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Molecular targeted therapy: A new avenue in glioblastoma treatment (Review)

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Abstract. Glioblastoma, also referred to as glioblastoma multiforme (GBM), is grade IV astrocytoma characterized by being fast-growing and the most aggressive brain tumor. In adults, it is the most prevalent type of malignant brain tumor. Despite the advancements in both diagnosis tools and therapeutic treatments, GBM is still associated with poor survival rate without any statistically significant improvement in the past three decades. Patient's genome signature is one of the key factors causing the development of this tumor, in addition to previous radiation exposure and other environmental factors. Researchers have identified genomic and subsequent molecular alterations affecting core pathways that trigger the malignant phenotype of this tumor. Targeting intrinsically altered molecules and pathways is seen as a novel avenue in GBM treatment. The present review shed light on signaling pathways and intrinsically altered molecules implicated in GBM development. It discussed the main challenges impeding successful GBM treatment, such as the blood brain barrier and tumor microenvironment (TME), the plasticity and heterogeneity of both GBM and TME and the glioblastoma stem cells. The present review also presented current advancements in GBM molecular targeted therapy in clinical trials. Profound and comprehensive understanding of molecular participants opens doors for innovative, more targeted and personalized GBM therapeutic modalities.

Contents

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1. Introduction

Glioblastoma (GBM) is the most aggressive and deadly form of malignant brain cancers. It accounts for ~80% of all primary brain gliomas and ~60% of all adult brain tumors (1). Currently, surgical resection of the tumor, followed by radiotherapy and temozolomide, is the typical treatment used for GBM (2). GBM is associated with poor survival rate, despite the enhancements in diagnosis tools, surgical techniques and therapeutic approaches; the median survival rate is ~14-20 months and fewer than 5% of the patients survive 5 years post-treatment (3). Moreover, the survival rates for patients with GBMs have not shown statistically significant improvements in the last three decades (4). Thus, there is a need of new therapeutic approaches that inhibit GBM growth, impair its migration and invasion ability and sensitize it to therapy.

The use of high throughput technology in the last decade allowed researchers to classify GBM according to their genomic signature. Based on The Cancer Genome Atlas analysis, four different GBM sub-classes are defined: i) The neural subtype that represents 16% of GBM and are characterized by the expression of neuron markers such as NEFL, GABRA1, SYT1, and SLC1A5, ii) the pro-neural subtype that shows an alteration of PGFRA, a point mutation in IDH1and TP3 and an overexpression in development genes such as NKX2-2, OLIG2, iii) the mesenchymal subtype that is distinguished with alterations in neurofibromatosis type 1 (NF1), phosphatase and tensin homolog (PTEN) point mutation and expression of mesenchymal genes such as MET, CD44, iv) the classical subtype that displays EGFR amplification, CDKN2 A deletion, and p53 mutations (5). Subsequently, researchers have detected several molecular and genomic alterations in core pathways that regulate GBM cell viability, growth, metastasis and invasion. It is thus important to target these altered molecules in order to inhibit GBM proliferation and progression. The present

review highlights current understanding of the molecular alterations commonly involved in GBM (Fig. 1). Thorough understanding of molecular alterations facilitates the development of current therapeutic targeted therapy, and opens the way to novel therapeutic approaches. The present review also highlight molecular elements that are currently targeted in GBM clinical trials and others that represent promising potential new targets.

2. Targeting intrinsic survival pathways

Receptor tyrosine kinases (RTKs). RTKs are transmembrane proteins that consist of an extracellular ligand-binding domain, a single transmembrane helix, and an intracellular catalytic domain (6). RTK superfamily includes epidermal growth factor receptor (EGFR), platelet-derived growth factors (PDGPR), hepatocyte growth factor receptor (c-MET), and fibroblast growth factor receptor (FGFR) (7). Under normal physiological conditions, RTKs are involved in maintaining cellular homeostasis by regulating cell-cell communication, cell survival, migration, proliferation, differentiation, metabolism, and cell cycle. Hence, dysregulation of the RTK pathway is thought to play an important role in GBM initiation, development, and progression (8,9).

EGFR. Genomic analysis revealed that 57% of GBM cells harbor EGFR genetic alterations (10). EGFR amplification and overexpression were identified in 40 and 60% of primary glioblastoma respectively. Other types of genetic alterations were also detected; these include EGFR rearrangement, point mutations, and deletions such as the deletion of exons 2-7 which leads to the truncated mutant variant III (EGFRvIII) (11). EGFR amplification and overexpression lead to constitutive activation of the receptor and enhance GBM cell proliferation, survival, invasion and resistance to treatments (12-15). Talasila *et al* (16) demonstrated that cells from patients with GBM and with EGFR gene amplification are able to invade the tumor microenvironment (TME) in an angiogenesis independent manner. Additionally, EGFRvIII mutations lacking an extra cellular domain, tend to maintain the EGFR signaling pathway constitutively active in a ligand independent manner. These mutations are detected in 25% of GBM cases and promote survival, tumor growth, migration, invasion and angiogenesis (17-21). Despite the lack of an extracellular domain, EGFRvIII mutation maintains the EGFR signaling It is important to note that 50-60% of GBMs overexpressing wild-type EGFR also express EGFRvIII (22,23). GFRvIII is therefore a potential therapeutic target for GBM.

EGFR is targeted in 77 clinical trials according to clinicaltrials.gov (August 10, 2022). The most common strategy for EGFR targeting is through the use of monoclonal antibodies (Table I). Several anti-EGFR antibodies have been developed since the first chimeric antibody Cetuximab. While Cetuximab and Panitumumab did not show promising results (24), Nimotuzumab and Depatuxizumab-mafodotin (ABT-414), an antibody-drug conjugate, showed survival benefits when combined with radiotherapy and chemotherapeutic Temozolomide (TMZ), respectively (25,26). EGFRs are also targeted by inhibitors of tyrosine kinase activity. Several inhibitors have been evaluated in clinical trials with minimal or no benefits such as Erlotinib, Gefitinib and Dacomitinib. However, using Afatinib, an irreversible pan-inhibitor of the ErbB family resulted in an increase in the progression-free survival (PFS) in patients with overexpressing EGFR or expressing EGFRvIII (27). Notably, EGFR could also be inhibited by anti-tumor vaccines such as ACTIVATe (Phase II NCT00643097), a vaccine against tumor-specific EGFRvIII. ACTIVATe reportedly induces immune responses and an elimination of EGFRvIII-expressing tumor cells (28).

PDGFR. The receptor tyrosine kinase PDGFR is the second therapeutic target in GBM proneural subtype. PDGFR gene amplification is found in 15% of GBM cases (10). Overexpression of PDGFR and its ligands (PDGF-AA/-AB/-BB/-CC/-DD) is observed in gliomas of all grades and associated with poor prognosis (10,29). Once activated, PDGFR triggers intracellular signaling cascades that regulate cancer cell survival, growth and progression (30,31). Therefore, dysregulation of PDGF signaling stimulates malignant transformation of normal neural stem cells into glioblastoma and enhances GBM cell growth and motility through autocrine signaling (32-34).

In clinical trials, PDGFR is either targeted by multikinase inhibitors or specific anti-PDGFR antibodies (Table I). To date, multikinase inhibitors such as Sunitinib, Imatinib and Dasatinib have not shown promising clinical benefits (24). In addition, two Phase II clinical trials assessed the tolerance and efficacy of Ramucirumab (IMC-3G3) and MEDI-575 anti-PDGFR antibodies (NCT00895180 and NCT01268566 respectively) in patients with recurrent glioblastoma multiforme. However, these monotherapies did not show improved survival.

cMET. MET is a transmembrane receptor with a tyrosine kinase activity. Different types of MET genetic alterations are detected in GBM cells. MET gene amplification and overexpression are detected in 5-13% of GBM cases (35), and MET fusion genes are found in pediatric GBM (36). Overexpression of MET and its ligand HGF promotes tumor growth, migration, invasion and drug resistance (37-40). Increased activation of MET/HGF pathway is strongly and selectively associated with highly anaplastic GBM cells. The c-MET pathway is either targeted directly by c-MET antibodies or inhibitors or through antibodies targeting its ligand, HGF (Table I). Treatment with MET inhibitors can potentially be viable from the standpoint of drug selectivity, thus avoiding toxicity to normal cells (41). However, and in order to avoid drug resistance, combination of both PI3K inhibitors along with MET inhibitors can be favorably considered in the upcoming trials to efficiently target patients with GBMs (42). On the other hand, one promising result for c-MET antibodies was shown by a randomized, double-blind, placebo-controlled, multicenter Phase II study (NCT01632228) assessing Onartuzumab (MetMAb, an anti-cMET antibody) with or without Bevacizumab. Survival benefits were reported in patients with high HGF expression (43).

FGFR. Genomic alterations in FGFR are rarely detected in GBM (44); however, the constitutive activation of its downstream pathways stimulate tumor progression, proliferation and resistance to apoptosis (44,45). Infigratinib (BGJ 398) selectively binds to and inhibits FGFRs and was used

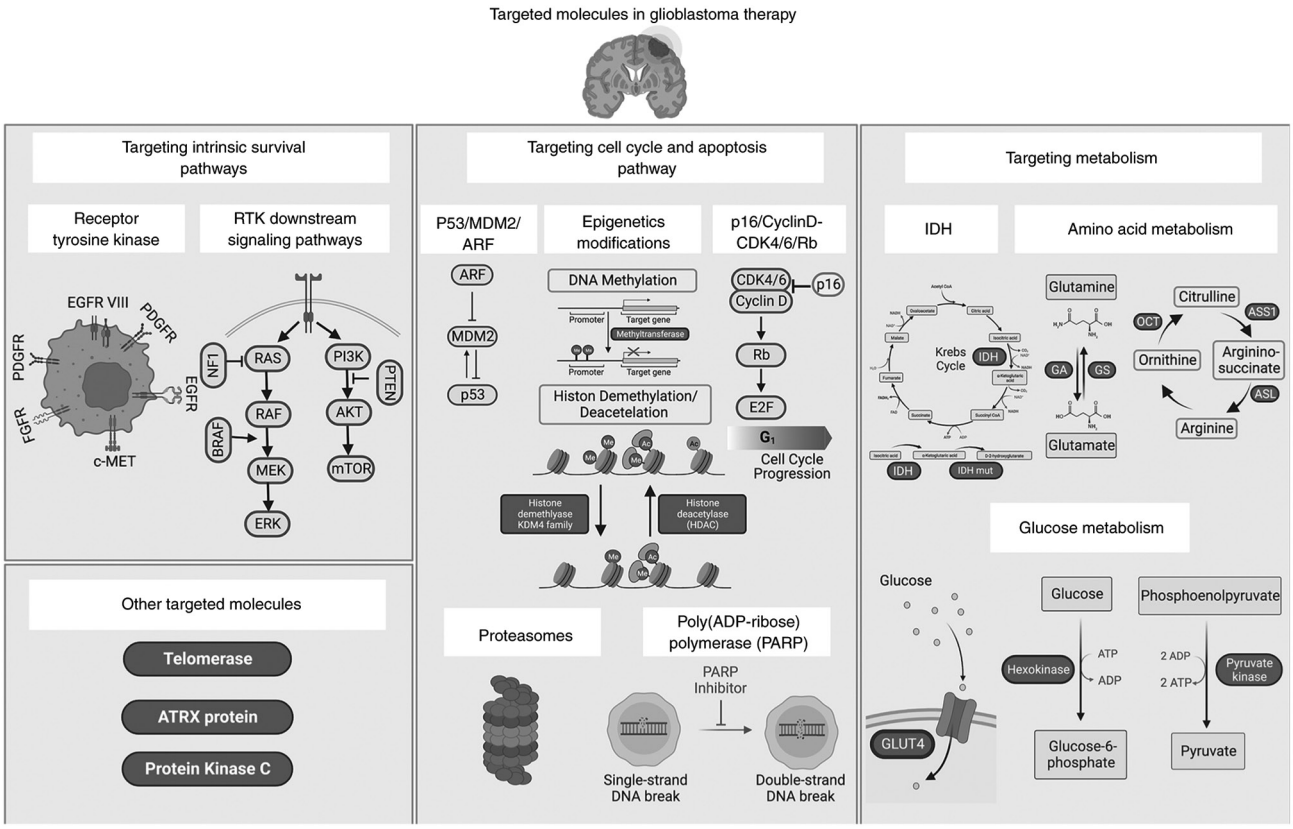

Figure 1. Overview of signaling pathways and intrinsically altered molecules implicated in GBM development. Several molecules are targeted in clinical trials for glioblastoma cancer therapy. These include molecules implicated in survival pathways (RTKs, BRAF and PI3K), cell cycle pathways (p53, MDM2, CDK4/6, proteasomes and PARP), and metabolism (IDH, hexokinase, glutamine and arginine), in addition to the hTERT and PKC. Increased understanding of glioblastoma biology revealed novel GBM molecular players that can be considered as potential new therapeutic targets for GBM treatment, such as PTEN, GLUT and PKM2. Created with Biorender.com. GBM, glioblastoma; RTKs, receptor tyrosine kinases; BRAF, B-Raf proto-oncogene MDM2, mouse double minute 2 homolog; PARP, Poly(ADP-ribose) polymerase; IDH, isocitrate dehydrogenase; hTERT, human telomerase reverse transcriptase; PKC, protein kinase C; PTEN, phosphatase and tensin homolog; GLUT, glucose transporter; PKM2, pyruvate kinase muscle 2. Created with Biorender.com.

as a monotherapy in Phase II NCT01975701 clinical trial in patients with recurrent glioblastoma or other glioma subtypes. However, Infigratinib was not licensed and the indication was abandoned (Table I).

Ras/Raf/MEK/ERK pathway. Ras/Raf/MEK/Erk (also known as the Ras/MAPK pathway) is a chain of effectors downstream of RTKs that regulate cell survival and proliferation (46). Alterations in various components of this pathway are detected in GBM.

B-Raf proto-oncogene (BRAF). BRAF is a serine/threonine kinase that belongs to the RAF family. Multiple BRAF gene alterations are associated with GBM; however, the BRAF V600E mutation is the most relevant. This missense mutation leads to constitutive activation of Ras/Raf/MEK/Erk pathway, promoting tumor cell proliferation, survival and inhibit apoptosis (47).

NF1. The NF1 gene encodes neurofibromin, a GTPase activating protein that controls cell growth and survival. It regulates the conversion of active GTP-bound Ras to its inactive GDP-bound form, thus inhibiting Ras/Raf/MEK/Erk signaling pathway (48). NF1 mutation or deletion is found in 10% of glioblastoma cases, especially in the mesenchymal GBM subtype (10). Despite the fact that genomic alterations of this tumor suppressor gene enhance the epithelial-mesenchymal transition in neurofibromatosis and can induce malignant transformation (49), its role in glioblastoma is not fully understood.

In glioblastoma, MEK inhibitors are common drugs used to target Ras/Raf/MEK/Erk signaling pathway (Table II). For instance, Atorvastatin, a Ras/MAPK inhibitor, showed encouraging results when evaluated in combination with radiotherapy and TMZ in patients with GBMs (Phase II NCT02029573) (50). Additionally, BRAF inhibitors such as Dabrafenib and Encorafenib are evaluated in clinical trials in combination with MEK inhibitors trametinib (Phase II NCT03919071) and Binimetinib, respectively (Phase II NCT03973918).

PI3K/AKT/mTOR pathway. Phosphatidylinositol-3-kinase (PI3K) is a family of lipid kinases that regulates cell survival, growth, motility and metabolism (51). It consists of three subclasses depending on their structure and substrate specificities (51). Activated PI3Ks phosphorylate the lipid phosphatidylinositol (4,5)-bisphosphate (PIP2) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (51). PI3K signaling pathway dysregulation is predominant in glioblastoma and is mainly caused by gain-of-function mutations in *PIK3CA* gene, *PIK3R1* gene and loss of *PTEN* gene.

Table I. Therapeutic agents targeting RTK pathways for the treatment of glioblastoma in interventional clinical trials. Only interventional Phase II, III and IV clinical trials are listed here for therapeutic agents targeting RTK[a].

Therapeutic agent	Specificity	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
Cetuximab	EGFR	Monotherapy	Newly diagnosed glioblastoma	Phase I/II NCT02861898	Recruiting	
		In combination with Bevacizumab	Recurrent glioblastoma multiforme	Phase II NCT02800486	Recruiting	
		In combination with Irinotecan and Bevacizumab	Recurrent glioblastomas	Phase II NCT00463073	Completed	
Depatuxizumab-mafodotin (ABT-414)	EGFR or mutant EGFRvIII	ABT-414 to concomitant radiotherapy and TMZ followed by combination of ABT-414 with adjuvant TMZ	Newly diagnosed Glioblastoma with EGFR Amplification	Phase II/III NCT02573324	Completed	
		ABT-414 alone or ABT-414 plus TMZ vs. Lomustine or TMZ	Glioblastoma	Phase II NCT02343406	Completed	(337)
Erlotinib (Tarceva)	EGFR	Monotherapy	Glioblastoma	Phase II NCT00337883	Completed	
		In combination with Bevacizumab	Glioblastoma	Phase II NCT00671970	Completed	(338)
		Monotherapy	Recurrent glioblastoma multiforme and anaplastic astrocytoma	Phase I/II NCT00301418	Completed	(339)
		Monotherapy	Recurrent malignant glioma or recurrent or progressive meningioma	Phase I/II NCT00045110	Completed	(340)
		In combination with radiotherapy	Newly diagnosed gliomas	Phase I/II NCT00124657	Completed	(341)
		In combination with TMZ during and following radiotherapy	Malignant glioma (glioblastoma or gliosarcoma)	Phase II NCT00187486	Completed	(342)
		Monotherapy	Recurrent or progressive glioblastoma multiforme	Phase II NCT00054496	Unknown	
Gefitinib (Iressa/ZD1839)	EGFR	Monotherapy	Recurrent glioblastoma	Phase II NCT00250887	Completed	(343)
		In combination with radiotherapy	Glioblastoma multiforme	Phase I/II NCT00052208	Completed	
		In combination with radiotherapy	Newly diagnosed gliomas	Phase I/II NCT00042991	Completed	

Table I. Continued.

Therapeutic agent	Specificity	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
GC1118	EGFR	GC1118 with standard concurrent chemoradiation	Recurrent glioblastoma with high EGFR amplification	Phase II NCT03618667	Unknown	
Sym004	EGFR	Monotherapy	Recurrent malignant glioma (glioblastoma or gliosarcoma)	Phase II NCT02540161	Completed	
Olaratumab (IMC-3G3)	PDGFRα	Compared to Ramucirumab (anti-VEGFR2)	Recurrent glioblastoma multiforme	Phase II NCT00895180	Completed	
MEDI-575	PDGFRα	Monotherapy	Recurrent glioblastoma multiforme	Phase II NCT01268566	Completed	
APL-101	c-MET	Monotherapy	Advanced solid tumors including glioblastoma multiforme	Phase I/II NCT03175224	Recruiting	
Onartuzumab (MetMAb)	c-MET	In combination with Bevacizumab compared with Bevacizumab alone or Onartuzumab monotherapy	Recurrent glioblastoma	Phase II NCT01632228	Completed	
Infigratinib (BGJ398)	FGFR1, FGFR2, and FGFR3	Monotherapy	Recurrent glioblastoma multiforme	Phase II NCT01975701	Completed	
Dacomitinib (PF-299804/ Vizimpro)	EGFR/HER1, HER2, and HER4	Monotherapy	Recurrent Glioblastoma With EGFR Amplification or Presence of EGFRvIII Mutation	Phase II NCT01520870	Completed	
		Monotherapy	Recurrent glioblastoma	Phase II NCT01112527	Completed	
Afatinib (BIBW 2992)	ErbB family	With or without daily TMZ	Recurrent malignant glioma	Phase II NCT00727506	Completed	
AEE788	ErbB & VEGFR family	Monotherapy	Glioblastoma Multiforme	Phase I/II NCT00116376	Completed	
Tesevatinib	EGF, HER2, and VEGF	Monotherapy	Recurrent glioblastoma	Phase II NCT02844439	Completed	
Anlotinib	VEGFR, FGFR, PDGFR, Kit	Monotherapy	Recurrent glioblastoma	Phase I/II NCT04004975	Unknown	(344)
		In combination with dose-dense TMZ	First recurrent or progressive glioblastoma	Phase II NCT04547855	Recruiting	

Table I. Continued.

Therapeutic agent	Specificity	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
Sorafenib	VEGFR, FLT-3, PDGFR-β, Kit	Radiotherapy and TMZ followed by TMZ plus Sorafenib	Glioblastoma Multiforme	Phase II NCT00544817	Completed	(345)
		A combination of Sorafenib tosylate, Valproic acid and Sildenafil	Recurrent high-grade glioma	Phase II NCT01817751	Active, not recruiting	(303)
Dasatinib	c-KIT, EPHA2, and PDGFR-β	Monotherapy	Recurrent glioblastoma multiforme or gliosarcoma	Phase II NCT00423735	Completed	(304)

[a]Data acquired from the U.S. National library of medicine (http://clinicaltrials.gov, accessed on 10 August 2022). Terminated studies are not included. c-MET, hepatocyte growth factor receptor; EGFR, epidermal growth factor receptor; EPHA2, Ephrin type-A receptor 2; ErbB, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FLT-3, fms-like tyrosine kinase 3; HER, human epidermal growth factor receptor; PDGFR, platelet-derived growth factors; RTK, receptor tyrosine kinase; TMZ, Temozolomide; VEGFR, vascular endothelial growth factor receptor.

PTEN. PTEN mediates the conversion of PIP3 to PIP2 which regulates cell proliferation, apoptosis, metabolism, motility and angiogenesis (52). PTEN deletion or mutation, found in 5-40% of GBM (53), inhibit AKT (protein kinase B) phosphorylation and induce the hyperactivation of PI3K signaling pathway, which in turn accelerates tumor growth, progression, and metastasis (54).

PI3K. Heterodimeric Class IA PI3Ks consist of a catalytic subunit (p110α, p110β, or p110) and a p85-type regulatory subunit (55). PIK3CA and PIK3R genes encode for p110α and p85α respectively (55,56). Several studies show that mutations in PIK3R1, identified in 8-10% of GBM cases, are found to be mutually exclusive with mutations in PIK3CA in primary GBM cases (57,58). Constitutively active PIK3CA and PIK3R1 upregulate the PI3K/AKT signaling pathway and promote tumorigenesis (59,60). Somatic mutations in PIK3CA occurs in 6-17% of GBM (57,58,61) and PIK3CA activating mutations are correlated with poor prognosis, aggressive and more disseminated phenotype and with shorter survival rate (62). Alterations in PIK3CD (p110δ) and PIK3CB (p110β) are also detected in GBM (56).

The PI3K pathway is generally targeted by several PI3K pan-inhibitors in clinical trials (Table III). One example is Pictilisib, a PI3K isoform inhibitor shown to sensitize tumors to radio- and chemotherapy. Effectiveness of Pictilisib is being compared with immunotherapeutic Pembrolizumab (MK-3475) in Phase II clinical trial in patients with glioblastoma (NCT02430363). Paxalisib (GDC-0084) is another small molecule inhibitor of PI3K currently evaluated as an adjuvant therapy after surgical resection and concomitant chemoradiation therapy with TMZ in patients with GBM and unmethylated MGMT promoter status (Phase II NCT03522298). Preliminary results show enhanced overall survival (OS; 17.7 months for treated group compared with 12.7 months for patients treated with TMZ) (24). The PI3K/AKT/mammalian target of rapamycin (mTOR) pathway is also targeted by mTOR inhibitors. While several mTOR inhibitors do not show clinical benefits, AZD2014 is proposed as a promising drug for radiosensitizing glioblastoma stem cells (GSCs) *in vitro* and *in vivo*, and is currently being evaluated in clinical trials (Phase I NCT02619864). Moreover, another clinical trial is evaluating the efficiency of ABI-009 (Nab-Rapamycin), a novel albumin-bound mTOR inhibitor, in recurrent and newly diagnosed glioblastoma (Phase II NCT03463265).

3. Targeting cell cycle and apoptosis pathways

p53/mouse double minute 2 homolog (MDM2)/ARF and p^{16}/cyclinD-CDK4/6/Rb signaling pathways regulate cell cycle and cell proliferation. According to the Cancer Genome Atlas Research Network, 87 and 77% of the analyzed GBM samples harbor a mutation in p53 and Rb signaling pathway respectively (57).

p53/MDM2/ARF pathway. The p53/MDM2/ARF pathway is one of the major core pathways deregulated in 84% of patients with GBMs. p53, the key protein of this pathway and also known as 'guardian of the genome', protects cells from external or internal stress signals by regulating cell processes such as cell cycle, DNA repair, angiogenesis, metabolism, cell death (apoptosis and autophagy) and senescence (63).

p53 genetic alterations were detected in 30% of primary GBM and 65% of secondary GBM (64). Mainly two different types of genomic alteration are detected in GBM cells; point mutation associated with overexpression of the mutant version of p53 and deletion that provokes the loss of function of p53. These alterations result in either gain or loss of p53 function (65). The oncogenic potential of p53 is mediated by the accumulation of p53 mutants in cells. Indeed, mutations in the MDM2 binding domain of p53 affect its regulation by MDM2, leading to the

Table II. Therapeutic agents targeting the Ras/MAPK pathway for the treatment of glioblastoma in interventional clinical trials[a].

Therapeutic agents	Therapeutic target	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
ABM-1310 and Cobimetinib	BRAF V600E (ABM-1310) and MEK1/2 (Cobimetinib)	ABM-1310 monotherapy or in combination with Cobimetinib	Advanced solid tumors	Phase I NCT04190628	Recruiting	
Dabrafenib and Trametinib	BRAF (Dabrafenib) and MEK1/2 (Trametinib)	Dabrafenib in combination with Trametinib	BRAF V600 mutation positive low grade glioma or relapsed or refractory high grade glioma.	Phase II NCT02684058	Active, not recruiting	(346)
			Dabrafenib Combined with Trametinib after radiotherapy Newly-diagnosed high-grade glioma	Phase II (NCT03919071	Recruiting	(346)
Encorafenib and Binimetinib	BRAF (Encorafenib) and MEK1/2 (Binimetinib)	Combination treatment with Encorafenib and Binimetinib	Recurrent BRAF V600-Mutated high grade glioma	Phase II NCT03973918	Active, not recruiting	
Atorvastatin	Ras/MAPK	In combination with Radiotherapy and TMZ	Glioblastoma	Phase II NCT02029573	Completed	(50)
Ulixertinib (BVD-523)	ERK1/2	Monotherapy	Advanced solid tumors	NCT04566393	Available	
LY2228820	p38 MAPK	With radiotherapy plus concomitant TMZ	Newly diagnosed glioblastoma	Phase I/II NCT02364206	Completed	
RSC-1255	Ras	Monotherapy	Advanced malignancies including glioblastoma	Phase I NCT04678648	Recruiting	

[a]Data acquired from the U.S. National library of medicine (http://clinicaltrials.gov, accessed on 10 August 2022). Terminated studies are not included. BRAF, B-Raf proto-oncogene; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; MEK, mitogen-activated protein kinase kinase; TMZ, Temozolomide.

accumulation of p53 inside the cell and the subsequent gain of function phenotype. This enhances tumorigeneses in GBM by promoting inflammation, genomic instability, tumor growth, invasion, metastasis and neo-angiogenesis (66,67). Pedrote *et al* (68) revealed that accumulation of amyloid-like p53 with p53 gain of function phenotype leads to increased chemo-resistant GBM cells. Thus, p53 gain of function mutations are associated with great oncogenic potential and aggressive GBM phenotype.

Loss of p53 tumor suppression function is not only mediated by p53 mutations. Previous studies demonstrated that different components in the p53/MDM2/ARF pathway, such as MDM2/MDM4 protein, negatively regulate the activity of p53 (69-72). Under normal physiological conditions, MDM2 protein, which is an E3 ubiquitin ligase, binds p53 transcriptional domain and controls its activity by preventing its transcriptional function and promoting its degradation. However, the activity of p53 is positively regulated by the alternative reading frame tumor suppressor (ARF), an upstream molecule that suppresses the MDM2 activity (73). p53 mutations or deletions, MDM2 amplification or overexpression and ARF homozygous deletion inactivate p53 and trigger the p53 loss of function phenotype in glioblastoma, thus contributing to tumor growth, progression and therapy resistance (65,74).

RG7388 (Idasanutlin) is a small molecule antagonist of MDM2 used to target p53/MDM2 pathway. Recent Phase I/II trials are recruiting to test for molecularly matched targeted therapies (APG101, Alectinib, Idasanutlin, Atezolizumab, Vismodegib, Temsirolimus and Palbociclib) in combination with radiotherapy in patients with newly diagnosed glioblastoma without MGMT promoter methylation (NCT03158389). A more recent Phase I clinical trial is studying the uptake and tolerance of BI 907828 (an MDM2 inhibitor) in combination

Table III. PI3K inhibitors applied for the treatment of glioblastoma in interventional clinical trials[a].

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status
Paxalisib (GDC-0084)	Adjuvant therapy following surgical resection and initial chemoradiation with TMZ	Newly-diagnosed glioblastoma with unmethylated MGMT promoter status	Phase II NCT03522298	Active not recruiting
	With metformin while maintaining a ketogenic diet	Glioblastoma	Phase II NCT05183204	Not yet recruiting
	Multiple drugs and drug combinations (TMZ, Lomustine, Regorafenib, Radiation, Paxalisib, VAL-083, VT1021, Troriluzole)	Newly diagnosed and recurrent glioblastoma	Phase II/III NCT03970447	Recruiting
Pictilisib	Compared with Pembrolizumab (MK-3475/anti-PD-1 monoclonal antibody)	Glioblastoma	Phase I/II NCT02430363	Unknown

[a]Data acquired from the U.S. National library of medicine (http://clinicaltrials.gov, accessed on 10 August 2022). Terminated studies are not included. MGMT, O^6-methylguanine DNA methyltransferase; PD-1, programmed cell death protein 1; PI3K, phosphatidylinositol-3-kinase; TMZ, Temozolomide.

with radiotherapy in newly diagnosed glioblastoma patients (NCT05376800). Markedly, several different strategies are currently employed for targeting p53 using gene therapy in different cancer types. In GBM, only one clinical trial reported using SGT-53, a human wild type p53 DNA sequence encapsulated in nanodelivery liposome. However, this study was terminated due to the very small number of participants (Phase II NCT02340156).

Epigenetic modifications. In addition to genetic alterations, epigenetic modulators affect expression by interacting with drivers of GBM cell proliferation, without causing any changes in the DNA sequence (75). In GBM, DNA methylation is strongly correlated with responses to TMZ treatment that methylates adenine in position N3 and guanine in position O6 and N7 (76). Guanine methylation at O6 position leads to strand breaks, activating p53-mediated apoptosis through Fas/CD95/Apo-1 receptor or by the mitochondrial pathway (77). To decrease the effects of O^6-methylguanine DNA methyltransferase (MGMT) methylation, synthetic inhibitors of MGMT entered human trials (78). Nevertheless, several studies revealed that inhibitors such as O6-benzylguanine and PaTrim-2 (Lomeguatrib) did not show survival improvement in response to TMZ (79-81).

Histone demethylases (KDM)4C is often overexpressed in GBM and is associated with epigenetic regulation of both tumor suppressor genes and oncogenes (82). Lee *et al* (82) showed that KDM4C binds to the promoter of c-Myc oncogene inducing its expression/activation and suppressing the functions of p53 by demethylating p53K372me1, thus inducing apoptosis. KDM4C knockdown significantly suppresses both the proliferation and tumorigenesis of glioblastoma cells *in vitro* and *in vivo*, thus suggesting KDM4C inhibition as a promising tool in targeting glioblastoma.

Histone deacetylases (HDAC) also serve an important role in regulating cell growth and survival of cancer cells (83). Inhibition of HDACs leads to cell cycle arrest as well as apoptosis, and, in GBM, causes the rebalance of histones acetylation (84,85). In clinical trials, testing Romidepsin (FR901228, an HDAC inhibitor) exhibited failure in treating patients with GBMs (NCT00085540) (24). Other HDAC inhibitors, such as Vorinostat. did not show improvement in the median OS or PFS both alone or in combination with Bortezomib (NCT00641706) or Bevacuzimab (NCT01738646) (24).

p16/CyclinD-CDK4/6/Rb pathway. The p^{16}/cyclinD-CDK4/6/Rb signaling pathway regulates cell cycle progression and is also disrupted in GBM cells (10). The cyclin D-CDK4/6-Rb axis, which is a crucial cell cycle checkpoint, controls cell transition from G_1 to S phase (86). Indeed, cyclin D forms a complex with CDK 4/6 inducing the phosphorylation of Rb and inhibiting Rb-E2F formation (87). Released E2F mediates transcription of genes that facilitate the transition into S phase and consequently cell cycle progression (88). Under carcinogenic conditions, p16 protein inhibits cyclin D-CDK 4/6 complex formation (74). Thus, Rb associates with E2F and triggers cell cycle arrest. It has been demonstrated that genetic alterations such as deletion in CDKN2A/B, amplification of CDK4/6 and mutations of Rb and p16 genes are detected in 79% of patients with GBM (10). Consequently, cells harboring such alterations undergo uncontrolled cell proliferation and tumor growth. Collectively, targeting cell cycle and apoptosis pathways could emerge as promising tool for treating GBM. There are three CDK4/6 inhibitors evaluated in clinical trials for GBM treatment in clinical trials (Table IV). Ribociclib and Abemaciclib are being evaluated in current studies, whereas PD 0332991 (Palbociclib) monotherapy was not effective in treating patients with recurrent glioblastoma (89).

Proteasomes. Proteasomes are protein complexes that are central for degradation of unneeded or damaged proteins (90). They regulate cell cycle and homeostasis in normal and cancer

Table IV. Therapeutic agents targeting CDK4/6, proteasomes and Poly(ADP-ribose) polymerase applied for the treatment of glioblastoma in interventional clinical trials[a].

A, CDK4/6 inhibitors

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
Abemaciclib (LY2835219)	In combination with TMZ and Irinotecan vs. Abemaciclib in combination with TMZ	Solid tumors	Phase I NCT04238819	Recruiting	
	In combination with Bevacizumab	Recurrent glioblastoma patients with loss of CDKN2A/B or gain or amplification of CDK4/6	Early Phase I NCT04074785	Active, not recruiting	
	With or without surgery	Recurrent glioblastoma	Phase II NCT02981940	Recruiting	
	In combination with LY3214996 (ERK Inhibitor)	Recurrent glioblastoma	Early Phase I NCT04391595	Recruiting	
Ribociclib (LEE011)	Before surgical tumor resection	Preoperative glioma and meningioma	Early Phase I NCT02933736	Recruiting	
	In combination with Everolimus	Preoperative recurrent high-grade glioma	Early Phase I NCT03834740	Active, not recruiting	
	Ribociclib and Everolimus following Radiotherapy	High grade gliomas	Phase I NCT03355794	Active, not recruiting	
	Monotherapy	Recurrent glioblastoma or anaplastic glioma	Phase I NCT02345824	Unknown	

B, Proteasome inhibitors

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
Bortezomib	In combination with TMZ	Glioblastoma	Phase I/II NCT03643549	Recruiting	(347)
	In combination with TMZ	Brain tumors or other solid tumor that have not responded to treatment	Phase I NCT00544284	Completed	
	In combination with bevacizumab and escalating doses of TMZ	Recurrent glioblastoma multiforme	Phase I NCT01435395	Completed	

Table IV. Continued.

B, Proteasome inhibitors

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
	In combination with TMZ and radiotherapy	Newly diagnosed glioblastoma multiforme or gliosarcoma	Phase II NCT00998010	Completed	
	In combination with Bevacizumab	Recurrent Malignant Glioma	Phase II NCT00611325	Completed	
Marizomib	In combination with TMZ and radiotherapy	Newly diagnosed glioblastoma	Phase III NCT03345095	Active, not recruiting	
	In combination with Bevacizumab	Malignant glioma and glioblastoma	Phase I/II NCT02330562	Completed	(348)
	In combination with TMZ and radiotherapy	Malignant glioma and glioblastoma	Phase I NCT02903069	Completed	(348)
Ixazomib (MLN9708)	Monotherapy	Glioblastoma	Early Phase I NCT02630030	Completed	(95)
Disulfiram (DSF)	After radiotherapy with TMZ	Glioblastoma multiforme	Early Phase I NCT01907165	Completed	
	DSF-Copper in combination with TMZ	Recurrent glioblastoma	Phase II NCT03034135	Completed	(97)
	DSF-Copper with concurrent radiotherapy and TMZ	Glioblastoma multiforme	Phase I/II NCT02715609	Active, not recruiting	

C, PARP inhibitors

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
Iniparib (BSI-201)	In combination with TMZ	Newly diagnosed malignant glioma	Phase I/II NCT00687765	Completed	
Olaparib	Cediranib maleate and Olaparib work compared with bevacizumab	Recurrent glioblastoma	Phase II NCT02974621	Active, not recruiting	
	In combination with TMZ	Relapsed glioblastoma	Phase I NCT01390571	Completed	(101)
	In combination with radiotherapy compared with Pamiparib with radiotherapy	Newly diagnosed and recurrent glioblastoma	Early Phase I NCT04614909	Recruiting	
	Monotherapy	Advanced glioma, cholangiocarcinoma, or solid tumors with IDH1 or IDH2 mutations	Phase II NCT03212274	Recruiting	

Table IV. Continued.

C, PARP inhibitors

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
	Combination therapy of Pembrolizumab, Olaparib and TMZ	Glioblastoma and recurrent glioblastoma	Phase II NCT05463848	Not yet recruiting	
	Afatinib, Dasatinib, Palbociclib, Everolimus or Olaparib based on patient's genetic profile	Glioblastoma and recurrent glioblastoma	Early Phase I NCT05432518	Not yet recruiting	
Veliparib	In combination with TMZ	Young patients with recurrent or refractory CNS tumors	Phase I NCT00946335	Completed	
	In combination with TMZ and radiotherapy	Younger patients with newly diagnosed diffuse pontine gliomas	Phase I/II NCT01514201	Completed	(103)
	In combination with TMZ	Recurrent glioblastoma	Phase I/II NCT01026493	Completed	(102)
	In combination with TMZ and radiotherapy	Newly diagnosed glioblastoma multiforme	Phase I NCT00770471	Completed	
	In combination with TMZ and radiotherapy	Newly diagnosed malignant glioma without H3 K27M or BRAFV600 mutations	Phase II NCT03581292	Active, not recruiting	
	TMZ with or without Veliparib	Newly diagnosed glioblastoma multiforme	Phase II/III NCT02152982	Active, not recruiting	(349)
Pamiparib	In combination with TMZ and radiotherapy	Newly diagnosed or recurrent glioblastoma	Phase I/II NCT03150862	Completed	(350)

[a]Data acquired from the U.S. National library of medicine (http://clinicaltrials.gov, accessed on 10 August 2022). Terminated studies are not included. CDK, cyclin-dependent kinase; CNS, central nervous system; DSF, Disulfiram; ERK, extracellular signal-regulated kinase; IDH, Isocitrate dehydrogenase; PARP, poly(ADP-ribose) polymerase; TMZ, Temozolomide.

cells by regulating p53 and endoplasmic reticulum stress. Proteasomes can thus influence drug resistance in tumor cells (91). Due to the complexity of GBM physiology, there is a need for therapeutic options that could target broad systems contributing to the tumorgenicity in GBM. Proteasome inhibition is thus identified as a promising strategy for GBM treatments. Currently, four proteasome inhibitors are tested in clinical trials to target proteasomes in GBM: Bortezomib, Ixazomib, Marizomib and Disulfiram (Table IV). Bortezomib is the first-generation proteasome inhibitor and showed promising results when studied in combination with TMZ and radiotherapy (NCT00998010) (92). Marizomib is a second-generation proteasome inhibitor that has the ability to cross the BBB (93). It is currently being evaluated in ongoing clinical trials combined with Bevacizumab, TMZ, or ABI-009 (Nab-rapamycin, nanoparticle albumin-bound rapamycin). Encouraging observations are reported in Phase III study (NCT03345095) for Marizomib administered in combination with radiotherapy and TMZ to treat patients with newly diagnosed glioblastoma (94). Ixazomib, on the other hand, was evaluated in early Phase I clinical trial (NCT02630030) for its ability to reach brain tumors due to its distinctive permeability to tumor tissues (95). Disulfiram is another interesting proteasome inhibitor that has an improved BBB penetration to employ its anti-tumor action (96). Disulfiram was well tolerated in Phase II clinical trial in combination with TMZ but showed limited activity for unselected population (97).

Poly(ADP-ribose) polymerase (PARP). Elements of DNA damage repair mechanisms are considered promising radio-sensitizing agents for cancer therapy (98). PARP is an important protein in DNA repair pathways and high PARP-1 mRNA expression is associated with poor survival in classic GBMs (99). A few PARP-1 inhibitors have been evaluated in clinical studies (Table IV). For instance, a Phase I/II clinical trial (NCT00687765) recently completed a study to evaluate the safety and efficiency of Iniparib (BSI-201), a PARP1 inhibitor (results as yet unpublished). Olaparib is another inhibitor of PARP that shows an effective radio-sensitizing result in GBM cell lines and preclinical glioma models (98,100). A Phase I trial OPARATIC (NCT01390571) reported that Olaparib penetrates core and margin regions of GBM at radio-sensitizing concentrations. It was also reported to be safe when used with continuous low-dose of TMZ (101). PARP inhibitor Veliparib was also evaluated in several clinical trials. However, results showed that Veliparib did not show improved clinical survival when combined with TMZ (Phase I/II NCT01026493) (102), nor when added to radiation followed by TMZ (Phase I/II NCT01514201) (103).

4. Targeting metabolism

Isocitrate dehydrogenase (IDH). WHO classifies GBM depending on IDH mutational status (104). The IDH enzyme plays a pivotal role in major cellular metabolic processes. It is involved in the Krebs cycle, lipid and glutamine metabolism and oxidative stress regulation. During the Krebs cycle, IDH catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate [α-KG, also known as 2-oxyglutarate (2OG)] and reduces the cofactor $NADP^+$ into NADPH which is essential to decrease the level of reactive oxygen species (ROS) (105-107).

Three different isoforms of IDH exist in human cells: IDH1, detected in the cytoplasm and in peroxisomes, whereas IDH2 and IDH3 are both present in mitochondria (108). IDH mutations were found in 6% of primary GBM and in 54% of secondary GBM (109). Missense mutations, caused by the substitution of arginine residue in codons 132 and 172 in the enzymatic site of the IDH1 and IDH2, are frequently detected in glioblastoma cells (58,110,111). IDH mutants catalyze the production of 2-hydroxyglutaric acid (2-HG) from isocitrate. 2-HG affects cellular metabolism, enhances the oxidation of NADPH in NADP+ which, in turn, disrupts cellular homeostasis and increases the level of ROS (112). It has been suggested that the accumulation of this oncometabolite inhibits the activity of α-KG dependent dehydrogenase, a family of enzymes involved in methylation of histones and DNA, which includes KDMs and 10-11 translocation (*TET*, a family of DNA hydroxylases that leads to a hypermethylation state and genetic instability) (113). Moreover, modification of the epigenetic profile is associated with the inhibition of glioma stem cell differentiation (114). Furthermore, overexpression of hypoxia-inducible factor 1-α (HIF-1α), VEGF and platelet-derived growth factor subunit A (PDGF-A) are detected in GBM cells holding IDH mutation (115-117). Hence, these alterations induced by the high levels 2-HG are associated with an aggressive and invasive carcinogenic phenotype.

Patients with GBMs and IDH mutations are often treated with inhibitors such as Olaparib targeting PARP (Phase II NCT03212274). More clinical trials are investigating other PARP inhibitors such as Nivolumab (Phase II NCT03718767, recruiting), Talazoparib (Phase II NCT04740190, recruiting) and BGB-290 (Phase II NCT03914742, active, not recruiting/Phase I NCT03749187) to treat patients with advanced gliomas including glioblastoma. Notably, a recently completed study investigated the safety and clinical activity of Enasidenib (AG-221), a small molecule inhibitor of IDH2 in patients with advanced solid tumors, including glioma (Phase I/II NCT02273739). Patients with similar cancer types were treated in a recently completed study (Phase I/II NCT03684811) with Olutasidenib (FT-2102), another agent that specifically inhibits mutant IDH1 at arginine R132. Currently, a number of other clinical trials are evaluating IDH inhibitors, therefore, more time and research are needed to conclude about their efficiency to treat gliomas (Table V).

Glucose metabolism. Normal cells rely on oxidative phosphorylation as the main energy source. However, cancer cells shift to aerobic glycolysis even in the presence of oxygen. This phenomenon, known as Warburg effect, protects cancer cells from apoptosis, stimulates the production of new precursors and increases invasion capacity (118-121). Therefore, targeting glucose transporters, such as glucose transporter (GLUT), and metabolic enzymes, such as Hexokinase 2 and Pyruvate kinase muscle, may provide a novel avenue in glioblastoma treatment.

GLUT. GLUT is a transmembrane protein that belongs to the major facilitator superfamily (122), Currently, 14 different isoforms are identified in human tissues and divided into three classes (123). Class I, which consists of GLUT 1/2/3/4, is mainly expressed in brain cells. In GBM, GLUT-1 and GLUT-3 are the predominantly expressed isoforms and associated with poor survival rates. These transporters, characterized by their high affinity for glucose, promote glycolysis metabolism by increasing glucose uptake to maintain the survival, proliferation and growth of cancer cells. GLUT-3, also known as neural glucose transporter, is highly expressed by brain tumor initiating cells (BTICs) and has higher affinity for glucose compared with GLUT-1 (124).

Overexpression of GLUT-3 enhances chemoresistance and survival of BTICs in glucose deficient microenvironment (124). Recently, Libby *et al* (125) proposed that overexpression of GLUT-3 is positively correlated with the invasion phenotype of GBM. They found that the C-terminal tail of GLUT-3 reduces invasion (125). Accordingly, the design of drugs targeting GLUT-3 could be improved to inhibit molecular functions outside its role in metabolism as a means to limit potential brain toxicity. This can be achieved by potentially targeting the C-terminal tail or the protein interactions driving GLUT-3 mediated invasion. While glucose transporters provide promising results in research laboratories, they are not yet targeted in clinical trials.

Hexokinase. Hexokinase 2 (HK2), the driver of the aerobic glycolysis, regulates the first step of glucose metabolism by converting glucose to glucose 6-phosphate. In GBM, HK2

Table V. Therapeutic agents targeting metabolism of gliomas (including glioblastoma) in interventional clinical trials[a].

A, IDH

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
Enasidenib (AG-221), Inhibitor of mutant IDH2	Monotherapy	Solid tumor, glioma, angioimmunoblastic T-cell lymphoma, intrahepatic cholangiocarcinoma, chondrosarcoma	Phase I/II NCT02273739	Completed	
Olutasidenib (FT-2102), Inhibitor of R132 mutant IDH1	In combination with other anti-cancer drugs (Azacitidine, Nivolumab, Gemcitabine and Cisplatin)	Advanced solid tumors and gliomas (glioblastomas)	Phase I/II NCT03684811	Completed	
AG-120, Inhibitor of R132 mutant IDH1	Monotherapy	Advanced solid tumors and gliomas	Phase I/II NCT02073994	Active, not recruiting	(351)
IDH305, Inhibitor of mutant IDH1	Monotherapy	Advanced malignancies with IDH1 R132 mutations	Phase I NCT02381886	Active, not recruiting	(352)
LY3410738, Inhibitor of R132 mutant IDH1, and R140 or R172 mutant IDH2	Monotherapy or in combination with gemcitabine and Cisplatin, or Durvalumab	Advanced solid tumors with IDH1 or IDH2 mutations	Phase I NCT04521686	Recruiting	

B, Hexokinase

Therapeutic agents	Therapeutic strategy	Cancer condition	Clinical phase	Status	(Refs.)
Posaconazole	Monotherapy	Glioblastoma	Early Phase I NCT04825275	Recruiting	
Ketoconazole	Monotherapy	Glioblastoma	Early Phase I NCT04869449	Recruiting	

[a]Data acquired from the U.S. National library of medicine (http://clinicaltrials.gov, accessed on 10 August 2022). Terminated studies are not included. IDH, Isocitrate dehydrogenase.

promotes tumor progression by enhancing cancer cell growth, lactate production and chemoresistance (126,127). It also binds to the mitochondrial membrane and controls the release of cytochrome c, protecting cancer cells from apoptosis (128). Notably, depletion of HK2 induces the shift to oxidative glucose metabolism, impairs cancer cell proliferation, reduces angiogenesis and sensitizes GBM cells to chemoradiotherapy (129). Microarray analysis reveals that HK2 expression is negligible in normal cells but predominant in GBM (126). Therefore, targeting HK2 or its activity may be a novel therapeutic strategy to selectively kill cancer cells without damaging normal cells. Ketoconazole and Posaconazole are antifungals known to inhibit tumor metabolism. These drugs also selectively target HK2 in glioblastoma cells (129). Two recent recruiting early Phase I clinical trials are studying the delivery and activity of Ketoconazole and Posaconazole in brain tumors (NCT04869449 and NCT04825275, respectively).

Pyruvate kinase muscle (PKM)2. PKM consists of two isoforms PKM1 and PKM2 (130). PKM1 is mainly expressed in muscle and brain cells while PKM2 is highly expressed in embryonic cells, stem cells and cancer cells (131). PKM converts phosphoenolpyruvate to pyruvate during the last irreversible step of glycolysis (132). Mukherjee *et al* reported an overexpression of PKM2 and an isoform switching between metabolic enzymes PKM1 and PKM2 in GBM (133).

Three different forms of PKM2 exist in mammalian cells: A tetrameric form, known as the metabolic/glycolytic form and is involved in aerobic glycolysis, and less active monomeric and dimeric forms. The latter two forms promote GBM tumor growth (134,135) and protect it from apoptosis (136,137). This is achieved either by diverting the glycolysis pathway to an anabolic pentose phosphate pathway, or by activating the transcription of a number of oncogenes such as c-Myc and cyclin D (138). The ratio of monomeric/dimeric and tetrameric

forms of PKM2 decides whether cells will undergo glycolysis or phosphate pathway (139). In addition, a study conducted by Sizemore *et al* (140) reveals that the ATM-PKM2-CtIP axis enhances DNA double-stand break repair efficiency and resistance to genotoxic damage caused by radiation. Overexpression of PKM2 is hence associated with a radio-resistant phenotype. Therefore, targeting PKM2 might be a promising therapeutic strategy for glioblastoma treatment.

Amino acid metabolism. Amino acid metabolism is another intriguing metabolism route important for GBM cells. Data suggest that GBM cells produce an increased pool of free amino acids associated with poor disease prognosis (141). Research provided evidence that the hypoxic stress in glioma and GBM cells increases protein catabolism (142,143). Under hypoxic conditions, and due to elevated levels of redox stress, hypoxic tumor cells maintain redox homeostasis that is dependent on increased glutathione synthesis. Thus, GBM cells want to maintain high levels of glutathione, consequently favoring conditions that are resistant to chemoradiotherapy (142). Arginine, asparagine, glutamine and lysine are among the relevant amino acids studied, however, the present review will discuss metabolic pathways of both glutamine and arginine that are targeted to therapeutically control cancer cell proliferation and spreading.

Glutamine metabolism. Glutamine is a non-essential amino acid that is highly concentrated in blood (144) and required for the tricarboxylic acid (TCA) cycle and the synthesis of nucleotide bases, amino acids, fatty acids and proteins. Glutamine metabolism regulates oxidative stress by regulating glutathione synthesis (145). Additionally, healthy neural cells in the brain synthesize glutamate, the main activator neurotransmitter, by the uptake of glutamine from astrocytes in the glutamine-glutamate cycle (146). Glutamine in the extracellular space is transported into neural cells through SNAT1 transporter where it is hydrolyzed by the glutaminase enzyme (GA) to form glutamate. Glutamate is then packed in synaptic vesicles and cleared during neurotransmission and transported into astrocytes via glutamate transporters GLT-1 and GLAST. In astrocytes, glutamine synthetase (GS) catalyzes the generation of glutamine from glutamate. The cycle is completed by the release of glutamine from astrocytes into the synaptic cleft by the SN1 transporter (147).

In GBM, glutamine is an important source for the TCA cycle and nucleotide and fatty acid synthesis, thereby sustaining tumor growth and stimulating glutathione synthesis (148). Concentrations of glutamine and its related metabolites are proportional to decreased cell survival (149). Due to developments in noninvasive image collection and analysis, *in vivo* glutamine measurement is now clinically feasible. Previous research demonstrated that glutamine imaging may have prognostic significance (150). In the line of the role of glutamine in cancer cell growth and promoting chemo-and radiotherapy resistance, targeting glutamine metabolism constitutes an attractive research area for the treatment of brain tumors.

Research strategies to target glutamine metabolism included targeting enzymes involved in their synthesis (GA and GS), or targeting transporters for glutamine/glutamate uptake (147,151-153). Modulating the GA enzyme was performed using the RNA interference approach or the use of allosteric inhibitors. *GLS*, a gene encoding GA, was targeted in GBM cell lines, patient derived GBM cells and mouse xenografts (147,154). Several research groups report a decreased cancer cell viability by silencing *GLS*, whereas GLS2 isoform shows an opposite effect as its overexpression reverses the aggressive GBM phenotype (155-157). These data suggest approached for a combinatorial therapeutic approach of both *GLS* inhibition and GLS2 overexpression thereby enhancing the antitumor effect. On the other hand, GS enzyme is also targeted, and its overexpression resulted in decreased proliferation and migration in rat glioma cell lines (158). Along with similar supporting data, GS is suggested to have an anti-glioma effect.

To date, targeting glutamine uptake by neural cells through silencing SNAT transporters has not shown significant effects on the proliferation of GBM cells (147). Conversely, targeting glutamate transporters such as GLT-1 and GLAST showed more promising results in GBM cell lines and xenografts [reviewed in details in (147)]. Another promising target is system x_c^- (SXC), a cysteine-glutamate exchanger that is shown to mediate >50% of glutamate transport in GBM cell lines (159). Notably, SXC is targeted and inhibited by an FDA approved sulfasalazine (SAS) for the treatment of bowl disease (159). While SAS resulted in increased cell death of GBM cells *in vitro* and in xenografts (160), Phase I/II clinical trials were terminated due to incidents of severe side effects (161). Research studies using SAS in gliomas, in combination with radiotherapy, are still in progress (162) and more studies are needed to reveal the effectiveness of the combined treatment.

There are several pharmacological strategies to inhibit glutamine metabolism (147,163). Telaglenastat (CB-839) is a GLS inhibitor currently being evaluated in a Phase I clinical trial (NCT03528642) combined with radiotherapy and TMZ for treatment of IDH-mutant astrocytomas and anaplastic astrocytomas. Collectively, several combinatorial strategies targeting various facets of glutamine cancer metabolism can be applied and this should lead to more research in this field (148).

Arginine metabolism. Arginine is a semi-essential amino acid synthesized from citrulline via urea cycle enzymes, argininosuccinate synthetase-1 (ASS1) and argininosuccinate lyase (ASL) (164). Cancer cells, which are characterized with a high proliferation rate, depend on exogenous arginine in their microenvironment (165). Therefore, reduced expression of urea cycle enzymes ASS1, ASL and OCT makes cancer cells auxotrophic and completely reliant on extracellular arginine sources (166,167). Arginine deprivation has been shown to promote cell death and impairs cell motility and invasion. Hence, arginine deprivation is a promising potential therapy target for selective destruction of tumor cells.

Arginine deprivation serves a role in enhancing endoplasmic reticulum stress and inducing the expression of genes involved in unfolded protein responses, thus resulting in cancer cell death (168). Furthermore, arginine deprivation reduces the extent of β-actin arginylation which disturbs cell cytoskeletal organization, alters cell adhesion and impairs cell migration and invasion (169). HuArgI (Co)-PEG5000 is human arginase I, characterized by the addition of two cobalt ions and polyethylene glycol that results in improved

enzymatic catalytic activity, increased stability, decreased immunogenicity and lower dissociation constant at neutral pH (170,171). Promising effects were shown when using HuArgI (Co)-PEG5000 to target arginine auxotrophy in different tumor types (166,172-175). Our laboratory research found evidence that HuArgI (Co)-PEG5000 induces autophagy cell death in hepatocellular carcinoma (176), pancreatic carcinoma (173,176), acute lymphoblastic leukemia (174), renal cell carcinoma (177), prostate cancer (178), colorectal cancer (CRC) (172), ovarian carcinoma (175) and breast cancer (179). In GBM, we observed that HuArgI (Co)-PEG5000 induces autophagy-dependent cancer cell death. The addition of exogenous citrulline failed to rescue any of the GBM cell lines from arginine depletion-induced cytotoxicity, indicating a high level of arginine dependence (166). A recent recruiting Phase I clinical trial (NCT04587830) is currently assessing the safety and tolerability of ADI-PEG 20, an arginine deprivation agent, in combination with radiotherapy and TMZ in patients newly diagnosed with GBM (180). Taken together, targeting GBM cells using arginine depletion is a potent and selective potential treatment for GBM.

5. Other targeted molecules

Other molecules such as human telomerase reverse transcriptase (hTERT), ATRX and protein kinase C, have also gained attention as potential targets for GBM cancer therapy. This is due to the finding that alterations in these molecules enhance GBM cells survival and progression.

Telomerase

hTERT. Telomerase is a ribonucleoprotein that consists of two subunits: i) the protein catalytic hTERT that mediates reverse transcriptase activity and ii) the human telomerase RNA template. Telomerase is mainly active in embryonic cells, stem cells and precursor cells. Notably, studies show that hTERT expression is upregulated in glioblastoma (181,182). In addition, ~80% of primary glioblastoma cells harbor a mutation or methylation in the hTERT promoter (183). Genomic sequencing revealed a high incidence of mutually exclusive point mutations C228T and C250T in hTERT promotor of glioblastoma cells (183). Arita *et al* (181) show that hTERT expression is 6.1 times higher in tumors carrying these mutations than in wild-type tumors and therefore leads to the upregulation of TERT. Upregulation of hTERT enhances telomerase activity and promotes an immortal phenotype (183). In addition, hTERT mutations are associated with poor prognosis and a multifocal invasive phenotype in GBM (184,185). Thus, hTERT promotor mutation can be recognized as a novel biomarker and therapeutic target molecule for GBMs (184,186,187). Currently, two immunization strategies are currently targeting hTERT in clinical trials. A Recruiting Phase II/III study (NCT03548571) is investigating the efficiency of immunization with IMP dendritic cells that are transfected with mRNA hTERT, as well as autologous tumor stem cells and survivin, compared with standard adjuvant chemoradiotherapy. Additionally, an active, not recruiting Phase I/II study, (NCT03491683) is evaluating the safety of INO-5401 (a combination of three separate DNA plasmids targeting hTERT, Wilms tumor gene-1 antigen, and prostate-specific membrane antigen) in patients with a glioblastoma tumor with an unmethylated MGMT promote.

Alpha thalassemia/mental retardation syndrome X-linked (ATRX). ATRX is a DNA helicase/ATPase that belongs to the SWI2/SNF2 family (188). It is involved in chromatin remodeling and telomere maintenance. ATRX mutations occur in 57% of secondary glioblastoma and are associated with IDH1 and p53 mutations (189). ATRX loss of function mutation triggers a telomerase length mechanism also known as 'alternative lengthening of telomeres' that maintains telomere length, thus enabling cancer cells to escape cell senescence and promoting tumor growth (190). In addition, ATRX mutations impair non-homologous end joining and pDNA-PKcs recruitment, causing genetic instability and leading to DNA damage (190). Therefore, ATRX is another potential target for GBM treatment, however, it is not yet targeted in clinical trials.

Protein kinase C (PKC). PKC is a serine threonine kinase that serves a crucial role in GBM growth and progression. PKC consists of 11 isoforms and is divided into three subfamilies: Classical, novel and atypical classes (191). Classical PKCs consist of two members, PKCα and PKCβ (192). Leirdal *et al* (193) show that PKC enhances GBM survival via MAK-ERK1\2 pathway. PKCα induces GBM progression via PKC-progesterone pathway (194). Once stimulated with 12-O tetradecanoylphorbol-13-acetate or lysophosphatidic acid, PKCa translocates to the nucleus, enhances progesterone receptor phosphorylation and promotes the migration and invasion capacity of GBM (194,195). PKCb serves an essential role in enhancing angiogenesis, thus facilitating GBM progression (196). Moreover, PKCβ leads to GBM cell death. Conversely, Liu *et al* (197) show that in an orthotopic mouse xenograft model, PKCβ II expression suppresses GBM tumor growth and extends mouse survival by inhibiting YAP/TAZ. Thus, PKCβ has two opposite roles in GBM.

The novel PKC subfamily contains four members: PKCδ, PKCε, PKCη and PKCθ. Inhibition of PKCδ suppresses tumor spheroid formation *in vitro* and tumor development *in vivo* (198). In glioblastoma, PKCδ is involved in tumor initiation (199), cancer cell migration and invasion (194,200) and radio-chemoresistance (199). Activation of PKCδ phosphorylates glycerol-3-phosphate dehydrogenase enzyme and serves an essential role in glucose metabolism and phospholipid synthesis (201). PKCε, on the other hand, is overexpressed in glioblastoma cell (202) and controls tumor survival (203) cancer cell adhesion and motility via activation of the ERK pathway (204,205). PKCη promotes GBM proliferation (206,207) and reduces cell sensitivity to radiotherapy (208).

Atypical PKCs ι and ζ are involved in GBM viability, migration, and invasion (209-211). Baldwin *et al* (211) show that inhibition of PKCi increases the activity of Rho B that, in turn, improves actin fiber formation and reduces GBM cell migration and invasion. Similarly, Guo *et al* (210) found that the suppression of PKCz enhances actin polymerization and reduces GBM cell migration and invasion.

Enzastaurin is a kinase inhibitor that particularly inhibits protein kinase C, thus decreasing tumor growth and cell proliferation (212). Results from recent clinical studies (Phase II NCT00586508 and Phase III NCT03776071) did not

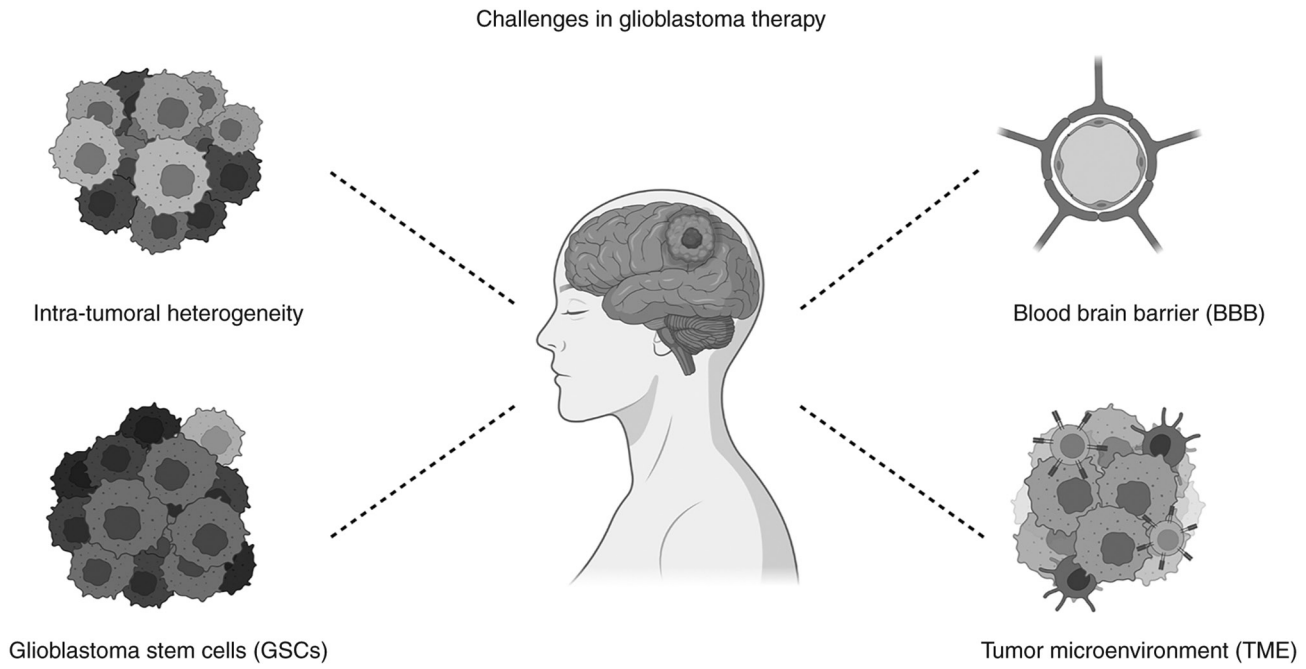

Figure 2. Challenges for glioblastoma treatment. Intra-tumoral and tumor microenvironment heterogeneity, the blood brain barrier and the glioblastoma stem cells are barriers against effective therapeutic strategies for glioblastoma patients. They also confer resistance to drugs and may obstruct proper drug deliver. Created with Biorender.com.

show improved PFS compared with bevacizumab and other therapeutic agents (212,213).

6. Challenges

Targeting intrinsically altered molecules that trigger GBM malignant phenotype is an innovative therapeutic strategy that aims to destroy tumor cells without damaging normal cells. However, despite the promising *in vitro* results, this novel therapy faces various barriers *in vivo* (Fig. 2).

Intra-tumoral heterogeneity. One reason behind the complexity of GBM management is that the genetic profiles of recurrent glioma cells and the initial cancer derived from the same patient are different (214). Thus, the plasticity and heterogeneity of GBM cell populations can explain the failure of clinical trials based on monotherapy *in vivo*. Clones of the genes that are identified by different number of chromosomal sets, exhibit a high level of heterogeneity in the expression of cell membrane markers. This leads to phenotypic heterogeneity at the level of the population providing an advantage for tumor survival (215). Neftel *et al* (216) found that gliomas rarely have identical proportions of single-cell transcriptomic states and this proportion is skewed by genetic associations that are specific to each patient leading to abundant heterogeneity. Therefore, it is recommended to carefully weigh the genetic, epigenetic, and molecular profile of each patient. Single-cell RNA-sequencing reveals the presence of various molecular and genetic cell subtypes within the same patient biopsy (215,217). These subtypes can change progressively and become more resistant to chemoradiotherapy (214). Neftel *et al* (216) describe multiple cellular states and determine a correlation between the cellular states, plasticity and genetic signature of tumor cells. They define four cellular states of malignant GBM tumor cells: Neural-progenitor-like, oligodendrocyte-progenitor-like, astrocyte-like and mesenchymal-like states. These states are characterized by the overexpression of CDK4, PDGPRα, epidermal growth factor (EGF) and NF1 alterations, respectively. Neftel *et al* (216) also showed the co-existence of multiple cellular states and their transition potential within the same patient sample and evidenced the ability of a single cell to generate all cellular states. Amplification of the EGFR and PDGFRA genes that specifically encode for RTKs has been recognized in GBM tumors (218). Snuderl *et al* (219) established that the amplification of different RTKs was infrequently found in the same region of the tumor; yet, different RTKs (MET, EGFR and PDGFRA) were amplified in diverse subpopulations of the GBM tumoral cells.

Another advancing technology that expands the understanding of the heterogeneity of GBM is the whole genome amplification (WGA) methods. WGA helps to identify different imbalances on the level of the chromosomes (218). In one study, Nobusawa *et al* (220) applied the WGA method on GBM samples (14 different primary samples) from two to five locations within each tumor. They recognized not only common modifications between all studied locations, but also changes that were specific to a certain region among the studied GBM tumor samples. A later study targeted 33 cancer genes with single molecular inversion probes and reported regional mutational heterogeneity among different patients with GBMs, as well as among the same patient (221).

Recently, Bhaduri *et al* (222) discovered the existence of 'cancer stem cell likes' that are able to give rise to heterogeneous cancer cell populations within the same tumor (discussed in details in the section below). In their study, Pang *et al* (223)

discovered a path characterized by the progressive renovation of the GBM stem cells to reach an invasive state, known as the 'stem-to-invasion path'. A gradual expression of the invasion signatures linked with GBM, failure in expressing the markers of GBM stem cells and different molecular cascades related to the invasion path were detected, along with different key factors such as transcription factors and long noncoding RNAs (223). Such findings help the understanding of the progression of glioma tumors and thus facilitate screening for efficient methods to therapeutically target GBM.

Efficient treatment options necessitate building a model the mimics a patient's GBM biology. Jacob *et al* (224) generated a new organoid model from patient-derived primary cancer cells that conserved the original tumor's histological, genetic and molecular signatures in addition to its cellular heterogeneity. In order to generate patient-derived glioblastoma organoids (GBOs), Jacob *et al* (224) obtained tissues along the tumor margin. These tissues were refined from necrosis and surrounding brain tissues, dissected and cultured in optimized serum-free GBO medium. When allowed to grow larger, GBO created gradients of hypoxia reflecting a hallmark of GBM. Immunohistological analyses reported markers for glia, immature neurons, neural progenitors and glioma stem cells, resembling the cellular composition of parental tumors. Also, single-cell transcriptome analysis confirmed cell-type heterogeneity and suggested that specific elements of the TME were preserved. Upon transplantation into adult rodent brains, GBOs showed reliable engraftment and aggressive infiltration (224). This suggested that a biobank of patient-derived GBO can be used as a prescreening drug model to investigate drug response and to evaluate its effectiveness on patient cells.

Glioblastoma stem cells (GSCs). 'Cancer stem cell likes', also referred to as 'cancer stem cells' (CSCs) are self-autonomous units that play a significant role in tumor initiation and growth as well as therapeutic resistance. GSCs are derived from the malignant transformation of normal neural stem cells or the dedifferentiation of tumor cells after radiotherapy or chemotherapy (225,226). GSCs exhibit prolonged proliferation and are able to metastasize and suppress anti-inflammatory responses and confer resistance to therapeutic treatments (227). Thus, GSCs are important factors that limit clinical options and treatments of GBM (228,229). Numerous studies that are currently being undertaken to target GSC were made possible by improved understanding of the biology of GCS (24). GSCs represent a hot topic for improved GBM treatment.

Some studies aim to target and kill GSCs directly by targeting stem cell biomarkers. For instance, GSCs that are CD133 positive are important for sustaining and spreading GBM (230). Patients with GBM have a higher survival rate when CD133 positive stem cells are eliminated (231). CD133 stem cells may be eliminated by BMI1 gene suppression, an oncogene involved in the control of stem cell self-renewal and differentiation (232). Inducing apoptosis in GSCs can be also achieved by targeting the signaling pathways that play a role in their self-renewal. For instance, the Wnt signaling pathway has been targeted since 2014 as it is involved in neural stem cell development (233). Targeting glycogen synthase kinase-3β (GSK-3β) and β-catenin is suggested to inhibit the Wnt pathway *in vitro* (234). AR-A01441, LiCl and SEN461 are examples of GSK-β inhibitors that lead to increased apoptosis of GBMs cells (24). Notably, Celecoxib is a β-catenin inhibitor that is already studied in clinical trials, however, no promising results have been reported yet (Table VI).

The Notch pathway is another targeted pathway for GBM treatment as it is involved in resistance to immunotherapies (235). γ-secretase is an enzyme that mediates Notch's cleavage and translocation to the nucleus. Therefore, γ-secretase is suggested as a plausible target for Notch inhibition (236). In fact, several clinical trials are testing the effect of RO4929097, a γ-secretase inhibitor, as a monotherapy for GBM treatment or in combination with other treatments. In addition to the aforementioned pathways, Hedgehog (SHH) pathway is also targeted to inhibit the renewal of GSCs, as it confers resistance to conventional GBM treatment. SHH pathway is targeted by inhibiting smoothened (SMO), its downstream effector (24). Vismodegib is one of the SMO inhibitors used in several clinical trials and it showed enhanced PFS when used before surgical resection (NCT00980343). Finally, the STAT3 pathway is also targeted to control neural stem development (237). WP1066 and Napabucasin (BBI608) are STAT3 inhibitors currently in Phase I (NCT01904123) and II (NCT02315534) clinical trials, respectively (no results are reported yet).

Notably, GSCs can be targeted by targeting the mitochondria or via specific antibiotics. For instance, oxidative phosphorylation (OXPHOS) is a major source of ATP in a number of types of cancer and plays a significant role in carcinogenesis and tumor growth (238). GSCs are OXPHOS-dependent GBM cells that require mitochondrial translation (239). The bacterial antibiotic quinupristin/dalfopristin (Q/D) blocks mitochondrial translation, which not only inhibits the formation of GSCs but also disrupts the cell cycle and increases apoptosis (239). These results suggest that Q/D may be taken into consideration for the treatment of GBM and that reducing mitochondrial translation may be examined to inhibit GSC growth. On the other hand, Salinomycin, is a K^+ ionophore antibiotic that causes lysosomal iron sequestration, and leads to the production of ROS and lysosome membrane permeabilization (240). Salinomycin is shown to favorably kill GSCs and other types of CSCs (240,241). Although clinical trials examining the possible efficiency of Salinomycin in the treatment of glioblastoma have yet to be published, Salinomycin and its derivatives are now being researched intensively as anti-CSCs treatments for a variety of malignancies (241). Collectively, these results imply that focusing on GSCs is a promising approach for treating GBM more effectively.

Blood Brain Barrier (BBB). The BBB forms an interface between the brain and the systematic blood circulation. It consists of endothelial cells (ECs) that line blood vessels, are surrounded by astrocytic perivascular pseudopodium and pericytes, and interconnected by neuronal ending and microglia (242,243). Under normal physiological conditions, ECs, connected by tight junction proteins, create a compact barrier. In addition, efflux transporters, carried by BBB cells, regulate molecular and cellular transport across the BBB and protect the brain from toxins, xenobiotics, and pathogens (243). The development and progression of GBM impair the integrity and function of the BBB, resulting in a heterogenous resistant barrier known as Blood Brain Tumor Barrier (BBTB). In

Table VI. Therapeutic agents targeting glioblastoma stem cells in interventional clinical trials[a].

Therapeutic target	Therapeutic agent	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
CD133 positive stem cells	ICT-121 DC vaccine	Four intradermal injections of the autologous ICT-121 dendritic cell vaccine	Recurrent glioblastoma	Phase I NCT02049489	Completed	
	Activated T cells	ATC against glioma CSC antigens administered intravenously at one timepoint	Recurrent glioblastoma	Phase I NCT05341947	Not yet recruiting	
Wnt pathway (b-catenin)	Celecoxib	TMZ alone or in combination with Thalidomide and/or Isotretinoin and/or Celecoxib after radiotherapy	Glioblastoma multiforme	Phase II NCT00112502	Completed	(353)
		Combination of Celecoxib, 6-Thioguanine, and Xeloda (Capecitabine), with TMZ or Lomustine (CCNU)	Recurrent anaplastic glioma and glioblastoma multiforme	Phase II NCT00504660	Completed	
		TMZ, Thalidomide, and Celecoxib after radiotherapy	Patients with newly diagnosed glioblastoma multiforme	Phase II NCT00047294	Completed	(354)
		Coordinated undermining of survival paths by 9 repurposed drugs (aprepitant, auranofin, captopril, celecoxib, disulfiram, itraconazole, minocycline, ritonavir and sertraline) combined with metronomic TMZ	Recurrent glioblastoma	Phase I/II NCT02770378	Completed	(355)
		Thalidomide, Celecoxib, and combination chemotherapy	Relapsed or refractory malignant glioma	Phase II NCT00047281	Completed	
Notch pathway (g-secretase)	RO4929097	RO4929097 in combination with Cediranib Maleate	Advanced solid tumors including glioblastoma	Phase I NCT01131234	Completed	
		RO4929097, TMZ, and radiotherapy	Advanced solid tumors including glioblastoma	Phase I NCT01119599	Completed	
Hedgehog (SHH) pathway (SMO)	Vismodegib (GDC-0449)	Monotherapy	Recurrent glioblastoma multiforme	Phase II NCT00980343	Completed	

Table VI. Continued.

Therapeutic target	Therapeutic agent	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
		Umbrella protocol of molecularly matched targeted therapies (APG101, Alectinib, Idasanutlin, Atezolizumab, Vismodegib, Temsirolimus, Palbociclib) in combination with radiotherapy	Glioblastoma without MGMT promoter methylation	Phase I/II NCT03158389	Recruiting	
	Sonidegib (LDE225)	Oral LDE225 in combination with BKM120	Advanced solid tumors including recurrent glioblastoma multiforme	Phase I NCT01576666	Completed	
		Molecularly-Driven doublet therapy for Gemcitabine, Ribociclib, Sonidegib, and Trametinib	Children and young adults with recurrent brain tumors	Phase I NCT03434262	Recruiting	
	Glasdegib (PF-04449913)	In combination with TMZ	Newly diagnosed glioblastoma	Phase I/II NCT03466450	Active, not recruiting	
STAT pathway	Napabucasin (BBI608)	In combination with TMZ	Recurrent or progressed glioblastoma	Phase I/II NCT02315534	Completed	
	WP1066	Monotherapy	Malignant glioma including recurrent glioblastoma	Phase I NCT01904123	Completed	

[a]Data acquired from the U.S. National library of medicine (http://clinicaltrials.gov, accessed on 10 August 2022). Terminated studies are not included. ATC, activated T cells; CSC, cancer stem cells; DC, dendritic cells; MGMT, O^6-methylguanine DNA methyltransferase; STAT, signal transducer and activator of transcription; TMZ, Temozolomide.

glioblastoma, the BBTB is characterized by the downregulation of tight junction proteins such as claudin and occludin and overexpression of efflux transporters (244-247). These tight junction proteins increase the permeability of the BBTB, enhance paracellular transport and thus facilitate the penetration of cells and small molecules to the interstitial space of the brain. Accumulation of such molecules might have neuro-toxic effects. In addition, the heterogeneous permeability of the BBTB caused by the 'leaking' structure of the new blood vessels as against the compact structure of the local blood vessels leads to unequal distribution of the drug (248-250). Several FDA-approved drugs, including chemotherapy drugs, have affinity for efflux transporters, including P-gp and BCRP (251-256). Hence, overexpression of efflux transporters in glioblastoma restricts drug delivery to the brain.

In the line of BBB heterogeneity and overexpression of efflux transporters, cellular, chemical and physical approaches are being investigated to enhance drug delivery through the BBTB. One of these advanced approaches is the nanomaterial system which is based on different types of nanocarriers including, polymer-based, viral, drug-conjugated, lipid-based and inorganic nanoparticles (NPs) (257). NPs are usually loaded with drugs and are known to have a small size within a range between 1-100 nm (258). Budama-Kilinc *et al* (259) used a nanoparticle system based on a poly (ε-caprolactone) to deliver a tripeptide, glycyl-L-histidyl-L-lysine (GHK), to the GBM cells. GHK is known as a natural growth modulating tripeptide *in vitro*; thus, it decreases the viability of GBM cells to ~65%. Another study was based on a nanobubble system that consists of NPs made up of iron and platinum. NPs were either surface-functionalized with transferrin to target the glioma cells or loaded with doxorubicin (DOX) in a mouse model. These NPs resulted in reduced growth of the tumor by ~70% (260). On the other hand, Ambruosi *et al* (261) used a rat glioma model to test poly (n-butyl cyanoacrylate) (PBCA) as a potential therapy for GBM. PBCA NPs were loaded with

DOX chemotherapeutic drug and coated with polysorbate 80 (surfactant). NPs successfully delivered the drug across the BBB and increased the median survival rate by 35% among tested rats living for the whole time of the study period.

On the molecular level, transferrin receptor (TfR) is a protein commonly targeted to enhance the delivery of therapeutical drugs through the BBB (262). Poly (lactic-co-glycolic acid) NPs loaded with TMZ and coated with TfR-monoclonal antibodies showed higher internalization in GBM cells compared with control cells (263). Kuang *et al* (264) examined the use of polymer-based NPs coupled to a peptide having the ability to target TfR on both the BBB and GBM cells to transport doxorubicin and RNA. Results showed higher efficiency in *in vitro* tumor targeting and cellular uptake, with a decline in the tumor growth leading to improved median survival time.

Focused ultrasound therapy (FUS) is another method used to facilitate transport through the BBB. It is a non-invasive and image-guided method that enhances the efficacy of drug delivery to GBM cells (265). Wei *et al* (266) showed an enhancement in the local delivery of TMZ to tumor cells through the FUS-mediated disruption of the BBB. Results reported an increase in the OS of rats with experimentally induced gliomas. Notably, MRI-guided FUS (MRgFUS) was evaluated to attain enhanced tissue delivery of TMZ in mice (267), liposome-encapsulated doxorubicin in rats (266) and cisplatin-conjugated gold NPs in mice (268). This method is advantageous as it reduces the systematic toxic effects of the drugs used (low doses of therapeutic drugs can be used) (269) and leads to temporary opening of the BBB rather than long-term opening associated with BBB disruptions (270). These studies show that the use of different NPs (nanomaterial system) or FUS has the potential to enhance the efficiency of therapeutical drug delivery in GBM management.

TME. The TME comprises fibroblasts, endothelial cells, immune cells, ECM and soluble factors surrounding the tumor. It provides biophysical and biochemical support for the tumor and contributes significantly to tumor initiation, growth, progression, and resistance to therapy (271).

GBM is classified as a hypoxic solid tumor where hypoxia is considered one of the non-cellular TME components. The hypoxic microenvironment is a major inducer of angiogenesis; a process in which ECs branch from preexisting small vessels to form sprouts of capillaries. Angiogenesis thus promotes tumor growth by supplying cancer cells with O_2 and nutrients and facilitates their metastasis (272,273). Moreover, hypoxia induces the activation of a number of cellular processes that promote the migration, invasion and radio-chemoresistance of GBM cells (274-280). This is due to the overexpression of VEGF/HIF-1α protein. Therefore, and in order to control the hypoxic environment, researchers developed Bevacizumab, an FDA approved anti-VEGF antibody, for GBM treatment. Bevacizumab showed encouraging results as monotherapy or when combined with traditional treatment, as reviewed in the study by Cruz Da Silva *et al* (24).

GBM secretes a wide range of chemo-attractants and cytokines that recruit immune cells to the TME. For instance, GBM stem cells recruit macrophages and promote their transition from the antitumor (M1) to the pro-tumor (M2) phenotype (281). Tumor associated macrophages (TAM) are the most abundant cells inflating tumor lesion and accounting for ≤40% of the tumor mass. Indeed, the disrupted structure of BBTB facilitates the infiltration of TAM. In addition, GBM cells inhibit the proliferation of cytotoxic T cells and activate regulatory T cells (282). Occurrence of immune cells in the TME leads to the formation of an extensively immune-suppressive microenvironment and enhances GBM cell migration and invasion.

At present, researchers are investigating new therapeutic approaches that aim to reactivate the immune system against GBM and restore the immune surveillance in GBM. These approaches include reversing the polarization of M2 macrophages to M1, targeting immune checkpoints using checkpoint inhibitors to rebut T cell responses, and implementing cytokine therapy. Yang *et al* (283), discovered that the dual-targeting of IL-6, which stimulates different macrophages activation in GBM, and CD40 may help in reversing tumor immunosuppression mediated by macrophages thus informing the immunotherapy based on T-cells against GBM. In their study, it was shown that CD40 stimulation and IL-6 inhibition reverse tumor immunosuppression mediated by macrophages and increase the survival rates of the animals in GBM models (283). Similar findings supported the use of macrophages-based therapies as effective therapeutic approaches when targeting GBM.

Cytokine therapy represents a hot topic for immunotherapeutic approaches since cytokines are molecular messengers of innate and adaptive immunity (284). This approach promotes human natural killer (NK) and T cell activity, survival and proliferation thus coordinating immune responses against malignancies (285). TGF-β, IFN-α, IFN-γ and IL-12 and other cytokines have anti-tumor effect in GBM, thus reducing tumor size and inhibiting carcinogenesis at early stages (286). Tumoral injection of Ad-RTS-hIL-12 in combination with veledimex (VDX, an hIL-12 activator) and an FDA approved antibody cemiplimab was tolerated well and exhibited increased circulating cytotoxic T cells in patients with recurrent or progressive GBM (Phase II NCT04006119). IFN-β immunotherapy in combination with TMZ was also evaluated in clinical trials (287). Han *et al* (288), used cytokine-induced killer therapy with TMZ on patients with GBMs and reported higher PFS rates in treated vs. control untreated group. The therapeutic efficacy of various cytokines in the treatment of gliomas is still being investigated. The cytokines' safety and tolerance are being defined, and some are thought to be a promising supplement to conventional treatments (289).

Glioma cells frequently employ a variety of strategies to avoid immune surveillance. Therefore, several therapeutic approaches have been developed to activate immune responses in the TME. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and PD ligand 1 (PD-L1) are immune checkpoint proteins targeted for monoclonal antibody inhibition (290,291). Monoclonal antibodies act by freeing T lymphocytes from their negative regulation thus suppressing cancer evasion of the immune system (292,293). Several immune checkpoint inhibitor antibodies have been approved by the FDA for the treatment of several types of cancer (294). Neoadjuvant therapy with pembrolizumab, an anti-PD-1 monoclonal antibody, resulted in improved OS and PFS in patients with recurrent

surgically respectable GBM (Phase II NCT02337686) (43). Wang *et al* (295) demonstrated that genetically engineered multifunctional NK cells (CD73.mCARpNKs) can lead to efficient anti-GBM activity mainly by bypassing the heterogeneity and the immunosuppressive structures of GBM tumors. Immune responses in the TME can be also stimulated by adoptive cell therapy (ACT). This method involves isolating autologous T lymphocytes with anti-tumor activity from cancer patients, expanding them *ex vivo*, and transferring amplified engineered T cells to the patients (296,297). Among the different ACT methods developed, chimeric antigen receptor (CAR)-T cell gene therapy has attracted the most attention for killing cancer cells. CAR-modified T cells may identify different types of antigens regardless of how they are presented on MHC molecules (298). To date, there are currently 24 clinical trials examining the efficacy of CAR-T cell treatment for GBM according to Clinicaltrials.gov (accessed on 10 August 2022; Table VII). Several CARs have been generated to specifically target GBM such as EGFRvIII, HER2, IL13R2, and CD70 (290). Collectively, studies using CAR-T cell immunotherapy exhibit encouraging results. However, obstacles such as tumor heterogeneity, the heterogenous expression of antigens, and the role of T cells at the tumor's sites make it challenging to efficiently eradicate the tumor (290).

7. Discussion

Glioblastoma is characterized with high cell proliferation and neovascularization leading to tumor infiltration reaching crucial structures in the brain. This increases the possibility of tumor recurrence, scoring low survival rates (299,300). Therefore, more effective treatment procedures are required to extend the lives of patients. Conventional treatments such as radiotherapy and chemotherapy are regarded as a two-edged sword. This is because these treatments are frequently accompanied with normal tissue damage or genetic changes, nonspecific drug distribution and multidrug resistance. However, chemoradiotherapy promises improved survival and quality of life that may outweigh their disadvantages; thus, these modalities remain the primary cancer weapons. Substantial research has been done, and continues to be done, to develop novel therapeutic approaches that are less toxic for normal tissues and endow increased survival and improved quality of the lives of patients. Researchers have discovered genetic and subsequent molecular changes influencing critical pathways that cause aggressive behavior of tumors (24,214). Therefore, targeting these altered molecules and pathways is seen as a promising new approach to GBM treatment.

Over the previous few decades, biomarker-driven methods, such as small molecule inhibitors and monoclonal antibodies, have been developed. These methods aim to target key altered players that are directly correlated with tumor progression and invasion. EGFRs constitute one of the mostly targeted molecules in clinical trials to treat patients with GBMs (Table I). However, targeted therapy has shown little to no promising results due the intra-tumoral heterogeneity of GBM and drug resistance (216,218). Tumor heterogeneity could be a plausible explanation for GBM resistance to EGFR-targeted treatments. Moreover, resistance to EGFR therapy can also be caused by upregulation of redundant RTKs and downregulation of EGFR downstream molecules (218). Consequently, co-targeting of EGFR and other RTKs such as c-MET could offer a solution as seen *in vivo* (301). Multi-targeted therapeutic approaches offer an advantage where drugs can target numerous important nodes for GBM growth and progression, compensating for the inefficiency and quick acquisition of resistance seen with monotherapies (302). Multi-targeted therapeutic approaches also include the administration of multi-kinase inhibitors (303,304). Moreover, more complicated pathways have been identified and targeted *in vitro*. In-depth-investigation of these pathways helps to identify novel drugs.

Glioblastomas are intrinsically immunologically 'cold' tumors due to presence of few T cells but predominant pro-tumor immune cells in the TME, constituting a common reason for drug resistance (305). Immunotherapies thus aim to use a patient's immune system to re-direct immune cells against a tumor. Researchers have established several other immunotherapeutic strategies to target patients with GBMs with immunostimulatory conditions. These include immune check-point inhibition, autologous stimulated lymphocytes, cytokine therapy and dendritic cell therapy (306). Except for certain cases, immunotherapy in glioblastoma clinical trials has been disappointing overall. CAR-T cell therapy, for instance, faces a significant hurdle since glioblastoma tumors can rapidly adapt via antigen escape (307). CAR-NK cell therapy, on the other hand, offers an advantage as it can be administered to an HLA-mismatched patient, and Phase I clinical trial is showing promising preliminary results (NCT03383978) (308). Yet, NK cell expansion and manufacturing are still time and money consuming (309). Akin to targeted therapy, tumor heterogeneity is also a limiting factor for immunotherapy. Moreover, combing immunotherapy with chemotherapy may also lead to drug resistance (310). Anti-inflammatory corticosteroids, for example, may counteract the therapeutic effects of immunotherapy (311). Additionally, glioblastoma cells can adapt to immune checkpoint inhibition by increasing the expression of alternative checkpoints (312). The immunosuppressive TME is another limitation for immunotherapy, thus, combinatorial immunotherapy approaches may overcome this issue (313,314). In fact, preclinical research on combination immunotherapy yielded promising outcomes [reviewed in (314)]. The most successful approaches are those that influence the cancer-immunity cycle at different times and/or sites, leading to the activation of immune response and the inhibition of immunosuppressive elements (314).

Cancer vaccines are one of the very promising immunotherapies and are being thoroughly researched as a method to reduce tumor development and eliminate tumor cells in cancer patients. Peptide-based, dendritic cell (DC)-based, and personalized vaccines are being explored as potential vaccine therapies in GBM (305,315) (Table VII). Cancer vaccine design depends on selecting an ideal antigen specifically expressed and present on all cancer cells, highly immunogenic and essential for cancer cell survival (316). These antigens are therefore processed by DCs aiding in recruiting and activating $CD8^+$ and $CD4^+$ T cells that will promote tumor eradication (317). DCVax®-L and ICT-107 are DC-based vaccines tested in clinical trials for safety, tolerance and efficacy in patients with GBMs (NCT00045968 and NCT01280552 respectively). Personalized vaccination is now made possible using multi- epitope-based patient-specific

Table VII. CAR-T cells and cancer vaccines applied in interventional clinical trials for the treatment of glioblastoma[a].

A, CAR-T cells

Therapeutic agent	Specificity	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
CAR-T Cell Receptor Immunotherapy	EGFRvIII	In combination with Aldesleukin, Fludarabine or Cyclophosphamide	Malignant gliomas expressing EGFRvIII	Phase I/II NCT01454596	Completed	(356)
CAR-T-EGFR-IL13 Ra2 cells	EGFR epitope 806 and IL13Ra2	CART-EGFR-IL13 Ra2 cells following lymphodepleting chemotherapy	Recurrent glioblastoma	Phase I NCT05168423	Not yet recruiting	
CAR-T-EGFRvIII cells	EGFRvIII	In combination with pembrolizumab (PD-1 Inhibitor)	With newly diagnosed, MGMT-unmethylated glioblastoma	Phase I NCT03726515	Completed	
CD147-CAR-T Cells	CD147	Monotherapy	Recurrent glioblastoma CD147 Positive	Early Phase I NCT04045847	Unknown	
B7-H3CAR-T	IFN-1	Monotherapy	Recurrent glioblastoma multiforme	Phase I NCT05474378	Completed	
NK-92/5.28.z Cells	NK-cells	With the anti-PD-1 antibody Ezabenlimab	Recurrent HER2-positive glioblastoma	Phase I NCT03383978	Recruiting	(308)

B, Cancer vaccine

Therapeutic agent	Specificity	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
DCVax®-L	DC based vaccines	Monotherapy	Newly diagnosed glioblastoma multiforme	Phase III NCT00045968	Active, not recruiting	(357)
ICT-107	DC based vaccines	After surgery and chemotherapy	Glioblastoma multiforme	Phase II NCT01280552	Completed	
ACTIVATe (PEP-3 vaccine)	EGFRvIII	In combination with sargramostim and TMZ	Newly diagnosed glioblastoma multiforme	Phase II NCT00643097	Completed	(28)
Rindopepimut (CDX-110, ACT IV vaccine)	EGFRvIII	With GM-CSF, TMZ and KLH	Newly diagnosed glioblastoma	Phase III NCT01480479	Completed	(358)
		With GM-CSF, Bevacizumab and KLH	Recurrent EGFRvIII-positive glioblastoma	Phase II NCT01498328	Completed	(359)
Personalized Neo Antigen Vaccine	Personalized neoantigens	After radiotherapy, with Pembrolizumab	Newly diagnosed glioblastoma multiforme	Phase I NCT02287428	Recruiting	(318)
GAPVAC (APVAC1 and APVAC2)	Personalized mutated and unmutated tumor antigens	With immunomodulators concurrent to TMZ	Newly diagnosed glioblastoma	Phase I NCT02149225	Completed	(319)

Table VII. Continued.

B, Cancer vaccine						
Therapeutic agent	Specificity	Therapeutic strategy	Cancer condition	Phase	Status	(Refs.)
AV-GBM-1		Following primary surgery plus concurrent chemoradiation	Newly diagnosed glioblastoma	Phase II NCT03400917	Active, not recruiting	

[a]Data acquired from the U.S. National library of medicine (http://clinicaltrials.gov, accessed on 10 August 2022). Terminated studies are not included. CAR-T, chimeric antigen receptor-T cell; EGFR, epidermal growth factor receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; INF-1, type I interferon; KLH, Keyhole Limpet Hemocyanin; MGMT, O^6-methylguanine DNA methyltransferase; NK, natural killer cells; PD-1, programmed cell death protein 1; TMZ, Temozolomide.

vaccine to target neoantigens only (Phase I NCT02287428) (318) or both neoantigens and unmutated tumor-specific antigens (Phase I NCT02149225) (319). Corresponding results reported neoantigen-specific $CD4^+$ and $CD8^+$ T cell responses migrating into the tumor with increased number of infiltrating T cells (318,319). AV-GBM-1 is another personalized cancer vaccine based on autologous dendritic cells full of autologous tumor neoantigens. Phase II clinical study (NCT03400917) reported an enhanced PFS (320) demonstrating a promising positive effect of vaccination in patients with GBM.

Most of the current therapeutic strategies need to overcome the obstacles of drug delivery due to BBB, tumor and TME heterogeneity and abundant GSC niches. Burkhardt *et al* (321) propose promising results for an intra-arterial brain administration of Bevacizumab after temporary destruction of the BBB by mannitol. Followed by intravenous administration, their technique is able to overcome the poor intracerebral availability of the drug and enhanced PFS in patients with recurrent GBMs. Several studies suggest that molecular subgroups exist within histologically similar GBM tumors (322,323). Research into the dynamics of various glioblastoma subtypes would help understand the survival features of the TME. This highlights that future treatment options should no longer be based exclusively on morphological assessments, but must now take molecular and cellular heterogeneity into account. Furthermore, the plasticity of GBM cells caused by the bidirectional communication between GSCs and their differentiated counterparts complicates heterogeneity (227). These two populations respond differently to radio-, chemo- and targeted therapies. Thus, targeting both components should be well considered during the course of treatment.

Due to genetic heterogeneity and tumor growth, single-target therapy generates recurrence and consequent resistance to initial treatment. Therefore, therapeutic strategies are implementing targeted therapy along with immunotherapy. Notably, immunostimulatory and immunosuppressive effects are also mediated by targeted therapy (324). Inhibition of several molecular targets, such as anti-angiogenic agents, has already been proved to improve immunotherapy clinical results in a variety of types of cancer (325). It is worth noting here that, when it comes to combinatorial therapeutic approaches, greater laboratory and clinical efforts are required to validate their efficiency.

The increased understanding of GBM biology outlined above was made possible in part by the growth of murine preclinical GBM models. These models were key for translatable research from preclinical to clinical studies. Glioblastoma cell-line xenografts, patient-derived xenografts, and genetically engineered mouse models are currently available. However, these models fail to recapitulate tumoral and TME heterogeneity of GBM. Thus, the effects of advanced therapies in animal models rarely replicate clinical data, which is a persistent issue in glioblastoma research. The success of such therapies may rely on the development of reliable GBM models. Several reproducible glioma models in pigs are evolving as preclinical large-animal models (326-329). Therefore, xenograft and genetically engineered porcine models are suggested as reliable prospective models to allow for a transitional step between murine models and human clinical trials (330).

Effective drug delivery systems that can cross the BBB are also attracting attention. This includes non-viral vectors such as nanocarriers and viral vectors such as oncolytic viruses. Due to advanced research and promising outcomes, chemoradiotherapy nanomedicine is expected to be progressively applied in the clinic in the coming years (331). Targeted gene therapy employing nano-based carriers is being explored and its applicability for a number of forms of cancer are currently under Phase I-III development (332). It is thus expected that combing monoclonal antibodies and nano-carriers could also improve the efficiency of immunotherapy. On the other hand, oncolytic viruses are vectors of targeted gene therapy that can cross the BBB and are considered a promising method for treating GBM (333-336). While drug and gene delivery via oncolytic viruses are currently in clinical trials, more research and large randomized controlled Phase II/III trials are needed to assess their beneficial clinical outcome.

8. Conclusion

An efficient GBM treatment starts by understanding the molecular alterations in cells derived from the patient and that are driving the malignant phenotype. This is followed by studying the effect of a selected treatment *in vitro* using the 'ideal' model that can conserve the molecular signature of patient cells. Prior to incorporating the patient in any clinical trial, it is also

recommended that researchers or physicians analyze the structure of the BBB to evaluate the ability of a selected drug to pass across the BBB. Finally, the use of a combined therapy strategy that targets tumor cells and cells of the surrounding microenvironment may increase the efficiency of the GBM treatment.

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Competing interests

The authors declare that they have no competing interests.

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