study did not differentiate WHO grade of the meningiomas, and more research with malignant meningiomas may be warranted [69]. Temozolomide has a similarly disappointing effect in humans. Progression-free survival was 0 at 6 months, and there were 27 Grade 3 and one Grade 4 toxicities over 29 cycles in 16 patients [68].

Of the three therapies endorsed by the NCCN, somatostatin and interferon-α appear to show the most promise. However, neither has been proven in a placebo-controlled study. This leaves question as to whether these medications are truly effective. However, a patient who requires a chemotherapeutic agent for a meningioma is likely to have exhausted treatments with surgery and radiosurgery. Additionally, though the aforementioned trials do not clearly demonstrate efficacy, they do demonstrate minimal toxicity. Thus using these compounds in the context of a maximally resected and radiation-refractory meningioma is reasonable. Ultimately, further research should be done to identify effective chemotherapeutics that can be used beyond the realm of last-resort or salvage therapy.

9.5 Development of Chemotherapy Resistance

Meningiomas were thought to be more vulnerable to chemotherapy than intraparenchymal tumors because they are located outside of the blood-brain barrier. Despite this there are limited successful approaches to meningioma management with chemotherapeutics, as seen in the chemotherapy section. To understand this contradiction, one must look to the mechanisms of tumor resistance seen in meningioma.

Tews et al. explored the expression of proteins associated with drug resistance in classic, atypical, and malignant meningiomas [75]. The studies use immunohistochemical assays to elicit the presence of proteins involved in membrane transport (P-glycoprotein [P-gp], multidrug resistance-associated protein [MRP1], and lung

resistance-related protein [LRP]), detoxification (metallothionein), and DNA proliferation (topoisomerase II). Interestingly, normal arachnoidal cells were found to be constitutively positive for P-gp and LRP. Additionally, half of the meningeal cells demonstrated nuclear expression of topoisomerase II. Arachnoidal cells did not show positive for MRP1 and metallothionein. In classic meningioma cells, there was continued expression of P-gp and LRP with additional expression of MRP1 and in 50 % metallothionein. There was some variable expression of topoisomerase. Compared to other meningiomas, the fibrous subtype of meningioma had lower expression of MRP1, and the meningotheliomatous meningiomas had metallothionein expression in all samples. Atypical meningiomas were remarkable for strong clustered expression of MRP and metallothionein around necrotic areas. Furthermore, there was continued but increased expression of P-gp. Of note, there was no difference in the expression patterns of meningiomas exposed to radiation [75].

Haroun et al. analyzed a number of different primary brain tumors for the level of resistance to various chemotherapies [76]. Drug resistance was defined as extreme if the percent cell inhibition (PCI) was one standard deviation below the median, intermediate if the PCI was within one standard deviation of the median, and low drug resistance if the PCI was greater than on standard deviation above the mean. Meningiomas demonstrated extreme drug resistance to vincristine, dacarbazine, carmustine (BCNU), and 4-hydroxy-cyclophosphamide. Meningioma demonstrated particular resistance to BCNU. This resistance is attributed to O6-alkylguanine-DNA alkyltransferase (AGAT) activity, which according to one study has higher expression in meningiomas as compared to other primary brain tumors [77].

With an appreciation for the wide variety of mechanisms for drug resistance in meningiomas, the difficulty in treating with chemotherapeutics can be understood. The use of chemotherapeutics in the future will have to take these resistances into account to counteract or avoid their effect.

9.6 Future Chemotherapy and Targeted Therapy Development

As previously discussed, the role of chemotherapy has been traditionally limited to tumor recurrence and after RT options are exhausted [4]. Results of cytotoxic chemotherapy for atypical and malignant meningiomas have not proved overly beneficial. Furthermore, development of new chemotherapeutic options has been slow by the understanding of molecular and genetic pathogenesis of high-grade meningiomas [78].

The future of chemotherapy and targeted therapy development lies with the identification of mitogenic or antiapoptotic pathways. Recent research has shown that multiple growth factors/receptors are overexpressed including epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), transforming growth factor-α (TGF-α), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) [79]. Overexpression of these factors leads to the activation of transforming growth factor-β (TGF-β), phospholipase C-gamma-1/protein kinase C (PLC-gamma-

1-PKC), Raf-1 mitogen-activated protein kinase/extracellular signal-regulated kinase cascade (Raf-MEK-1-MAP-K/ERK), and phosphatidylinositol 3-kinase/protein kinase B (PI3K-Akt/PKB) [79].

Preclinical and clinical trials currently exist for therapies directed at disruption of growth factor receptor activation and downstream effects [79]. Although these therapies have not been specifically evaluated for efficacy in high-grade meningiomas, they hold promise for other high-grade neoplasms and may be translated into meningioma treatment. Examples of these therapies include inhibitors of vascular endothelial growth factor (VEGF), VEGF receptor, PDGF, and EGFR [9, 62, 78]. With the development potential targets and potential therapies, preclinical trials become increasingly important.

As our knowledge of genetic and molecular tumor alterations grows, cell signaling pathways, hormones, and chromosomal changes become possible target sites for therapy. Development of novel targeted therapies to these signaling molecules and receptors may lead to new, fruitful treatment options. Future prospective clinical studies will be necessary to determine the effect on local control, PFS, and overall survival.

9.7 Conclusion

WHO Grades II and III meningiomas comprise a minority of the overall prevalence of meningiomas. However, treatment options are lacking in the event of failure of standard resection and adjuvant radiation or in the setting of recurrence. Multiple chemotherapeutic agents have been initially studied including mifepristone, hydroxyurea, somatostatin analogues, interferon-α, irinotecan, and temozolomide. Current outcomes have not proven to be significantly beneficial with these agents, and they remain last-resort options. Continued research in mitogenic and antiapoptotic pathways and in agents used with other high-grade neoplasms may lead to the discovery of agents that will be successful in the treatment of high-grade meningiomas.

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Chapter 10
Recurrence of Low-Grade Glioma: Have the Targeted Therapies Improved for Better Outcomes?

Zaitun Zakaria

Abstract Low-grade gliomas (LGG) in adult, despite being less aggressive than high-grade gliomas, eventually will recur. For many decades, the standard radiological imaging to treatment planning has evolved. Nowadays, clinicians are leaning toward early surgical resection followed by adjuvant chemo- and/or radiotherapy rather than waiting for the tumor to recur before the treatment commences. This will put patients at risk of unavoidable treatment-related side effects and increase of resistance to further treatment following tumor recurrence. The current chapter gives an overview of changes that have been made, together with limitations that we still encounter in managing patients with LGG in order to improve progression-free survival (PFS) and overall survival (OS).

Keywords Low-grade glioma • Radiological imaging • Radiotherapy • Chemotherapy • Cognitive decline

Abbreviations

ADC	Apparent diffusion coefficient
DWI	Diffusion-weighted MRI
EORTC	The European Organization for Research and Treatment of Cancer
FET	O-(2-[18F]Fluoroethyl)-L-tyrosine
FDG	18F-Fluorodeoxyglucose
FLAIR	Fluid-attenuated inversion recovery
FMT	L-3-[18F]Fluoro-α-methyltyrosine

Z. Zakaria (✉)
Royal College of Surgeons in Ireland, 123 St Stephen's Green, Dublin 2, Ireland
e-mail: zaitunzakaria@rcsi.ie

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_10

Gy	Gray
GTR	Gross total resection
LGG	Low-grade glioma
MET	[11C]Methyl-L-methionine
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
MRS	MR spectroscopy
OS	Overall survival
PCV	Procarbazine, lomustine (CCNU), and vincristine
PET	Positron emission tomography
PFS	Progression-free survival
RTOG	The Radiation Therapy Oncology Group
STR	Subtotal resection
TMZ	Temozolomide

10.1 Introduction

Low-grade gliomas (LGGs) include a diverse group of tumors within the brain, brainstem, and spinal cord, with distinct characteristics, patterns of occurrence, response to treatment, and survival [1]. The commonly accepted World Health Organization classification for brain tumors identified four inclusion analyses of surgical specimen; these include atypical cells, mitoses, endothelial proliferation, and necrosis. Grade I gliomas, such as pilocytic astrocytoma, dysembryoplastic neuroepithelial tumor, pleomorphic xanthoastrocytoma, and ganglioglioma, possess none of these characteristics. Diffuse grade II gliomas, which represent approximately 15 % of gliomas and include astrocytoma, oligodendroglioma, and oligoastrocytoma, possess one of these characteristics. The presence of up to 50 % astroglial component is accepted to make the diagnosis of oligoastrocytoma. LGG shows a preference in the frontal and temporal lobes [2], more frequently in the right hemisphere than in the left. The diagnosis can be delayed due to vague symptoms such as headache, lethargy, or personality changes that are present for months before the tumor mass causes an increase in intracranial pressure. In contrast to malignant gliomas, most patients with LGG present at younger age between the second and fourth decades of life with epileptic seizure experience as the most common presenting symptom [1, 3], occurring in up to 80 % of the patients [4]. Patients with oligodendrogliomas and oligoastrocytomas, which more often involve the cortex, are more prone to seizures than those with astrocytomas, which tend to be situated in the white matter [5, 6]. Despite the indolent nature and slow growing (3–4 mm of mean diameter per year) [7], these tumors have a propensity to recur and ultimately undergo malignant transformation [8]. It is crucial that the tumor is detected and diagnosed early for a proper future therapy. This chapter gives an overview of changes that have been made, together with limitations that we still encounter in managing patients with LGG from diagnosis to personalized targeted therapies in order to improve progression-free survival (PFS) and overall survival (OS).

10.2 Limitation of Current Radiological Imaging

Radiological imaging is an essential pre- and postoperative workup of patients with brain tumors. On computerized tomography, LGG usually appears as a heterogeneous region of low attenuation that may or may not enhance on contrast study or induced mass effect on adjacent structures [1]. A conventional 1.5 Tesla magnetic resonance imaging (MRI) is a gold standard of radiological investigation and is a more helpful tool in distinguishing between low- and high-grade gliomas [9]. The information achieved from this imaging includes contrast material enhancement with gadolinium, perienhancement edema, distant tumor foci, hemorrhage, necrosis, and mass effect [10]. LGGs are usually non-enhancing tumors with an increase signal on T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences. FLAIR sequences show the best contrast between presumed infiltrating tumor margins and the normal brain [1]. The advantage of FLAIR, in comparison to T2-weighted sequence, is that the FLAIR has subtracted the extracellular fluid and, thus, provides a better delineation between tumor and edema or tumor and cerebrospinal fluid [11]. An irregular calcification seen on imaging is a feature of oligodendrogliomas [12], rather than astrocytomas [13].

Conventional MRI comes with its limit; hence, further development of advance imaging techniques can reliably justify the decision-making of initial diagnosis, response to treatment, or recurrence of tumor. Therefore, certain treatment centers have integrated perfusion-weighted MRI, diffusion-weighted MRI (DWI), or MR spectroscopy (MRS), since each has shown their potential usefulness for diagnostic purposes, estimation of prognosis, and assessment of early treatment response [14]. DWI, such as apparent diffusion coefficient (ADC), is useful in solid parts of gliomas. ADC values show a relationship between high-grade versus low-grade gliomas, where high values are used as indicators of low-grade tumors, with accuracy [14, 15]. MRS quantifies variation in metabolites within regions and tissue types of the brain [16, 17]. LGGs are generally characterized by a relatively high concentration of N-acetylaspartate, a low level of choline, and an absence of lactate and lipids. An increase in choline levels over time is compatible to malignant degeneration in LGG [18]. However, this method relatively has a high sensitivity but low specificity [19] and, therefore, is included as a supplementary to a standard MR examination.

Non-enhancement of supratentorial brain tumors in adults does not equate with a low-grade tumor, with 40 % of these non-enhancing lesions being histologically found to be anaplastic [20]. There is a trend to overcome this limitation with positron emission tomography (PET) using radiolabeled amino acids, such as O-(2-[18F]fluoroethyl)-L-tyrosine (FET), ^{18}F-fluorodeoxyglucose (^{18}F-FDG), [11C] methyl-L-methionine (MET), or L-3-[^{18}F]fluoro-α-methyltyrosine (FMT). PET has gained much scientific interest and has increasingly been applied to increase diagnostic accuracy [21–23]. In patients where the MRI did not show evidence of contrast enhancement with gadolinium, metabolic imaging with PET tracers may help to locate tumor "hot spots," which should be targeted when doing a minimally invasive tumor biopsy [24]. The presence of a small hot spot has been associated with a worse prognosis despite aggressive treatment post surgery [9].

^{18}FET-PET is a more powerful tool than MRI for the detection of tumor recurrence in patients with glioma after radiotherapy, radiosurgery, or multimodal treatment. While the specificity for ^{18}FET-PET (92.9 %) was quite similar to MRI (93.5 %), a marked contrast is seen in sensitivity (100 % versus 50 %, respectively) [25]. Amino acid PET has also previously been shown to be superior to MRI for evaluating temozolomide (TMZ) responses in LGG patients. Following MET-PET, metabolic active tumor volume during chemotherapy yielded a reduction of 25 % as early as 2.3 months. In contrast, MRI responses on T2-weighted MRI of the same patients were delayed, and volume reduction of 25 % was only evident at 16.8 months [26]. Of different radiolabeled amino acid, ^{18}F-FDG-PET is of limited use in the assessment of therapy in LGG [17]. FDG shows a high uptake in gray matter, resulting in a poor tumor to background ratio [27]. Furthermore, radiation necrosis may be indistinguishable from recurrent tumor because ^{18}F-FDG can accumulate in macrophages which may infiltrate the sites having received radiation therapy [28]. A delay in diagnosing a recurrent tumor could potentially minimize further treatments available for these patients, and by the time the disease was detected, the tumor may have transformed into a malignant grade. Recently, the integration of PET-MRI allows a more accurate histological diagnosis [29]. However, while MRI is widely accessible, PET is limited as yet due to lack of experiences and expensive production of radiolabeled isotopes [24, 30].

10.3 Surgery: From Conservative to Radical Approach

The concept of aggressive treatment in LGG remains controversial, and conservative treatment or "watch and wait" strategy by active surveillance, i.e., follow-up MRI imaging, should still be proposed in asymptomatic patients. Whittle has previously suggested that if the lesion is surgically inaccessible, the patient does not wish to have surgery, or if there are doubts about the evidence basis for surgery, then a "watch and wait" policy may be appropriate [31]. Nonetheless, there is no evidence from randomized controlled trials to either support or reject this strategy [31, 32], and with evolved diagnostic and surgical techniques, it is very rarely good practice [33]. The incidence and timing of malignant progression are variable [34], and the tumor may progress very slowly before the evidence of progression. In grade II astrocytoma, ~70 % transform to glioblastoma within 5–10 years of diagnosis [35]. This can be detected either by MRI, worsening of current symptoms, or the presence of new symptoms. According to the mathematical three-dimensional (3D) MRI model, glioma growth does not obey an exponential evolution because many newly produced tumor cells diffuse into the surrounding parenchyma and their density does not reach the minimal threshold required to appear on MRI [36]. Furthermore, it has been suggested that oligodendroglioma cells have indistinct borders and microscopic invasion and can extend to a margin beyond MRI-defined abnormalities [37].

The stereotactic biopsy of tumor, either single or serial sampling of tumor areas, is a minimally invasive procedure to obtain tissue for histopathological and molecular genetics evaluation. The biopsy is performed by radiological guided of a careful selection of target point. This technique, on a careful hand of experienced neurosurgeons, is shown to have a better rate of morbidity and mortality [38]. However, the technique is limited to the fact that an inadequate tissue sample can lead to inaccuracy in histopathological diagnosis, which could affect the postoperative management plans, overall prognosis, and misinterpretation of clinical trials. Of the previous articles, Jackson et al. reviewed the limitation of stereotactic biopsy in comparison to surgical resection and found that 12 of 19 (63 %) cases of low- or intermediate-grade tumors were upgraded to malignant tumors. Overall, of the 80 specimens that were analyzed, 21 (26 %) discrepancies would probably have affected the treatment plan and 30 (38 %) cases would have affected prognosis [39]. While this review was performed more than a decade ago, it is still worrying that more than half of the tumors were initially misdiagnosed and these patients could be have subjected to different treatment regimens or clinical trials.

A decade later, Reithmeier et al. disclosed the incomplete correlation of histopathological results in 11 of 33 (33 %) of patients who had open surgery within 30 days of stereotactic biopsy. The discrepancies were either from tumors with the same grading but different cell types, different grading but same cell type, or different grading and different cell types [40]. Since the intratumoral heterogeneity of LGG could contribute to the undersampling or error in the histopathological diagnosis that is not reflective of the tumor as a whole [41], they suggested that a careful interpretation of histopathological finding should correlate with the neuroradiological and clinical history of patients and a consensus of further treatment should come from a multidisciplinary meeting. Indeed, it was the quality of biopsy rather than the quantity of tissue obtained that determined the diagnostic yield and accuracy rate [38]. Even though some have thought that the stereotactic biopsy is perhaps an unnecessary procedure [39], a recent recommendation had suggested that when the risk of open surgery outweighs the potential benefits, perhaps the stereotactic biopsy should be recommended such as when the tumor is in the eloquent cortex or patients are medically unfit for craniotomy [42].

The volume of tumor resection affects the PFS and OS of LGG patients. The Norwegian group examined the survival of LGG patients who had biopsies and watchful waiting versus early surgical resection. Early resection is associated with better 7-year survival when compared to watchful waiting (68 % versus 44 %, respectively). Particularly, in patients with astrocytoma, early resection gives an additional 4.1-year survival benefits than watchful waiting (median survival of 9.7 versus 5.6 years, respectively). Later malignant transformation was more common when biopsy only was performed than early surgical resection [43]. Preoperative tumor volume has also been inversely associated with PFS and OS due to the reason that tumors that are larger at presentation may have an inherently faster growth rate [44].

Therefore, similar to high-grade glioma, the extent of maximal safe tumor resection has evidently been associated with longer life expectancy [45]. More aggressive resections also have the advantage of treating the disease while the neoplasm is

smaller, which may decrease the risk associated with malignant differentiation, prevent the emergence of intractable seizure disorder [46], and improve seizure control particularly in patients who presented these symptoms [33, 47, 48]. The notion that surgical resection is therapeutic is valid when the tumor volume produced mass effect, and without resection further increased intracranial pressure could occur from radiotherapeutic effects which often induced further peritumoral edema and swelling [49, 50]. Nevertheless, resection of these tumors that have no clear defined boundaries and often occurring in functional regions is less likely to be completely resected and carries a high risk of a permanent deficit [51–53].

For the last two decades, awake craniotomy has become a frequent procedure. This surgical approach helps to identify and preserve functional areas during cortical and subcortical tumor resections of eloquent areas for maximum patient safety [54]. Further refinement with neurophysiological mapping of the cortex and white matter tract additionally helps to extend the radicality of resection and preserve essential language sites, even in the setting of negative mapping data [55, 56]. Indeed, with the addition of intraoperative MRI, awake craniotomy can give further maximal safe resection with an acceptable morbidity profile [57, 58]. This is an encouraging learning curve for neurosurgeons to master the technique of awake craniotomy but a potentially positive step to prepare patients for future adjuvant treatment(s).

10.4 Adjuvant Treatment Post Surgery

The management of adult patients with supratentorial LGG remains a dilemma, as whether to start early adjuvant treatment after surgery, e.g., radiotherapy, chemotherapy, or chemo- and radiotherapy, or delay the treatment until the time of tumor recurrence. From several clinical trials that are still ongoing and awaiting maturation, we will look at the results of the study, particularly the PFS and OS of patients and what are the limitations that we will still encounter.

10.4.1 Early Adjuvant Radiotherapy Post Surgery

Clinical trials of radiotherapy in LGG are still controversial and so far have only proven a modest benefit. An overall survival (median follow-up between 5 and 10 years) from three separate randomized trials, the European Organization for Research and Treatment of Cancer (EORTC) 22844, EORTC 22845, and the North Central Cancer Treatment Group (NCCTG) 86-72-51, did not show an OS benefit from radiotherapy, when compared to no treatment, delay radiotherapy, or treatment at higher dosage. The US study, EORTC 22844, involved 379 adult patients with cerebral LGGs in two radiotherapy arms, either 45 gray (Gy) in 5 weeks or 59.4 Gy in 6.6 weeks. Both 5-year overall survival (58 % versus 59 % years, respectively) and

progression-free survival (47 % versus 50 %, respectively) were similar between the treatment arms [59]. Nine years later, EORTC 22845 was conducted and completed on 311 patients; this study assessed the efficacy of early radiotherapy versus deferred radiotherapy until the time of progression, with a total radiation dose of 54 Gy in fractions of 1.8 Gy for 6 weeks [60, 61]. Although OS did not differ between the two groups (7.4 years versus 7.2 years, respectively), the median PFS for early radiotherapy was superior to the deferred treatment (5.3 years versus 3.4 years, respectively). A significant benefit was observed with regard to seizure control at 1 year in the early radiotherapy group. This study recommended delay radiotherapy to be given when in younger patients presenting with seizures only, while withholding radiotherapy until tumor progression does not jeopardize survival.

A prognostic scoring system was developed using MRI interpretations, patients, and tumor characteristics derived from the database of phase III EORTC 22844 and 22845 studies. The following five negative risk factors are identified and validated: age 40 years or older, astrocytoma histology subtype (rather than oligodendroglioma or oligoastrocytoma), largest tumor diameter of ≥6 cm, tumor crossing the corpus callosum (midline), and presence of neurological deficit before surgery. The presence of up to two of these factors identified the low-risk group with median OS of 7.7 years, whereas higher scores identified the high-risk group with median OS of 3.2 years [4]. The Intergroup Clinical Trial NCCTG 86-72-51 was a study to compare survival and toxicity in 203 patients with supratentorial LGGs who were randomized after surgery to a low-dose (50.4 Gy in 28 fractions) versus a high-dose (64.8 Gy in 36 fractions) radiotherapy. No dose response was seen at a median follow-up of 6.43 years, while OS at 5 years did not find an advantage for high-dose over low-dose radiotherapy (72 % versus 64 %, $p=0.48$). However, grades 3–5 toxicity occurred in 5 % of patients in the high-dose arm as compared with 2.5 % in the low-dose arm. Patients in the high-dose group also were found to have a significantly increased risk of developing radionecrosis [62]. Multivariate analysis identified the histologic subtype, tumor size, and age as the most significant prognostic factors. Patients with oligodendrogliomas or oligodominant mixed oligoastrocytomas had a 5-year survival rate of ~75 %, compared with 55 % for astrocytomas or astrodominant mixed oligoastrocytomas. Furthermore, survival is significantly better in patients <40 years than those ≥40 years (77 % versus 60 %, respectively) [62].

Caution interpretations are needed of the three trials mentioned above. The result is hampered by the apparent histological heterogeneity of the tumors studied. In the EORTC 22844, most (68 %) of the LGGs were oligodendrogliomas or oligoastrocytomas [62]. In the second EORTC 22845 trial, 22 % of the tumors were reclassified as high-grade astrocytomas after a histopathological review [60]. It is also important to recognize that, unlike the EORTC trials (i.e., EORTC 22845), all patients in the NCCTG 86-72-51 received immediate postoperative radiotherapy [63]. The summary of these trials has suggested that a radiation dosage of 40–50 Gy gives a similar outcome and is better tolerated than treatment up to a dosage of 60–65 Gy. The currently accepted treatment for LGG includes a radiation dose of 50–54 Gy in fractions of 1.8 Gy to the tumor bed as well as a 1–2 cm surrounding the margin [1].

10.4.2 Early Adjuvant Chemotherapy Post Surgery

TMZ is recognized as the standard regime for glioblastoma, and the clinical benefit of this alkylating agent has currently been examined in LGGs. A phase III study, EORTC 22033-26033 that is still awaiting maturation, compares the benefit of radiotherapy only (total of 50.4 Gy) or TMZ only (75 mg/m^2 daily for 21 days every 28 days for maximum of 12 cycles) in patients with at least one of the following criteria: age ≥40 years, radiologically proven progressive lesion, new or worsening neurological symptoms other than seizures only, or the presence of intractable seizures. During the presentation at the American Society of Clinical Oncology in 2013 [64], at a median follow-up of 45.5 months and after 246 patients (52 %) had progressed, there was no statistically significant difference in the PFS between the treatment arms. The dosage of TMZ treatment was previously categorized into conventional regimes and protracted metronomic regimes. Unfortunately, no randomized control trials comparing different regimens of TMZ in patients with LGGs exist. It was only on a systemic review analysis where metronomic regimens were associated with an increased objective response rate and superior PFS rates at 6 months and 12 months, respectively, when compared to that of the standard regimes [65]. Similar to early radiotherapy, TMZ also resulted in a better seizure control [26, 61]. In in vivo studies carried out in xenografted athymic rats, when comparing between these two regimes, Zhou et al. suggest that the metronomic regimen may be superior by preventing tumors from progressing to a pro-angiogenic state because of differences of expression levels of vascular endothelial growth factors and hypoxia-inducible factor-1α between the two regimens [66].

The dilemma with aggressive chemotherapy treatment in patients with LGGs was due to two important findings. First, the alkylating agent can exert a mutagenic effect influencing the risk of malignant transformation or secondary malignancy [67–69]. As explained by Johnson et al. when comparing between primary and recurrent tumor samples from the same patients who progressed to malignant gliomas and who previously were treated with TMZ, six of ten patients exhibited hypermutated phenotypes, carrying many more mutations per million base pairs compared with their initial tumors. Predominantly identified within the tumor exposed to TMZ were C>T/G>A transition, and more than 98.7 % are thought being due to TMZ-induced mutagenesis. The authors also suggested that TMZ-associated mutations compromise the retinoblastoma and mTOR signaling pathways.

Second, despite TMZ's action in inducing apoptosis [70], TMZ-induced hypermutation is the consequence of a TMZ resistance mechanism in LGGs [71]. Resistance to TMZ develops, in part, through the sequential acquisition of genetic and epigenetic changes in MGMT and inactivation of the DNA mismatch repair (MMR) pathway. The compromised DNA repair contributes to the mutagenic potential and subsequent malignant transformation, indicating that while the addition of TMZ plus radiotherapy provides a better survival than radiotherapy alone in treating GBM [72], a careful decision-making should be considered when treating LGG patients [73].

10.4.3 Early Adjuvant Chemotherapy and Radiotherapy Post Surgery

The phase III RTOG 9802, which enrolled patients between October 1998 and June 2002, is the paradigm of a recent trial in LGGs and opens the clinician's perception on early upfront chemo- and radiotherapy post surgery, rather than initial radiotherapy and further chemotherapy at the time of relapse in patients with supratentorial LGG. Patients were dichotomized into two risk groups: a favorable group, defined as 18–39 years with surgeon-defined gross total resection (GTR) of their tumor, and an unfavorable group, defined as either >40 years or older, irrespective of the extent of resection plus all 18–39 years with a subtotal resection (STR) or biopsy. Patients with favorable LGG were observed postoperatively (no adjuvant therapy was given), whereas patients with unfavorable LGG were randomized to radiotherapy (total of 54 Gy) only or the same radiotherapy followed by six cycles of procarbazine, lomustine (CCNU), and vincristine (radiotherapy + PCV) [74]. The OS rates at 5 years were significantly higher for patients with favorable LGG than the unfavorable LGG (93 % versus 66 %, respectively, $p < 0.0001$). However, the PFS rates at 5 years were 48 % for favorable LGG, which were similar (50 %) to those of the patients with unfavorable LGG. The median PFS time was 4.9 years for patients with favorable LGG versus 5.5 years for patients with unfavorable LGG [75]. This study also identified prognostic factors associated with recurrence rate; these include preoperative tumor diameter ≥4 cm and postoperative histological diagnosis of astrocytoma/oligoastrocytoma and the size of residual tumor on MRI. Particularly, patients with GTR (<1 cm residual tumor) had a recurrence rate of 26 %, STR (between 1 and 2 cm residual tumor) had a recurrence rate of 68 %, and STR (>2 cm residual tumor) had a recurrence rate of 89 %.

When the updated data were presented at the American Society of Clinical Oncology 2014, a long-term follow-up showed a widen survival benefit between the two groups of unfavorable patients. Having the addition of PCV to radiotherapy significantly increased PFS (10.4 versus 4.0 years) and median OS (13.3 years versus 7.8 years) than having radiotherapy alone. Overall, survival is worse in males with astrocytoma/oligoastrocytoma histology. This study brought a message that upfront chemo- and radiotherapy at earlier treatment is far superior to having radiotherapy only followed by chemotherapy at the time of relapse [76]. The LGG trial E3F05 further recruited high-risk patients, as defined by age more than 40 with clinically or radiographic evidence of progressive disease. This trial randomly assigns LGG patients to receive combined radiotherapy (total dose of 50.4 Gy) with TMZ and followed by a year of TMZ or to receive radiotherapy alone. However, the trial accrual has recently been temporarily closed when the overall survival after radiotherapy alone was found inferior to radiotherapy with PCV in the RTOG 9802. The study was halted on the assumption that results with radiotherapy alone would be inferior to radiotherapy with TMZ [77].

The RTOG 0424 trial is a single-arm phase II study to compare OS at 3 years in high-risk patients who received combined radiotherapy (total dose of 54 Gy) and TMZ treatment to the 3-year OS rate of the high-risk EORTC LGG patients reported

by Pignatti et al. [4]. With a median follow-up of 4.1 years, the 3-year OS rate of 73.1 % for these high-risk LGG patients is significantly higher than those reported for historical controls with p-value <0.0001. The median PFS was 4.5 years with a 3-year PFS rate of 59 % [78]. However, adjuvant radiotherapy and TMZ appear inferior to those from the RTOG 9802 trial. With a 3-year OS on both RT alone and RT plus PCV which was nearly 80 % [79], adjuvant radiotherapy and PCV demonstrated a median PFS of 10.4 years and 3-year PFS of 75–80 % [76, 77]. While there are differences with regard to PFS and OS, cross trial comparisons can raise questions with regard to study designs or inclusion/exclusions criteria. This includes a different definition of "high risk", and perhaps higher PFS in the RTOG 9802 is likely from the inclusion of lower-risk patients. While these data have translated the management of patients with LGG, they are new to other neuro-oncology centers that still preferred upfront TMZ than PCV post surgery [80].

10.4.4 Treatment-Related Cognitive Decline

The quality of life of patients who received adjuvant treatment has long been debated. The EORTC 22844 assessment on quality of life evaluated the psychological, physical, social, and symptom domains over time among patients who received a low dosage (45 Gy) to a high dosage (59.4 Gy) of radiotherapy. The authors concluded that, since there are no major differences in the quality of life between the groups, significantly higher levels of fatigue/malaise and worsening emotional functioning in the high-dose group were detected [81]. In addition, radiotherapy treatment affects neurocognitive functions. The deleterious effect was experienced in long-term survivors of glioma patients who had early radiotherapy that was recognized more than 30 years ago [82]. Klein et al. in their report performed cognitive function tests to compare among healthy controls, LGG patients, and hematological patients for a mean of 6 years after diagnosis. While glioma patients generally had a lower baseline cognitive assessment when compared to the other two groups, the administration of radiotherapy (total dose of 55.6 Gy) resulted to a poorer cognitive outcome. This has been made worse when antiepileptic medications were coadministered [83]. After 12 years from diagnosis, his coworker further calculated cognitive assessment to detect differences between patients who had radiotherapy and patients who did not have radiotherapy [84]. Late radiation-induced cognitive deterioration, such as attentional functioning, and radiological abnormalities, such as global cortical atrophy, were significantly worse in patients who received radiotherapy. In view of the higher survival rate in LGG patients, it is imperative that this treatment-related side effect be discussed to patients prior to the informed consent.

Other studies, such as the RTOG 9802, used the Mini-Mental State Examination (MMSE) to assess cognitive functions in high-risk patients [85]. Over 5 years of follow-up, they found that the addition of PCV and radiotherapy (total dose of 54 Gy) did not result in significantly higher rates of MMSE score decline than radiotherapy alone. Furthermore, regardless of any treatment arms, patients were

more likely to experience significant MMSE gain than decline, suggesting that intensive therapy did not affect the MMSE. Similarly, the result of the intergroup clinical trial also reported that, after median follow-up of 7.4 years, most patients maintain a stable MMSE score after receiving radiotherapy [86]. In another analysis, an abnormal baseline MMSE score was a predictor of poorer PFS and OS for patients with a LGG [87]. Despite that, the rationale of using the MMSE test was refuted by others who believed that this method is a poor assessment of cognitive functions. MMSE does not measure cognitive functions that are likely to be affected by radiotherapy [88, 89]. In an assessment of the diagnostic accuracy of the MMSE (using the same criteria as Brown et al. to detect abnormality), in comparison to neuropsychological test of brain tumor patients, MMSE yielded a poor sensitivity of 50 % [88]. The discrepancy of these two methods underlines the importance of using standardized neuropsychological test protocols, which in the future are more useful for comparing studies of adjuvant-related damage.

10.5 Conclusion

From the clinical trials that categorized LGG patients into either low risk or high risk, the treatment paradigm is therefore not equal. Clearly, the gross total resection is the best possible operative approach in order to remove the tumor, but the morbidity associated with neurological deficit is always a concern to neurosurgeons. The chances of leaving too much tumor behind during surgery or misdiagnosing the tumors are unfortunate events that leave the patient to a higher risk of tumor recurrence or even an inappropriate treatment plan/clinical trial. With further sophisticated radiological imaging that continues to improve, this technology remains not widely accessible. After surgery, as shown by the clinical trials, early adjuvant treatment is recommended rather than waiting for the tumor to recur before the treatment commences. However, we are still balancing the best treatment options and how to tailor this to treatment-related side effects, particularly future cognitive decline and intolerable toxicities. What we understand is that the best result so far is from the RTOG 9802, which means that the adjuvant radiotherapy and PCV give the best PFS and OS. Nevertheless, it depends on the clinician's preferences within neuro-oncology institutions, treatment availability, and sometimes the patient's clinical status. With those conclusions we must realize that glioma will recur and become more aggressive and resistant to treatment; hence, there will always be an urgent need for new active chemotherapeutic agents.

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Chapter 11
Host–Tumor Interactions in Brain Cancer Metastasis Leading to Drug Resistance

Robert R. Langley and Isaiah J. Fidler

Abstract An estimated 200,000 cases of brain metastases occur in the United States each year. Brain metastasis is associated with poor prognosis, neurological deterioration, and reduced quality of life. At present, there are no targeted therapies solely for brain metastasis, and patients are more likely than not to be excluded from clinical trials evaluating new therapeutic agents. Treatment efforts are hindered by the blood-brain barrier, which prevents the entry of many cytotoxic agents and antibody-based therapies into the central nervous system (CNS). Accumulating evidence suggests that tumor-associated endothelial cells and reactive astrocytes also limit the efficacy of chemotherapy by signaling for activation of antiapoptotic programs in cancer cells. Consequently, a diagnosis of brain metastasis usually signifies a fatal outcome for patients with solid tumors. Improved clinical outcomes are dependent on an enhanced understanding of the brain metastasis-initiating population of cancer cells, a greater enrollment of patients with brain metastases into clinical trials, and the development of new therapeutic strategies to overcome the mechanisms that mediate resistance to therapy.

Keywords Brain metastasis • Astrocyte • Endothelial cell • Endothelin

Abbreviations

BCL2L1	BCL2-like 1
BCRP	Breast cancer resistance protein
GSTA5	Glutathione S-transferase alpha 5
MDR1	Multidrug resistance
MRP	Multidrug resistance protein

R.R. Langley (✉) • I.J. Fidler
Department of Cancer Biology, Unit173, Metastasis Research Laboratory, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
e-mail: Rlangley@mdanderson.org

A. Tivnan (ed.), *Resistance to Targeted Therapies Against Adult Brain Cancers*,
Resistance to Targeted Anti-Cancer Therapeutics,
DOI 10.1007/978-3-319-46505-0_11

TWIST1	TWIST-related protein 1
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

11.1 Introduction

Estimates indicate that approximately 200,000 cases of brain metastases occur in the United States each year [1], and between 20 % and 40 % of patients with disseminated cancers will develop brain metastases during the course of their disease [2]. Accumulating evidence suggests that the incidence of brain metastasis may be increasing due to improved control of primary tumors, advances in imaging technologies, and an increase in melanoma cancers [3–5]. Brain metastases most frequently arise from tumors that originate in the lung (40–50 %), breast (15–20 %), and skin (5–10 %) [6]. The distribution of metastases parallels cerebral blood flow with 80 % of tumors developing in the cerebral hemispheres, 15 % in the cerebellum, and 5 % in the brainstem [7]. Some cancer cells preferentially spread to the posterior fossa, particularly those that originate from the uterus, prostate, and gastrointestinal tract [7]. Metastatic emboli tend to lodge at the junction of the gray and white matter and at vascular border zones where there is a sudden reduction in blood vessel diameter [8]. Contrast-enhanced magnetic resonance imaging (MRI) is the most sensitive imaging modality for determining the presence, location, and number of cerebral metastases [9]. Results from MRI series indicate that at the time of diagnosis, most patients with brain metastasis harbor multiple lesions [10]. Unfortunately, many of these patients will suffer some degree of neurocognitive impairment during the course of their disease. Brain metastasis-associated neurocognitive disorders may result from the destruction or displacement of normal brain tissue, formation of peritumoral edema, increased intracranial pressure, and/or vascular compromise [11].

The prognosis for patients diagnosed with brain metastasis is extremely poor. Median survival times for untreated patients have been reported to be as short as 5 weeks post clinical diagnosis [12]. Current treatment approaches for brain metastases include surgery, whole brain radiotherapy, and chemotherapy [11]. Multimodality therapy may extend overall survival in a subset of patients to 3–18 months [13]. To date, there are no targeted therapies solely for brain metastases, and the blood-brain barrier poses a physiologic impediment to cytotoxic drugs and antibody-based therapies [14]. In addition, many patients with brain metastases are excluded from clinical trials, particularly from those trials that are sponsored by industry [15]. Recent studies have determined that brain microvascular endothelial cells and reactive astrocytes also hinder treatment efforts by signaling for upregulation and activation of antiapoptotic programs in cancer cells [16]. In the following sections, we review the pathogenesis of brain metastasis and then discuss how brain endothelial cells and astrocytes contribute to therapeutic resistance.

11.2 Pathogenesis of Brain Metastasis

The proximity of a transformed cell to a capillary determines its fate [17, 18]. Fidler and coworkers [18] reported that in order for human lung cancer cells to proliferate in vivo, they must reside within 75 μm of a capillary and that cancer cells located beyond a distance of 150 μm from a blood vessel are not viable. It is worth noting that this latter value exceeds the diffusion distance of oxygen in tumor tissue, which has been measured and is approximately 120 μm [19]. Early reports examining the relationship between the microvascular density of neoplasms and tumor size predicted that any tumor growth beyond 1–2 mm in diameter must be preceded by the formation of a new vascular supply (i.e., angiogenesis) [20, 21]. However, studies have since shown the extent of angiogenesis varies widely among different tumors [22] and that some tumors actually progress in the absence of a neovascularization response [23]. Angiogenesis-independent tumor growth has been documented in experimental melanoma brain metastasis models [24] and in a subset of human gliomas [25] and non-small cell lung cancers [26]. One way that tumors progress without invoking a neovascularization response is by proliferating along the length of preexisting blood vessels in a process that is referred to as vessel co-option [27, 28]. There are also data showing that when experimental brain metastases are treated with antiangiogenic therapies, the cancer cells revert to vessel co-option as a means to ensure their survival [29].

The process of metastasis begins when a cancer cell detaches from a primary mass and begins to invade the surrounding tissue. In normal tissues, adjacent epithelial cells are tightly interconnected by several calcium-dependent cell-cell adhesion molecules that function to maintain the structural integrity of the tissue. E-cadherin is one of the more widely studied epithelial adhesion proteins and is a member of the cadherin superfamily of proteins [30]. Examinations of carcinoma cells at the invasive front have shown that these cells repress E-cadherin expression and adopt morphological features and patterns of gene expression that are characteristic of mesenchymal cells as they undergo epithelial-mesenchymal transition (EMT) [31]. Other epithelial proteins, such as β-catenin and cytokeratin, are downregulated during EMT, whereas expression levels of the mesenchymal-associated proteins N-cadherin, vimentin, and fibronectin are upregulated. EMT occurs physiologically during embryonic development and is also observed during tissue repair processes in the adult [32]. When this developmental program is activated in cancer cells, it provides cells with properties that are characteristic of hematopoietic stem cells [33] and enhances their resistance to therapy [34].

The first barrier encountered by invading cancer cells is the basement membrane that separates the epithelial compartment from the connective tissue. The basement membrane is freely permeable to small solutes, but not to cells. Cancer cells attach to the basement membrane using laminin and integrin receptors and initiate localized proteolysis at the cancer cell-basement membrane interface [35]. The destruction of the basement membrane represents the transition from a benign carcinoma in situ to an invasive tumor [36]. Cancer cells gain access to the systemic circulation by penetrating thin-walled venules or lymphatic vessels, which offer little resistance to invading cancer cells [17].

Tumor-associated blood vessels are structurally deficient and hyperpermeable [37], which make them vulnerable to penetration by cancer cells [17]. Results derived from experimental tumor models indicate that a significant correlation exists between cancer cell intravasation and the tumor neovascularization process [38].

Once cancer cells gain access to the vascular compartment, they may circulate as individual entities or they may form homotypic or heterotypic aggregates with other circulating cells. The ability of cancer cells to circulate as aggregates increases their likelihood of forming metastases [39, 40]. Cancer cell retention in distal organs may be due to size restriction (e.g., mechanical trapping) or it may result from selective adhesive interactions between cancer cells and microvascular endothelial cells. Several lines of evidence support a role for the inducible endothelial glycoprotein, vascular cell adhesion molecule-1 (VCAM-1), in melanoma brain metastasis. VCAM-1 is minimally expressed in most vascular beds, but is dramatically upregulated in response to inflammatory cytokines, such as tumor necrosis factor-alpha, interleukin-1, and lipopolysaccharide [41]. VCAM-1 is the endothelial ligand for VLA-4 (very late antigen-4 or integrin α4β1), and interactions between receptor and ligand normally function to support the adhesion of monocytes, eosinophils, and lymphocytes to the microvascular surface of inflamed tissues [42]. Studies examining integrin expression in melanoma determined that there is a direct correlation between α4β1 integrin expression and the occurrence of metastasis, reduced disease-free overall survival, and decreased overall survival [43]. The formation of melanoma metastases in the lungs of mice was associated with an increased VCAM-1 expression in their cerebral vasculature and the formation of brain metastases [44]. VCAM-1 expression was upregulated on tumor-associated vessels in two experimental models of breast cancer brain metastasis and, moreover, on the endothelium of human brain metastasis tissues [45]. Rebhun and coworkers [46] reported that VCAM-1 was constitutively expressed on brain endothelial cells and lymphatic endothelial cells growing in culture and that melanoma cell adhesion to lymphatic endothelial cells was largely VCAM-1 dependent. However, monoclonal antibodies targeting either the integrin α4β1 expressed on melanoma cells or VCAM-1 on brain endothelial cells had no effect on the adherence of melanoma cells to brain endothelial cells, suggesting the existence of an alternative receptor-ligand pair that can mediate the adhesion of melanoma cells to brain endothelial cells.

Studies examining the kinetics of the glial response to experimental brain metastases indicate that astrocytes are one of the first cells to respond to brain-homing cancer cells [47]. Indeed, there is evidence suggesting that astrocytes are capable of detecting a single disseminating cancer cell once it arrests in the cerebral microvasculature [48]. Arrested cancer cells may proliferate within cerebral capillaries or they may extravasate and gain access to the underlying tissue parenchyma. Ultrastructural studies of the extravasation process indicate that it takes longer for cancer cells to enter into the brain parenchyma than to extravasate into other organs [49]. Bos and colleagues [50] performed comparative genome-wide expression analysis on MDA-MB-231 breast cancer brain metastatic variants and parental cells and found that the cyclooxygenase COX2, the epidermal growth factor receptor (EGFR) ligand HB-EGF, and the α2,6-sialyltransferase ST6GALNAC5 were important mediators of MDA-MB-231 breast cancer cell extravasation through the

blood-brain barrier. However, it remains unclear whether other types of cancer cells that metastasize to the brain utilize the same set of genes to enter into the CNS. Accumulating evidence suggests that the information garnered on primary tumors and extracranial metastases may be insufficient for identifying clinically actionable targets for cancer cells residing in the brain. Whole exome sequencing of 86 matched clinical brain metastases, primary tumors, and normal tissues revealed that 53 % of patient brain metastases harbored genetic alterations that were not present in their primary tumors [51]. Brain metastases were also genetically distinct from regional lymph nodes and extracranial metastases.

Studies examining the fate of extravasated cancer cells indicate that the ability of cancer cells to maintain communication with resident microvascular endothelial cells is a key determinant that governs their survival in the brain. Real-time imaging of experimental brain metastases revealed that the failure of melanoma cells to co-opt cerebral blood vessels signals for their demise, whereas the inability to evoke a neovascularization response is fatal for lung cancer cells in the brain [52]. These findings underscore the decisive role that cerebral microvascular endothelial cells play in mediating the outcome of experimental brain metastases. Examinations of clinical brain metastases have yielded similar conclusions. A recent study of human brain metastases originating from various tissues concluded that approximately 98 % of early human brain metastasis growth occurs through physical interactions with the existing neurovasculature [53].

11.3 Mechanisms of Therapeutic Resistance

11.3.1 Blood-Brain Barrier

In addition to supporting the nutritional demands of cancer cells, the cerebral microvasculature also protects cancer cells by preventing many anticancer agents from entering into the CNS. The cerebral vasculature is responsible for maintaining the constancy of the CNS and for protecting neuronal tissues from toxins and pathogens. The movement of ions, molecules, and cells between the blood and the brain is so tightly regulated that the brain vasculature is frequently referred to as the blood-brain barrier [54]. Brain endothelial cells perform the majority of the barrier function and receive critical induction signals from a number of other different cell types, including astrocytes, neurons, smooth muscle cells, and pericytes. Brain endothelial cells are distinct from endothelial cells present in other anatomic locations in several respects. One major difference is that brain endothelial cells are joined by both tight junctions and adherens junctions, which create an impermeable barrier that severely restricts the paracellular transport of solutes [55]. Brain capillaries also lack fenestra that is present in capillaries in other organs, and they possess very few transport vesicles [56]. Brain endothelial cells do, however, contain a much greater number of mitochondria than endothelial cells in other vascular beds in order to fuel the active transport of nutrients to the brain. Carrier-mediated transport systems catalyze the bidirectional movement

between blood and brain of small molecule polar nutrients, such as glucose, amino acids, monocarboxylic acids, choline, purine nucleobases, and nucleosides [57]. Circulating endogenous peptides, such as insulin and transferrin, are transferred into the brain by receptor-mediator transport. In addition, brain endothelial cells also express a number of efflux transporters for xenobiotics and endogenous metabolites. The expression of these transporters poses a major obstacle for the delivery of drugs into the CNS, and they are considered in additional detail in the following section.

11.3.2 ABC Transporters

The ATP-binding cassette (ABC) superfamily of transporters are multidomain integral membrane proteins that use the energy of ATP hydrolysis to translocate solutes across cellular membranes in all mammalian species [58]. P-glycoprotein (Pgp) is a product of the human multidrug resistance (MDR1) gene and is perhaps the most studied efflux transporter. Pgp appears to be predominantly expressed on the luminal membranes of brain capillary endothelial cells in mammals and has a number of anticancer substrates, including doxorubicin, daunorubicine, vinblastine, vincristine, etoposide, paclitaxel, methotrexate, and others [59]. In addition to Pgp, brain endothelial cells also express members of the multidrug resistance protein (MRP) family and breast cancer resistance protein (BCRP). MRP transporters are located on both the apical/luminal (MRP2, MRP4) and basolateral (MRP1, MRP3, MRP5, MRP6) membranes of endothelial cells [58]. ABC transporters have also been implicated in tumor resistance to small molecule tyrosine kinase inhibitors (e.g., sunitinib, erlotinib, imatinib).

Lockman and colleagues [60] studied the extent that the tumor-associated blood vessels supporting brain metastases limit chemotherapy delivery to tumors by measuring the uptake of 14C-paclitaxel and 14C-doxorubicin in over 2,000 brain metastases from two experimental models (human MDA-MB-231-BR-Her2 and 4 T1-BR5 cancer cells). The investigators found that while varying degrees of vascular permeability were evident in more than 89 % of lesions and that drug uptake in metastases was greater than that in normal brain, the drug uptake in brain metastases was less than 15 % that of other tissues or peripheral metastases. Moreover, drug concentrations only reached cytotoxic concentrations in a small subset (~10 %) of the most permeable metastases.

11.3.3 Activation of Redundant Signaling Pathways

Given that the tumor-associated blood vessels play a critical role in the progression and protection of cerebral metastases, it is not surprising that they have emerged as a primary target for therapeutic intervention. Most efforts to suppress angiogenesis as a means to control tumor growth have focused on the vascular endothelial cell growth factor (VEGF) signaling pathway. VEGF belongs to a family of growth

factors that includes placental growth factor, VEGFB, VEGFC, and VEGFD. VEGF proteins mediate their effects by binding to three distinct VEGF receptors: VEGFR1, VEGFR2, and VEGFR3 [61]. VEGFA is sometimes regarded as the archetypical angiogenic cytokine inasmuch as it can elicit all of the endothelial cell processes required for the formation of new blood vessels (i.e., migration, protease production, and cell division) [62]. VEGF also signals for survival of endothelial cells by upregulating the phosphatidylinositol-3 kinase/Akt signal transduction pathway [63]. Results generated from experimental tumor models suggest that VEGF-mediated signaling plays an important role in the progression of metastases in the brain. Yano and coworkers [64] reported that cancer cell expression of VEGF is directly correlated with angiogenesis and growth of several types of experimental brain metastases. In other studies, targeting of VEGF-mediated signaling with a small molecule tyrosine kinase inhibitor of VEGFR reduced angiogenesis and significantly impaired the growth of experimental breast cancer brain metastases [65].

Inhibitors that target the VEGF signaling pathway are now in clinical use, including bevacizumab, a chimeric monoclonal antibody that binds to VEGF and inhibits its function, and small molecule inhibitors of the VEGFR2 tyrosine kinase receptor (e.g., sorafenib, sunitinib). When bevacizumab was added to standard therapy, it was found to significantly increase overall survival in patients with advanced non-small cell lung cancers and colorectal cancers [66, 67]. Unfortunately, however, not all patients benefit from anti-VEGF therapies, and the majority of tumors that demonstrate an initial response to treatment eventually become refractory to therapy.

To better understand the mechanisms that mediate resistance to antiangiogenic therapy, we examined the effects of simultaneously blocking signaling of VEGFR2, EGFR, and HER2 (human epidermal growth factor receptor 2) using the small molecule multitargeted tyrosine kinase inhibitor, AEE788, in experimental models of lung adenocarcinoma (PC14-PE6) brain metastases [14]. We noted that vehicle-treated (control) tumors possessed relatively few blood vessels, most of which were large with dilated lumens (Fig. 11.1a). The microenvironment of control tumors also contained modest levels of basic fibroblast growth factor (bFGF). However, these large blood vessels were absent from tumors of mice that were treated for four weeks with AEE788 therapy and were instead replaced by a greater number of small, irregular blood vessels to the extent that there were no significant differences in the total microvascular surface area between control and AEE788-treated tumors. We also noted a dramatic increase in levels of bFGF in the microenvironment of AEE788-treated tumors. However, there were no significant differences in tumor volume or overall survival between control and AEE788-treated mice. To determine a possible cellular source of bFGF, we treated monolayers of PC14-PE6 lung cancer cells and brain microvascular endothelial cells with AEE788 and measured levels of bFGF using ELISA. We noted that both the cancer cells and brain endothelial cells significantly increased their secretion of bFGF in response to treatment with AEE788 (Fig. 11.1b).

bFGF is a member of the fibroblast growth factor family of proteins and activates the “angiogenic switch” of some tumors [68]. Accumulating evidence suggests that upregulation of bFGF is a common compensatory response of tumors that are

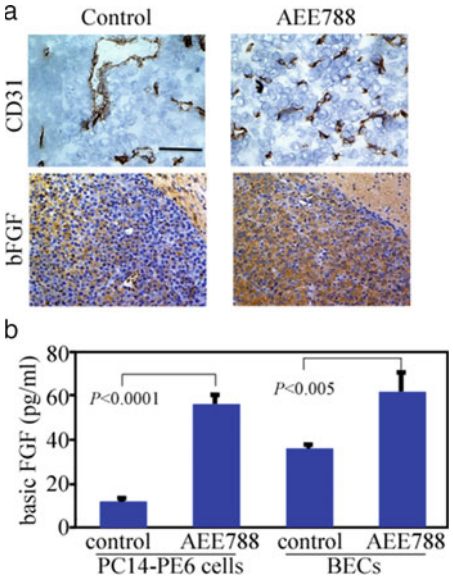

Fig. 11.1 (**a**) Immunohistochemical staining for blood vessels (CD31) and bFGF in PC14-PE6 experimental brain tumor models that had been treated with vehicle (*left panel*) or the multi-tyrosine kinase inhibitor AEE788 (*right panel*) for a period of 28 days. The large, dilated tumor blood vessels observed in control tumors were replaced by an increased number of small, irregular blood vessels in the AEE788 treatment group. Levels of bFGF appear increased in the AEE788 treatment group. (**b**) PC14-PE6 lung adenocarcinoma cells and brain microvascular endothelial cells (BECs) were treated with either vehicle or 1 μM AEE788 for 48 h and expression levels of bFGF measured by ELISA. Reproduced from Langley RR and Fidler IJ [14] with permission from the American Association for Clinical Chemistry

treated with anti-VEGF therapies. Cascone and colleagues [69] used cross-species hybridization of microarrays to determine the patterns of human and mouse gene expression in non-small cell lung cancers that had become resistant to bevacizumab therapy. These investigators discovered that prolonged administration of bevacizumab leads to upregulation and activation of components of the EGFR and bFGF signaling pathways in tumor-associated stromal cells, which restored angiogenesis and tumor progression. In that model, the overwhelming majority of gene expression alterations in tumors that acquired resistance to bevacizumab therapy occurred in the stromal compartment. Casanovas and coworkers [70] reported similar findings in late-stage pancreatic islet tumors that were treated with antibodies that blocked the function of VEGFR1 and VEGFR2. Tumor resistance to VEGF blockade in the pancreatic islet tumors was due to hypoxia-mediated induction of other proangiogenic factors, including members of the bFGF family, by cancer cells and vascular endothelial cells. Collectively, these studies emphasize the important contribution of stromal cells in mediating tumor resistance to antiangiogenic therapy.

11.3.4 Astrocyte- and Endothelial Cell-Mediated Chemoprotection of Cancer Cells

In addition to being a primary mediator of physiologic and pathologic angiogenesis, VEGF also enhances endothelial cell permeability [71]. Indeed, VEGF was originally characterized based on its ability to induce protein extravasation from tumor vessels [72]. An examination of eight different human tumor lines growing in the brains of nude mice indicated that the blood-brain barrier was intact in tumors smaller than 0.2 mm2, but that tumor-associated blood vessels were hyperpermeable when tumors exceeded this diameter [73]. This finding led us to hypothesize that there are likely additional mechanisms that contribute to the resistance of brain metastases.

Astrocytes play a key role in maintaining cerebral homeostasis by reinforcing the blood-brain barrier, participating in neural signal transduction, buffering the ionic balance in the central nervous system, and modulating local blood flow in the brain [74–76]. Under pathological conditions, astrocytes become activated and express GFAP and alter their patterns of gene expression [77]. Astrogliosis is considered the most important hallmark of CNS injury [78] and is thought to be a defensive mechanism that limits the extent of tissue damage [79]. Several studies have demonstrated that astrocytes protect neurons from injury-induced cell death and cytotoxic agents [80, 81], but no information was available regarding the ability of astrocytes to protect cancer cells from the cytotoxic effects of chemotherapy. Bidirectional communication between astrocytes and cancer cells was a conceivable possibility in that reactive astrocytes surround and infiltrate both primary [82] and secondary brain tumors [47, 83]. To begin to study whether reactive astrocytes could affect survival-related signaling pathways of cancer cells, we first generated an immortalized astrocyte cell line derived from the brains of transgenic H-2Kb-tsA58 mice [47]. This mouse line is unique in that each tissue harbors a temperature-sensitive SV40 large T antigen, which allows the user to control the kinetics of cell division in cells derived from these animals [84]. We then developed an approach to pattern reactive astrogliosis in vitro by coculturing these astrocytes with cancer cells. When we modeled astrogliosis of PC14-PE6 lung cancer tumors, we discovered that astrocytes induce phosphorylation of the mitogen-activated protein kinase (MAPK) prosurvival signaling pathway on cancer cells [47].

Lin and coworkers [85] extended that initial work by demonstrating that the coincubation of murine astrocytes with several different melanoma cell lines significantly protected the cancer cells from paclitaxel- and fluorouracil (5-FU)-induced apoptosis. Kim and colleagues [86] applied cross-species hybridization of microarrays to human cancer cells that were co-incubated with murine astrocytes and discovered that contact between the two different cell types leads to marked alterations in the cancer cell transcriptome, including upregulation of the antiapoptotic gene glutathione S-transferase alpha 5 (GSTA5), BCL2-like 1 (BCL2L1), and TWIST-related protein 1 (TWIST1). The authors verified the expression of antiapoptotic genes in cancer cells at the protein level and, moreover, in clinical samples of breast and lung cancer brain metastases. Genetic modulation of the survival genes using small inter-

fering RNAs targeting GSTA5, BCL2L1, and TWIST1 alone had no effect on astrocyte-mediated protection of cancer cells, but the chemoprotective effect was abolished when the cancer cells were transfected with siRNA targeting all three genes. Collectively, these studies demonstrated that astrocytes protect cancer cells growing in culture from the cytotoxic effects of chemotherapy by upregulating expression of antiapoptotic genes in cancer cells. However, the astrocyte-releasing signal that promotes upregulation of antiapoptotic genes in cancer cells remained unknown.

A review of the literature suggested a potential role for the endothelin peptides in mediating the astrocyte-induced upregulation of antiapoptotic genes in cancer cells. The endothelin signaling pathway is comprised of the three small peptides (ET-1, ET-2, and ET-3) that mediate their effects by binding to two G-protein-coupled receptors, ETAR and ETBR [87]. Endothelin proteins are most widely recognized for their ability to evoke potent vasoconstrictor responses from vascular smooth muscle cells [88]. However, recent studies have shown that endothelin proteins play multiple complex roles in cardiovascular, renal, pulmonary, reproductive, and neural physiology [89]. Aberrant endothelin has also been implicated in the initiation and perpetuation of several diseases processes [87]. Activation of endothelin signaling was found to stimulate cell division and invasion in some types of cancer cells [90, 91]. Upregulation of ETAR was associated with chemoresistance and EMT in epithelial ovarian cancers [92].

An expanding body of evidence suggests that endothelin-mediated signaling may be particularly important in CNS pathologies. Immunohistochemical analyses on a large number of human brain metastasis cases revealed that tumor-associated astrocytes overexpress endothelin in 85 % of brain metastases [83]. Reactive astrocytes were also found to overexpress endothelin peptides in several other CNS disease processes, including Alzheimer's disease, progressive multifocal leukoencephalopathy, infarctions, and subacute panencephalitis [93]. A recent study comparing patterns of gene expression between melanoma cell variants that spontaneously metastasize to the brain and parental cells led to the identification of ETBR as a critical determinant in the stepwise progression of melanoma to the brain metastatic phenotype [94].

The abovementioned studies provided a rationale for studying the potential role of the endothelin axis in mediating astrocyte-induced chemoprotection MDA-MB-231 breast cancer cells and H226 non-small cell lung cancer cells. Co-incubation of the breast cancer cells and lung cancer cells with astrocytes led to marked upregulation in astrocyte ET-1 gene expression and significantly increased ETAR and ETBR expression levels on cancer cells [16]. The administration of exogenous ET-1 to cancer cells or co-incubating cancer cells with astrocytes elicited a profound increase in expression of the phosphorylated forms of AKT and MAPK and marked upregulation of the GSTA5, BCL2L1, and TWIST1 survival genes that protected the cancer cells from taxol. To determine whether the chemoprotective-stimulating ability was unique to murine astrocytes, we incubated the cancer cells with human astrocytes, murine brain endothelial cells, murine fibroblasts, and murine microglial cells. Only human astrocytes and murine brain endothelial cells could protect cancer cells from taxol. The finding that brain endothelial cells protected cancer cells through an endothelin-dependent signaling mechanism was not

totally unexpected, given that endothelial cells are the primary cellular source of endothelin in the body [87]. Both ETAR and ETBR were found to contribute to astrocyte- and brain endothelial cell-mediated protection of cancer cells from taxol.

To determine the therapeutic efficacy of targeting ETAR and ETBR in mice harboring brain tumors, we compared survival of nude mice bearing established orthotopically implanted LN-229 glioblastomas or temozolomide (TMZ)-resistant (LN-229Res and D54Res) glioblastomas that were treated with macitentan, TMZ, or both. Macitentan is a dual endothelin receptor antagonist that is currently used in the clinic for the treatment of pulmonary arterial hypertension [95]. We rationalized that glioblastoma was a suitable choice for macitentan therapy because of the heterogeneous expression of ETAR and ETBR on high-grade glioma cells and tumor-associated endothelial cells [96]. Moreover, a recent study determined that an intact ETBR signaling pathway on glioblastoma stems cells was essential for their viability [97]. Indeed, we noted that combined macitentan and TMZ therapy produced marked apoptosis of both glioma cells and tumor-associated cells, which led to glioblastoma regression and long-term responses in the different models, including the TMZ-resistant tumors [98]. Macitentan alone had no effect on overall survival, but instead downregulated expression of the survival-related proteins Bcl2L1, Gsta5, and Twist1 on glioma cells and tumor-associated endothelial cells (Fig. 11.2). The elimination of these protective barrier rendered glioma cells and their supporting blood vessels sensitive to TMZ therapy.

Increasing evidence suggests that tumor blood vessels provide a niche for brain cancer stem cells, where signal-releasing endothelial cells promote their renewal and ensure their survival [99, 100]. Disruption of the communication between endothelial cells and glioma cells may have been responsible for the profound glioma cell death observed in our models. Recently, we found that combined therapy of macitentan and paclitaxel produced dramatic improvements in the survival of mice harboring experimental breast cancer and lung cancer brain metastases (in press, Neuro-Oncol). Similar to our results with GBM, a combination treatment downregulated the expression of the antiapoptotic proteins Bcl2L1, Gsta5, and Twist1 in both cancer cells and tumor-associated endothelial cells. This observation is consistent with demonstrating the ET-1 functions as a survival factor for different types of cancer cells [90, 97, 101] and for vascular endothelial cells [102, 103].

11.4 Conclusion

It has become increasingly apparent that reciprocal signaling between cancer cells and resident host cells in the organ microenvironment plays a decisive role in determining the growth of tumors and their response to treatment. Therapeutic targeting of both cancer cells and components of the microenvironment provides a greater benefit than targeting either compartment alone. Continued examinations of the cross talk between cancer cells and the brain microenvironment will undoubtedly lead to the identification of new therapeutic targets for patients with brain metastases.

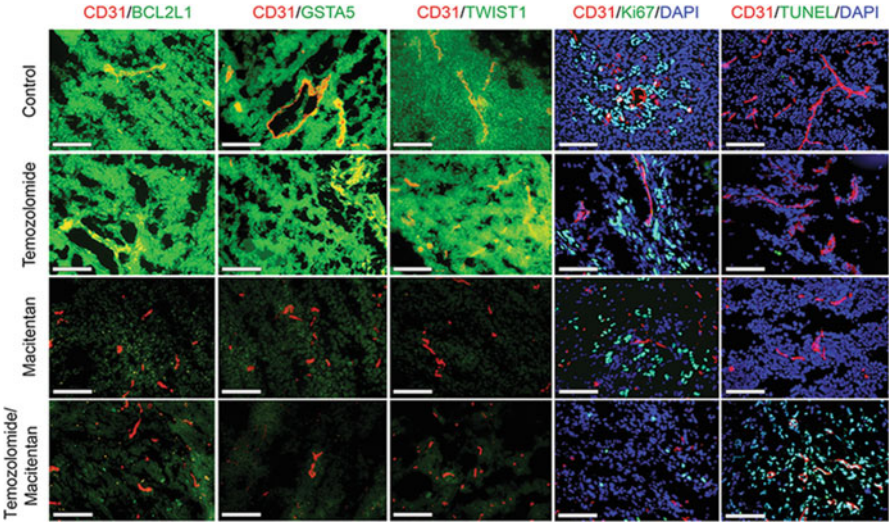

Fig. 11.2 Combination therapy with macitentan and temozolomide leads to targeted destruction of glioma cells and tumor-associated endothelial cells. Images were collected from the different experimental groups following 21 days of therapy. Macitentan downregulates expression levels of Bcl2L1, Gsta5, and Twist1 proteins, which are labeled *green*. Proliferating (Ki67) and apoptotic (TUNEL) cells are also depicted in *green*. Blood vessels were labeled with an antibody directed against CD31 (*red*): scale bar, 50 μm. Reproduced from Kim et al. [98] with permission from the American Association of Cancer Research

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